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INSTYTUT FIZJOLOGII ROŚLIN im. *FRANCISZKA GÓRSKIEGO*
POLSKIEJ AKADEMII NAUK

Zakład Biologii Rozwoju

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Proces rozhartowania rzepaku – podłoże fizjologiczno-biochemiczne i konsekwencje dla mrozoodporności roślin

Rozprawa doktorska

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Składam serdeczne podziękowania:

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Spis treści

Wykaz prac naukowych wchodzących w skład cyklu publikacji do rozprawy doktorskiej

Niniejsza praca stanowi podsumowanie badań przeprowadzonych w latach 2020−2024, a opracowanie zostało przygotowane na podstawie wyników opisanych w następujących publikacjach:

A. **Stachurska, J.**, Rys, M., Pociecha, E., Kalaji, H.M., Dąbrowski, P., Oklestkova, J., Jurczyk, B., Janeczko, A. 2022. Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and *BRI1* Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance. International Journal of Molecular Sciences, 23, 5224.
   $IF_{(2023)}$ 4,9; 140 pkt (MNiSW)

B. **Stachurska, J**., Sadura, I., Rys, M., Dziurka, M., Janeczko, A. 2023. Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.). Agriculture 13, 641.
   $IF_{(2023)}$ 3,3; 100 pkt (MNiSW)

C. Rys M., **Stachurska J**., Rudolphi-Szydło E., Dziurka M., Waligórski P., Filek M., Janeczko A. 2024. Does deacclimation reverse the changes in structural/physicochemical properties of the chloroplast membranes that are induced by cold acclimation in oilseed rape? Plant Physiology and Biochemistry, Volume 214, 108961
   $IF_{(2023)}$ 6,1; 70 pkt (MNiSW)

D. **Stachurska J**., Sadura I., Jurczyk B., Rudolphi-Szydło E., Dyba B., Pociecha E., Ostrowska A., Rys M., Kvasnica M., Oklestkova J., Janeczko A. 2024. Cold acclimation and deacclimation of winter oilseed rape − special attention being paid to role of brassinosteroids. International Journal of Molecular Sciences 25, 6010.
   $IF_{(2023)}$ 4,9; 140 pkt (MNiSW)

E. **Stachurska, J.**; Janeczko, A. 2024. Physiological and Biochemical Background of Deacclimation in Plants, with Special Attention Being Paid to Crops: A Minireview. Agronomy 14, 419.
IF$_{(2023)}$ 3,3; 100 pkt (MNiSW)

F. **Stachurska J**., Janeczko, A. 2024. Zjawisko hartowania i rozhartowania roślin w kontekście zmian klimatu. Kosmos, tom 73, nr 1, 37−46.
20 pkt (MNiSW)

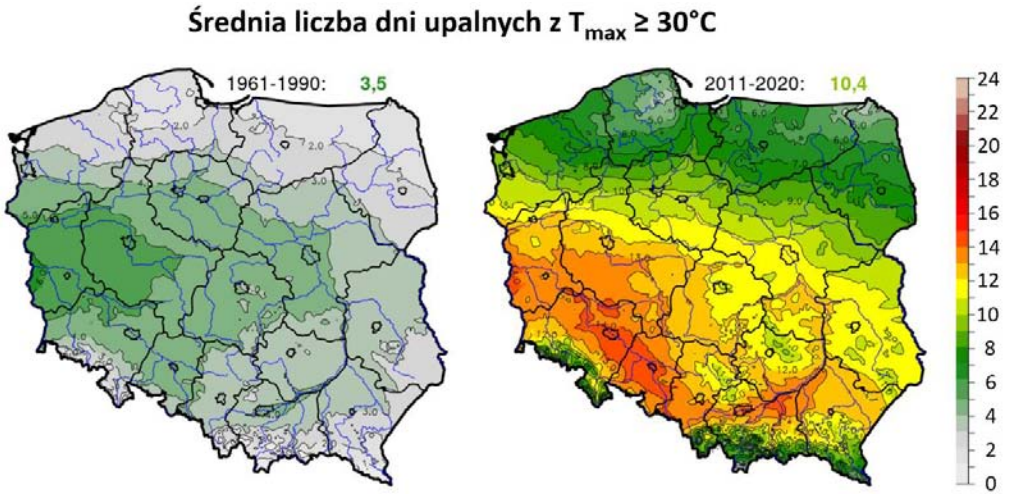
Suma IF$_{(2023)}$ publikacji: 22,5
Średni IF$_{(2023)}$ publikacji: 4,5

Prace wykonano w Instytucie Fizjologii Roślin im. *F. Górskiego* Polskiej Akademii Nauk w Krakowie w ramach projektu badawczego NCN Opus nr 2019/35/B/NZ9/02868 (doktorant-stypendysta).

1. Wprowadzenie

1.1. Zmiany klimatu i ich potencjalny wpływ na ozime rośliny uprawne – zjawisko rozhartowania

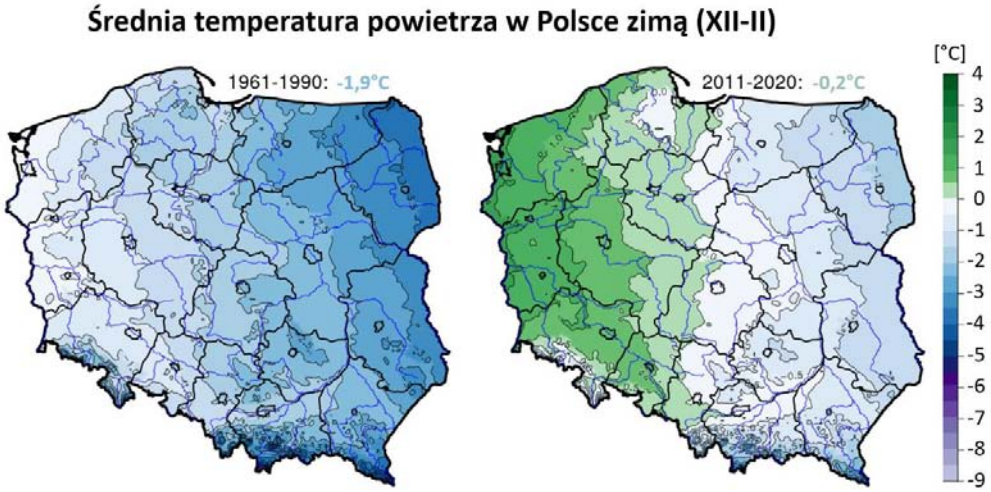
Zmianom klimatu, bez względu na ich przyczyny jakimi mogą być działalność człowieka, zmiany aktywności Słońca, erupcje wulkanów itp., towarzyszy globalne ocieplenie. Modele klimatyczne przewidują wzrost temperatur na świecie o co najmniej 2°C do końca XXI wieku (Fan et al., 2021). W Polsce, w ostatnich latach zaobserwowano podwyższenie średniej temperatury w każdej z pór roku. Latem zwiększa się liczba dni upalnych (z temperaturą powyżej 30°C) (Źródło internetowe 1 − Nauka o klimacie). W zależności od regionu Polski, w latach 1961−1990 notowano średnio 3,5 dnia upalnego rocznie, zaś w latach 2011−2020 zanotowano średnio 10,4 dni upalnych rocznie (rycina 1). W niektórych rejonach było to nawet 14−15 dni upalnych rocznie. Równocześnie spadała liczba dni z temperaturą niższą niż 0°C (Źródło internetowe 1 − Nauka o klimacie).



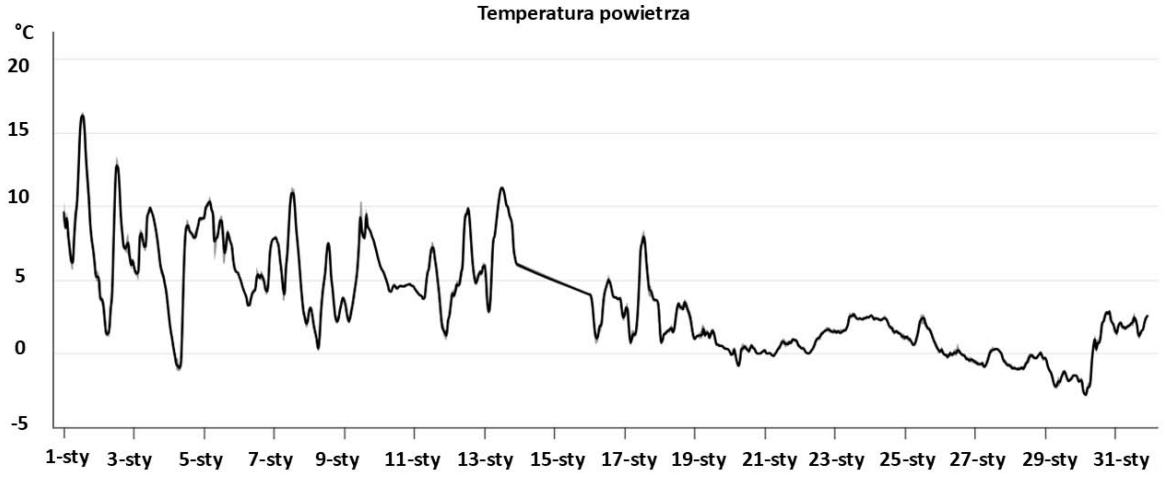
Rycina 1. Średnia liczba dni upalnych (z temperaturą powyżej 30°C) w Polsce − zmiany na przestrzeni ostatnich 60-ciu lat. Źródło: https://naukaoklimacie.pl/aktualnosci/zmiana-klimatu-w-polsce-na-mapkach-468

W porównaniu do lat 1961−1990, w latach 2011−2020 odnotowano znaczne zwiększenie się średniej temperatury powietrza zimą, średnio o 1,7°C (rycina 2) (Źródło internetowe 1 − Nauka o klimacie). Wraz ze wzrostem średnich temperatur, częściej

obserwowane są zmiany wzorców pogodowych występujących w poszczególnych porach roku. Na przykład, początek stycznia 2023 roku był wyjątkowo ciepły. W niektórych dniach odnotowano temperaturę sięgającą około 16°C (Źródło internetowe 2 − Meteo AGH) (rycina 3). Wrzesień tego roku był z kolei cieplejszym miesiącem niż czerwiec (IMGW, 2023).



Rycina 2. Średnia temperatura powietrza w Polsce zimą (XII−II) − zmiany na przestrzeni ostatnich 60-ciu lat. Źródło: https://naukaoklimacie.pl/aktualnosci/zmiana-klimatu-w-polsce-na-mapkach-468



Rycina 3. Zmiany temperatury powietrza w styczniu 2023 roku w Krakowie. Źródło: http://meteo2.ftj.agh.edu.pl/meteo/archiwalneWykresyMeteo

Wahania temperatur w poszczególnych porach roku mogą wpływać negatywnie na cykle rozwojowe roślin, co może być szczególnie niekorzystne w przypadku ozimych roślin uprawnych. Odmiany ozime roślin uprawnych (np. rzepak czy pszenica) są uprawiane w Polsce częściej niż odmiany jare, ponieważ dają wyższe plony. Do prawidłowego przebiegu cyklu rozwojowego rośliny ozime potrzebują okresu wzrostu w warunkach chłodu – tzw. wernalizacji, która jest ważna dla indukcji rozwoju generatywnego (Chouard, 1960). Jednocześnie, podczas kilkutygodniowego wzrostu roślin w chłodzie jesienią (zwykle w temperaturze 2–4°C), zachodzi proces hartowania, który umożliwia roślinom zwiększenie mrozoodporności i ułatwia przetrwanie zimy. Dobrze zahartowane rośliny rzepaku mogą przetrwać nawet w temperaturze rzędu −20°C (Rapacz i Janowiak, 1998). Proces hartowania roślin jest związany z wieloma zmianami biochemicznymi i metabolicznymi (Fürtauer et al., 2019). Dochodzi wówczas między innymi do zwiększonej akumulacji cukrów, które zagęszczają sok komórkowy i obniżają temperaturę jego zamarzania (Sasaki et al., 1996) oraz zwiększenia płynności błon komórkowych (m.in. przez zwiększenie ilości nienasyconych kwasów tłuszczowych), co ułatwia ich funkcjonowanie w chłodzie (Uemura et al., 1995; Filek et al., 2017). Obserwuje się również zwiększenie ilości białek ochronnych, takich jak białka szoku cieplnego HSP [ang. ***H**eat **S**hock **P**roteins*] (Zhang et al., 2008; Sadura et al., 2020) oraz białek, których ekspresja jest regulowana przez chłód – tzw. COR [ang. ***C**old-**R**egulated* proteins] nazywanych niekiedy także LTI [ang. ***L**ow **T**emperature **I**nduced proteins*] (Giorni et al., 1999; Kmieć et al., 2005). Istotnym zmianom ulega gospodarka hormonalna roślin − często notuje się podwyższoną zawartość hormonów stresu, takich jak kwas abscysynowy (ABA) (Kosová et al., 2012). Wzrost roślin w warunkach chłodu/ niskich temperatur skutkuje zazwyczaj obniżeniem intensywności fotosyntezy (Stitt i Hurry, 2002).

Coraz częstsze występowanie zimą okresów z podwyższoną temperaturą (np. powyżej 9°C) może stanowić zagrożenie dla wielu gatunków ozimych roślin uprawnych, ponieważ taka temperatura (utrzymująca się kilka dni) może prowadzić do rozhartowania (dehartowania/ deaklimacji), obniżenia mrozoodporności, a nawet do stymulacji/wznowienia wzrostu roślin (Rapacz, 2002a). Rozhartowanie może być szczególnie niebezpieczne, gdy wystąpi po nim nagły silny mróz, znacznie bowiem zwiększa to ryzyko uszkodzenia roślin (Rapacz et al., 2017; Rys et al., 2020), co pociąga za sobą możliwość strat ekonomicznych (Wałkowski, 2016). Z danych literaturowych wynika, że tempo spadku mrozoodporności w wyniku rozhartowania zależy od temperatury rozhartowującej (Rapacz, 2002a). Tempo rozhartowania rzepaku było wyższe w temperaturze 20°C niż w 12°C zarówno w roślinach rzepaku odmiany jarej, jak

i ozimej (Rapacz, 2002a), a także w temperaturze 20/12°C (dzień/noc) niż w 12/20°C (dzień/noc) (Rapacz, 2002b). Zjawisko rozhartowania może zajść w ciągu kilku dni ekspozycji roślin na podwyższoną temperaturę. Przykładowo, rozhartowanie rzepaku potwierdzono po siedmiu dniach wzrostu w temperaturze 16°C w dzień i 9°C w nocy (Rys et al., 2020). Badania genetyczne ujawniły jednak, że zmiany w ekspresji genów pojawiają się już po kilku godzinach działania podwyższonej temperatury (Pagter et al., 2017).

Rozhartowanie jest procesem odwracalnym, mówimy wtedy o zjawisku rehartowania. Rehartowanie roślin zachodzi (zwykle późną jesienią czy zimą), gdy wystąpią ponownie niskie, hartujące temperatury, a jednocześnie nie miało miejsca istotnie silne wznowienie wzrostu roślin (w szczególnym przypadku − rzepaku ozimego − niebezpieczne jest wybicie pędu kwiatowego).

1.2. Aktualny stan wiedzy w zakresie fizjologiczno-biochemicznych zmian zachodzących w czasie rozhartowania roślin ze szczególnym uwzględnieniem gatunków z rodziny *Brassicaceae*

Badania zjawiska rozhartowania prowadzono na licznych gatunkach, m.in. na drzewach (Taulavuori et al., 2004) i trawach (Hoffman et al., 2014), ale przede wszystkim na roślinach istotnych gospodarczo, takich jak herbata chińska (*Camellia sinensis* L. Kuntze) (Ding et al., 2023), kapusta (*Brassica olearea* L.) (Sasaki et al., 1996), jęczmień (Pociecha et al., 2020), ryż (Cen et al., 2018), pszenica (Vaitkevičiūtė et al., 2022) oraz pszenżyto (Rapacz et al., 2022). Szczegółowe zmiany metaboliczne towarzyszące rozhartowaniu tych i innych gatunków zostały omówione w pracy przeglądowej **(Stachurska i Janeczko, 2024**).

1.2.1. Roślina modelowa *Arabidopsis thaliana*

Z danych literaturowych wynika, iż rozhartowanie najczęściej prowadzi do odwrócenia zmian metabolicznych, które zostały wcześniej wywołane poprzez hartowanie chłodem (Kalberer et al., 2006; Rapacz et al., 2017; Rys et al., 2020), jednak zjawisko rozhartowania jest słabiej poznane niż hartowanie (Vyse et al., 2019). Zmiany fizjologiczno-biochemiczne zachodzące w roślinach w czasie rozhartowania badano dotychczas m.in. na modelowej roślinie, pochodzącej z tej samej rodziny co rzepak − *Arabidopsis thaliana* L. Heynh. (m.in. Byun et al., 2014; Zuther et al., 2015; Pagter et al., 2017; Kutsuno et al., 2023). Rozhartowane rośliny *A. thaliana* po wykonanym teście mrozowym charakteryzował zwiększony w porównaniu do roślin hartowanych wypływ elektrolitów, a zatem zwiększona

przepuszczalność membran, która dowodzi większych uszkodzeń mrozowych (Miki et al., 2019).

W rozhartowanych roślinach *A. thaliana* zwiększona była ekspresja genów kodujących białka, które związane są ze ścianą komórkową, takie jak np. ksylozydaza i pektynesteraza (Oono et al., 2006). Po rozhartowaniu, zwiększyła się także ekspresja zahamowanych w czasie hartowania genów kodujących białka *AGP12* [ang. *Arabinogalactan Protein 12*] (Byun et al., 2014), a zmianom uległa akumulacja białek arabinogalaktanowych (Kutsuno et al., 2023). Opisane przez Kutsuno et al., (2023) zmiany w strukturach ścian komórkowych wskazują jednak, że rozhartowanie w przypadku ścian komórkowych nie odwraca całkowicie zmian indukowanych chłodem tj. nie przywraca ich do poziomu charakterystycznego dla roślin niehartowanych. Raczej powoduje powstanie nowej, specyficznej struktury, która zdaniem autorów może z jednej strony umożliwiać/sprzyjać wznowieniu wzrostu, ale z drugiej, nie wyklucza ponownego zahartowania (rehartowania).

Z kolei, jeżeli chodzi o białka błon komórkowych, analizy proteomiczne wykazały, że u *A. thaliana* wzrost lub spadek wybranych białek w czasie hartowania wykazuje najczęściej odwrotną tendencję w czasie rozhartowania, a większość zmian w akumulacji białek zaindukowanych chłodem wraca do poziomu obserwowanego w roślinach przed hartowaniem (Miki et al., 2019).

W rozhartowanych roślinach *A. thaliana*, zaobserwowany został spadek akumulacji transkryptów *COR*, kodujących białka COR (Zuther et al., 2015). COR to białka, których akumulacja zwiększa się w niskiej temperaturze przyczyniając się do poprawy mrozoodporności roślin (Artus et al., 1996). Mają one działanie stabilizacyjne poprzez fałdowanie i wiązanie się do m.in. membran chloroplastów (Thalhammer i Hincha, 2014).

Rozhartowanie wpływa również na akumulację aminokwasów mających role ochronne. Zawartość proliny (aminokwasu, który jest akumulowany przez rośliny w stresie i pełni antyoksydacyjne funkcje (Hayat et al., 2012)), akumulowanej w rozhartowanych roślinach *A. thaliana* obniżała się, w porównaniu do zawartości tego aminokwasu w roślinach hartowanych (Zuther et al., 2015).

Rozhartowanie wywołuje zmiany także w gospodarce węglowodanowej i wodnej roślin *A. thaliana*. Głównie dochodzi do odwrócenia zmian zaindukowanych hartowaniem, tj. np. obniżenia zawartości cukrów, które u hartowanych roślin zagęszczają sok komórkowy. W rozhartowanym *A. thaliana* zaobserwowano obniżoną zawartość cukrów takich jak glukoza,

fruktoza, sacharoza i rafinoza (Zuther et al., 2015) oraz skrobia (Kutsuno et al., 2023). Dodatkowo, zwiększyła się ekspresja genów związanych z gospodarką węglowodanową, takich jak β-galaktozydaza i syntaza sacharozy, które są zaangażowane w metabolizm m.in. sacharozy (Oono et al., 2006). Zmiana ekspresji tych genów również była odwróceniem zmian indukowanych hartowaniem w chłodzie (Oono et al., 2006).

Pod wpływem rozhartowania, zmiany zachodzą także w homeostazie hormonalnej. W rozhartowanych roślinach *A. thaliana* zaobserwowano zwiększoną ekspresję genów związanych z biosyntezą hormonów odpowiedzialnych za wzrost i rozwój, m.in. giberelin i auksyn (Pagter et al., 2017), co można skorelować z indukowanym rozhartowaniem zjawiskiem wznowienia wzrostu roślin, o którym była mowa wcześniej. Również ekspresja genów związanych z biosyntezą brasinosteroidów była wyższa w porównaniu do poziomu ekspresji obserwowanej w roślinach hartowanych (Pagter et al., 2017).

W rozhartowanych roślinach *A. thaliana* stwierdzono wzmożoną, w porównaniu do hartowanych roślin, ekspresję genów powiązanych z fotosyntezą, genów kodujących podjednostkę D2 kompleksu PSII oraz genów zaangażowanych w reakcje fazy jasnej fotosyntezy (Byun et al., 2014). Wykryto także zwiększoną, w porównaniu do roślin hartowanych, ekspresję genów kodujących małą podjednostkę RuBisCO oraz białka związane z PSI i PSII (Oono et al., 2006).

1.2.2. Rzepak (*Brassica napus* L.)

Rzepak jest rośliną, dla której przeprowadzono już wcześniej badania dotyczące zjawiska rozhartowania (Rapacz, 2002a; Rapacz, 2002b; Rapacz i Hura, 2002; Rapacz et al., 2003; Trischuk et al., 2014; Rys et al., 2020). Pozwoliło to na poznanie/opisanie u tego gatunku części zmian fizjologiczno-biochemicznych indukowanych rozhartowaniem, wciąż jednak wiele pozostało do wyjaśnienia.

Równowaga pomiędzy ilością hormonów wzrostowych (takich jak gibereliny, auksyny i cytokininy), a ilością hormonów stresowych (np. ABA, JA, SA) jest istotna dla utrzymania mrozoodporności hartowanych roślin. Szczególnie dotyczy to giberelin i ABA. Rapacz et al. (2003) badali zawartość tych hormonów w hartowanych i rozhartowanych roślinach rzepaku. Po rozhartowaniu (trwającym jeden lub dwa tygodnie) stwierdzono niewielki wzrost zawartości ABA oraz różnice pomiędzy odmianą ozimą, a jarą − odmiana jara charakteryzowała się wyższą zawartością ABA niż odmiana ozima. Poziom ABA obniżył się dopiero

po trzech−czterech tygodniach rozhartowania (Rapacz et al., 2003). Podczas rozhartowania generalnie zaobserwowano wzrost stężenia giberelin. W pierwszym tygodniu rozhartowania, zaobserwowano wzrost stężenia giberelin w odmianie ozimej i jarej, niezależnie od warunków rozhartowujących zastosowanych w eksperymencie. W odmianie ozimej, po tygodniu rozhartowania wzrost stężenia giberelin obserwowano w warunkach wyższej temperatury w ciągu dnia: 20/12°C i 20/20°C (d/n). W niższych temperaturach w ciągu dnia: 12/12°C i 12/20°C, zawartość giberelin była podobna do zawartości obserwowanej w roślinach przed hartowaniem. W przypadku odmiany jarej, zawartość giberelin wzrosła w pierwszym tygodniu rozhartowania o około 10 razy w porównaniu do odmiany ozimej, a najszybszy wzrost stężenia tych hormonów zaobserwowano w roślinach rozhartowanych w wyższej temperaturze za dnia.

W ramach niniejszej pracy doktorskiej znacznie poszerzono profil hormonalny poddany analizie u hartowanych i rozhartowanych roślin rzepaku, włączając w badania także m.in. auksyny, cytokininy, kwas jasmonowy, ich prekursory, pochodne i koniugaty, a także dość szczegółowo zanalizowano grupę hormonów steroidowych − brasinosteroidów (BR), wraz z ich receptorem BRI1 oraz genami, których ekspresja jest indukowana przez te związki (Stachurska et al., 2022; Stachurska et al., 2023; Stachurska et al., 2024).

Zmiany gospodarki hormonalnej pociągają za sobą pośrednio lub bezpośrednio inne zmiany metaboliczne. Na szczególną uwagę zasługuje fotosynteza, gospodarka węglowodanowa i wodna.

Rozhartowanie rzepaku prowadzi do zmian w obrębie aparatu fotosyntetycznego (Rapacz i Hura, 2002; Rys et al., 2020). Wzrost temperatury w czasie rozhartowania (zarówno w dzień, jak i w nocy) skutkował poprawą aktywności fotosyntetycznej ocenionej poprzez pomiary szybkiej kinetyki fluorescencji chlorofilu *a* (Rapacz i Hura, 2002). Pomiary te wykazały m.in., że po rozhartowaniu wartość parametru F_v/F_m, informującego o maksymalnej kwantowej wydajności PSII, była znacząco wyższa niż wartość obserwowana w roślinach hartowanych, a nawet wyższa od wartości charakterystycznej dla roślin niehartowanych (Rys et al., 2020). Generalnie, podczas rozhartowania dochodzi u rzepaku do intensyfikacji reakcji jasnej fazy fotosyntezy. Zmiany obserwowano również w zawartości chlorofilu – hartowanie rzepaku obniżało zawartość tego barwnika, a rozhartowanie zwiększało (Rys et al., 2020).

Pomiary opóźnionej fluorescencji chlorofilu *a* [ang. *delayed fluorescence, DF*] oraz odbiciowości modulowanej [ang. *modulated 820 nm reflection, MR820]* nie były do tej pory

badane w aspekcie rozhartowania roślin rzepaku i są przedmiotem analiz w ramach niniejszej pracy doktorskiej (Stachurska et al., 2022).

Jeżeli chodzi o fazę ciemną fotosyntezy i aktywność niektórych enzymów związanych z biosyntezą cukrów to, jak stwierdzają Rapacz i Hura (2002), podczas rozhartowania dochodzi do obniżenia aktywności enzymów RuBPCO i syntazy fosforanu sacharozy (SPS). W liściach rozhartowanego rzepaku dochodzi także do spadku koncentracji cukrów rozpuszczalnych, nawet do poziomu podobnego jak obserwowany w roślinach niehartowanych (Rys et al., 2020).

Liście rozhartowanego rzepaku charakteryzowało (częściowo zależne od odmiany) zwiększenie względnej zawartości wody [ang. *RWC, **R**elative **W**ater **C**ontent*] oraz podwyższenie potencjału osmotycznego (Rys et al., 2020). W hartowanych roślinach, potencjał osmotyczny wynosił średnio około −1.3MPa i obniżył się o około 20−25% w porównaniu do roślin niehartowanych. W roślinach rozhartowanych wzrósł on o 23−45% w porównaniu do roślin hartowanych (Rys et al., 2020). Dodatkowo, w liściach rzepaku poddanego rozhartowaniu stwierdzono obniżenie akumulacji akwaporyny BnPIP1 (Rys et al., 2020). Obniżenie akumulacji akwaporyn było odwróceniem zmian zaindukowanych hartowaniem, jednakże poziom transkryptu kodującego BnPIP1 nie uległ zmianie po rozhartowaniu i pozostał na niskim poziomie podobnym jak w roślinach hartowanych (Rys et al., 2020).

Zmiany wywołane rozhartowaniem wpływają także na akumulację innych ważnych białek, przykładem są tu dehydryny i białka COR. Dehydryny pełnią rolę ochronną względem innych białek i membran przed niekorzystnymi zmianami wywołanymi odwodnieniem tkanek (Kosová et al., 2007). W rozhartowanych roślinach rzepaku akumulacja dehydryny (47 kD) uległa znacznemu obniżeniu, do poziomu podobnego jak w roślinach niehartowanych (Trischuk et al., 2014). Po rozhartowaniu zaobserwowano niższą w porównaniu do roślin hartowanych akumulację białka COR78 (Trischuk et al., 2014).

Na tym tle, badania wykonane w ramach niniejszej pracy doktorskiej wzbogacają wiedzę o szczegółową analizę zmian akumulacji białek szoku cieplnego (HSP) u rozhartowanych roślin rzepaku (Stachurska et al., 2023).

Spośród innych zmian biochemicznych, warto dodać iż zawartość pektyn po rozhartowaniu roślin rzepaku obniżyła się do poziomu podobnego jak u roślin niehartowanych, w porównaniu do zwiększonej ich akumulacji w czasie hartowania (Solecka et al., 2008). Pektyny to polisacharydy występujące w ścianach komórkowych roślin, które pełnią funkcje m.in. kontrolując porowatość ścian komórkowych i reakcje na stres. Spadek akumulacji pektyn po rozhartowaniu mógł być powiązany z rozrastaniem się liści (Solecka et al., 2008).

Z danych literaturowych wiadomo, że hartowanie do niskich temperatur powoduje zmiany w strukturze membran i kompozycji lipidów. Zmiany te zwykle oznaczają zwiększenie płynności membran, co ma na celu poprawę ich funkcjonowania w chłodzie (Uemura et al., 1995; Ogweno et al., 2009; Filek et al., 2017). Większa płynność membran związana jest ze zwiększeniem zawartości nienasyconych kwasów tłuszczowych włączonych w strukturę membrany. Płynność membran może być także modyfikowana poprzez wbudowanie do membran innych komponentów, takich jak sterole, tokoferole czy karotenoidy, co ma miejsce szczególnie w membranach chloroplastów (Ford i Barber, 1983; Munné-Bosch, 2005). **Wpływ rozhartowania na strukturę oraz własności fizykochemiczne membran komórkowych roślin rzepaku po rozhartowaniu nie został dotąd poznany/opisany, dlatego też badania takie stały się przedmiotem niniejszej pracy doktorskiej (Rys et al., 2024).**

1.3. Metody detekcji rozhartowania roślin i możliwości przeciwdziałania skutkom rozhartowania

W związku z nasilaniem się zmian klimatu i występowaniem anomalii pogodowych takich jak okresy podwyższonej temperatury zimą, warto poszukiwać prostych i szybkich sposobów oceny stopnia rozhartowania roślin. Miałoby to na celu umożliwienie zastosowania odpowiednich środków prewencyjnych np. preparatów ochronnych zapobiegających uszkodzeniom mrozowym roślin w razie gwałtownego spadku temperatury poniżej zera po rozhartowaniu. Podjęcie takich działań może być istotne zwłaszcza w przypadku ważnych gatunków uprawnych, które stanowią źródło pożywienia dla wciąż rosnącej liczby ludności na świecie. Monitorowanie zmian zachodzących podczas rozhartowania upraw jest skomplikowane, ponieważ najczęściej zmiany te (zwłaszcza na początku) nie są widoczne gołym okiem, zaś analizy laboratoryjne wymagają zebrania prób liści oraz są czasochłonne. Z pomocą przychodzą nieinwazyjne metody pomiarowe, takie jak np. pomiar bezpośredniej fluorescencji chlorofilu *a* [ang. ***p**rompt **f**luorescence,* ***PF***], której skuteczność została udowodniona u zbóż i rzepaku (Rapacz et al., 2017; Rys et al., 2020). Co istotne, metody tej można używać na większą skalę (np. całych pól uprawnych), przy użyciu dronów, bezzałogowych statków powietrznych lub satelitów specjalnie dostosowanych do monitorowania zmian fluorescencji (Lednev et al., 2022). **Pomiary szybkiej kinetyki fluorescencji chlorofilu *a* są popularnym narzędziem do oceny stresu roślin, jednak pomiary opóźnionej fluorescencji (DF) i odbiciowości modulowanej (MR820) nie były do tej pory stosowane do stwierdzenia rozhartowania roślin rzepaku. Przetestowania**

wymagają również inne, nieinwazyjne metody pomiarowe, np. pomiar własności spektralnych liści dający możliwość uzyskania licznych parametrów obliczanych na podstawie m.in. refleksji liści. Z tego względu jednym z celów niniejszej pracy było przetestowanie tych metod pod kątem przydatności do oceny rozhartowania roślin rzepaku (Stachurska et al., 2022, Stachurska et al. 2024).

Jak wspomniano wyżej, wczesna detekcja stanu rozhartowania uprawy stwarza możliwość podniesienia jej mrozoodporności przy użyciu preparatów o działaniu ochronnym. W niniejszej pracy doktorskiej po raz pierwszy badano aktywność w tym kierunku kilku regulatorów steroidowych (hormonów steroidowych i ich analogów) oraz dostępnego komercyjnie preparatu Asahi SL, starając się także wyjaśnić niektóre/wybrane mechanizmy ich działania (Stachurska et al., 2024).

Mając na uwadze iż wykonywanie dodatkowych zabiegów (oprysków) zawsze jest kosztowne ekonomicznie, w przypadku pogłębiania się zmian klimatycznych innym podejściem mogło by być **uprawianie odmian, które będą charakteryzować się zdolnością utrzymania wysokiej mrozoodporności mimo występowania warunków rozhartowujących,** tj. będą charakteryzować się lepszą tolerancją warunków rozhartowujących **(Stachurska et al., 2022; Stachurska i Janeczko, 2024)** lub wolniejszym tempem rozhartowywania (Rowland et al., 2005). **Przekonanie to przyświecało badaniom w tej części pracy doktorskiej, której celem była charakterystyka mrozoodporności wybranych odmian rzepaku ozimego (a także jarego) po rozhartowaniu i wskazanie odmian słabiej/lepiej tolerujących rozhartowanie (Stachurska et al., 2022).**

2. Cele pracy

Celem doświadczeń przeprowadzonych w ramach rozprawy doktorskiej było:

- scharakteryzowanie mrozoodporności wybranych odmian rzepaku po rozhartowaniu, w tym znalezienie odpowiedzi na pytanie, czy odmiany różnią się tolerancją na rozhartowanie
- określenie fizjologiczno-biochemicznego podłoża procesu rozhartowania oraz powiązanie ich ze zmianami mrozoodporności
- przetestowanie możliwości zastosowania nieinwazyjnych metod pomiarowych do monitorowania rozhartowania
- zweryfikowanie potencjalnej, ochronnej roli regulatorów zwiększających mrozoodporność rozhartowanych roślin

Szczegółowe cele zostały przedstawione w publikacjach wchodzących w skład rozprawy doktorskiej.

3. Materiał roślinny oraz schematy eksperymentów

3.1. Materiał roślinny

Materiał roślinny wykorzystany w eksperymentach stanowiły rośliny rzepaku. Wybrano dziesięć odmian, w tym dziewięć odmian ozimych (Birdy, Bojan, Darcy, Finley, Graf, Monolit, Pantheon, President i Rokas) oraz jedną odmianę jarą (Feliks).

Odmiany Birdy, Bojan, Darcy, Feliks, Finley, Monolit i Rokas są odmianami populacyjnymi, a Graf, Pantheon i President są odmianami hybrydowymi (F1). W momencie rozpoczęcia badań, w krajowym rejestrze COBORU znajdowało się pięć odmian, charakteryzujących się następującą wysokością roślin: Birdy −139 cm, Bojan − 160 cm, Feliks − 121 cm, Graf − 140 cm, Monolit − 138 cm, Rokas − 131 cm. Inne odmiany, nieujęte w rejestrze, zostały scharakteryzowane przez producentów: Pantheon i President charakteryzują wysokie rośliny, a Darcy i Finley to odmiany półkarłowe.

Według producentów, odmiany Birdy, Bojan, Finley, Graf, Monolit, Pantheon, President i Rokas charakteryzują się wysoką i bardzo wysoką zimotrwałością, odmiana Darcy – dobrą zimotrwałością.

Według producentów, odmiany Bojan, Finley, Monolit, Pantheon, President, Rokas charakteryzują się wysoką mrozoodpornością, a odmiany Darcy i Birdy charakteryzują się dobrą mrozoodpornością. Mrozoodporność odmiany Graf nie została podana przez producenta.

Nasiona odmian Bojan, Monolit i Feliks otrzymano z Hodowli Roślin Strzelce Sp. z o.o. Grupa IHAR (Strzelce, Polska). Nasiona odmian Darcy, Finley, Pantheon i President uzyskano z firmy Saatbau (Środa Śląska, Polska). Nasiona odmiany Rokas uzyskano z firmy Syngenta (Warszawa, Polska). Nasiona odmiany Birdy uzyskano z firmy KWS (Poznań, Polska), a nasiona odmiany Graf uzyskano z firmy Obrol (Kruszewnia, Polska).

Obecnie, w krajowym rejestrze COBORU (dostęp: 25.04.2024 r.) znajdują się następujące odmiany: Birdy, Graf, Monolit, Rokas.

W eksperymencie opisanym w publikacji **Stachurska et al., 2022** badaniom poddano 10 następujących odmian: Birdy, Bojan, Darcy, Feliks, Finley, Graf, Monolit, Pantheon, President, Rokas. Wykonano badania porównawcze mrozoodporności roślin po wzroście w kontrolowanych warunkach komór wegetacyjnych. Do eksperymentów opisanych w publikacjach **Stachurska et al., 2023** i **Rys et al., 2024** wybrano odmiany ozime Bojan, President i Rokas oraz jarą Feliks na podstawie oceny mrozoodporności (dane RT50). Rokas

wykazywał wysoką mrozoodporność, zaś Feliks niską. Mrozoodporność odmian Pantheon i President zależna była od warunków wzrostu − rośliny tych odmian niehartowane, hartowne chłodem i rozhartowane reagowały nieco inaczej [tabela w rozdziale 5]. W publikacji **Stachurska et al., 2024** użyto do eksperymentów już tylko odmiany ozime Pantheon i President.

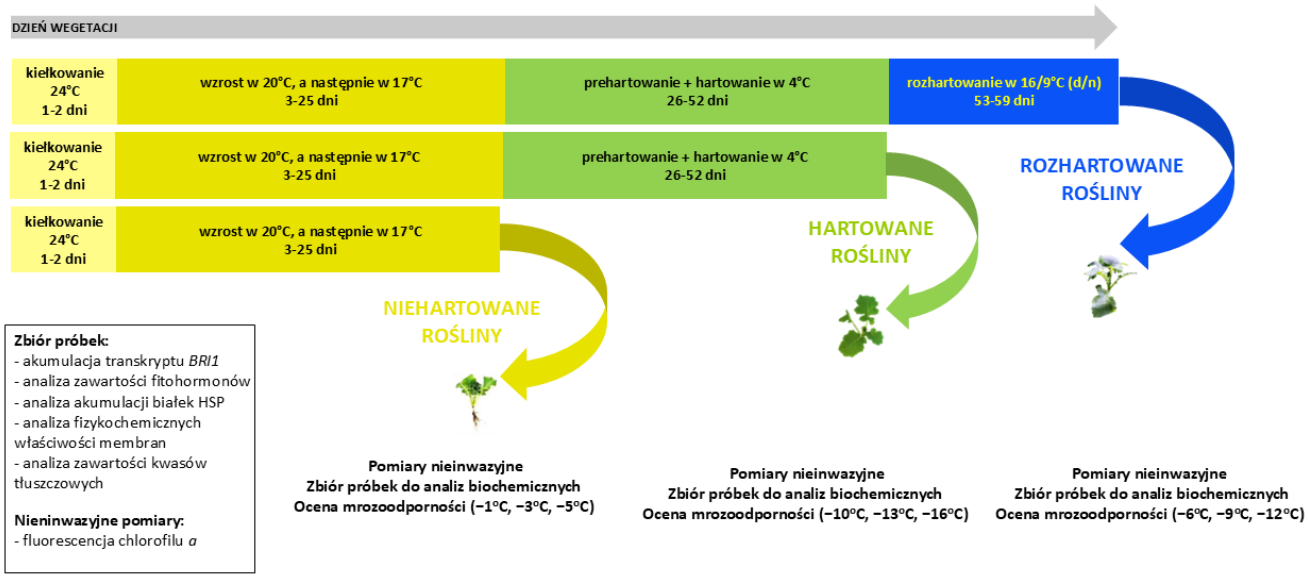
3.2. Przebieg eksperymentów

3.2.1. Schemat eksperymentów opisanych w publikacjach Stachurska et al., 2022; Stachurska et al., 2023; Rys et al., 2024

Uproszczony schemat eksperymentu przedstawia rycina 4. Nasiona rzepaku kiełkowano w temperaturze 24°C przez dwa dni, a następnie siewki zostały wysadzone do doniczek z mieszanką ziemi i piasku i rosły w temperaturze 20°C przez 4 dni, po czym temperaturę obniżono do 17°C (trzy tygodnie). Po trzech tygodniach wykonano pomiary i pobrano próbki do analiz biochemicznych, a tak pozyskane rośliny określono jako niehartowane (kontrolne). Następnie, stopniowo obniżano temperaturę stosując prehartowanie (jeden tydzień). Po prehartowaniu temperaturę obniżono do 4°C (trzy tygodnie). Po upływie trzech tygodni, wykonano pomiary oraz pobrano próby do analiz biochemicznych, a rośliny określono jako hartowane chłodem. Następnie, poddano je rozhartowaniu w temperaturze 16/9°C (dzień/noc) przez jeden tydzień, po czym wykonano pomiary oraz pobrano próby do analiz. Rośliny tej trzeciej grupy określono jako rozhartowane.

Ze względu na stosowanie języka angielskiego w cyklu publikacji, na potrzeby niniejszego opracowania przyjęto następujące skróty do oznaczania w/w trzech grup roślin, które były podawane pomiarom nieinwazyjnym i z których pobierano próbki materiału roślinnego:

- **NA** – rośliny niehartowane (kontrolne) [ang. *non-acclimated*]
- **CA** – rośliny hartowane [ang. *cold-acclimated*]
- **DA** – rośliny rozhartowane (dehartowane, deaklimowane) [ang. *deacclimated*]



Rycina 4. Schemat eksperymentu opisanego w publikacjach **Stachurska et al., 2022; Stachurska et al., 2023; Rys et al., 2024,** przedstawiający grupy roślin niehartowanych, hartowanych i rozhartowanych, ze wskazaniem momentu wykonania pomiarów nieinwazyjnych (**Stachurska et al., 2022**).

3.2.2. Schemat eksperymentu opisanego w publikacji Stachurska et al., 2024

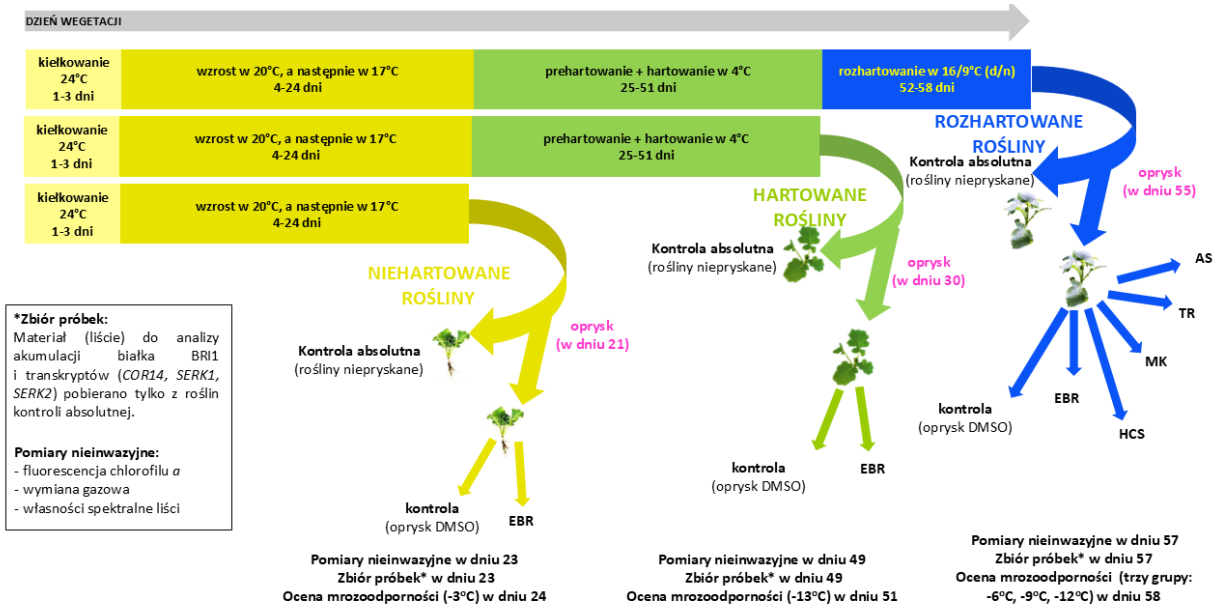
Uproszczony schemat eksperymentu przedstawia rycina 5. Generalnie, schemat eksperymentu był podobny jak opisany w publikacjach **Stachurska et al., 2022; Stachurska et al., 2023; Rys et al., 2024**, z tą różnicą, iż stosowano opryski regulatorami, co miało na celu poprawę mrozoodporności. Nasiona kiełkowano w 24°C w ciemności przez trzy dni. Następnie, siewki przeniesiono do doniczek z mieszanką ziemi i piasku i pozostawiono je w 20°C przez cztery dni, a następnie w 17°C przez 17 dni. W 21 dniu wegetacji rośliny podzielono na trzy grupy.

Pierwszą grupę, 21-dniowych roślin, którą stanowiły rośliny niehartowane (NA), podzielono na kolejne trzy podgrupy: rośliny, które zostały opryskane wodnym roztworem zawierającym hormon steroidowy z grupy brasinosteroidów (24-epibrasinolid; stosowany skrót EBR), rośliny opryskane roztworem wodnym zawierającym rozpuszczalnik steroidów – DMSO (kontrola) i rośliny niepryskane (kontrola absolutna).

Drugą grupę roślin stanowiły rośliny, które po 21 dniach wegetacji pozostawiono by kontynuowały wzrost przez kolejne trzy dni w 17°C, a następnie zostały poddane prehartowaniu przez sześć dni. Po tym okresie rośliny były hartowane w 4°C przez trzy tygodnie. Tę grupę roślin określono mianem hartowanych (CA). Jeden dzień przed

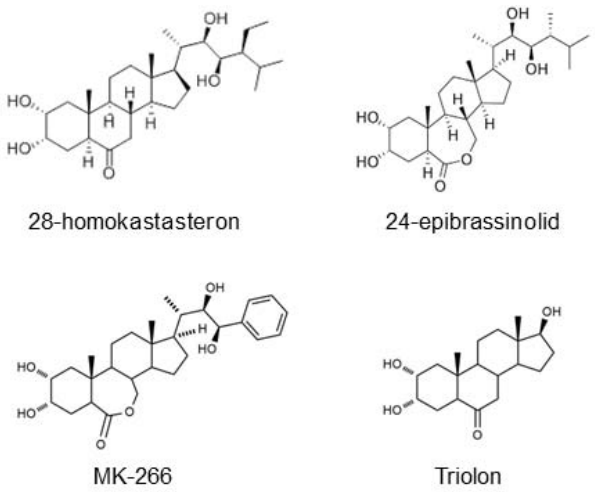
rozpoczęciem procesu hartowania, rośliny zostały podzielone na trzy podgrupy i podobnie j.w. opryskane roztworami zawierającymi EBR, rozpuszczalnik DMSO (kontrola), a jedna podgrupa roślin pozostała nie opryskana (kontrola absolutna).

Trzecią grupę stanowiły rośliny, które po 21 dniach wegetacji zostały poddane prehartowaniu, i hartowaniu (jak opisano powyżej), a następnie zostały rozhartowane w 16/9°C (dzień/noc) przez sześć dni. Jest to grupa roślin określana mianem rozhartowanych (DA). W 55 dniu wegetacji, rośliny podzielono na sześć grup i opryskano roztworami wodnymi zawierającymi brasinosteroidy – 24-epibrasinolid (EBR) i 28-homokastasteron (HCS), roztworami z syntetycznymi analogami brasinosteroidów – MK-266 (MK) i triolonem (TR), roztworami z DMSO (kontrola) oraz (dodatkowo) roztworem komercyjnego preparatu Asahi SL (AS) (Agrecol, Wieruszów, Polska). Grupę roślin niepryskanych pozostawiono jako kontrolę absolutną.



Rycina 5. Uproszczony schemat eksperymentu opisany w publikacji **Stachurska et al., 2024**, przedstawiający grupy roślin niehartowanych, hartowanych i rozhartowanych wraz z podziałem na poszczególne traktowania regulatorami; EBR − 24-epibrasinolid; HCS − 28-homokastasteron; analogi brasinosteroidów (MK − MK-266; TR − triolon); AS − regulator Asahi SL; DMSO − rozpuszczalnik steroidów; kontrola absolutna (rośliny niepryskane). Pomiary nieinwazyjne i ocena mrozoodporności zostały wykonane na wszystkich grupach/podgrupach roślinach. Zbiór próbek do analiz akumulacji białka BRI1 i transkryptów (*COR14, SERK1, SERK2*) wykonano tylko z roślin niepryskanych (kontrola absolutna).

Struktury chemiczne brasinosteroidów stosowanych w publikacji **Stachurska et al., 2024** oraz ich analogów przedstawiono na rycinie 6. Brasinosteroidy i ich analogi stosowano w stężeniu 0,5 mg/L. Stężenie preparatu Asahi SL dobrano wg wskazań producenta. Szczegóły techniczne przygotowania wszystkich roztworów podano w publikacji **Stachurska et al., 2024**.



Rycina 6. Struktura brasinosteroidów: 28-homokastasteronu i 24-epibrasinolidu oraz struktura syntetycznych analogów brasinosteroidów: MK-266 i Triolon. MK-266 i Triolon zsyntetyzowane wg Kohout et al., 1987; Kvasnica et al., 2016.

W publikacji **Stachurska et al., 2024**, niewielka część badań (wypływ elektrolitów zmierzony metodą konduktometryczną) została wykonana na roślinach, które uzyskano wg nieco zmodyfikowanego schematu przedstawionego na rycinie 5. Modyfikacja ta dotyczy przede wszystkim terminów stosowania oprysków, co szczegółowo opisano w publikacji **Stachurska et al., 2024.**

4. Metody badawcze

Szczegółowy opis wszystkich przedstawionych w tabeli 1 metod analitycznych, pomiarów i obserwacji znajduje się w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej.

Tabela 1. Wykaz metod analitycznych wykorzystanych w rozprawie doktorskiej.

	Metoda	Analizy	Publikacja
1.	Pomiar fluorescencji chlorofilu *a*	Analiza wydajności reakcji fazy jasnej fotosyntezy	Stachurska et al., 2022
			Stachurska et al., 2024
2.	Wysokosprawna chromatografia cieczowa sprzężona ze spektrometrią masową	Analiza zawartości hormonów steroidowych − brasinosteroidów [BR] (tyfasterol, kastasteron, brasinolid, dolichosteron, dolicholid, homokastasteron) w materiale roślinnym	Stachurska et al., 2022
		Analiza zawartości pozostałych hormonów i ich metabolitów, prekursorów i koniugatów: auksyn (indolilo-3-acetamid [IAM]; idolilo-3-acetonitryl [IAN]; kwas indolilooctowy [IAA], kwas indolilo-3-acetyloasparaginowy [IAAsp], kwas 2-oksindolo-3-octowy [OxIAA], kwas indolilo-3-karboksylowy [I3CA]), giberelin (GA_{15}, GA_9, GA_4, GA_7, GA_{53}, GA_{19}, GA_{20}, GA_3, GA_6), cytokinin (*cis*-zeatyna [*cis*-zea], rybozyd *cis*-zeatyny [*cis*-zea-rib]), hormonów stresu (kwas abscysynowy [ABA],	Stachurska et al., 2023

		kwas 12-oksofitodienowy [12-oxo-PDA], kwas jasmonowy [JA], kwas benzoesowy [BA], kwas salicylowy [SA]) w materiale roślinnym	
		Analiza zawartości tokoferoli (α-, β-, γ-, δ-tokoferolu) i karotenoidów (β-karotenu i zeaksantyny) w materiale roślinnym (liście i izolowane chloroplasty)	Rys et al., 2024
3.	Quantitative Real-Time PCR	Analiza akumulacji transkryptu *BRI1* w materiale roślinnym	Stachurska et al., 2022
		Analiza akumulacji transkryptów *COR14, SERK1, SERK2* w materiale roślinnym	Stachurska et al., 2024
4.	Western Blotting, Immunoblotting	Analiza akumulacji białek HSP70 cytoplazmatycznego, HSP70 chloroplastowego i HSP90 w materiale roślinnym	Stachurska et al., 2023
		Analiza akumulacji białka receptorowego BRI1 w materiale roślinnym	Stachurska et al., 2024
5.	Chromatografia gazowa	Analiza zawartości kwasów tłuszczowych w materiale roślinnym	Rys et al., 2024
6.	Waga Langmuira	Analiza właściwości fizykochemicznych membran chloroplastów (monowarstw lipidowych)	Rys et al., 2024
		Analiza odziaływań analogów brasinosteroidów (Triolon i MK-266) z modelowymi membranami (zmiany własności fizykochemicznych monowarstw lipidowych)	Stachurska et al., 2024

7.	Pomiar właściwości spektralnych (refleksji) liści	Analiza własności spektralnych liści	Stachurska et al., 2024
8.	Pomiar wymiany gazowej liści	Analiza wymiany gazowej liści (faza ciemna fotosyntezy)	Stachurska et al., 2024
9.	Konduktometria	Ocena uszkodzeń membran komórkowych na podstawie wypływu elektrolitów	Stachurska et al., 2024

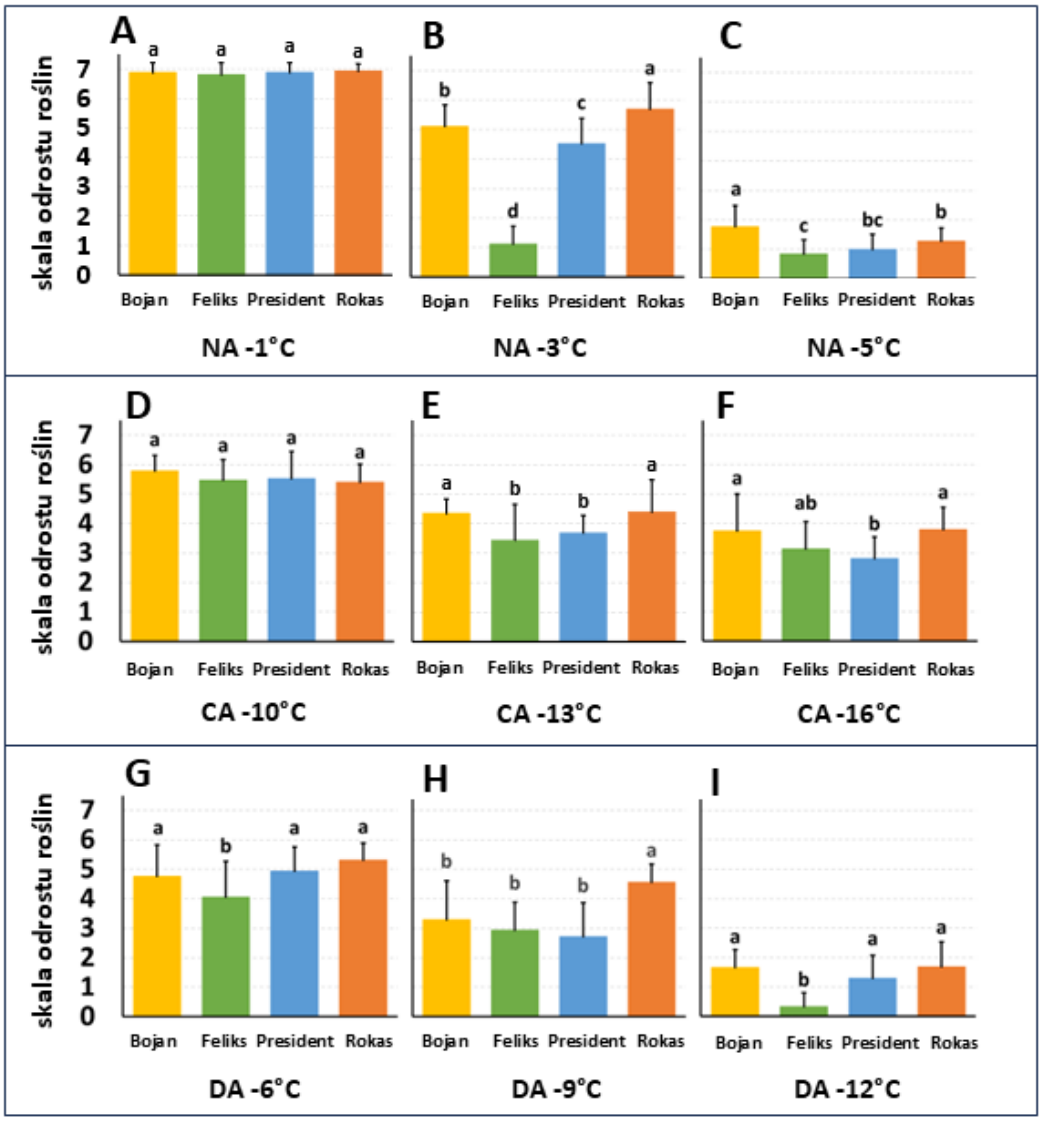
5. Podsumowanie najważniejszych wyników

5.1. Zjawisko rozhartowania a mrozoodporność wybranych odmian rzepaku

1. Dziesięć badanych odmian rzepaku, charakteryzujących się m.in. różnymi cechami morfologicznymi (wysokością), wykazywało także różnice w tzw. bazowej mrozoodporności (rycina 7A−C). Zgodnie z przewidywaniami najniższa bazowa mrozoodporność charakteryzowała odmianę jarą (Feliks). Różnice występowały także między odmianami ozimymi i uwidaczniały się w temperaturach −3°C i −5°C. Najwyższą bazową mrozoodpornością charakteryzowały się odmiany Rokas i Bojan (rycina 7B−C).

2. Po hartowaniu mrozoodporność wszystkich odmian ozimych, zgodnie z oczekiwaniami, znacznie wzrosła (rycina 7D−F). Co ciekawe, mrozoodporność wzrosła również u odmiany jarej (Feliks), tak iż nie odbiegała ona od mrozoodporności niektórych odmian ozimych (np. President). Stosunkowo najwyższą mrozoodpornością wykazywały się odmiany Bojan i Rokas, co uwidoczniło się szczególnie po testach w temperaturze −13°C (rycina 7E).

3. Rozhartowanie (siedem dni w temperaturze 16/9°C d/n) obniżało mrozoodporność wszystkich badanych odmian (rycina 7G−I). Po rozhartowaniu odmiana jara charakteryzowała się najniższą mrozoodpornością, co szczególnie uwidoczniło się po testach w temperaturze −6° i −12°C (porównanie z tabelą 2). Na podstawie wartości RT50 można przyjąć iż odmiana Rokas, wykazywała się najwyższą mrozoodpornością po rozhartowaniu.



Rycina 7. Mrozoodporność czterech przykładowych odmian rzepaku ozimego: Bojan, President i Rokas oraz odmiany jarej Feliks, charakteryzująca rośliny niehartowane (NA), hartowane (CA) i rozhartowane (DA). Ocenę mrozoodporności przeprowadzono na podstawie obserwacji odrostu roślin po ich ekspozycji na mróz od −1°C do −5°C (rośliny NA); od −10°C do −16°C (rośliny CA); od −6°C do −12°C (rośliny DA). Odrost roślin po mrozie oceniano wg skali wizualnej (0−7 punktów) po dwóch tygodniach wzrostu w warunkach szklarniowych w temperaturze około 12°C, gdzie 7 − rośliny bez uszkodzeń, a 0 − rośliny martwe. *Średnie wartości ± SD oznaczone tymi samymi literami nie różnią się istotnie na poziomie p<0.05.*

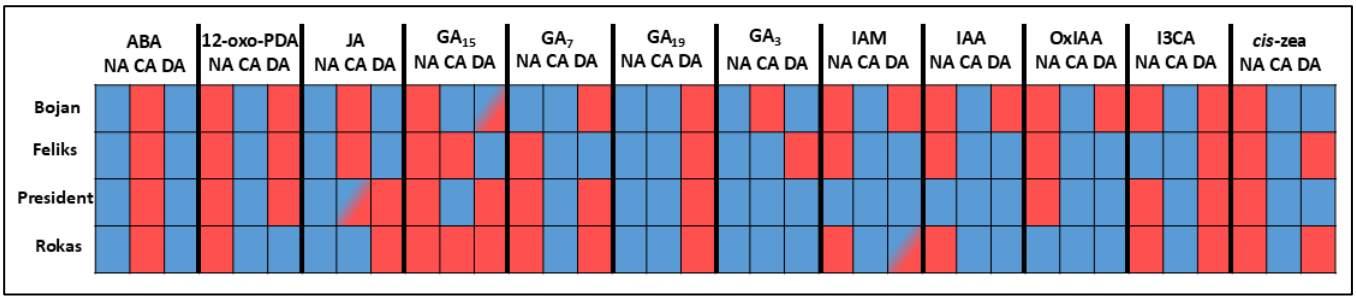
Tabela 2. Przewidywana/estymowana temperatura [°C], która zredukuje potencjalny odrost po wykonaniu testów mrozowych o 50% (RT50). Wartości RT50 obliczono na podstawie trzech temperatur stosowanych do oceny mrozoodporności.

Rośliny niehartowane		**Rośliny hartowane**		**Rośliny rozhartowane**	
odmiana	**RT50**	**odmiana**	**RT50**	**odmiana**	**RT50**
Bojan	−3.68	Bojan	−13.54	Rokas	−8.91
Rokas	−3.34	Rokas	−13.50	Pantheon	−8.84
Monolit	−3.23	Darcy	−13.26	Graf	−8.41
President	−3.21	Graf	−13.24	Bojan	−8.40
Darcy	−3.12	Pantheon	−13.09	Monolit	−8.35
Birdy	−3.07	Monolit	−12.98	Finley	−8.31
Finley	−2.94	Feliks	−12.92	Darcy	−8.29
Graf	−2.69	President	−12.80	Birdy	−8.14
Feliks	−2.54	Finley	−12.76	President	−7.96
Pantheon	−2.35	Birdy	−12.44	Feliks	−7.63

5.2. Fizjologiczno-biochemiczne podłoże obniżonej mrozoodporności rzepaku po rozhartowaniu

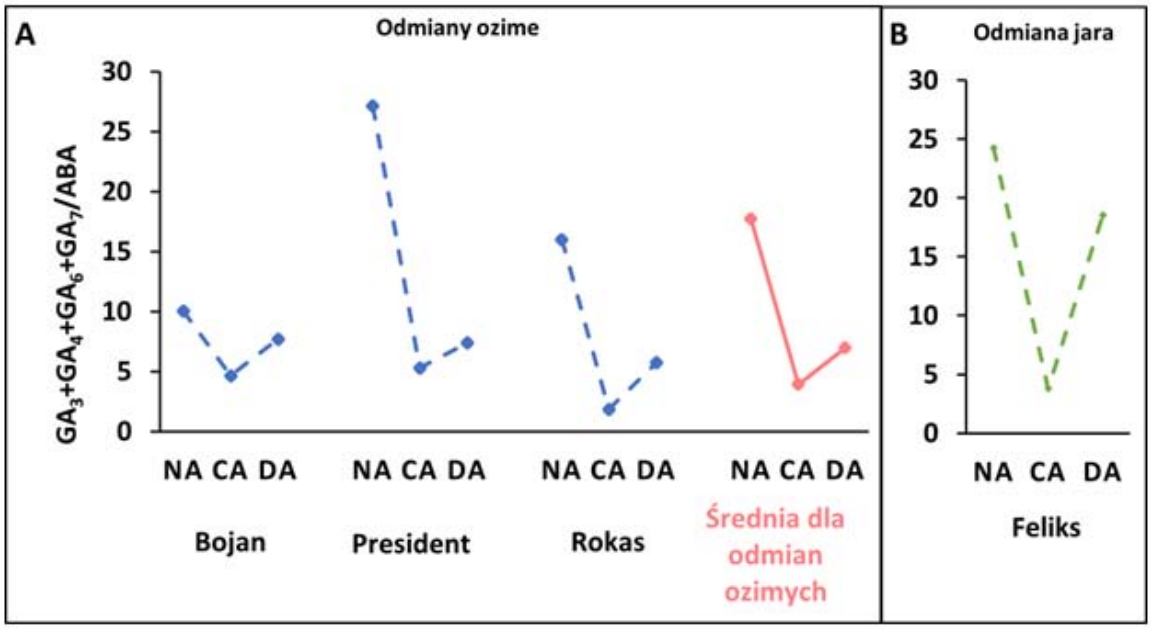
5.2.1. Gospodarka hormonalna

1. W pracy badano wpływ rozhartowania na zmiany koncentracji ponad 20 fitohormonów, ich metabolitów, prekursorów i koniugatów (ABA, 12-oxo-PDA, JA, BA, SA, GA_{15}, GA_9, GA_4, GA_7, GA_{53}, GA_{19}, GA_{20}, GA_3, GA_6, IAM, IAN, IAA, IAAsp, OxIAA, IAGlu, I3CA, *cis*-zea, *cis*-zea-rib) (**Stachurska et al., 2023**). W większości przypadków analizy tych związków w aspekcie rozhartowania przeprowadzono po raz pierwszy. Na rycinie 8 przedstawiono uproszczoną wizualizację zmian stężeń wybranych hormonów w liściach roślin rzepaku niehartowanych, hartowanych i rozhartowanych. Spośród zbadanych związków szczególną uwagę warto zwrócić na wyniki dotyczące ABA, giberelin oraz auksyn i I3CA.



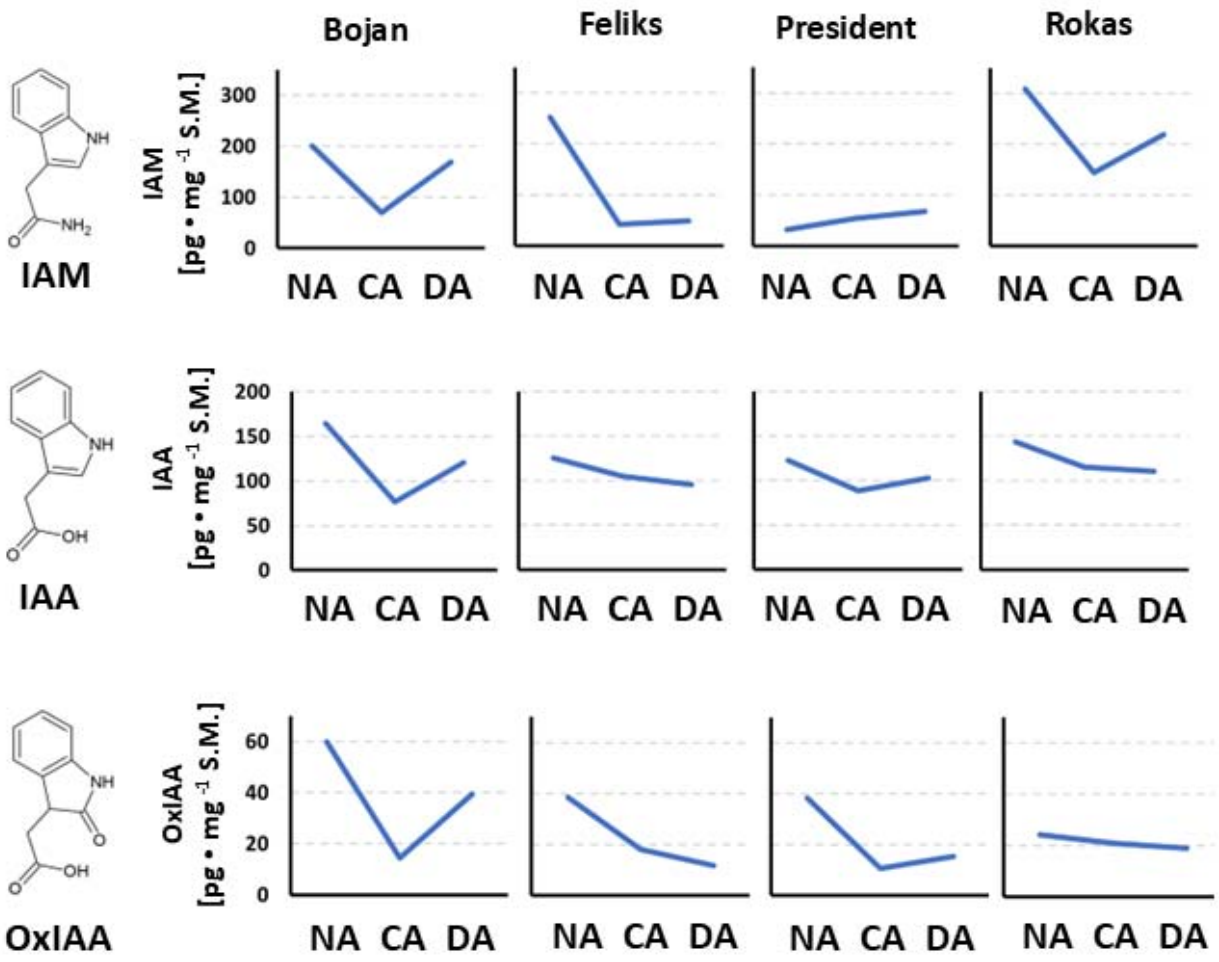
Rycina 8. Uproszczona wizualizacja zmian stężeń wybranych hormonów w liściach roślin rzepaku niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) odmian Bojan, Feliks, President i Rokas. ABA – kwas abscysynowy; 12-oxo-PDA – kwas 12-oksofitodienowy; JA – kwas jasmonowy; GA_{15} – giberelina A15; GA_7 – giberelina A7; GA_{19} – giberelina A19; GA_3 – kwas giberelinowy; IAM – indolilo-3-acetamid; IAA – kwas indolilo-3-octowy; OxIAA − kwas 2-oksindolo-3-octowy; I3CA – kwas indolilo-3-karboksylowy; *cis*-zea – *cis*-zeatyna. *Kolorem niebieskim symbolicznie oznaczono statystycznie istotnie niskie stężenia hormonów, a kolorem czerwonym oznaczono wysokie stężenia. Gradientem oznaczono nieistotne statystycznie tendencje (wzrost stężenia).* Dane na podstawie publikacji **Stachurska et al., 2023.**

2. Hartowanie chłodem powodowało zmiany równowagi hormonalnej rzepaku. W porównaniu do roślin niehartowanych zwiększeniu uległa akumulacja tzw. hormonów stresu (głównie ABA, a także JA i jego prekursora, kwasu 12-oksofitodienowego), natomiast u większości badanych odmian obniżała się zawartość hormonów związanych ze wzrostem i rozwojem − niektórych aktywnych giberelin, GA_7 oraz GA_4, a także ich prekursora, GA_{15}, czy cytokininy − *cis*-zeatyny (**Stachurska et al., 2023**). Na tym tle wykazano, iż rozhartowanie odwracało indukowany hartowaniem kierunek zmian hormonalnych (**Stachurska et al., 2023**). U większości badanych odmian obserwowano zwiększenie koncentracji wymienionych wyżej hormonów związanych ze wzrostem i rozwojem oraz obniżenie koncentracji hormonu stresu (ABA). Model zmian równowagi hormonów z grupy giberelin oraz ABA u ozimych odmian rzepaku oraz odmiany jarej ilustruje rycina 9A−B.



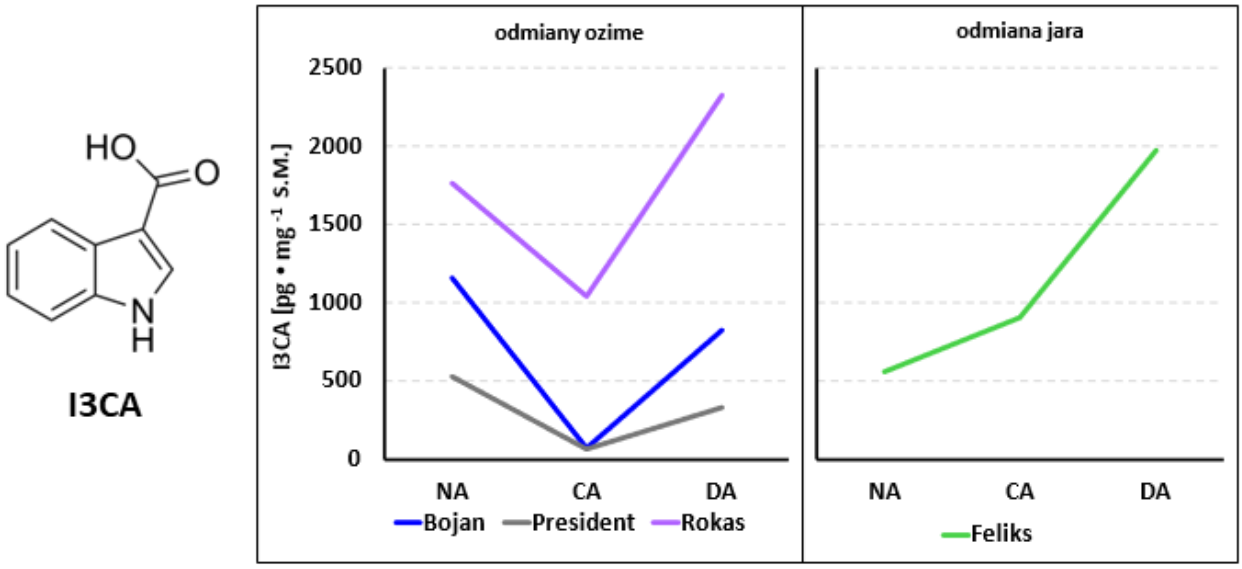
Rycina 9. Stosunek stężenia hormonów odpowiedzialnych za wzrost i rozwój − aktywnych giberelin (GA_3, GA_4, GA_6, GA_7) i hormonu stresu − kwasu abscysynowego (ABA) w niehartowanych (NA), hartowanych chłodem (CA) i rozhartowanych (DA) liściach roślin rzepaku. Zmiany równowagi hormonalnej zilustrowano u trzech odmian ozimych Bojan, President i Rokas [A] oraz u odmiany jarej Feliks [B] (**Stachurska et al., 2023)**.

3. W odróżnieniu od wspomnianych cytokinin oraz części giberelin, w przypadku auksyn, u trzech z czterech badanych odmian rzepaku, nie stwierdzono istotnego wpływu rozhartowania na zawartość aktywnej auksyny IAA (**Stachurska et al., 2023**). Jej poziom był zbliżony u roślin hartowanych i rozhartowanych. Wyjątek stanowiła jedynie odmiana Bojan, u której rozhartowanie zwiększało poziom IAA. Na rycinie 10 przedstawiono wpływ hartowania i rozhartowania na (zależne od odmiany) zmiany równowagi pomiędzy jednym z prekursorów biosyntezy auksyn (IAM), aktywną auksyną IAA oraz oksydowaną formą auksyny (OxIAA) o obniżonej aktywności biologicznej, powstającą zwykle w odpowiedzi na wysokie stężenia IAA jako element regulacyjny poziomu auksyn w tkankach.



Rycina 10. Zależne od odmiany modele zmian stężeń prekursora auksyn (IAM), formy aktywnej (IAA) i pochodnej (OxIAA) w niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) liściach roślin rzepaku odmiany Bojan, Feliks, President i Rokas. Dane na podstawie publikacji **Stachurska et al., 2023**.

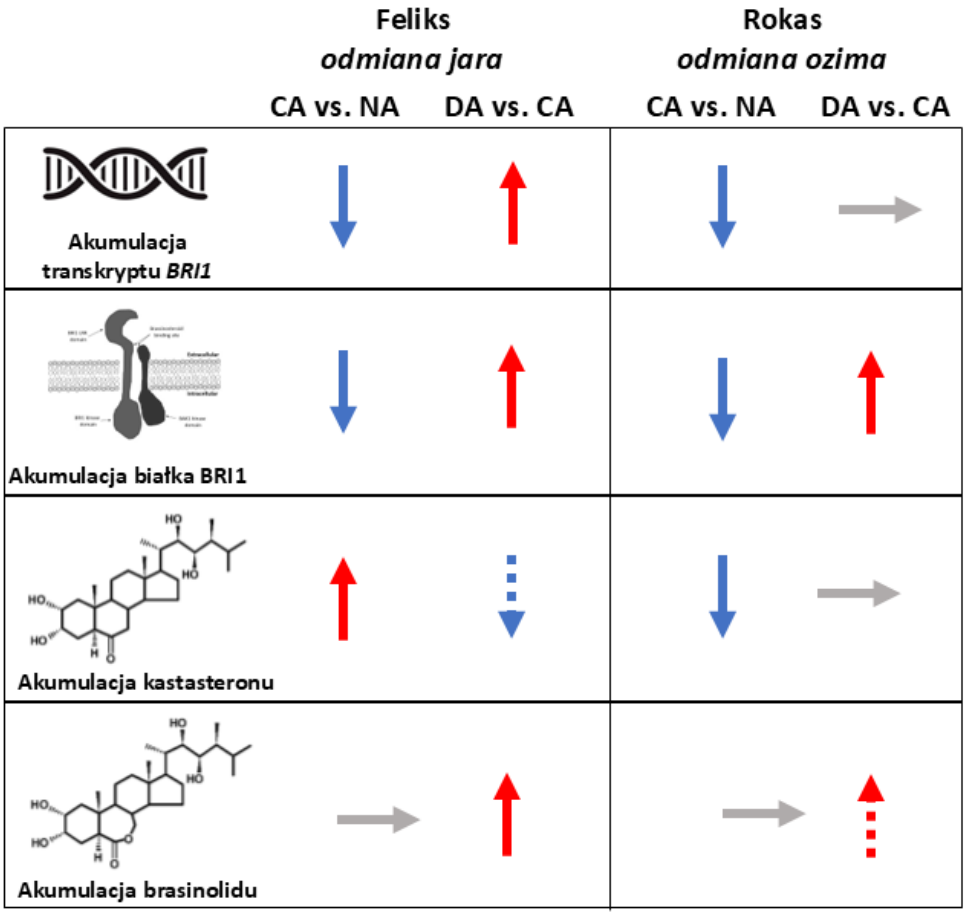
4. W niniejszej pracy po raz pierwszy zidentyfikowano w rzepaku kwas indolilo-3-karboksylowy (I3CA, ang. *indole-3-carboxylic acid*), który jest pochodną indolową uznawaną za regulator auksyno-podobny. I3CA jest zaangażowany w procesy kształtowania się odporności na patogeny oraz udział w regulacji podziałów komórkowych (szerzej na ten temat w publikacji **Stachurska et al., 2023).** Obserwowany silny wzrost poziomu I3CA w wyniku rozhartowania rzepaku (zarówno ozimego jak i jarego; rycina 11) można zatem próbować powiązać z intensyfikacją procesów wzrostowych a zarazem z koniecznością lepszej ochrony rośliny przed patogenami łatwiej atakującymi w warunkach podwyższonej temperatury.



Rycina 11. Dynamika zmian stężenia kwasu indolilo-3-karboksylowego (I3CA) w liściach roślin rzepaku − odmian ozimych (Bojan, President, Rokas) i jarej (Feliks) w wyniku procesu hartowania i rozhartowania. NA – rośliny niehartowane, CA – rośliny hartowane chłodem, DA – rośliny rozhartowane. Dane dla odmian Bojan, President, Rokas i Feliks na podstawie publikacji **Stachurska et al., 2023.**

5. W niniejszej pracy po raz pierwszy w kontekście rozhartowania rzepaku badano także zmiany akumulacji roślinnych hormonów steroidowych − brasinosteroidów (BR) wraz z ekspresją ich receptora błonowego BRI1. W rzepaku wykryto następujące brasinosteroidy: tyfasterol, kastasteron, brasinolid, dolichosteron, dolicholid i homokastasteron (**Stachurska et al., 2022).** Wszystkie odmiany miały podobny skład (profil) BR, lecz zauważa się duże między-odmianowe zróżnicowanie koncentracji poszczególnych BR. Podobnie, w zróżnicowany sposób na akumulację BR oddziaływał proces hartowania i rozhartowania **(Stachurska et al., 2022)**. Tylko w niektórych przypadkach obserwowano wzrost akumulacji wybranych BR w czasie hartowania, a następnie ich spadek w czasie rozhartowania − odmiana Feliks (tyfasterol, kastasteron, dolichosteron i homokastasteron); odmiana Rokas (tyfasterol, dolicholid, dolichosteron); odmiana President (brasinolid) (**Stachurska et al., 2022**). Znacznie bardziej jednoznaczne wyniki uzyskano w przypadku ekspresji *BRI1,* a w szczególności akumulacji białka BRI1. Akumulacja transkryptu *BRI1* (kodującego membranowy receptor brasinosteroidowy BRI1) była niższa po hartowaniu i pozostała na niskim

poziomie po rozhartowaniu u dwóch odmian (Pantheon i Rokas), natomiast u pozostałych dwóch (Feliks i President) wzrosła po rozhartowaniu. Akumulacja białka receptorowego BRI1 wyraźnie spadła w roślinach hartowanych i wzrosła po rozhartowaniu, a zjawisko to było jednoznacznie niezależne od odmiany i zostało potwierdzone u 5 przebadanych pod tym kątem odmian, w tym u odmiany Bojan, Feliks, President i Rokas **(Stachurska et al., 2024** + materiały uzupełniające − rycina S4) (rycina 12). Wysoka akumulacja białka receptora BRI1 obserwowana w roślinach rozhartowanych (mimo fluktuacji, w tym spadków, koncentracji BR stanowiących ligandy BRI1) może oznaczać, że w rozhartowanych roślinach rzepaku wzmagała się transdukcja sygnału w kierunku pobudzenia wzrostu. Wspomniane hormony steroidowe wykazują bowiem aktywność stymulującą wzrost, a w tym przypadku jest to niekorzystne z punktu widzenia mrozoodporności. Rozhartowanie prowadziło także do odwrócenia wywołanych hartowaniem zmian w ekspresji genów *COR14* (regulowanych m.in. przez BR) i *SERK1 (*ale nie *SERK2)*, biorących udział w szlaku transdukcji sygnału od receptora brasinosteroidów (**Stachurska et al., 2024**). Ekspresja *SERK1* wykazywała spadek w chłodzie i wzrost w czasie rozhartowania. *COR14* charakteryzowało zwiększenie akumulacji w czasie hartowania i obniżenie w czasie rozhartowania.

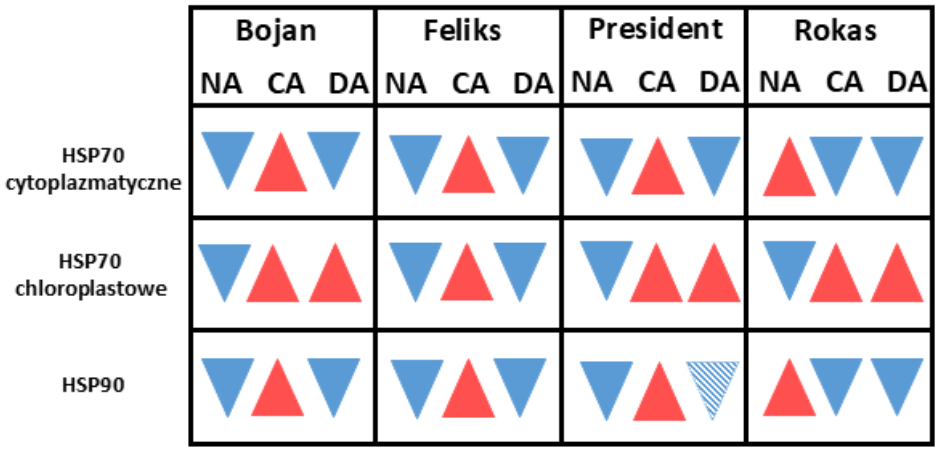


Rycina 12. Schematyczne przedstawienie zmian akumulacji transkryptu genu *BRI1*, akumulacji białka receptorowego BRI1 oraz akumulacji brasinosteroidów: kastasteronu (prekursora brasinolidu) i brasinolidu (aktywnej formy BR) zachodzących w roślinach niehartowanych (CA) i rozhartowanych (DA) dla dwóch wybranych odmian: jarej Feliks i ozimej Rokas. Zmianę w roślinach hartowanych przedstawiono w odniesieniu do wartości notowanej u roślin niehartowanych (CA vs. NA); zmianę w roślinach rozhartowanych przedstawiono względem wartości zanotowanej u roślin hartowanych (DA vs. CA). *Czerwona strzałka w górę oznacza istotny statystycznie wzrost, niebieska strzałka w dół – spadek; linia przerywana oznacza występowanie tendencji (wzrost lub spadek) nieistotnej statystycznie; szara strzałka pozioma – brak zmian.* Dane na podstawie publikacji **Stachurska et al., 2022; Stachurska et al., 2024.**

5.2.2. Białka chaperonowe

Analizy akumulacji białek szoku cieplnego (HSP) wykazały, że rozhartowanie najczęściej prowadziło do odwrócenia wywołanych hartowaniem zmian. Akumulacja białka HSP70

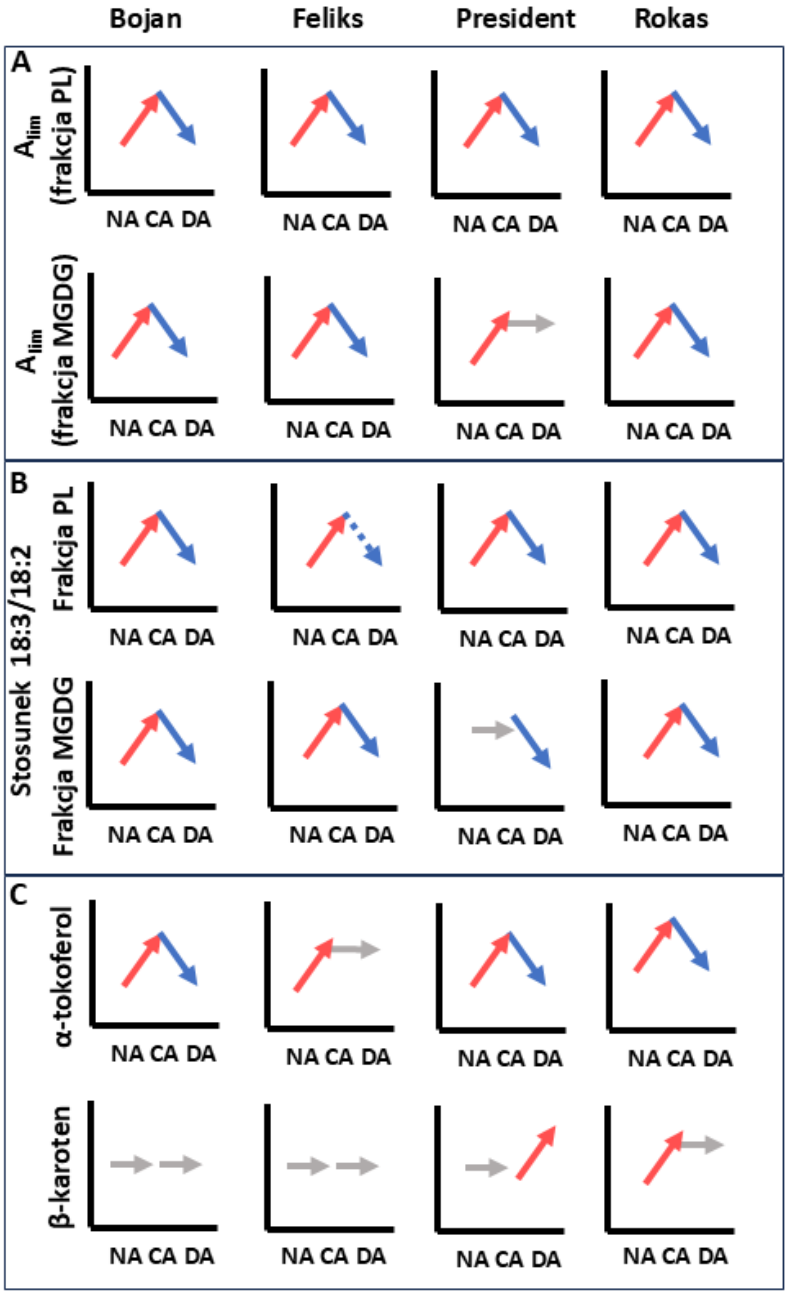
cytoplazmatycznego [HSP cyt.], HSP70 chloroplastowego [HSP chl.] oraz białka HSP90 w trzech z czterech badanych odmian wzrosła po hartowaniu, a obniżała się po rozhartowaniu (rycina 13) (**Stachurska et al., 2023**). Zjawisko to było wyraźnie widoczne w przypadku wszystkich badanych białek u wrażliwej na rozhartowanie odmiany jarej Feliks, natomiast u ozimych odmian Bojan i President obserwowano je w przypadku HSP70 cyt. i HSP90. Interesujący wyjątek stanowiła najbardziej mrozoodporna po rozhartowaniu odmiana Rokas, u której taka prawidłowość nie występowała. Odmianę tą cechowała wysoka bazowa (tj. notowana przed hartowaniem) zwartość HSP70 cyt. i HSP90, która później systematycznie obniżała się w czasie hartowania, a następnie rozhartowania. Z kolei poziom HSP70 chl. u tej odmiany wzrastał po hartowaniu i dalej w czasie rozhartowania. Generalnie zauważyć można prawidłowość, że zawartość białka HSP70 chl. nie spadała po rozhartowaniu u odmian ozimych, spadała natomiast u podatnej na rozhartowanie odmiany jarej Feliks (rycina 13) (**Stachurska et al., 2023**). Biorąc pod uwagę, że występujące w stromie chloroplastu białko HSP70 odpowiada za fotoprotekcję i naprawę PSII zjawisko to mogło by się przyczyniać do lepszej ochrony białek aparatu fotosyntetycznego u odmian ozimych niż u odmiany jarej po rozhartowaniu.



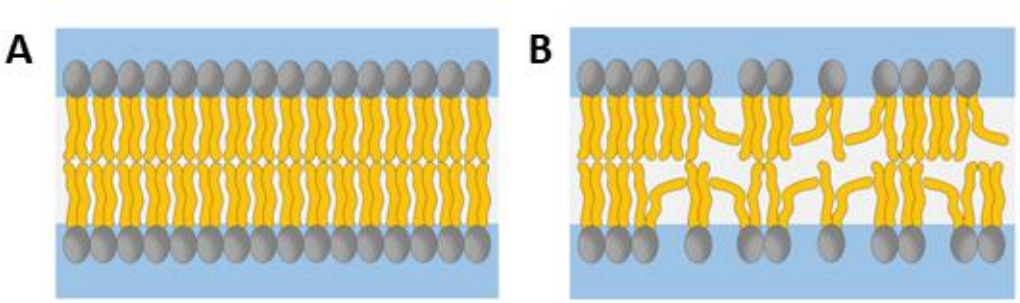
Rycina 13. Schematyczny model zmian akumulacji białek szoku cieplnego (HSP70 cytoplazmatycznego i chloroplastowego oraz HSP90) w liściach czterech odmian rzepaku − Bojan, Feliks, President i Rokas − niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA). *Niebieskim trójkątem symbolicznie zwizualizowano istotny statystycznie niski poziom akumulacji białka, czerwony trójkąt oznacza istotnie podwyższoną akumulację białka, występowanie tendencji spadkowej nieistotnej statystycznie zilustrowano trójkątem z niebieskimi skośnymi liniami.* Dane na podstawie publikacji **Stachurska et al., 2023.**

5.2.3. Własności membran chloroplastów

1. Rozhartowanie generalnie powoduje odwrócenie wywołanych hartowaniem zmian we właściwościach membran chloroplastów w kierunku obniżenia ich płynności, co może zwiększać szanse uszkodzenia membran w wyniku nagłego wystąpienia ujemnych temperatur (**Rys et al., 2024)**. Informacje o zmianie płynności membran uzyskano na podstawie analizy wartości parametru A_{lim} z wykorzystaniem wagi Langmuira dla monowarstw lipidowych zbudowanych z frakcji fosfolipidów (PL) i galaktolipidów (MGDG). Parametr A_{lim} [$Å^2$] opisuje pole powierzchni przypadające na cząsteczkę lipidu w maksymalnie upakowanej monowarstwie. Wartości A_{lim} zwiększały się dla monowarstw lipidów ekstrahowanych z roślin hartowanych (rycina 14A), co interpretuje się jako zwiększenie płynności membran. Rozhartowanie odwróciło tę zmianę, co świadczyło o obniżeniu płynności membran. Kierunek zmian wartości A_{lim} był zgodny z kierunkiem zmian stosunku kwasów tłuszczowych 18:3/18:2 we frakcjach PL i MGDG (**Rys et al., 2024) (**rycina 14B), którego wartość generalnie u wszystkich odmian zwiększała się w wyniku hartowania, a następnie spadała w wyniku rozhartowania. Wzrost udziału długołańcuchowych wielonienasyconych kwasów tłuszczowych zmienia własności fizykochemiczne błony w kierunku zwiększenia jej płynności (rycina 15).



Rycina 14. Uproszczony model zmian płynności membran chloroplastów − monowarstw zbudowanych z fosfolipidów (PL) i galaktolipidów (MGDG) [A] w powiązaniu ze zmianami stosunku kwasów tłuszczowych 18:3/18:2 [B]. Frakcje izolowano z chloroplastów niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) roślin rzepaku. Płynność określano na podstawie parametru A_{lim} [Å^2] opisującego pole powierzchni przypadające na cząsteczkę lipidu w maksymalnie upakowanej monowarstwie. [C] Kierunki zmian stężeń α-tokoferolu i β-karotenu zachodzące w roślinach pod wpływem hartowania i rozhartowania. *Czerwona strzałka w górę oznacza wzrost wartości parametru lub wyższą akumulację związku; niebieska strzałka w dół − spadek; szara strzałka pozioma − brak zmian.* Dane dla odmian Bojan, Feliks, President i Rokas na podstawie publikacji **Rys et al., 2024**.



Rycina 15. Uproszczony model struktury błony komórkowej: dwuwarstwa lipidowa złożona z nasyconych kwasów tłuszczowych [A], dwuwarstwa lipidowa zawierająca nasycone oraz nienasycone kwasy tłuszczowe [B]. Dwuwarstwę zawierającą kwasy nienasycone charakteryzuje większa płynność, co jest korzystne z punktu widzenia funkcjonowania błony u roślin rosnących w niskiej temperaturze. Źródło grafik: https://ib.bioninja.com.au/membrane-fluidity/

2. Na właściwości membran chloroplastów mogą mieć wpływ także występujące w nich komponenty, takie jak tokoferole i karotenoidy (**Rys et al., 2024)** (rycina 14C). W chloroplastach hartowanych roślin rzepaku stwierdzono wyższą akumulację tokoferoli, w tym wyższą zawartość α-tokoferolu niezależnie od odmiany. W przypadku karotenoidów, wzrost akumulacji β-karotenu był zależny od odmiany. Warto zaznaczyć, że znaczący wzrost β-karotenu stwierdzono w chloroplastach odmiany Rokas, o wysokiej mrozoodporności. Zwiększona akumulacja tych związków może wpływać na płynność membran poprzez lokalizowanie się ich pomiędzy łańcuchami kwasów tłuszczowych, co ma na celu ich ochronę przed oksydacją przez reaktywne formy tlenu. Obniżenie akumulacji tokoferoli w chloroplastach po rozhartowaniu może przyczyniać się do osłabienia ochrony antyoksydacyjnej membran chloroplastów i sprzyjać ich uszkodzeniu w temperaturach ujemnych. W odniesieniu do karotenoidów, dla których nie obserwowano tak jednoznacznych zmian (NA vs. CA vs. DA) jak dla tokoferoli, na uwagę zasługuje jednak fakt, iż odmiana o wyższej mrozoodporności po rozhartowaniu (Rokas) akumulowała jednocześnie wysokie ilości karotenoidów. Akumulacja β-karotenu, szczególnie po rozhartowaniu, może być korzystna z punktu widzenia ochrony membran. Zjawisko to może dodatkowo mieć znaczenie dla ochrony aparatu fotosyntetycznego przed stresem oksydacyjnym u rozhartowanych roślin w przypadku wystąpienia nagłego mrozu i wysokiej intensywności nasłonecznienia. Wyższą akumulację β-karotenu obserwowano także u odmiany President oraz

(tendencję) u odmiany Bojan, a zatem u odmian ozimych, natomiast nie u odmiany jarej bardziej podatnej na rozhartowanie.

3. Płynność membran mogą modyfikować także związki steroidowe. W niniejszej pracy badania takie przeprowadzono jedynie na membranach modelowych tj. złożonych z kwasów 18:3 i/lub 16:0 (**Stachurska et al., 2024**). Po raz pierwszy przetestowano oddziaływanie z membranami dwóch syntetycznych analogów brasinosteroidów (MK-266 i triolon). Wykazano, że steroidy te lokalizowały się w monowarstwach lipidowych i zmieniały ich właściwości fizykochemiczne w kierunku zwiększenia płynności, o czym świadczył wzrost wartości parametru A_{lim}. Zjawisko to występowało przede wszystkim dla stosunku lipid:analog 4:1. W dalszej części pracy testowano aktywność tych analogów w kontekście poprawy mrozoodporności rzepaku poddanego rozhartowaniu.

4. Zmiany w płynności i budowie membran chloroplastów mogą być powiązane ze zmianami w procesie fotosyntezy (fazy jasnej) roślin poddanych hartowaniu i rozhartowaniu. Różnice w wydajności fotosyntetycznej PSII i PSI widoczne były głównie pomiędzy roślinami niehartowanymi, hartowanymi i rozhartowanymi, a w mniejszym stopniu obserwowano różnice międzyodmianowe (**Stachurska et al., 2022**).

5.3. Wykrywanie stanu rozhartowania roślin metodami nieinwazyjnymi

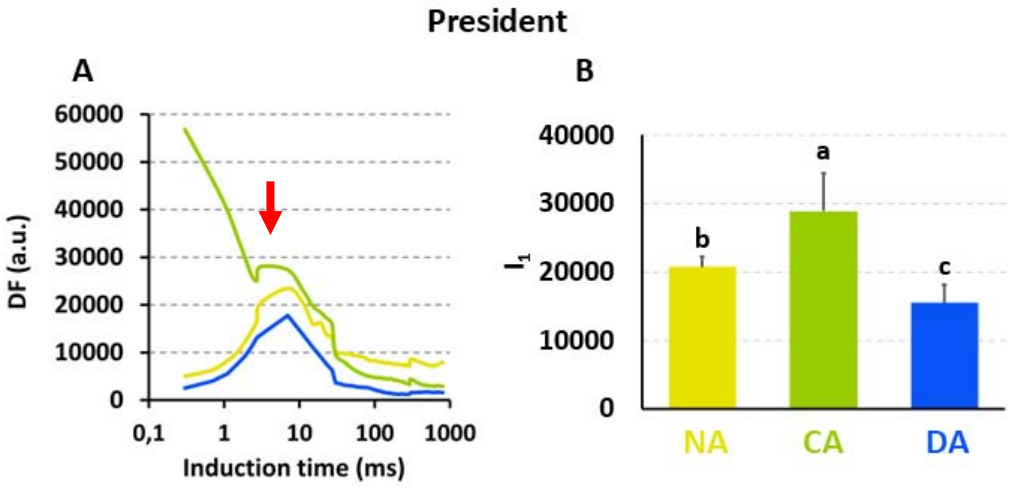
1. Metoda pomiaru szybkiej kinetyki fluorescencji chlorofilu *a* pozwalająca na charakterystykę wydajności fotosystemu II u hartowanych i rozhartowanych roślin stosowana była już przez innych autorów. W niniejszej pracy potwierdzono tylko obniżoną wydajność PSII u roślin hartowanych chłodem. Z kolei wzrost temperatury w czasie rozhartowania wiązał się ze zwiększeniem wydajności PSII. Ten kierunek zmian obserwowano np. w przypadku wartości parametru F_v/F_m, który informuje o maksymalnej fotochemicznej wydajności PSII, wartości PI_{ABS} (ogólnego wskaźnika sprawności funkcjonowania PSII w relacji do absorpcji energii) oraz tzw. przepływów

fenomenologicznych takich jak np. parametr DIo/CSm, informujący o rozpraszaniu cieplnym energii wzbudzenia przez PSII (**Stachurska et al., 2022**).

W niniejszej pracy wykonano jednak pomiary dodatkowe − różnych sygnałów związanych z fluorescencją chlorofilu nie tylko bezpośrednią (PF), ale także opóźnioną (DF) oraz odbiciowością modulowaną (MR820) (**Stachurska et al., 2022**). Opóźniona fluorescencja jest formą emisji światła w zakresie światła czerwonego-podczerwonego po wystawieniu roślin na ekspozycję świetlną. Emisja DF z PSII następuje przez krótki czas po zaniknięciu PF. Z kolei sygnały odbiciowości modulowanej (MR820) informują o transporcie elektronów po plastochinonie i do akceptorów PSI, wskazując w ten sposób na zmiany stanu redoks centrów reakcji PSI i plastocyjaniny (Salvatori et al., 2014 i literatura tam cytowana). Uzyskane w pracy wyniki pokazują, że rozhartowanie u rzepaku generalnie powoduje odwrócenie zmian wywołanych hartowaniem.

Po rozhartowaniu roślin, zmiany obserwowane były także w przypadku intensywności fazy ciemnej fotosyntezy, którą zbadać można wykonując nieinwazyjne pomiary wymiany gazowej liści. Przykładowo, parametr P_N informuje o intensywności asymilacji CO_2 (fotosyntezy netto). W wyniku hartowania intensywność asymilacji CO_2 obniżyła się, a na skutek wzrostu temperatury (rozhartowania), zgodnie z przewidywaniami, zwiększyła się.

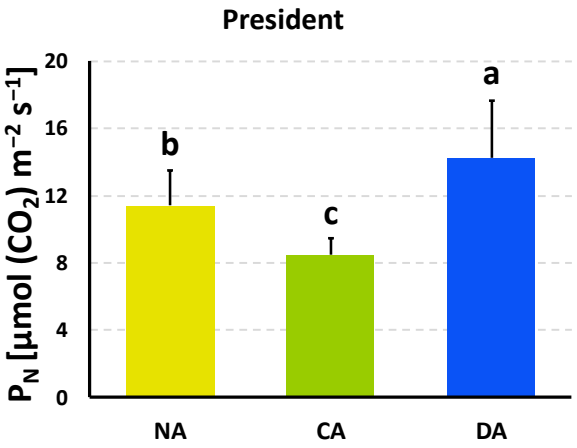
2. Metody fluorescencyjne (szybka kinetyka fluorescencji chlorofilu *a*) były już z powodzeniem zastosowane do wykrywania stanu rozhartowania roślin. Na podstawie wyników z niniejszej pracy do metod tych dodać można także możliwość wykorzystania pomiaru opóźnionej fluorescencji chlorofilu (DF). Krzywe opóźnionej fluorescencji chlorofilu dla roślin niehartowanych i rozhartowanych charakteryzował podobny przebieg, zaś krzywe dla roślin hartowanych charakteryzowały się innym kształtem w przedziale od 0,1 do kilku ms (rycina 16A). Jednym z parametrów, który byłby użyteczny do oceny stanu rozhartowania roślin może być parametr I_1, określający maksimum krzywej DF w przedziale czasu od 1 do 10 ms (rycina 16A). Najwyższe wartości parametru I_1, charakteryzowały rośliny hartowane chłodem, a najniższe (u większości badanych odmian) rośliny rozhartowane. Na przykład, dla odmiany President, wartość parametru I_1 zwiększyła się o prawie 40% na skutek hartowania, a następnie, po rozhartowaniu, obniżyła się o 47% (rycina 16B).



Rycina 16. Krzywe indukcji fluorescencji opóźnionej (DF) dla niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) roślin rzepaku ozimego, odmiana President [A]. Wartości maksimum krzywej opóźnionej fluorescencji (parametr I_1) [B]. *Średnie wartości ± SD oznaczone tymi samymi literami nie różnią się na poziomie p≤0.05 wg testu Duncana.* Dane na podstawie publikacji **Stachurska et al., 2022.**

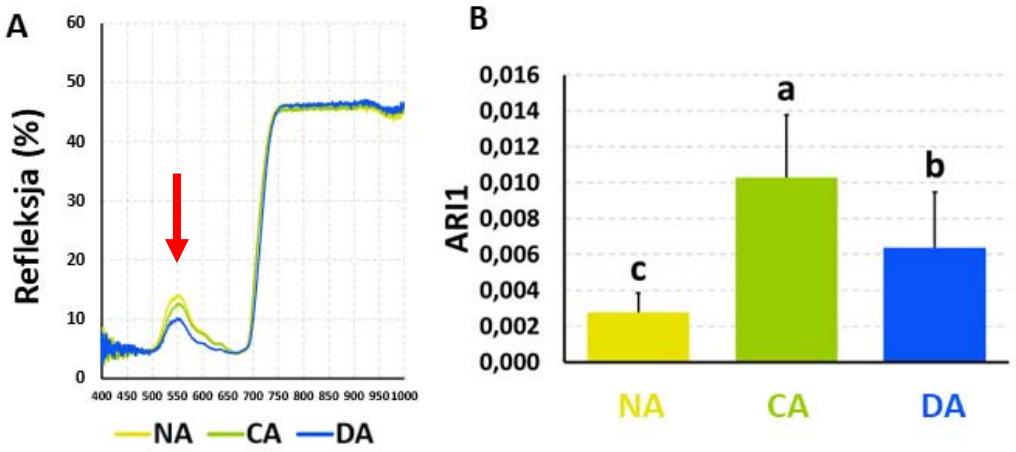
3. Sygnały odbiciowości modulowanej (MR820) także różniły się pomiędzy poszczególnymi traktowaniami – podobne wartości charakteryzowały rośliny niehartowane i rozhartowane, a inne były charakterystyczne dla roślin hartowanych. Istotne różnice stwierdzono w przypadku parametru MR_{max}, który informuje o maksymalnej odbiciowości [ang. *maximum of modulated 820 nm reflection intensity*]. Najczęściej wartości parametru MR_{max} obniżały się po hartowaniu, a po rozhartowaniu obserwowano tendencję do zwiększania wartości MR_{max}, co było dobrze widoczne u roślin odmian Rokas i Pantheon (**Stachurska et al., 2022**).

4. Nieinwazyjny pomiar wymiany gazowej liści również wydaje się być użyteczny do wykrywania rozhartowania roślin (**Stachurska et al., 2024**). Mierzona tą metodą wartość fotosyntezy netto (P_N) np. u roślin hartowanych odmiany President, spadła o 25%, zaś po rozhartowaniu wartość ta wzrosła i była wyższa o około 25% w porównaniu do wartości charakteryzującej rośliny niehartowane (rycina 17). Jednak ze względu na zróżnicowane dane uzyskane przed innych autorów, kwestia ta wymaga dalszych badań.



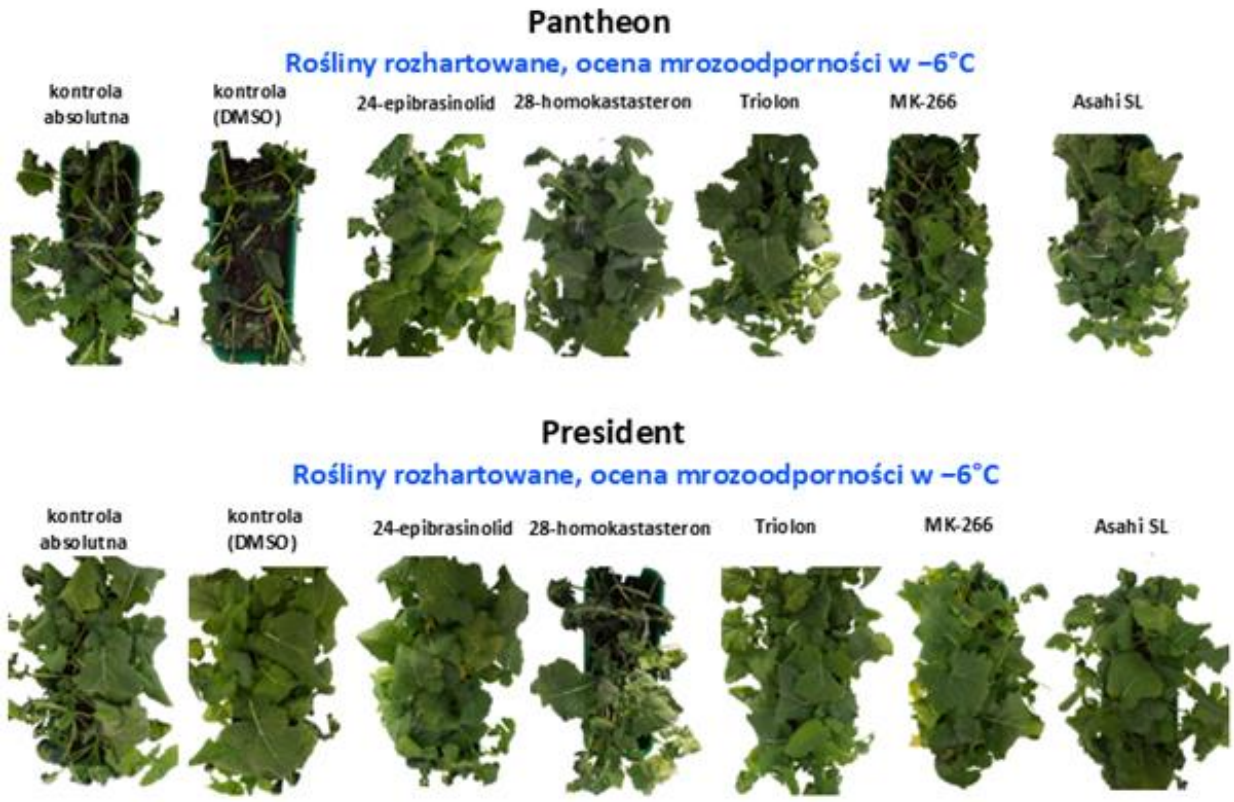
Rycina 17. Intensywność fotosyntezy netto (P_N) zmierzona dla liści roślin niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) odmiany President. *Średnie wartości ± SD oznaczone tymi samymi literami nie różnią się istotnie na poziomie p<0.05 wg testu Duncana.* Dane na podstawie publikacji **Stachurska et al., 2024.**

5. Pomiar własności spektralnych liści pozwolił na uzyskanie i zanalizowanie dużej liczby parametrów refleksji (WBI [***W**ater **B**and **I**ndex*], SIPI [***S**tructure **I**nsensitive **P**igment **I**ndex*], RENDVI [***R**ed-**E**dge **N**ormalised **D**ifference **V**egetation **I**ndex*], ARI1 [***A**nthocyanin **R**eflectance **I**ndex 1*], ARI2 [***A**nthocyanin **R**eflectance **I**ndex 2*], TVI [***T**riangular **V**egetation **I**ndex*], SRPI [***S**imple **R**atio **P**igment **I**ndex*], NDVI [***N**ormalised **D**ifference **V**egetation **I**ndex*], G [***G**reenness Index*] oraz CRI1 [***C**arotenoid **R**eflectance **I**ndex 1*]) (**Stachurska et al., 2024**). Sam przebieg krzywej refleksji pozwala wskazać charakterystyczne zmiany w zakresie 500−650 nm, które różnicują rośliny NA, CA i DA (rycina 18A). Spośród przeanalizowanych parametrów jako nadające się do wykrywania stanu rozhartowania roślin rzepaku wytypowano – ARI1, ARI2, RENDVI, G (**Stachurska et al., 2024**). Szczególnie przydatne w przypadku rzepaku wydają się jednak parametry z grupy ARI (np. ARI1 – *Anthocyanin Reflectance Index 1*) (rycina 18B).



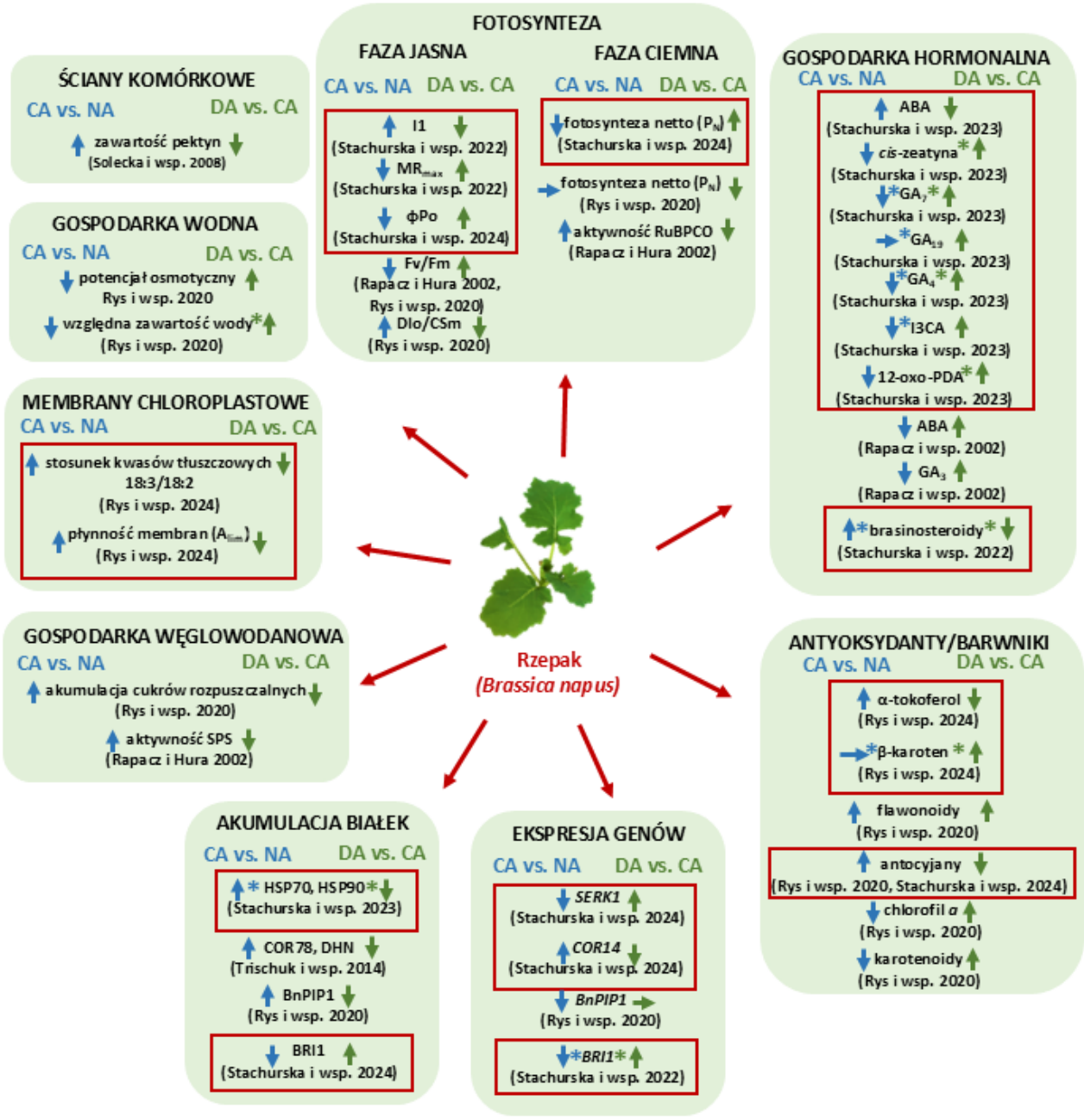
Rycina 18. Własności spektralne liści roślin rzepaku. Przebieg krzywej refleksji liści, charakterystyczny dla roślin odmiany President [A]. Strzałką wskazano różnice w spektrum 500−650 nm. Wartości parametru ARI1 (*Anthocyanin Reflectace Index 1*) charakterystyczne dla niehartowanych (NA), hartowanych (CA) i rozhartowanych (DA) roślin rzepaku ozimego odmiany President [B]. *Średnie wartości ± SD oznaczone tymi samymi literami nie różnią się istotnie na poziomie p<0.05 wg testu Duncana.* Dane na podstawie publikacji **Stachurska et al., 2024.**

6. Monitorowanie upraw metodami nieinwazyjnymi i stwierdzenie stanu rozhartowania mogłoby pozwolić na przedsięwzięcie środków ochronnych takich jak zastosowanie preparatów poprawiających mrozoodporność, zwłaszcza w momencie kiedy po ciepłej przerwie prognozowany jest gwałtowny spadek temperatury. W niniejszej pracy testowano brasinosteroidy, ich analogi i preparat komercyjny Asahi SL pod kątem poprawy mrozoodporności rzepaku po jego rozhartowaniu. Przykładowo, zastosowanie brasinosteroidu (24-epibrasinolidu) prowadziło do poprawy mrozoodporności rozhartowanych roślin, jednak efekt ten był zależny od różnych czynników (**Stachurska et al., 2024)**. Ochronny efekt działania steroidów zaobserwowano w przypadku lżejszego mrozu (−6°C) (rycina 19). Wśród czynników modyfikujących aktywność testowanych regulatorów wymienić należy odmianę, dodatek rozpuszczalnika steroidu do roztworu roboczego, a nawet termin, w którym wykonywano oprysk roślin (**Stachurska et al., 2024**).



Rycina 19. Wpływ wybranych regulatorów na mrozoodporność rozhartowanych roślin rzepaku odmian President i Pantheon (**Stachurska et al., 2024**). Rozhartowane rośliny rzepaku poddano testom mrozowym w temperaturze −6°C. Po mrożeniu rośliny odrastały w szklarni w temperaturze −6°C. Po mrożeniu rośliny odrastały w szklarni w temperaturze 12°C przez około dwa tygodnie; obserwowano i oceniano stopień uszkodzeń liści wg wcześniej opracowanej skali (**Stachurska et al., 2022**).

Na rycinie 20 zebrano i przedstawiono osiągnięcia omówione w niniejszym opracowaniu na tle wyników prac innych autorów dotyczących rzepaku.



Rycina 20. Wybrane fizjologiczne i biochemiczne zmiany zachodzące w roślinach rzepaku w wyniku procesu hartowania chłodem, a następnie rozhartowania w podwyższonej temperaturze. CA vs. NA – zmiany obserwowane w roślinach hartowanych w porównaniu do roślin niehartowanych; DA vs. CA – zmiany obserwowane w roślinach rozhartowanych w porównaniu do hartowanych. *Czerwoną ramką zaznaczono wyniki badań uzyskane w ramach niniejszej pracy doktorskiej. Strzałka w górę oznacza wzrost, strzałka w dół oznacza spadek, strzałka pozioma oznacza brak zmian. Gwiazdką zaznaczono parametry, dla których występowała zależność odmianowa*. Dane na podstawie publikacji podanych na rycinie.

6. Wnioski

Nasilanie się zmian klimatycznych i zwiększanie częstotliwości zjawisk powodujących rozhartowanie roślin skłania do zastanowienia się, czy i jakie środki zaradcze można/trzeba będzie podjąć w przyszłości w uprawach ozimin. W pracy wykazano, że występowanie ciepłych przerw generalnie powoduje odwrócenie wielu istotnych zmian fizjologiczno-biochemicznych wywołanych hartowaniem, dlatego jedną z opcji może być dobór i uprawa odmian charakteryzujących się mniejszą podatnością na rozhartowanie, a zatem utrzymujących jak najwyższą mrozoodporność mimo występowania ciepłych przerw. Alternatywą może być także zastosowanie preparatów zapobiegających uszkodzeniom mrozowym roślin ozimych w trakcie wegetacji po wystąpieniu ciepłych przerw – należy jednak pamiętać iż jest to koszt dodatkowy. Ponadto, w takim przypadku stan upraw należałoby monitorować przy pomocy nieinwazyjnych metod umożliwiających szybką ocenę stopnia rozhartowania roślin (np. refleksji liści). Jak pokazały wyniki uzyskane w niniejszej pracy istnieje kilka tego typu metod pomiarowych, które (po koniecznym zweryfikowaniu ich przydatności w warunkach polowych) powinny pozwolić na dostrzeżenie nawet wczesnych etapów rozhartowania roślin, a dodatkowo ich atutem byłaby możliwość użycia ich do oceny rozhartowania roślin na szeroką skalę za pomocą dronów i satelitów.

7. Spis literatury

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8. Streszczenie rozprawy doktorskiej w j. polskim

W ostatnich latach, z powodu postępujących zmian klimatu, coraz częściej obserwuje się występowanie w czasie późnej jesieni, a także zimy okresów z podwyższoną temperaturą (np. powyżej 9°C), która zaburza proces hartowania powodując rozhartowanie roślin i prowadząc do obniżenia ich mrozoodporności. Problem ten dotyczy szczególnie odmian ozimych, jednak odmiany jare również mogą być narażone na wystąpienie nagłego mrozu po okresie wyższej temperatury w okresie wczesnowiosennym.

Na podstawie oceny mrozoodporności rozhartowanych roślin w warunkach kontrolowanych stwierdzono, że odmiany rzepaku różnią się stopniem tolerancji warunków rozhartowujących, przez którą należy rozumieć utrzymanie jak najwyższej mrozoodporności pomimo rozhartowania. Spośród odmian ozimych, wyższa tolerancja charakteryzowała np. odmianę Rokas. Odmianą podatną na rozhartowanie była odmiana jara Feliks. Przyczyną spadków mrozoodporności roślin rzepaku na skutek rozhartowania (7 dni w temperaturze 16°C/9°C d/n) są liczne zmiany fizjologiczno-biochemiczne. Generalnie bowiem rozhartowanie w w/w warunkach wywołuje częściowe lub całkowite odwrócenie zmian indukowanych hartowaniem. W rozhartowanych roślinach rzepaku dochodzi m.in. do przesunięcia równowagi hormonalnej w kierunku zwiększenia akumulacji hormonów związanych ze wzrostem (np. giberelin, cytokinin) i obniżenia akumulacji ochronnych hormonów stresu (ABA). Na skutek rozhartowania zwiększa się akumulacja białka receptora brasinosteroidów (BRI1) wraz ze wzrostem ekspresji *SERK1*, którego produkt bierze udział w szlaku transdukcji brasinosteroidów. Obniżona jest akumulacja chaperonów − białek szoku cieplnego (HSP). Rozhartowanie przyczynia się do spadku stosunku kwasów tłuszczowych (18:3/18:2) membran chloroplastowych oraz obniżenia ich płynności. Dodatkowo, dochodzi do zmniejszenia akumulacji związków o działaniu ochronnym/antyoksydacyjnym (tokoferoli) w błonach chloroplastowych oraz intensyfikacji reakcji fazy jasnej i ciemnej fotosyntezy.

Stan rozhartowania roślin można wykryć poprzez analizy markerów biochemicznych (np. zmianę koncentracji ABA czy I3CA), ale również za pomocą szybkich, nieinwazyjnych pomiarów fizjologicznych. W tym celu można zastosować nie tylko pomiary fluorescencji chlorofilu (bezpośredniej, opóźnionej i odbiciowości modulowanej), ale także pomiar własności spektralnych roślin (np. refleksji). Mierzone lub obliczane parametry takie jak I_1, MR_{max} czy ARI1 informują o rozhartowaniu roślin. Wykrycie stanu rozhartowania umożliwia zastosowanie regulatorów poprawiających mrozoodporność rozhartowanych roślin, takich jak np. 24-epibrasinolid. Niemniej, biorąc pod uwagę iż wykonywanie dodatkowych oprysków

upraw podnosi koszty, w przypadku dalszego nasilania się zmian klimatycznych i globalnego ocieplenia wydaje się bardziej uzasadnione zalecanie do uprawy odmian, które charakteryzują się wyższą tolerancją warunków rozhartowujących.

9. Streszczenie rozprawy doktorskiej w j. angielskim (Summary)

In recent years, due to ongoing climate change, there have been more frequent periods with higher temperatures (e.g., above 9°C) in late autumn and winter, for example, in the Eastern European region. This disrupts the cold acclimation process and causes deacclimation leading to a decrease in plant frost tolerance. Although this problem particularly concerns winter cultivars, spring cultivars may also be exposed to sudden frost after a period of higher temperatures in early spring. Based on an assessment of the frost tolerance of deacclimated plants under controlled conditions, it was found that oilseed rape cultivars differ in their tolerance to deacclimating conditions. Plants with a higher tolerance of deacclimating conditions should be able to maintain the highest possible frost tolerance despite deacclimation. Among the winter cultivars, the cv. Rokas was characterised by higher tolerance of deacclimating conditions. The cultivar that was most susceptible to deacclimation was the spring cultivar Feliks. The reasons for the decrease in frost tolerance of oilseed rape plants due to deacclimation (7 days at 16°C/9°C d/n) are numerous physiological and biochemical changes. In general, deacclimation in the above-mentioned conditions caused a partial or complete reversal of the changes that had been induced by cold acclimation. In the deacclimated oilseed rape plants, among other things, there was a shift in the hormonal balance towards an increase in the accumulation of the growth-related hormones (e.g., gibberellins, cytokinins) and a decrease in the accumulation of the protective stress hormones (ABA). As a result of deacclimation, the accumulation of the brassinosteroid receptor protein (BRI1) increased along with an increase in the expression of *SERK1*, the product of which is involved in the brassinosteroid transduction pathway. The accumulation of the chaperones – heat shock proteins (HSP) – also decreased. Deacclimation contributed to a decrease in the fatty acid ratio (18:3/18:2) of the chloroplast membranes as well as a decrease in their fluidity. In addition, there was a decrease in the accumulation of the protective/antioxidant compounds (tocopherols) in the chloroplast membranes and an intensification of the light and dark phase reactions of photosynthesis.

The deacclimation of plants can be detected by analysing the biochemical markers (e.g., changes in the concentration of ABA or I3CA) and also by quick, non-invasive physiological measurements. In this case, not only can the measurements of chlorophyll *a* fluorescence (prompt, delayed and modulated reflection) be used, but also the measurements of the leaf spectral properties (for example reflection). The measured or calculated parameters such as I_1, MR_{max} or ARI1 provide information about the deacclimation of plants. Detecting deacclimation

enables the use of regulators that improve the frost tolerance of deacclimated plants such as 24-epibrassinolide. However, taking into account that additional crop spraying increases costs, in the case of any further intensification of climate change and global warming, it would seem to be more justified to recommend the cultivation of cultivars that are characterised by a higher tolerance of deacclimating conditions.

10. Załączniki

Oświadczenie Promotora pracy

Oświadczam, że niniejsza rozprawa doktorska została przygotowana pod moim kierunkiem i stwierdzam, że spełnia ona warunki do przedstawienia jej w postępowaniu o nadania stopnia doktora nauk rolniczych.

Kraków 2.12.2024 .....

Oświadczenie Autora pracy

Świadoma odpowiedzialności prawnej oświadczam, że niniejsza rozprawa doktorska została napisana przeze mnie samodzielnie i nie zawiera treści uzyskanych w sposób niezgodny z obowiązującymi przepisami.

Oświadczam ponadto, że niniejsza wersja pracy jest identyczna z załączoną wersją elektroniczną.

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Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and *BRI1* Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance

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Abstract: The objective of this study was to answer the question of how the deacclimation process affects frost tolerance, photosynthetic efficiency, brassinosteroid (BR) homeostasis and *BRI1* expression of winter oilseed rape. A comparative study was conducted on cultivars with different agronomic and physiological traits. The deacclimation process can occur when there are periods of higher temperatures, particularly in the late autumn or winter. This interrupts the process of the acclimation (hardening) of winter crops to low temperatures, thus reducing their frost tolerance and becoming a serious problem for agriculture. The experimental model included plants that were non-acclimated, cold acclimated (at 4 °C) and deacclimated (at 16 °C/9 °C, one week). We found that deacclimation tolerance (maintaining a high frost tolerance despite warm deacclimating periods) was a cultivar-dependent trait. Some of the cultivars developed a high frost tolerance after cold acclimation and maintained it after deacclimation. However, there were also cultivars that had a high frost tolerance after cold acclimation but lost some of it after deacclimation (the cultivars that were more susceptible to deacclimation). Deacclimation reversed the changes in the photosystem efficiency that had been induced by cold acclimation, and therefore, measuring the different signals associated with photosynthetic efficiency (based on prompt and delayed chlorophyll fluorescence) of plants could be a sensitive tool for monitoring the deacclimation process (and possible changes in frost tolerance) in oilseed rape. Higher levels of BR were characteristic of the better frost-tolerant cultivars in both the cold-acclimated and deacclimated plants. The relative expression of the *BRI1* transcript (encoding the BR-receptor protein) was lower after cold acclimation and remained low in the more frost-tolerant cultivars after deacclimation. The role of brassinosteroids in oilseed rape acclimation/deacclimation is briefly discussed.

Keywords: brassinosteroids; brassinosteroid insensitive 1; dehardening; delayed chlorophyll fluorescence; frost tolerance; homocastasterone; photosystem I; photosystem II; prompt chlorophyll fluorescence; stress tolerance

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1. Introduction

Oilseed rape (*Brassica napus* ssp. *oleifera* L.) is a major crop and is an important source of vegetable oil for the food, chemical and fuel industries. There are winter and spring cultivars of oilseed rape that differ with the season of growth and in crop yield. The yield of the winter cultivars is higher, and in Poland, winter oilseed rape is cultivated more often. The winter growth of the plants carries the risk of frost injuries that may lead to severe economic losses. Ice forms outside or inside a plant's cells, causes membrane damage and initiates frost injures. However, the winter species have developed mechanisms that enable them to survive temperatures below 0 °C. A few weeks of cold acclimation (cold hardening usually at +2–+5 °C) improves the ability of plants to survive winter frost [1]. Cold acclimation causes many biochemical and physiological changes in the lipid and protein components of the cell membranes, soluble sugar content, osmotic potential and many other changes [2]. Sugar management is a particularly important element in the hardening process. The leaves of plants that have been cultivated in the cold retain an overexpression of the enzymes that are involved in sugar production and a have higher level of activity of the Calvin cycle enzymes compared to the leaves that have been grown before cold acclimation [3]. Well-cold-hardened plants of oilseed rape can survive at temperatures as low as about −20 °C. However, if the period of cold acclimation is interrupted by episodes of higher temperatures (deacclimation), the frost tolerance of plants decreases [1,4]. In recent years, such warm periods during late autumn have occurred more often due to climate change. Generally, deacclimation can occur when the temperature is higher than 9 °C, and in some circumstances, there can be an impulse to resume growth and development [1]. The rate of deacclimation depends on the temperature, plant species and plant genotype [5]. Warm periods have a negative effect on frost tolerance when they occur in late autumn, in winter and sometimes in early spring. Spring frost events increase the risk of frost injuries to plants [6]. During spring, when the temperature rises and then suddenly falls below zero, deacclimated plants are threatened, among others, because of a decrease in the amount of soluble carbohydrates that are necessary to survive frost [7]. Deacclimation has been well studied in woody plants [8], grasses [9] and the model plant *Arabidopsis* [3,10]. Although the physiological and biochemical changes that occur during deacclimation in oilseed rape are less understood, deacclimation definitely reduces the freezing tolerance in winter rape cultivars [4,11] which can cause stem elongation or even the development of buds [11]. After deacclimation, many of the biochemical/physiological parameters reached values that were on the level observed in the non-acclimated control [4]. Deacclimation was accompanied, among others, by a decrease in soluble sugar content (and osmotic potential), a decrease in the accumulation of the aquaporin protein (cellular water channels), and a decrease in the anthocyanin level, while there was an increase in the chlorophyll content, which was also associated with an increase in the efficiency of the light reactions of photosynthesis.

Photosynthesis is generally widely understood to be a highly sensitive indicator that mirrors the interaction of plants with environmental stressors, including the stress that is associated with an exposure to low temperatures. For example, cold slows down the Calvin cycle enzymes in *Arabidopsis* [12]. The cold-acclimated seedlings of *Pinus concorta* L. had an inhibited photosynthesis that was accompanied by a partial loss of the PSII reaction centers, which was indicated by the decreased levels of the reaction center D1 protein and the loss of chlorophyll. Conversely, the cold-acclimated winter wheat maintained a high level of photosynthesis and a chlorophyll content at the same level [13]. The cold-acclimated winter wheat cultivars had a CO_2 assimilation and O_2 evolution that were similar to or greater than the non-acclimated plants [14], and this was associated with, among others, an increased capacity for PSI cyclic electron transport [15]. Similar to many other physiological processes, the process of photosynthesis functions under the influence of phytohormones. Extensive studies have already been devoted to explaining the role of brassinosteroids (plant steroid hormones; BR) in photosynthesis [16]. A brassinosteroid-insensitive 1 (*bri1*) *Arabidopsis* mutant showed downregulation of the

genes connected to the regulation of photosynthesis and was also characterized by reduced growth, lower photosynthetic activity and a disrupted PSII assemblage [17]. Studies of BR mutants of *Arabidopsis* that were conducted by [18] showed that brassinosteroids control the thylakoid membrane architecture and PSII function. *Arabidopsis cyp51A2* mutants that were defective in an early stage of the sterol biosynthesis pathway element, sterol 14α-demethylation, are lethal, and their genes, which are connected to the photosynthesis processes such as with the Rubisco large subunit, chlorophyll *a/b* binding protein and photosystem components, are downregulated and have reduced chlorophyll content and photosynthetic activity [19]. Conversely, BR deficiency can also lead to an increased accumulation of chlorophyll and the photosynthetic proteins that change the leaf color from green to dark green [20–22]. As for the effect of brassinosteroids on photosynthesis in plants that have been exposed to temperature stress (particularly low temperature), the knowledge is much more limited (reviewed in [23]). For example, in *Secale cereale*, BR stimulate the photoprotective mechanisms during a prolonged exposure to cold via the temporary suppression of the quantum efficiency of PSII, which is a consequence of energy dissipation in the form of non-photochemical quenching [24]. The duration of cold acclimation (three or six weeks) has a slightly different and cultivar-dependent effect on the regulation of the photosynthetic activity that is induced by BR in *Secale cereale* [25]. The brassinosteroid signaling pathways in plants are still being discovered, but an important element is the brassinosteroid membrane receptor protein (BRI1—brassinosteroid-insensitive 1) [26]. *BRI1* encodes a putative leucine-rich repeat receptor kinase. Mutations in *BRI1* result, among others, in semi-dwarfness or dwarfness in plants of *Arabidopsis thaliana* L. or barley [27,28]. The timing of flowering is also delayed in the BR-insensitive *bri1* mutants of *Arabidopsis* [29].

One of the most recent articles, in the section of the issue "expert views", [8] clearly states that deacclimation after cold acclimation is "a crucial, but widely neglected part of plant winter survival". Considering that the mechanisms of deacclimation are relatively poorly explained, especially regarding crop plants such as oilseed rape (where this phenomenon can cause severe injuries and economic losses), the main goal of our work was to study the deacclimation-induced changes in photosynthetic efficiency (PSI and PSII), brassinosteroid homeostasis and *BRI1* expression in winter oilseed rape. These changes were discussed in relation to the deacclimation-induced loss of frost tolerance. Moreover, we attempted to answer the question of whether the fluorescence measurements, which describe PSI and PSII efficiency, enable the potential changes in frost tolerance that are caused by the deacclimation process to be non-invasively predicted, which is well described in winter cereals [1] and can also be useful from the practical point of view in oilseed rape. Deacclimated plants were compared to plants that were non-acclimated and cold-acclimated. Ten cultivars were used for chlorophyll *a* fluorescence measurements and in frost tests. Based on the frost tests, four cultivars were selected and further examined for their BR content and *BRI1* accumulation.

2. Results and Discussion

2.1. Frost Tolerance

The data about the frost tolerance of the plants were obtained by examining their regrowth—resumption of growth and the appearance of new leaves two weeks after freezing. A temperature of −1 °C did not seriously affect the non-acclimated (NA) plants, and all of the regrowth of the cultivars was on a level of between six and seven points (Figure 1A). The NA plants were barely able to survive a temperature of −5 °C, and they had the lowest frost tolerance at a level of below one point. Cold acclimation significantly improved the frost tolerance of the plants, which was expected. The cold-acclimated (CA) plants were able to survive temperatures of −13 and −16 °C, and regrowth was at a level of about three to four points (Figure 1B). The deacclimated plants (DA) were more sensitive to frost than the cold-acclimated plants (Figure 1C). Freezing the DA plants at a temperature of −12 °C enabled the regrowth at a level below two points in all of the cultivars. The frost tolerance of the deacclimated plants was, however, higher compared to

the non-acclimated plants. A temperature of −5 °C caused regrowth in the non-acclimated plants at a level of about one point, while a temperature of −6 °C caused regrowth in the deacclimated plants at a level of about four to six points. The photographs of the oilseed rape plants that had been exposed to frost and then left to regrow (14 days at 12 °C) are presented in Figures 2 and 3. Generally, our results are in agreement with the results that were obtained for oilseed rape by [4] and [11], where the authors found that deacclimation decreased the frost tolerance of oilseed rape. The current work, however, brings some new information due to the use of not one or two but ten cultivars with different traits. The estimated temperature that is required to reduce plant regrowth by 50% (RT50) was calculated for all of the cultivars, and this enabled the cultivars to be ranked (Table 1). As shown in Table 1, testing the ten different oilseed rape cultivars enabled us to observe that some oilseed rape cultivars with a high frost tolerance after cold acclimation such as Rokas also maintained a higher frost tolerance after deacclimation. The cultivar Feliks, which had a lower tolerance after cold acclimation, was characterized by low tolerance also after deacclimation. However, there were also cultivars that had a higher frost tolerance after cold acclimation but a decreased tolerance to frost after deacclimation (or opposite) as is clearly shown in Table 1. The higher basal frost tolerance (observed in the non-acclimated plants) did not result in a higher frost tolerance after deacclimation (see the cultivar President). Similarly, a low basal frost tolerance did not result in a low frost tolerance after deacclimation (see the cultivar Pantheon). Tolerance to deacclimation seems to be a cultivar-dependent trait. As tolerance to deacclimation, we generally understand that plants maintain a satisfactory level of frost tolerance (as much as possible similar to the level of frost tolerance acquired after cold acclimation) after warm periods that interrupt the process of cold hardening (acclimation) in autumn or after warm periods that appear in winter or even early spring.

Table 1. Estimated temperature (°C) required to reduce plant regrowth by 50% (RT50). Ten cultivars of the non-acclimated, cold-acclimated and deacclimated oilseed rape were exposed to frost. The cultivars that were selected for a detailed analysis of the chlorophyll *a* fluorescence curves, analysis of brassinosteroid profile and *BRI1* expression are indicated with color.

Non-Acclimated Plants		Cold-Acclimated Plants		Deacclimated Plants	
Cultivar	**RT50**	**Cultivar**	**RT50**	**Cultivar**	**RT50**
Bojan	−3.68	Bojan	−13.54	Rokas	−8.91
Rokas	−3.34	Rokas	−13.50	Pantheon	−8.84
Monolit	−3.23	Darcy	−13.26	Graf	−8.41
President	−3.21	Graf	−13.24	Bojan	−8.40
Darcy	−3.12	Pantheon	−13.09	Monolit	−8.35
Birdy	−3.07	Monolit	−12.98	Finley	−8.31
Finley	−2.94	Feliks	−12.92	Darcy	−8.29
Graf	−2.69	President	−12.80	Birdy	−8.14
Feliks	−2.54	Finley	−12.76	President	−7.96
Pantheon	−2.35	Birdy	−12.44	Feliks	−7.63

Finally, based on the ranking (Table 1), four cultivars that differed in their frost tolerance were selected for a more detailed analysis of chlorophyll *a* fluorescence curves —Rokas, Feliks, Pantheon and President. The cultivar Rokas had a high frost tolerance of the NA, CA and DA plants. The cultivar Feliks had one of the lowest frost tolerances, especially in the non-acclimated and lowest tolerance in the deacclimated plants. The cultivar Pantheon had the lowest frost tolerance for NA plants, medium for CA plants, and high for DA plants. The plants of cultivar President had a relatively high frost tolerance if non-acclimated, a relatively low frost tolerance after cold acclimation and a low frost tolerance after deacclimation.

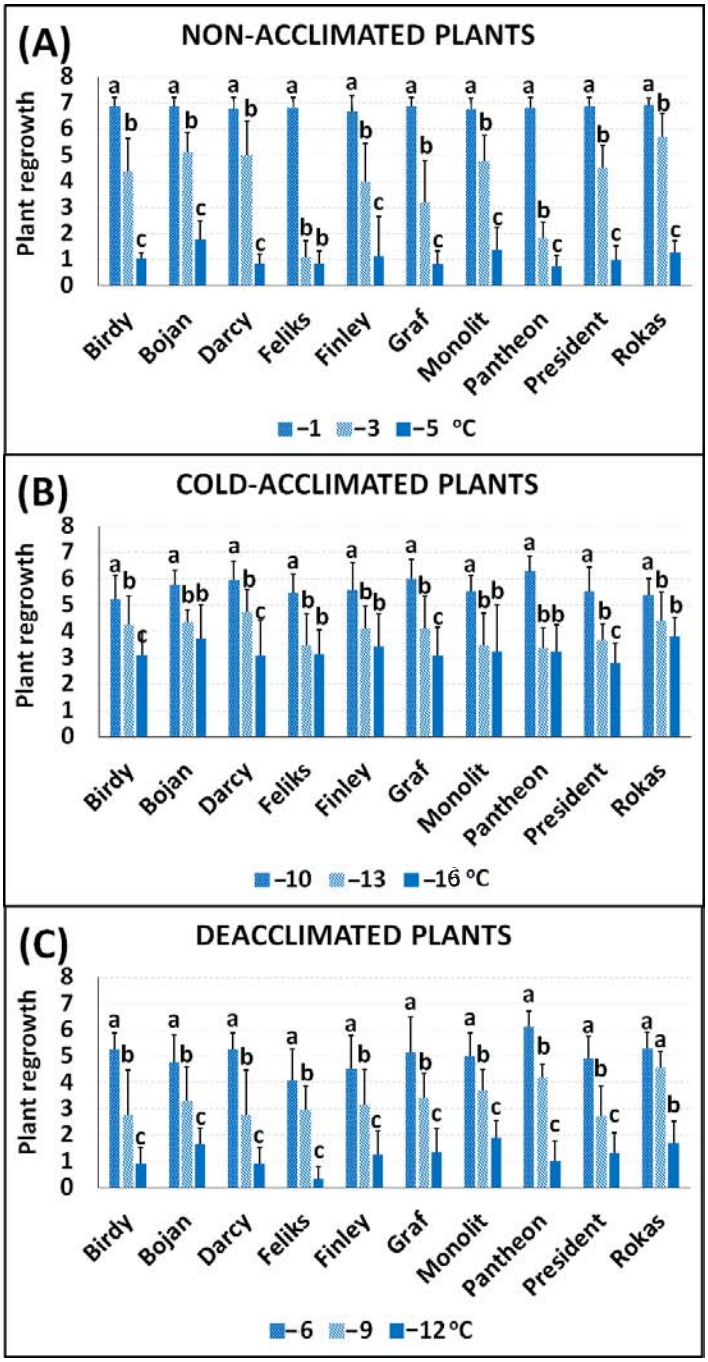

Figure 1. Frost tolerance of ten different cultivars of oilseed rape—Birdy, Bojan, Darcy, Feliks, Finley, Graf, Monolit, Pantheon, President and Rokas—that characterized the (**A**) non-acclimated plants, (**B**) cold-acclimated plants and (**C**) deacclimated plants. Frost tolerance based on the regrowth scale (0–7 points) after frost treatment (−1 to −16 °C); more detailed explanations of scale are in Section 3.3. Mean values ± SD that are marked with the same letters (separately for each cultivar) did not differ significantly at $p < 0.05$ according to Duncan's test, $n = 15$.

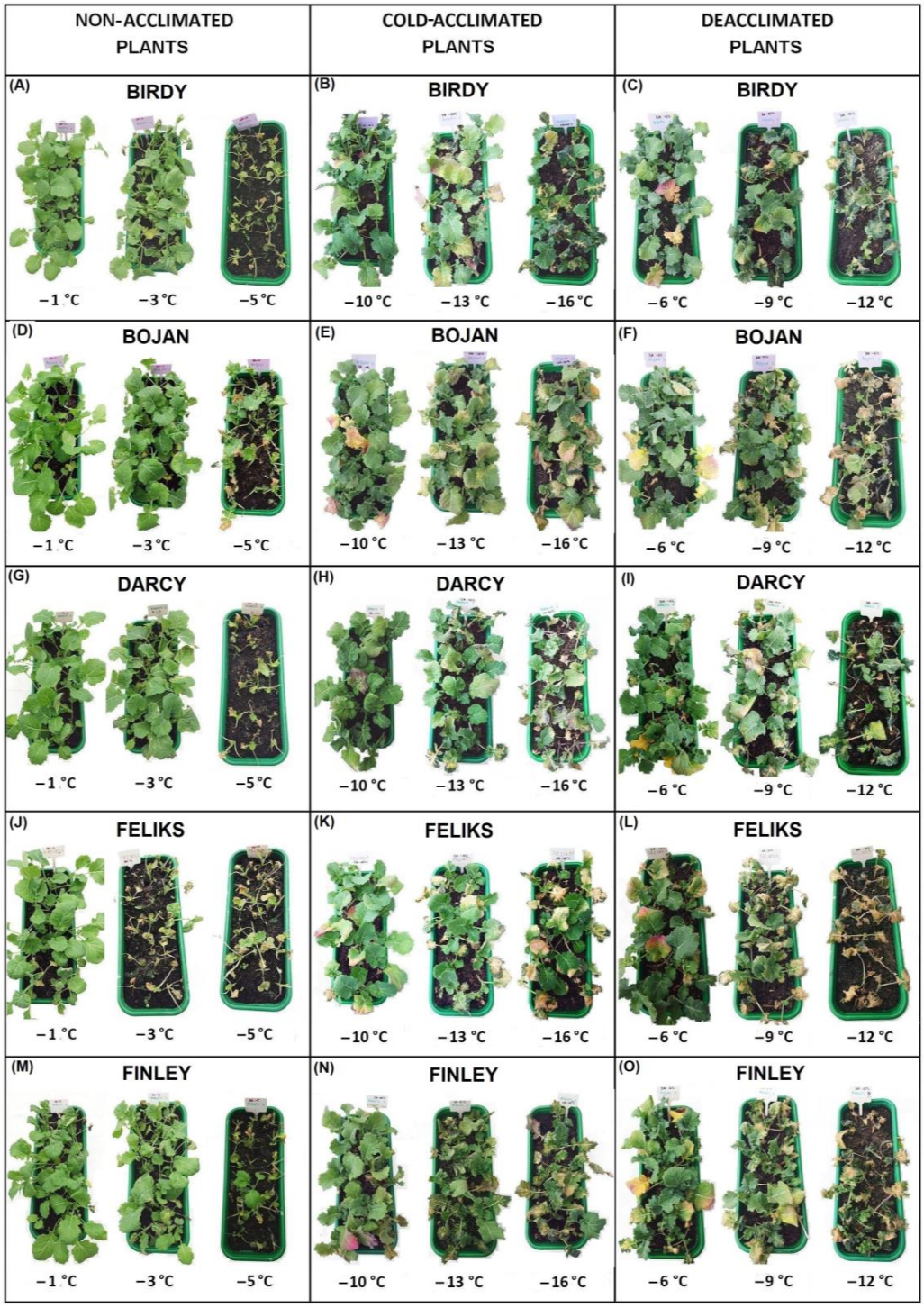

Figure 2. Plants of the non-acclimated, cold-acclimated and deacclimated oilseed rape (cultivars Birdy, Bojan, Darcy, Feliks, Finley) after exposure to frost. After frost treatment, the plants were left to regrow for two weeks at 12 °C. (**A**,**D**,**G**,**J**,**M**) non-acclimated plants; (**B**,**E**,**H**,**K**,**N**) cold-acclimated plants; (**C**,**F**,**I**,**L**,**O**) deacclimated plants.

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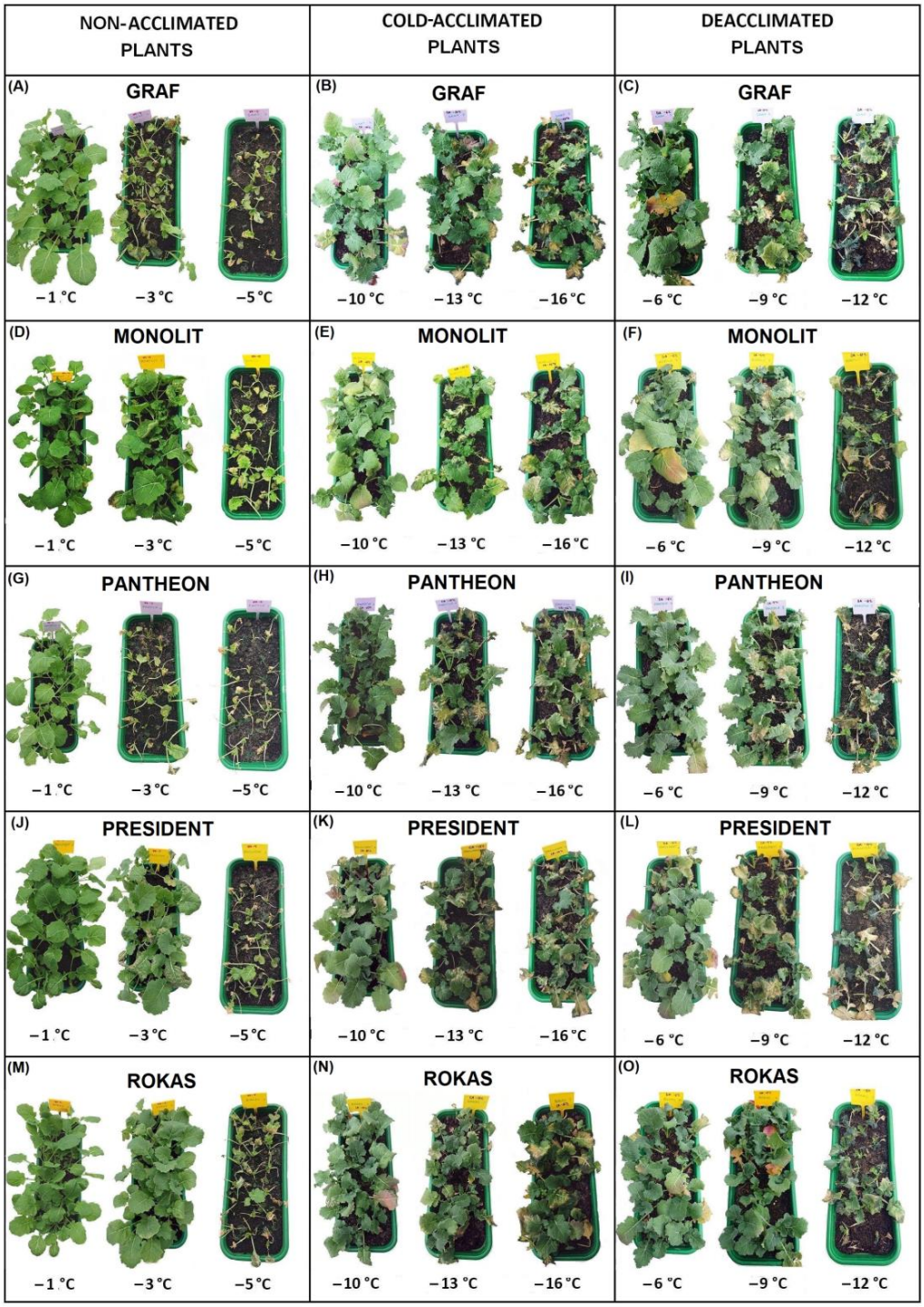

Figure 3. Plants of the non-acclimated, cold-acclimated and deacclimated oilseed rape (cultivars Graph, Monolith, Pantheon, President, Rokas) after exposure to frost. After frost treatment, the plants were left to regrow for two weeks at 12 °C. (**A**,**D**,**G**,**J**,**M**) non-acclimated plants; (**B**,**E**,**H**,**K**,**N**) cold-acclimated plants; (**C**,**F**,**I**,**L**,**O**) deacclimated plants.

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2.2. Prompt Chlorophyll a Fluorescence (PF)

Based on the PF transients and their normalization, differences between the tested treatments (NA, CA, and DA) were significant in all cultivars (Figure 4, Tables S1 and S2). The cold-acclimation (CA) caused higher intensity of chlorophyll fluorescence than the NA and DA treatments, especially in the J–I phase. However, there were no significant

differences between the cultivars. The differences between the cultivars were noticeable only after the normalization of the induction curves in the specific phases (O–J and O–K). The $_{\Delta}W_{(O–J)}$ parameter revealed that the CA transient was most pronounced in the Rokas cultivar (Figure 5). The transient curves that were measured in DA plants had a lower intensity than those in the CA treatment. The $_{\Delta}W_{(O–K)}$ parameter showed that the DA treatment had higher values compared to the CA treatment in all of the tested cultivars. During this phase, the Rokas cultivar showed the lowest intensity of chlorophyll fluorescence for both treatments. As can be seen in Figure 6, during the $_{\Delta}W_{(J–I)}$ phase, the DA treatment caused a decrease in the intensity of chlorophyll fluorescence as well as in $_{\Delta}W_{(I-P)}$, although there were no significant differences between the cultivars.

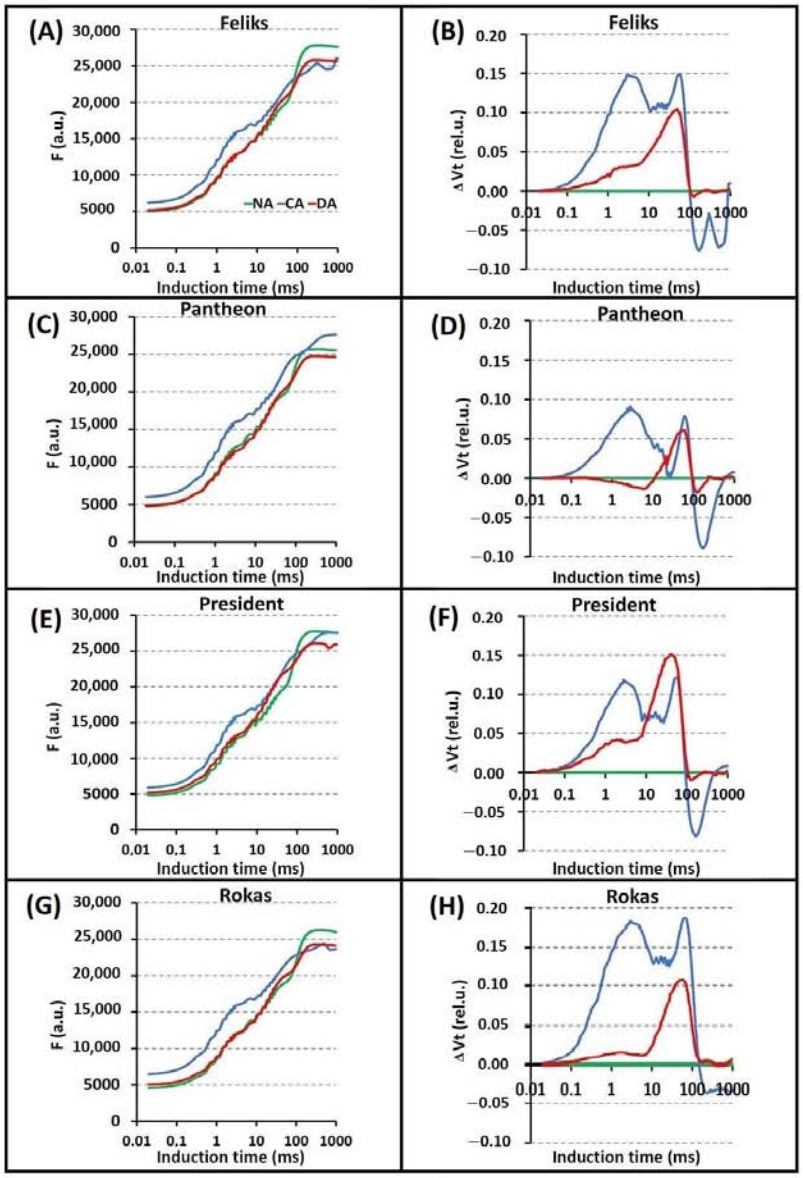

Figure 4. Induction curves of the chlorophyll *a* fluorescence (**F**) and the differential curves of $_{\Delta}V_t$ (obtained by subtracting the control curve (non-acclimated plants—NA)) of oilseed rape. Plants that were not acclimated (NA), cold acclimated (CA) and deacclimated (DA). (**A**,**B**) cultivar Feliks; (**C**,**D**) cultivar Pantheon; (**E**,**F**) cultivar President; (**G**,**H**) cultivar Rokas. Statistical analysis of values of typical transient points for curves in figures (**A**,**C**,**E**,**G**) are given in Table S2.

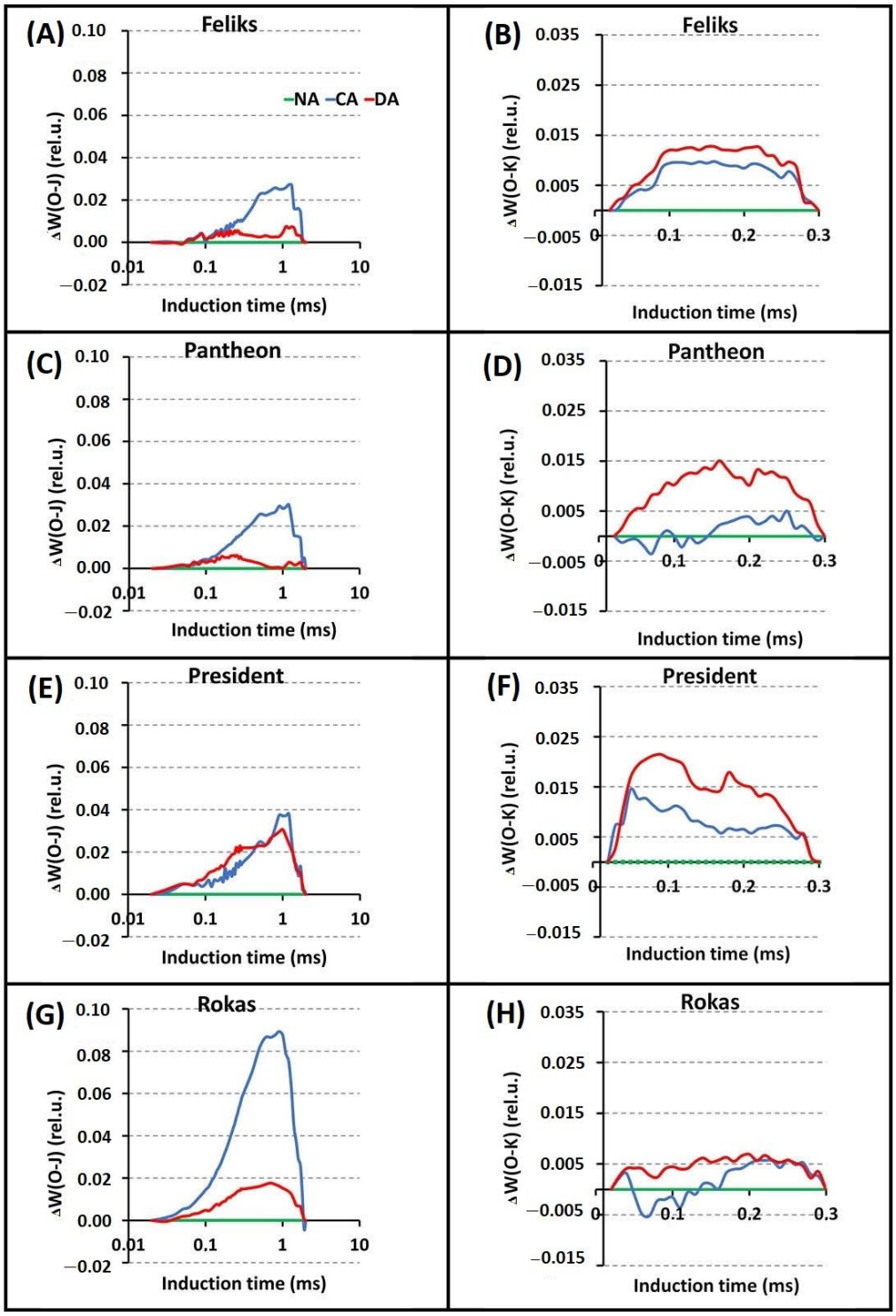

Figure 5. Differential curves of $_{\Delta}W_{(O\text{-}J)}$ and $_{\Delta}W_{(O\text{-}K)}$ (obtained by subtracting the control curve (non-acclimated—NA)) of oilseed rape. Plants not acclimated (NA), cold acclimated (CA) and deacclimated (DA). (**A**,**B**) cultivar Feliks; (**C**,**D**) cultivar Pantheon; (**E**,**F**) cultivar President; (**G**,**H**) cultivar Rokas.

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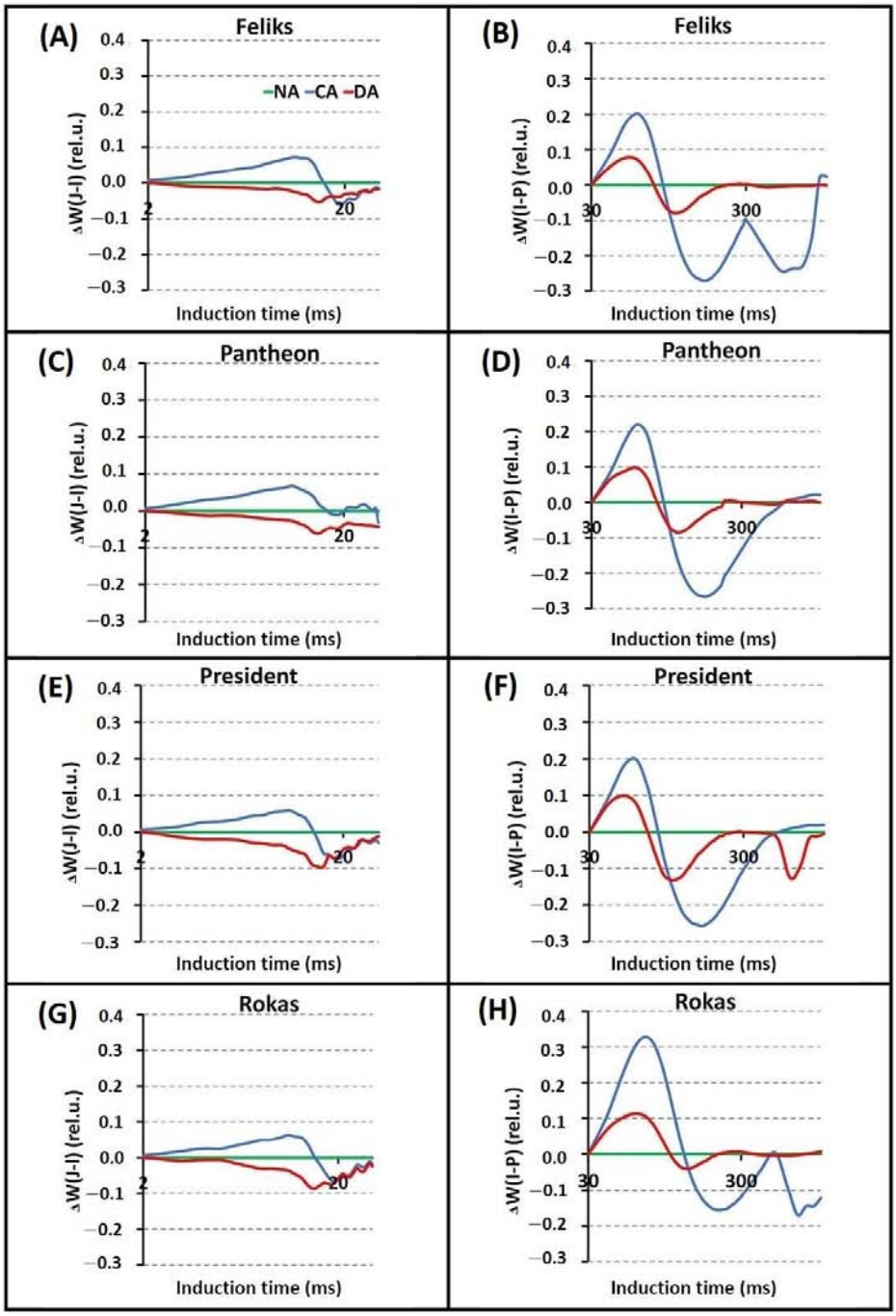

Figure 6. Differential curves of the $_{\Delta}W_{(J\text{-}I)}$ and $_{\Delta}W_{(I\text{-}P)}$ (obtained by subtracting the control curve (non-acclimated plants—NA)) of oilseed rape. Plants not acclimated (NA), cold acclimated (CA) and deacclimated (DA): (**A**,**B**) cultivar Feliks; (**C**,**D**) cultivar Pantheon; (**E**,**F**) cultivar President; (**G**,**H**) cultivar Rokas.

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The increase in the $_{\Delta}W_{(O\text{-}K)}$ might be connected with the inactivation of the oxygen-evolving complex (OEC) and/or the inhibition of the electron transport on the donor or acceptor side of photosystem II [30]. The $_{\Delta}W_{(O\text{-}K)}$ also provides information about the grouping or connectivity—the relative position of the antenna complexes of the different RCs relative to each other [31]. A positive change in the course of the analyzed curve might indicate a greater distance between the PSII antennas and thus to less efficient energy exchange [32,33]. Conversely, the significant increase in the O–J step could be correlated

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with a reduction in the acceptor side of PSII [34], while $_{\Delta}W_{(I-P)}$ provides information about the electron flow to the end electron acceptors of PSI [35].

Looking at the selected values of the parameters of the JIP test, which are expressed as a radar plot (spider), it can also be concluded that there were differences between the three applied treatments (NA, CA and DA), and some differences were between the cultivars (Figure 7). Generally, deacclimation caused the changes that had been induced by cold acclimation to be reversed, which agrees with our earlier findings [4]. The pattern of the changes in the chlorophyll fluorescence parameters presented in Figure 7 (for four cultivars) shows that the response of the DA plants was more similar to the NA plants than to the CA plants. This phenomenon can also be well tracked based on the values of the F_v/F_m and PI_{abs} and the parameters of the phenomenological fluxes and specific energy fluxes, which are presented in Table S1 for all ten tested cultivars. In all of the cultivars, most of these measured and/or calculated parameters (especially the F_v/F_m and phenomenological fluxes), which are presented in Table S1, were similar in the NA and DA plants compared to the CA and DA plants.

Table 2. The description of fluorescence parameters, modified from [36].

T_{Fm}	Time (in ms) to reach the maximal fluorescence intensity F_m
Area	Area above the curve
F_o	Minimal fluorescence, where all RC (reactive centers) are open
F_m	Maximal fluorescence, where all RC are closed
$F_v/F_m = (F_m - F_o)/F_m$	Maximum quantum yield of PSII photochemistry
$V_J = (F_J - F_o)/(F_m - F_m)$	Relative variable fluorescence at the J-step
$Sm = Area/(F_m - F_o)$	Normalized total area above the curve
$N = S_m M_o (1/V_J)$ turn-over number Q_A	Amount of Q_A reduction from 0 to T_{fm}
$ABS/RC = (1 - \gamma_{RC})/\gamma_{RC}$	Absorption flux (of antenna Chls) per RC
$DI_o/RC = (ABS/RC - TR_o/RC)$	Dissipated energy flux per RC (at t = 0)
$TR_o/RC = M_o(1/V_J)$	Trapping flux (leading to Q_A reduction) per RC
$ET_o/RC = M_o(1/V_J)\Psi_o$	Electron transport flux (further than Q_A^-) per RC
$RE_o/RC = M_o(1/V_J)(1 - V_I)$	Electron transport beyond PSI
$ABS/CS_m = F_m$	Absorption flux per excited cross section (CS_m)
$DIo/CS_m = (ABS/CS_m) - (TRo/CS_m)$	Energy dissipation per CS_m
$TR_o/CS_m = F_v/F_m\ (ABS/CS_m)$	Energy flux for trapping per CS_m
$ET_o/CS_m = (F_v/F_m)(1 - V_J)\ F_m$	Energy flux for electron transport per CS_m
RE_o/CS_m	Electron transport beyond PSI per CS_m
$PI_{abs} = \gamma_{RC}/(1 - \gamma_{RC}) \times \varphi_{Po}/(1 - \varphi_{Po}) \times \Psi_{Eo}/(1 - \Psi_{Eo})$	Performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors
I_1 and I_2	Maxima of DF induction curve
D_2	Minimum of DF induction curve
MR_o	Modulated 820 nm reflection intensity at time "0"
MR_{min}	Minimum of modulated 820 nm reflection intensity
MR_{max}	Maximum of modulated 820 nm reflection intensity
ΔMR_{fast}	Fast phase (oxidation) of reflection intensity = $MR_o - MR_{min}$
ΔMR_{slow}	Slow phase (reduction) of reflection intensity = $MR_{max} - MR_{min}$

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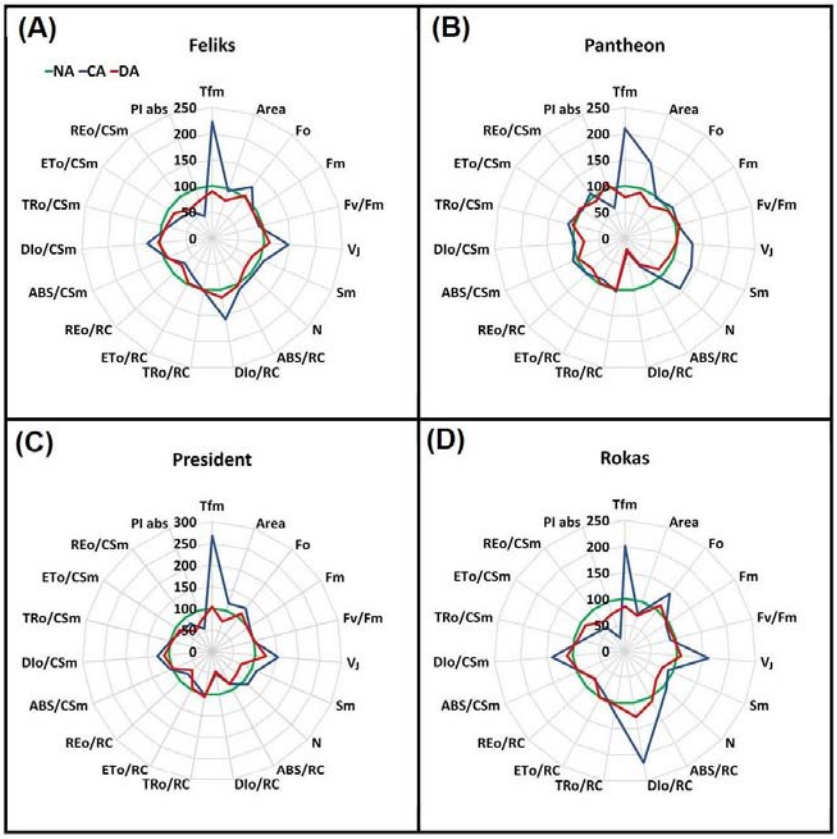

Figure 7. JIP—test parameters for the cold-acclimated (CA) and deacclimated (DA) plants of oilseed rape that were normalized to the values of the NA plants (non-acclimated control) as radar plots; NA is expressed as 100%. (**A**) cultivar Feliks; (**B**) cultivar Pantheon; (**C**) cultivar President; (**D**) cultivar Rokas. T_{Fm}—time (in ms) to reach the maximal fluorescence intensity F_m; Area—area above the curve; F_o—minimal fluorescence, where all RC are open; F_m—maximal fluorescence, where all RC are closed; F_v/F_m—maximum quantum yield of PSII photochemistry; V_J—relative variable fluorescence at the J-step; Sm—normalized total area above the curve; N—amount of Q_A reduction from 0 to T_{Fm}; ABS—absorption flux (of antenna Chls); DI_o—dissipated energy flux (at t = 0); TR_o—trapping flux (leading to Q_A reduction); ET_o—electron transport flux (further than Q_A^-); RE_o—electron transport beyond PSI_o; RC_o—calculation in relation to reaction center; CSm—calculation in relation to excited cross section; PI_{abs}—performance index (potential) for energy conservation from exciton to the reduction of intersystem electron acceptors. The description of fluorescence parameters is modified from [36] Detailed equations are also given in Table 2.

Some cultivar-dependent changes were also observed. For example, the time it took to reach maximal fluorescence (T_{fm}) significantly increased in the CA plants compared to the NA and DA plants (Figure 7). At the same time, the DI_o/RC increased in the CA plants only in the Feliks and Rokas cultivars, while in the President and Pantheon cultivars, the values of these parameters were below the values that were observed for the non-acclimated control plants. The parameter PI_{abs} was usually lower in the CA and DA experimental variants and was similar in all four cultivars (the one exception was that the PI_{abs} parameter in cultivar Pantheon was similar to the one in the NA and DA plants).

The main visible effects of the acclimation/deacclimation procedures caused on the photosynthetic machinery were a decrease in the excitation energy migration toward the PSII reaction centers (an increase in the Fo in the CA compared to both the NA and DA plants), a slight fluorescence quenching in the CA and DA samples (decreased Fm values), a decrease in the electron transport rates on the PSII acceptor side and from the PQH_2 pool to the PSI end acceptors, and deacclimation that partially restored the electron transport in the PSII acceptor side (the appearance of a J band).

The delayed chlorophyll fluorescence curves are presented in Figure 8. The beginning of the curves (before I_1) in all of the tested cultivars that had been grown under the CA treatment had a high course. This might have been due to the cold-acclimation procedure, which induced structural changes in the photosynthetic machinery. Thus, it was reflected

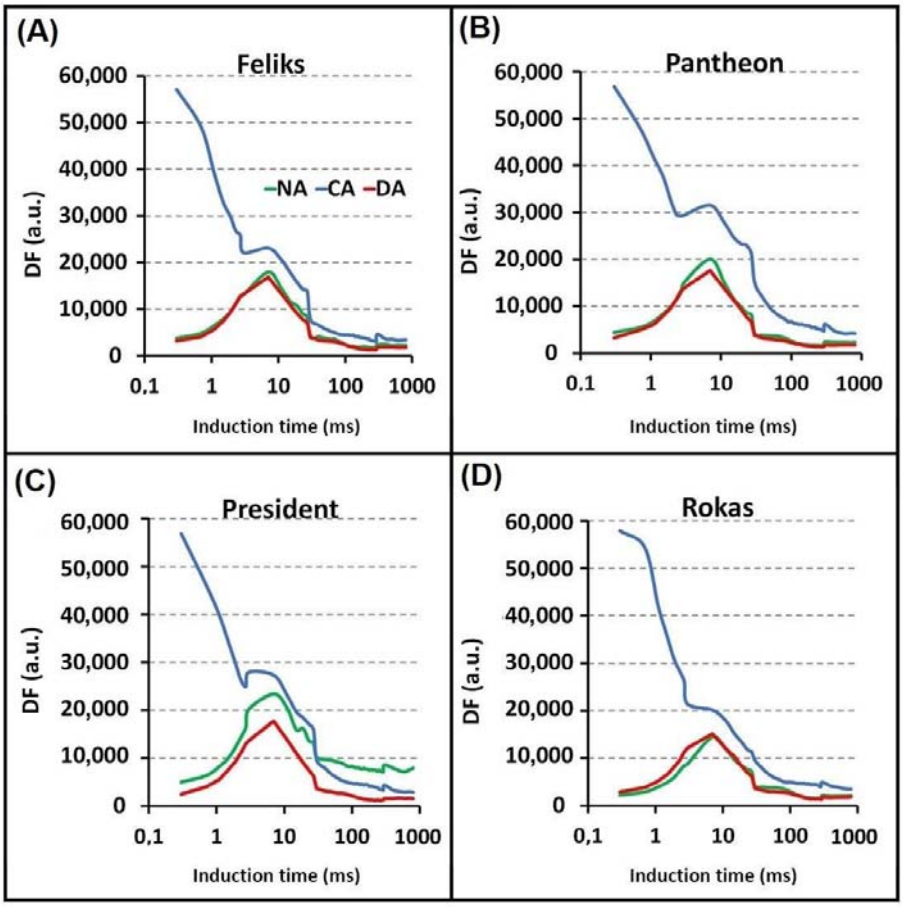

Figure 8. Delayed fluorescence induction curves for non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**) cultivars Feliks; (**B**) cultivar Pantheon; (**C**) cultivar President; (**D**) cultivar Rokas. Statistical analysis of values of characteristic points of DF curves are given in Table S3.

Figure 8. Delayed fluorescence induction curves for non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**) cultivars Feliks; (**B**) cultivar Pantheon; (**C**) cultivar President; (**D**) cultivar Rokas. Statistical analysis of values of characteristic points of DF curves are given in Table S3.

Based on statistical analysis (Table S3), it can be stated that in all tested cultivars, the I_1 point showed higher values under CA treatment as compared to NA. Simultaneously, DA treatment triggered the lowest value in all tested cultivars, except in Rokas. In Feliks and Rokas cultivars, the parameter I_1–D_2/D_2 had lower values under CA treatment as compared to NA and DA treatments. The curves for the other cultivars had a similar trend in the I_1, I_2 and D_2 points. Per the available literature, the changes in the I_1 point might be related to two phenomena: (1) photochemical—accumulation of certain light-emitting states of the PSII reaction centers, and (2) non-photochemical—increase in the DF due to the electrical gradient formed by PSI when P700 is oxidized [37].

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The I_2 maximum (usually only a shoulder) is probably related to the prolonged reopening of PSII RCs by the electron transfer from the reduced Q_B to PQ before the full reduction of the PQ pool [38]. The changes in values of the D_2 point correlate with the processes of reduction of the PQ pool, and the time when this point is reached can be an indicator of the reducing activity of the PSII complex [39].

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The modifications of the first induction maximum reflect the formation and dissipation of the "light-emitting states" of PSII RC, i.e., the concentration of the precursors of the re-excited states of P680, which are the sources of the excitation energy for the DF emission. As a precursor to the micro-s DF, the PSII RC state $P_{680}^{+}Q_A^{-}$ has been proposed.

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The modulated infrared light reflection at the 820 nm (MR820) signals, which are primarily influenced by the rate of the electron flow from PSII to PSI, showed differences between the treatments and cultivars (in the CA) (Figure 9). The 820 nm reflection of the sample at the onset of the actinic illumination (MR_0), the minimum (MR_{min}) and the maximum (MR_{max}) were analyzed (Table S4). There were significant changes between the values in particular treatments and between cultivars. In all tested cultivars, the values of the parameter MR_{max} were lower under CA, and here compared to NA. However, in Rokas, the values of this parameter were lower in CA, as compared to NA. In addition, significant changes were noted in the MR_{max} parameter in all tested cultivars. The values were lower under CA as compared to NA. The observed changes could have been the result of a limitation on the acceptor side of PSI [40,41]. Concluding remarks for all fluorescence measurements are given in Section 2.3.

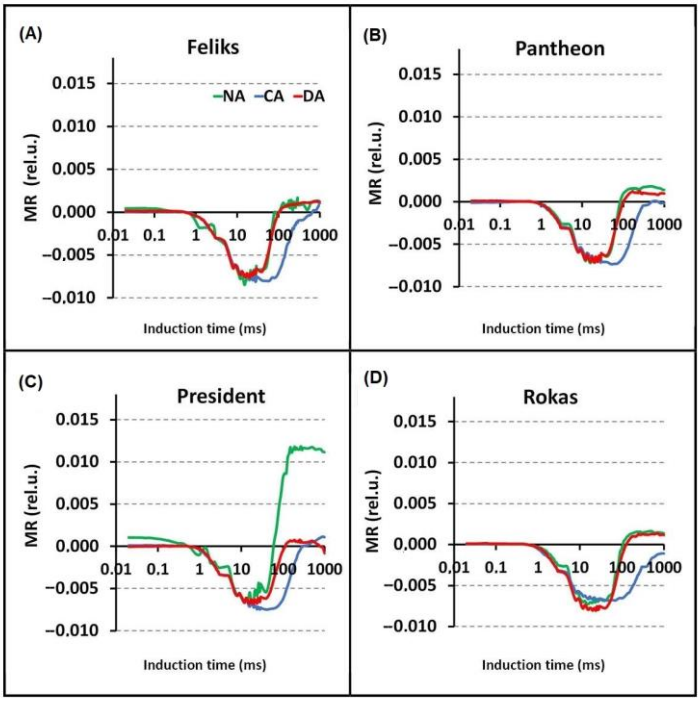

Figure 9. The kinetics of the modulated light reflection at 820 nm for the non-acclimated (NA), cold-acclimated (CA) and the deacclimated oilseed rape. The data were normalized to the initial measured value of the signal. (**A**) cultivar Feliks; (**B**) cultivar Pantheon; (**C**) cultivar President; (**D**) cultivar Rokas. Statistical analysis of values of characteristic points of curves are given in Table S4.

Figure 9. The kinetics of the modulated light reflection at 820 nm for the non-acclimated (NA), cold-acclimated (CA) and the deacclimated oilseed rape. The data were normalized to the initial measured value of the signal. (**A**) cultivar Feliks; (**B**) cultivar Pantheon; (**C**) cultivar President; (**D**) cultivar Rokas. Statistical analysis of values of characteristic points of curves are given in Table S4.

2.3. Brassinosteroid Profile and the Transcript Level of BRI1

The studies revealed the presence of typhasterol, castasterone, brassinolide, dolichosterone, dolicholide and homocastasterone (28-homocastasterone) in the leaves of oilseed rape (Figure 10). Homocastasterone and typhasterol were accumulated in the highest amounts. The other four BR were present in amounts that were even 1000-fold lower. Usually in oilseed rape, the content of brassinosteroids such as typhasterol, dolichosterone, dolicholide, and homocastasterone increases after cold acclimation, which agrees with previous results that had been obtained for winter wheat [42] and barley [21]. For these species was also reported an elevated level of BR after exposure to cold. Additionally, in winter wheat, there was correlation between the level of frost tolerance that was acquired after cold acclimation and brassinosteroid content. The more tolerant cultivars accumulated more BR (homocastasterone, teasterone) after cold acclimation. The dependency of the level of frost tolerance and BR content is generally observable in oilseed rape, although it is not

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rape, although it is Rokas clearly in wheat. Rokas and Pantheon were more frost tolerant as clearly in wheat. Rokas and Pantheon were more frost tolerant than Feliks or President after cold hardening (Table 1). After cold acclimation, the total BR content (sum of all measured BR), which was generally determined by the high content of homocastasterone and typhasterol (Figure 10A,F), was higher (in Rokas and Pantheon, respectively, about 229 and 170 pmol·g^{-1} F.W., about 129 and 100 in Feliks and President, and about 607 and 371 pmol·g^{-1} F.W., respectively). After deacclimation, Feliks and President definitely had lower frost tolerance (total BR content of about 265 to 531 pmol·g^{-1} F.W.) than Rokas and Pantheon (about 920 to 1471 pmol·g^{-1} F.W.), which had a higher frost resistance and retained a high content of BR. It seems likely that a higher content of BR may be more desirable for better frost tolerance of oilseed rape in both the case of cold acclimated and, more importantly, in deacclimated plants. In this aspect, the findings of [43], who observed that the exogenous application of BR in *A. thaliana* (the same family as oilseed rape) resulted in a delay of generative development, are interesting. Since, as was mentioned in the Introduction, deacclimation often causes oilseed rape to develop too early, a naturally higher content of endogenous BR could be beneficial for preventing/limiting such phenomenon. This issue will need more detailed studies.

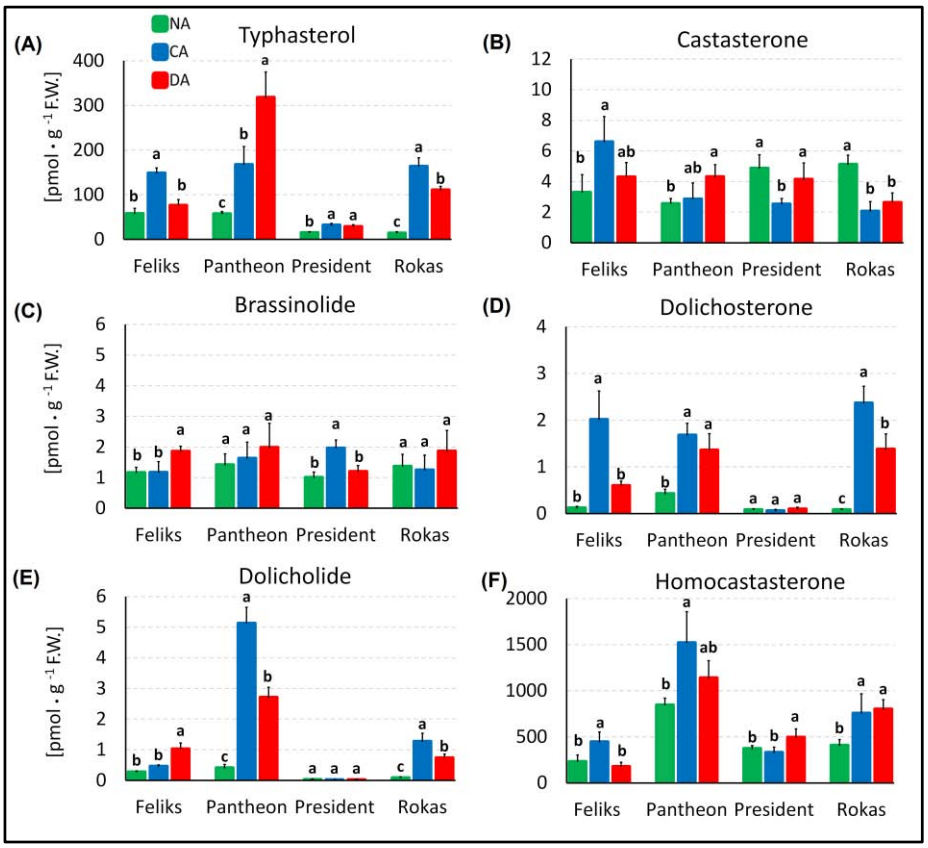

Figure 10. Content of brassinosteroids in the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape (cultivars Feliks, Pantheon, President, Rokas). (**A**) Typhasterol; (**B**) castasterone; (**C**) brassinolide; (**D**) dolichosterone; (**E**) dolicholide; (**F**) homocastasterone (28-homocastasterone). Mean values ± SD that are marked with the same letters (separately for each cultivar) did not differ significantly at $p < 0.05$ according to Duncan's test, $n = 5$.

It is also worth mentioning a mechanism that was proposed by [44]. The authors described a model that had a BR concentration-dependent balance between growth and the stress response of plants. The model was focused on the interplay of BR, BRI1, ROS and ABA, where a higher concentration of BR was associated with a stress reaction, while a lower concentration was associated with a growth response. The role of brassinosteroids in the stress response in oilseed rape was also confirmed by [45]. The overexpression of

the brassinosteroid biosynthetic gene *DWF4* increased stress tolerance (drought, high temperature) as well as resistance to some pathogens. Thus, our studies, which suggest that a higher BR content seems to be preferable for better frost tolerance, both after cold acclimation and deacclimation, are somewhat in agreement with these findings.

In the experiment, the transcript level of *BRI1* (an encoding receptor protein for brassinosteroids) was also studied. Cold hardening unambiguously lowered the accumulation of transcript *BRI1* in three of four of the tested cultivars (Figure 11). In one cultivar (President), the same tendency was noted, although it was statistically insignificant. After deacclimation, the accumulation of this transcript increased once again in the Feliks and President plants (in the frost-susceptible cultivars). In Rokas and Pantheon, the accumulation of *BRI1* remained at the same low level as after cold acclimation, and these cultivars also maintained a high frost tolerance after deacclimation. Earlier, [46] observed that the *bri1-9 Arabidopsis* mutant had a higher tolerance to cold than the wild type, while the *BRI1*-overexpressing transgenic plants were more sensitive to cold. The higher tolerance to cold in the *bri1* mutant was associated with the constitutive activation of the stress-inducible genes. Conversely, according to [47], the *BRI1*-overexpressing plants of *Arabidopsis* had a higher frost tolerance after cold acclimation (compared to the wild type), while the *bri1-301* mutant was more susceptible to frost than the wild type. In our studies on oilseed rape, a higher level of the *BRI1* transcript was accompanied by a lower tolerance to low temperature. The results are then closer to the findings of [46], where the *BRI1*-overexpressing transgenic plants were more sensitive to low temperature. The amount of the transcript decreased after cold acclimation (together with an increase in frost tolerance), and simultaneously, the deacclimation-sensitive cultivars had increases in the transcript accumulation after a period of higher temperatures. We think that to obtain a more complete picture, the data about the accumulation of the BRI1 protein would be required in all of these studies.

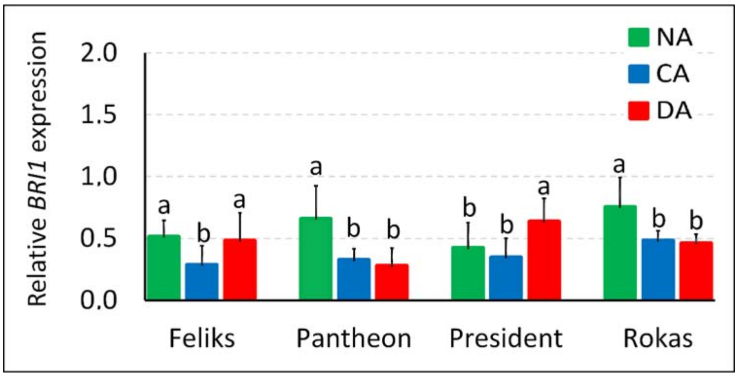

Figure 11. Relative transcript level of *BRI1* (brassinosteroid-insensitive 1, encoding the brassinosteroid membrane receptor) in the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape (cultivars Feliks, Pantheon, President, Rokas). The transcript level was calculated relative to actin (endogenous reference gene). Mean values ± SD that are marked with the same letters (separately for each cultivar) did not differ significantly at $p < 0.05$ according to Duncan's test, $n = 3$.

Finally, a few comments should be made to the relationship between brassinosteroids and photosynthesis. The regulation of the photosynthesis process by BR is complex, and sometimes stimulatory or inhibitory activity of BR can be, which is dependent on the plant growth conditions or other factors [20–22,48]. In light of our results, it is interesting that the *bri1* mutant of *Arabidopsis* had a downregulation of the genes associated with the regulation of photosynthesis and that it was also characterized by reduced growth, lower photosynthetic activity and a disrupted PSII assemblage [17]. As was mentioned earlier, this *bri1* mutant also had a better tolerance to low temperature than the wild-type plants [46]. Consequently, in our oilseed rape, a lower accumulation of *BRI1* transcript was accompanied by a lower PSII efficiency during cold acclimation (compare Figure 11

and Table S1). The phenomenon was, however, not so clear after deacclimation. Despite the low level of *BRI1* in Rokas, for example, the PSII efficiency in this cultivar was higher compared to the efficiency observed after cold acclimation. Generally, the dependency of the brassinosteroid level, *BRI1* transcript (and protein level) and photosynthetic efficiency in cold-acclimated and particularly deacclimated plants would be an interesting matter for further, more in-depth studies.

To summarize and conclude, frost tolerance of oilseed rape after deacclimation was lower than frost tolerance after cold acclimation. Regarding tolerance to deacclimation, we generally understand that plants maintain a satisfactory level of frost tolerance (as much as possible similar to the level of frost tolerance acquired after cold acclimation) after warm periods that interrupt the process of cold hardening (acclimation) in autumn or after warm periods that appear in winter or even early spring. Tolerance to deacclimation seems to be a cultivar-dependent trait. As presented in the rankings (Table 1), some of the cultivars that had acquired a high frost tolerance after cold acclimation also maintained a high frost tolerance after deacclimation (Rokas). However, there were also cultivars that had a high frost tolerance after cold acclimation but partially lost it after deacclimation (Bojan) and/or cultivars with a lower frost tolerance after cold acclimation (compared to the other cultivars) but that better handled the deacclimation (Pantheon). Cold acclimation resulted in a particular pattern of changes in fluorescence; delayed fluorescence and deacclimation largely reversed those changes. The measurements of the various signals that are associated with photosynthetic efficiency (based on the prompt and delayed chlorophyll fluorescence signals) of plants can be a tool for monitoring the process of deacclimation (and potential changes in frost tolerance) in oilseed rape. However, we have to remember that although the fluorescence parameters of deacclimated plants resembled the control (not acclimated plants), they still had some remaining level of frost tolerance (higher than characteristic for not acclimated plants). This is probably because not all metabolic changes induced by cold acclimation (and responsible for frost tolerance after cold acclimation) were fully reversed during the seven days of deacclimation. Regardless, using drones, unmanned aerial vehicles (UAV) or satellites to monitor the changes in the fluorescence of crop fields, such measurements may enable the moment when plants are deacclimated to be estimated in late autumn/winter/early spring and therefore to be more sensitive to sudden frost. It can even enable precautions, such as spraying plants with regulators to improve their frost tolerance, to be undertaken. A higher content of brassinosteroids was more characteristic for the better frost-tolerant cultivars of oilseed rape in both the case of the cold-acclimated and deacclimated plants. The relative expression of the *BRI1* transcript (an encoding protein of the BR receptor) was lower after cold acclimation and remained low after deacclimation in the cultivars that were more tolerant to frost after deacclimation.

3. Materials and Methods

3.1. Plant Material

The study was conducted on ten cultivars of oilseed rape (*Brassica napus* ssp. *oleifera* L.), which are available for cultivation in Poland—Birdy, Bojan, Darcy, Feliks, Finley, Graf, Monolit, Pantheon, President and Rokas. Among the selected cultivars, nine are winter forms and only Feliks is a spring cultivar, which served as a kind of reference point for the lowest frost tolerance. Moreover, Birdy, Bojan, Darcy, Feliks, Finley, Monolit and Rokas are population cultivars. Graf, Pantheon and President belong to the hybrid (F1) cultivars.

Five of the ten cultivars that were used are characterized in the COBORU database (Development of Polish Official Variety Testing) in terms of plant height, the percentage of dead plants after winter 2015/16 and 2017/18 and the general condition of the plants after winter (nine-point scale of winter survival).

According to COBORU, the height of the plants of each cultivar is as follows: Birdy 139 cm, Bojan 160 cm, Feliks 121 cm, Graf 140 cm, Monolit 138 cm and Rokas 131 cm. As for other cultivars, we obtained general information from the breeder (Saatbau Poland),

that the Pantheon and President cultivars are characterized by a tall plant, while the Darcy and Finley cultivars are semi-dwarf.

As was mentioned above, for some of the cultivars, data about winter survival are also available in the COBORU database. The percentage of dead plants after the winter of 2015/16 was 59% for Birdy, 41% for Graf, 21% for Monolit and 33% for Rokas. The percentage of dead plants after the winter of 2017/18 was 26% for Birdy, 19% for Monolit and 8.4% for Rokas. The general condition of the plants after the winter (on a nine-point scale) was 7.2 for Birdy, 8.0 for Bojan, 6.4 for Graf, 7.2 for Monolit and 7.0 for Rokas.

The seeds of the Bojan, Monolit and Feliks cultivars were derived from The Plant Breeding and Acclimatization Institute (IHAR), the National Research Institute in Strzelce (Poland). The seeds of the Darcy, Finley, Pantheon and President cultivars were derived from Saatbau (Poland). The seeds of Rokas were derived by Syngenta (Poland). The seeds of Birdy were derived from KWS (Poland), and the seeds of Graf were derived from the Obrol company (Poland).

3.2. Experimental Model/Plant Growth Conditions

Similar to the details that were described in an earlier work [4], the seeds were sown in Petri dishes in the dark at 24 °C (two days) for germination. The seeds were then transferred into 90 pots (40 × 15 × 15 cm; 18 plants/pot) with a soil mixture: the universal soil "Eco-Ziem Universal soil" (Eko-Ziem s.c., Jurków, Poland) pH = 5.5–7, the soil from the cultivation plots at the University of Agriculture (Kraków) and sand (2:1:1). The plants were cultured in a growth chamber in a controlled environment for three weeks (17 °C day/night (d/n); 12 h photoperiod). Three plants were removed from every pot to obtain a group of 15 plants of a uniform size. Next, for the pre-hardening, the plants were grown at 14 °C d/n (12 h photoperiod; two days); 12 °C d/n (8 h photoperiod; three days) and 10 °C d/n (8 h photoperiod, two days). Then, the temperature was lowered to 4 °C (8 h photoperiod, three weeks) for cold acclimation. Next, the plants were deacclimated at 16 °C/9 °C, d/n (8 h photoperiod, one week). The light intensity in the growth chamber was the same (at a canopy level of 300 µmol $m^{-1}s^{-1}$) during the entire experiment. Light source—LED lamps HORTI A (PERFAND LED, Trzebnica, Poland), which had been modified to emit light at a constant intensity and a blue light: red light spectrum (46%:54%). The experiment was conducted in the autumn/winter season (November/December 2020).

During the experiment, fluorescence measurements were taken three times: (1) after three weeks of growth at 17 °C (non-acclimated plants—control group), (2) after growth at 4 °C (cold-acclimated plants) and finally (3) in the deacclimated plants. The leaf samples for the analyses of the brassinosteroid content and transcript *BRI1* accumulation were collected at the same time points. After each time point, some of the pots with the plants were selected and placed in a freezing chamber to conduct the frost tests and to estimate the frost tolerance of the plants (for more details, see Section 3.3).

3.3. Testing of Freezing Tolerance of Cultivars

The test of the freezing tolerance was performed for each group of plants (treatments): non-acclimated, cold-acclimated and deacclimated plants (NA, CA and DA plants). The temperatures of freezing were selected based on the experience of the authors and were matched to the predicted level of frost tolerance of a specific group of plants. The non-acclimated plants were tested at −1, −3 and −5 °C; the cold-acclimated plants were tested at −10, −13 and −16 °C and the deacclimated plants were tested at −6, −9 and −12 °C. All of the pots were placed into the freezing chamber at 2 °C in darkness. Next, the temperature was decreased by 3 °C per hour until the required temperature (frost) was reached. The plants were kept at this temperature for 6 h. Afterward, the temperature was increased by 3 °C per hour to reach 2 °C, and the plants were transferred into the growth chamber at 12 °C (8 h photoperiod, 150 µmol m^{-1} s^{-1} light intensity). After two weeks of growth at 12 °C, the plant survival rate was evaluated using a visual score of:

0—a completely dead plant with no signs of leaf growth;

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0—a completely dead plant with no signs of leaf growth;

1—a dead plant without leaves (dead leaves had dropped). There was some small elongation of the leaves that were growing from the apical bud before it had died;

2—a plant that might not survive; there were leaves from the apical bud but they were small, discolored, or deformed;

3—a plant that has a chance of surviving but is badly injured (75% of the leaves are dead), elongation occurred, new leaves are green but their blades are small;

4—a plant that has survived but has severe injuries; about 50% of its leaves are dead (brown and shriveled) or with necrosis spots, leaf elongation occurred, new leaves are green and healthy;

5—a plant that is alive, but some symptoms of freezing injuries are visible; about 25% of the leaves have visible damage such as drying around their edges, yellowing or with necrosis spots;

6—a plant where only 5% to 10% of the leaves show minor symptoms of freezing injuries such as small necrosis spots or dried leaves edges;

7—a plant with no symptoms of injury.

Because there are some morphological/architectural differences between younger (non-acclimated) and older (cold-acclimated and deacclimated) plants that are more compact and shorter; photographs of exemplary plants at scale are presented separately for the non-acclimated plants (Figure 12A) and cold-acclimated and deacclimated plants (Figure 12B).

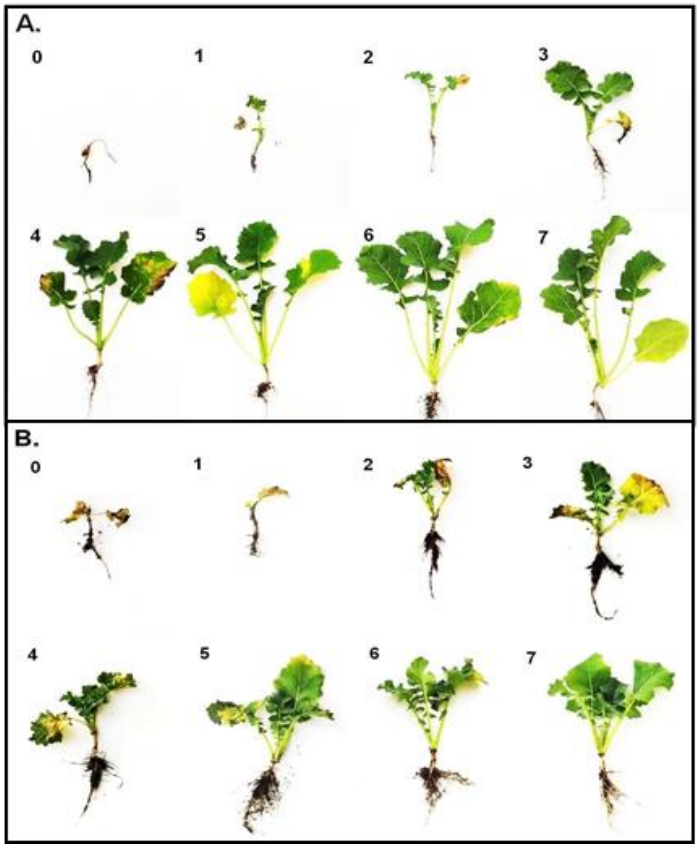

Figure 12. Scale showing the plant injuries after 14 days of their regrowth from the moment of frost exposure; (**A**) non-acclimated plants (**B**) cold-acclimated and deacclimated plants. See more details including an explanation of points 0–7 in Section 3.3.

Finally, based on data of the frost tolerance for all three groups of plants (treatments NA, CA, DA), the estimated temperature that was required to reduce plant regrowth by

50% (RT50) was calculated as was described earlier in [48], and the cultivar ranking is presented in Table 1.

3.4. Chlorophyll a Fluorescence Measurements

Chlorophyll *a* fluorescence was measured using a MPEA+ Multi-Function Plant Efficiency Analyser (PEA, Hansatech Ltd., King's Lynn, UK) for the analyses of photosystem I (PSI) and photosystem II (PSII). This apparatus measures prompt fluorescence, delayed fluorescence and modulated reflectance.

The leaves were adapted to dark for 30 min. using special clips. The measurements were taken in 15 replicates for each cultivar and treatment, using one leaf of an individual plant as one replicate. The measurements were always taken on the best-developed healthy leaves for a specific treatment (non-acclimated, cold-acclimated and deacclimated plants). Fluorescence measurements were taken for all ten cultivars, but more detailed analyses are presented in the article for four cultivars (Rokas, Feliks, Pantheon and President). Cultivars were selected based on calculated RT50 (Table 1).

The fluorescence signal was recorded with a maximum frequency of 105 points s^{-1} (each 10 ms) within 0–0.3 ms. In the next steps, the frequency of the recording decreased gradually, which resulted in the collection of 118 points within 1 s. The PF kinetics of the fluorescence were described by a fluorescence induction curve (fluorescence transient) in which its normalized analysis is known as the JIP-test [35,49]. The minimum curve is Fo, which is the initial fluorescence level measured at time 0.05 ms after actinic light, and maximum is Fm when the saturating light is applied to the leaf. There are also characteristic points between the minimum and maximum, which are labeled K (300 µs), J (2–3 ms), I (30 ms) and P (500–800 ms—1 s). The OJIP transients were induced by a short pulse (1 s) of saturating red light (650 nm and 3000 µmol s^{-1} m^{-2} of intensity) and were plotted on a logarithmic time scale.

In order to better visualize the effect of salt stress on the dynamics of the chlorophyll transients, the relative variable fluorescence was calculated. In the next stage, the differences in the relative variable fluorescence curves were calculated by subtracting the normalized fluorescence values (between the O and P steps) recorded in the control plants and plants under stress. The relative variable fluorescence intensity (V_t) and the difference of the relative variable fluorescence intensity (ΔV_t) were also calculated:

$$V_t = (F_t - F_o)/(F_m - F_o) \quad (1)$$

$$\Delta V_{t,\,NA} = V_{t,CA\ or\ DA} - V_{t,\,control} \quad (2)$$

$$W_{(O\text{-}J)} = (F_t - F_o)/(F_j - F_o) \quad (3)$$

$$W_{(O\text{-}K)} = (F_t - F_o)/(F_k - F_o) \quad (4)$$

$$W_{(J\text{-}I)} = (F_t - F_j)/(F_i - F_j) \quad (5)$$

$$W_{(I\text{-}P)} = (F_t - F_i)/(F_p - F_i) \quad (6)$$

$$\Delta W_{t,\,NA} = W_{t,CA\ or\ DA} - W_{t,\,control} \quad (7)$$

The JIP-test was also used to recalculate the characteristic points of the photoinduced chlorophyll fluorescence transients to the specific parameters of the light phase of photosynthesis [49].

The DF and MR signals were recorded simultaneously with the PF. The characteristic points (I_1, I_2 and D_2) of the DF curve were assessed according to [39]. The I_1 point is the first maximum of a curve, the I_2 point is the second maximum, and D_2 is the second minimum of the curve. To better illustrate the changes of the DF induction curves, two ratios were calculated: $(I_1 - D_2)/D_2$ and I_1/I_2.

The ratio MR_t/MR_o was assessed from the MR signal. MR_t is the modulated 820 nm reflection intensity at time t, and MR_o is the value of the 820 nm reflection of a sample

at the onset of the actinic illumination. MR_{min} is the minimum of the modulated 820 nm reflection intensity. MR_{max} is the maximum of the modulated 820 nm reflection intensity.

In order to better compare non-acclimated, cold-acclimated and deacclimated plants, the technical parameters were extracted from the curves of the fast fluorescence kinetic of chlorophyll *a*. The technical parameters and fluorescence parameters calculated based on them are presented on radar plots (Figure 7) for the four cultivars (Feliks, Pantheon, President and Rokas): T_{fm}, Area, F_o, F_m, F_v/F_m, V_J, Sm, N, ABS/RC, DI_o/RC, TR_o/RC, ET_o/RC, RE_o/RC, ABS/CSm, DIo/CSm, TR_o/CSm, ET_o/CSm, RE_o/CSm and PI_{abs}. T_{fm}—time to reach maximal fluorescence (F_m); Area—the area above the fluorescence induction curve from Fo to Fm; value is proportional to the size of the electron acceptors; F_o—minimal fluorescence when all PSII reaction centers are open; Fm—maximal fluorescence when all PSII reaction centers are closed; F_v/F_m—maximum quantum yield of the photosystem II primary photochemistry; V_J—relative variable fluorescence in step J (after 2 ms); Sm—normalized total area above the OJIP curve; N—turnover number (the number of Q_A reductions from time 0 to T_{fm}); ABS—energy absorption by the antenna chlorophylls, TR_o— trapping flux leading to Q_A reduction; ET_o—electron transport flux (further than Q_A^-); DI_o—dissipation of energy as heat; RE_o—electron transport beyond PSI; PI_{abs}—represents the general performance of PSII. Parameters (ABS, TR_o, ET_o, DI_o) were expressed on the CS_m (the sample cross section) (phenomenological energy flues) and on the reaction center (RC) (specific energy fluxes). The detailed equations for particular parameters can be found in [36,50], and they are also presented in Table 2.

In Figure 7, the values for the non-acclimated plants are expressed as 100%, while the values given for the cold-acclimated and deacclimated plants are given as the percent changes compared to 100%. Original, average values of selected parameters (F_v/F_m, ABS/RC, TR_o/RC, ET_o/RC, DI_o/RC, ABS/CSm, TR_o/CSm, ET_o/CSm, DI_o/CSm, PI_{abs}) for all ten cultivars are additionally presented in Table S1.

3.5. Analysis of the Brassinosteroid Content

BR analysis was performed as described in [51]. Plant leaves were powdered in liquid nitrogen, and then 60% acetonitrile was added. The samples were enriched by deuterium-labeled internal standards of brassinosteroids (25 pmol/sample, Olchemim s.r.o., Olomouc, Czech Republic). The samples were centrifuged, and the supernatant was passed through Discovery DPA-6S columns (Supelco, Bellefonte, PA, USA) and immunoaffinity (IA) columns (Laboratory of Growth Regulation, Olomouc, Czech Republic). The brassinosteroids were eluted from the IA columns using cold 100% methanol. The samples were dried and resuspended in 40 µL of methanol in order to measure the brassinosteroid content on a UHPLC using a tandem mass spectrometer (UHPLC-MS/MS) with an ACQUITY UPLC® I-Class System (Waters, Milford, MA, USA) and a Xevo™ TQ-S MS triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK). The detailed conditions of measurements are given in [51,52]. The analyses were performed in five repetitions, and each repetition included about 100 mg of fresh weight of the first and second leaf in the case of non-acclimated plants, the second and third leaf in the case of cold-acclimated plants and the fourth or fifth leaf of the deacclimated plants. This was because the period of growth of a culture was a total of about three months, and therefore, new leaves were developing systematically while the first leaves became senescent.

3.6. BRI1 Transcript Accumulation

The Quantitative Real Time PCR analysis for *BRI*1 expression was performed using QuantStudio 3 (ThermoFisher Scientific, Waltham, MA, USA). After collection, the leaf material was frozen in liquid nitrogen. RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) was used for the RNA extraction and was enriched for mRNA according to the manufacturer's protocol. Each RNA sample concentration and quality were determined spectrophotometrically (UV–Vis Spectrophotometer, Quawell, San Jose, CA, USA). Approximately 700 ng of RNA was subjected to a genomic DNA elimination, and immediately afterward, a re-

verse transcription reaction was performed (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany), according to the manufacturer's protocol. The PCR primers and probes for *BRI*1 and actin *Brassica napus* genes (Table S5) were designed using Primer Express Software v.3.0.1 (Applied Biosystems by Life Technologies, Foster City, CA, USA). The PCR amplifications for *BRI*1 and *Actin* as the endogenous control genes were conducted in triplicate as was described by [53]. The PCR data were analyzed using QuantStudio Design and Analysis Software v.1.5.0. The relative standard curve method (Applied Biosystems) was used to calculate the relative gene expression. The *BRI*1 expression level was determined relative to the *actin*. The analyses were performed in three biological repetitions, and for each repetition, four technical repetitions were made. The leaf material (50 mg per sample) was collected as follows: fresh weight of first and second leaf in the case of the non-acclimated plants, the second and third leaf in the case of the cold-acclimated plants and the fourth or fifth leaf of the deacclimated plants. This was because the period of growth of a culture was a total of about three months, and therefore, new leaves were developing systematically while the first leaves became senescent.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/ijms23095224/s1.

Author Contributions: J.S. and M.R. cultured the plants and took the fluorescence measurements; J.S. and E.P. performed the frost tests and took the photos; H.M.K. and P.D. calculated, visualized and interpreted the results of the fluorescence measurements; J.S. prepared the figures/tables under the supervision of A.J.; J.O. analyzed the brassinosteroids, B.J., J.S. and M.R. analyzed the *BRI1* transcript accumulation (including the method for the optimization); J.S., A.J., H.M.K., P.D., E.P., M.R. and B.J. participated in writing the manuscript; A.J. conceived the studies and participated in experimental design, article vision, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Table S1. Values of selected parameters of chlorophyll *a* fluorescence (Photosystem II efficiency) of non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) plants of oilseed rape (ten cultivars). *Average values ±SD marked with the same letters did not differ significantly at p≤0.05 according to Duncan's test, n=15.*

Treatment	Fv/Fm	ABS/RC	TRo/RC	ETo/RC	DIo/RC	ABS/CSm	TRo/CSm	ETo/CSm	DIo/CSm	P.I. $_{ABS}$
Birdy NA	0.826±0.01 a	1.04±0.04 b	0.86±0.04 b	0.58±0.03 b	0.18±0.01 b	27783±793 a	22961±751 a	15676±848 a	4823±161 b	10.05±1.57 a
Birdy CA	0.711±0.06 b	1.44±0.34 a	1.01±0.19 a	0.51±0.03 c	0.43±0.17 b	23080±5330 b	16645±4954 c	8634±2923 c	6435±916 a	2.45±1.79 b
Birdy DA	0.806±0.01 c	1.09±0.12 b	0.88±0.08 b	0.62±0.04 a	0.21±0.04 a	24509±1509 b	19773±1424 b	13956±1335 b	4736±312 b	9.66±2.54 a
Bojan NA	0.822±0.01 a	1.01±0.07 b	0.83±0.05 b	0.59±0.02 a	0.18±0.02 b	27099±1553 a	22291±1388 a	15943±1183 a	4808±264 b	12.12±3.33 a
Bojan CA	0.726±0.11 b	1.61±1.07 a	1.06±0.33 a	0.50±0.08 b	0.54±0.79 a	25450±5352 ab	18914±5551 b	9822±3789 c	6536±993 a	3.20±2.64 c
Bojan DA	0.784±0.03 a	1.24±0.26 ab	0.97±0.16 a	0.62±0.04 a	0.27±0.09 ab	23803±1935 b	18647±1577 b	12138±1788 b	5156±815 b	6.67±3.46 b
Darcy NA	0.827±0.01 a	1.08±0.06 b	0.89±0.04 a	0.65±0.04 a	0.19±0.02 b	27700±1411 a	22914±1328 a	16735±953 a	4786±169 b	12.16±1.59 a
Darcy CA	0.746±0.08 b	1.33±0.47 a	0.96±0.22 a	0.53±0.05 b	0.37±0.27 a	25462±4296 b	19268±4906 b	11488±4200 c	6194±883 a	5.04±3.77 b
Darcy DA	0.807±0.03 a	1.15±0.23 ab	0.93±0.14 a	0.64±0.05 a	0.23±0.09 b	26274±1638 ab	21227±1758 a	14948±2221 b	5047±645 b	10.03±4.08 a
Feliks NA	0.824±0.02 a	1.11±0.10 a	0.92±0.07 a	0.65±0.04 a	0.20±0.04 b	27811±1605 a	22939±1661 a	16430±1870 a	4871±368 b	11.26±3.08 a
Feliks CA	0.763±0.05 b	1.23±0.37 a	0.92±0.20 a	0.52±0.06 b	0.31±0.18 a	26026±3502 b	19981±3711 b	11672±2873 c	6044±928 a	5.01±3.39 c
Feliks DA	0.805±0.02 a	1.15±0.15 a	0.92±0.10 a	0.63±0.04 a	0.23±0.05 b	25789±1244 b	20771±1159 b	14241±1278 b	5018±404 b	8.44±2.86 b
Finley NA	0.822±0.01 a	1.08±0.12 a	0.89±0.09 a	0.61±0.05 a	0.19±0.03 b	26049±1999 a	21418±1781 a	14712±1703 a	4631±401 b	10.20±3.31 a
Finley CA	0.775±0.03 b	1.11±0.25 a	0.86±0.17 a	0.48±0.03 c	0.26±0.09 a	24425±4102 ab	19049±3955 b	10963±3039 b	5376±318 a	4.88±2.36 b
Finley DA	0.810±0.01 a	1.02±0.07 a	0.83±0.05 a	0.58±0.02 b	0.19±0.02 b	23891±1744 b	19365±1485 b	13622±1376 a	4527±332 b	10.39±3.04 a
Graf NA	0.826±0.01 a	1.07±0.05 b	0.88±0.04 a	0.61±0.03 a	0.19±0.01 b	26979±1386 a	22291±1286 a	15400±1048 a	4688±196 b	10.08±1.57 a
Graf CA	0.753±0.05 c	1.20±0.20 a	0.90±0.09 a	0.52±0.04 b	0.31±0.12 a	25344±4126 ab	19266±4377 b	11406±3012 c	6078±509 a	4.37±2.22 b
Graf DA	0.801±0.02 b	1.15±0.25 ab	0.92±0.17 a	0.64±0.05 a	0.23±0.08 b	24749±1574 b	19828±1298 b	13950±1415 b	4921±632 b	9.67±3.50 a
Monolit NA	0.830±0.01 a	1.06±0.08 b	0.88±0.06 a	0.63±0.04 a	0.18±0.02 b	27204±1228 a	22567±1048 a	16178±1005 a	4637±276 b	12.21±3.50 a
Monolit CA	0.750±0.06 b	1.31±0.42 a	0.96±0.22 a	0.49±0.04 b	0.35±0.21 a	26217±3728 a	19852±4142 b	10735±3734 b	6365±924 a	3.78±2.59 b
Monolit DA	0.814±0.02 a	1.10±0.18 b	0.89±0.13 a	0.62±0.05 a	0.21±0.05 b	26485±1863 a	21576±1673 a	15082±1749 a	4909±449 b	10.46±4.23 a
Pantheon NA	0.754±0.21 a	1.89±2.69 a	0.88±0.07 a	0.62±0.05 a	1.01±2.64 a	25527±2180 b	19459±5838 a	13581±4173 a	6068±4533a	9.20±3.75 a
Pantheon CA	0.784±0.04 a	1.17±0.26 a	0.91±0.16 a	0.55±0.06 b	0.26±0.11 a	27648±4017 a	21799±4040 a	13302±2698 a	5850±545 a	5.69±2.74 b
Pantheon DA	0.808±0.02 a	1.08±0.20 a	0.87±0.13 a	0.60±0.04 a	0.21±0.07 a	24775±1634 b	20015±1493 a	14052±1553 a	4760±528 a	10.09±3.10 a
President NA	0.797±0.16 a	1.34±1.39 a	0.87±0.05 a	0.64±0.07 a	0.48±1.41 a	26608±5730 a	22058±5024 a	15879±3630 a	4550±728 c	11.61±3.02 a
President CA	0.788±0.04 c	1.13±0.27 a	0.88±0.16 a	0.52±0.04 b	0.25±0.11 b	27566±3035 a	21811±3253ab	13329±3186 b	5755±686 a	6.47±3.31 c
President DA	0.807±0.02 b	1.14±0.21 a	0.92±0.14 a	0.61±0.04 a	0.22±0.06 ab	26079±1255 b	21037±1099 b	14267±1564 b	5042±507 b	8.80±3.62 b
Rokas NA	0.829±0.01 a	1.01±0.03 b	0.84±0.03 b	0.60±0.03 a	0.17±0.01 b	26253±1201 a	21753±1077 a	15695±850 a	4500±158 c	12.62±1.99 a
Rokas CA	0.739±0.05 c	1.37±0.47 a	1.00±0.27 a	0.53±0.05 b	0.37±0.21 a	24597±4712 a	18328±4414 b	10128±2792 c	6268±887 a	3.40±1.97 c
Rokas DA	0.801±0.02 b	1.09±0.16 b	0.87±0.10 b	0.61±0.04 a	0.22±0.06 b	25097±2117 a	20121±1952ab	14134±1667 b	4976±448 b	9.50±3.19 b

Table S2. Statistical analysis of values of typical transient points O, K, J, I and P of the chlorophyll *a* fluorescence induction curves presented on figure 3 A, C, E and G. Oilseed rape plants of cultivar Feliks, Pantheon, President and Rokas were not acclimated (NA), cold acclimated (CA) and deacclimated (DA). Average values ±SD marked with the same letters did not differ significantly at $p \leq 0.05$ according to Duncan's test.

Treatment	O	K	J	I	P
Feliks NA	4998±407 a	6018±525 a	11397±956 a	14044±1075 a	27848±1646 a
Feliks CA	6173±973 b	7486±1433 b	14227±2632 b	16692±2549 b	24589±6640 b
Feliks DA	5131±440 a	6160±665 a	11515±1083 a	14810±1351 a	25741±1283 a
Pantheon NA	4788±236 a	5729±374 a	11070±889 a	14060±1188 a	25569±2152 a
Pantheon CA	6020±598 b	7339±1010 b	14346±2174 b	17021±2432 b	27553±4097 a
Pantheon DA	4871±565 a	5776±892 a	10723±1468 a	14421±1809 a	24718±1646 a
President NA	4831±142 a	5751±176 a	11152±564 a	14073±789 a	27703±1196 a
President CA	5920±738 b	7197±1131 b	14237±2054 b	16716±2075 b	27564±3039 a
President DA	5181±552 a	6259±912 a	11812±1637 a	20720±1967 c	26070±1255 a
Rokas NA	4605±172 a	5461±259 a	10564±827 a	13556±1158 a	26217±1206 a
Rokas CA	6456±988 b	7895±1711 b	14468±3526 b	16831±3753 b	23544±6869 b
Rokas DA	5030±478 c	5899±695 a	10618±1205 a	14309±1445 a	24241±2116 a

Table S3. Statistical analysis of values of characteristic points of DF curves measured for oilseed rape leaves and presented on figure 7. Oilseed rape plants of cultivar Feliks, Pantheon, President and Rokas were not acclimated (NA), cold acclimated (CA) and deacclimated (DA). Average values ±SD marked with the same letters did not differ significantly at $p \leq 0.05$ according to Duncan's test.

Treatment	I_1	I_2	D_2	I_1/I_2	$(I_1-D_2)/D_2$
Feliks NA	19100±882 a	4301±1892 a	1602±810 a	5.3±2.3 a	14.4±8.1 a
Feliks CA	25278±3945 b	5199±2702 a	3338±1491 b	6.2±3.1 a	8.2±4.6 b
Feliks DA	15968±2005 c	3452±2267 a	863±150 c	7.5±5.5 a	18.2±4.9 a
Pantheon NA	20114±602 a	3503±2321 a	1198± 169 a	8.1±4.1 a	16.3±2.3 a
Pantheon CA	32204±3972 b	10604±6322 b	2180±553 b	4.1±2.3 a	14.5±3.8 a
Pantheon DA	16502±2077 c	3443±2259 a	991±217 a	7.1±4.2 a	16.5±4.8 a
President NA	20758±1500 a	4371±2943 a	1369±376 a	7.2±4.6 a	15.3±4.6 a
President CA	28892±5533 b	8739±6833 b	1768±223 b	5.6±3.5 a	15.4±3.0 a
President DA	15472±2660 c	2278±1516 c	1144±425 a	10.1±6.2 a	14.7±7.6 a
Rokas NA	15096±961 a	3300±2219 a	956±247 a	6.9±4.6 a	15.5±3.3 a
Rokas CA	22298±3949 b	4388±1949 a	2594±431 b	5.7±1.9 a	7.9±2.4 b
Rokas DA	16644±886 a	2331±1494 a	910±202 a	10.1±5.7 a	17.9±3.3 a

Table S4. Statistical analysis of the modulated infrared light reflection at the 820 nm (MR820) signals measured for oilseed rape leaves (curves presented on figure 8). Oilseed rape plants of cultivar Feliks, Pantheon, President and Rokas were not acclimated (NA), cold acclimated (CA) and deacclimated (DA). Average values ±SD marked with the same letters did not differ significantly at $p \leq 0.05$ according to Duncan's test.

Treatment	MR_0	MR_{min}	MR_{max}
Feliks NA	0.00044 a	-0.00874 a	0.00240 a
Feliks CA	0.00006 b	- 0.00813 a	0.00100 b
Feliks DA	0.00013 a	- 0.00764 a	0.00125 b
Pantheon NA	0.00002 a	-0.00720 a	0.00182 a
Pantheon CA	-0.00010 b	-0.00734 a	0.00009 b
Pantheon DA	0.00009 a	-0.00715 a	0.00725 c
President NA	0.00101 a	-0.00673 a	0.0128 a
President CA	0.00011 b	-0.00749 a	0.00114 a
President DA	-0.00001 c	-0.02026 b	0.00077 b
Rokas NA	0.00005 a	-0.00726 a	0.00163 a
Rokas CA	0.00006 a	-0.00683 a	0.00010 b
Rokas DA	0.00012 a	-0.00847 a	0.00136 c

Table S5. Genes, sequence origins and the designed primers and probes used in the study.

Gene name	**GenBank ID**	**Forward primer**	**Reverse primer**	**Probe**
*BRI*1	JX871217.1	GATGTTCAAGCAATCCGGGAAAA	TCTTTCTTCATCCCGTCGTTTTTTATGTA	FAM-TCGCTGTGAATTTCA-NFQ
Actin	AF111812.1	GCTATCCTCCGTCTCGATCTC	GTGGTGAACATGTACCCTCTCT	FAM-ACCTCACTGATTCCC-NFQ

OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 10.05.2024 r.

Dr Magdalena Ryś

Zakład Biologii Rozwoju

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Oświadczam, że w pracy: Stachurska, J., Rys, M., Pociecha, E., Kalaji, H.M., Dąbrowski, P., Oklestkova, J., Jurczyk, B., Janeczko, A. (2022) Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and BRI1 Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance. International Journal of Molecular Sciences, 2022, 23, 5224 mój udział polegał na: współpracy z doktorantką (J. Stachurską) przy zakładaniu i prowadzeniu doświadczenia, współpracy przy pomiarach fluorescencji chlorofilu *a* i analizie akumulacji transkryptu *BRI1*.

Magdalena Ryś

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Kraków, 11.06.2024 r.

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Ewa Pociecha

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……

(czytelny podpis współautora)

STATEMENT OF THE CO–AUTHOR

Olomouc, 3.5.2024

Mgr Jana Oklestkova, Ph.D.

Laboratory of Growth Regulators

Palacký University Olomouc & Institute of Experimental Botany AS CR

I declare that in work: Stachurska, J., Rys, M., Pociecha, E., Kalaji, H.M., Dąbrowski, P., Oklestkova, J., Jurczyk, B., Janeczko, A. (2022) Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and BRI1 Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance. International Journal of Molecular Sciences, 2022, 23, 5224 my participation was: to analyse the content of brassinosteroids in oilseed rape with UHPLC–MS/MS technique.

Jana Oklestkova

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(czytelny podpis współautora)

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Kraków, 10.06.2024 r.

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OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 10.05.2024 r.

Prof. dr hab. inż. Anna Janeczko

Zakład Biologii Rozwoju

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.....

(czytelny podpis współautora)

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Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.)

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Abstract: The aim of the current work was to characterize disturbances in the hormonal balance and changes in the accumulation of the protective heat shock proteins (HSP) as a result of deacclimation in a few cultivars of oilseed rape. Samples for both analyses were collected from plants that had not been acclimated (before cold acclimation—control), cold acclimated (at 4 °C d/n, three weeks) and then deacclimated at 16/9 °C d/n (one week). The tested hormones included abscisic acid, jasmonic acid, salicylic acid, gibberellins, auxins and cytokinins (including their precursors, intermediates and conjugates). Unambiguous results were obtained for a stress hormone, abscisic acid, whose concentration increased in the leaves of all of the tested cultivars during cold acclimation while it strongly decreased during deacclimation. Deacclimation resulted also in an elevated level of the typical growth hormones. As a result of cold acclimation, the accumulation of protective proteins such as cytoplasmic HSP70 and HSP90 increased in three of the four tested cultivars. The HSP content most often decreased in the deacclimated plants compared to the cold-acclimated plants. The hormonal and protein changes are discussed relative to the frost tolerance changes of the tested cultivar.

Keywords: ABA; auxins; cytokinins; deacclimation; gibberellins; HSP70; HSP90; indole-3-carboxylic acid

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1. Introduction

Cold acclimation (cold hardening) is a low-temperature-induced process that is especially important/characteristic for winter cultivars of crops because their vegetation season includes the autumn/winter months and, thus, requires specific metabolic adjustments. After a few weeks of growth at a temperature between +2 °C to +5 °C (in autumn), well cold-acclimated crop plants can survive temperatures that are as low as −20 °C, especially when they are under snow cover [1,2]. The process of cold acclimation of winter crops occurs in autumn. However, due to changes in climate conditions, in some regions where winter crops are cultivated, periods of warm breaks that interrupt the cold-hardening process are occurring more and more often. This phenomenon is called deacclimation (dehardening) and it disturbs the natural process of acquiring a high level of frost tolerance. Deacclimation can also occur in the middle of winter or in very early spring when the temperature starts to rise and plants begin to resume their growth and development, thereby losing their frost tolerance. In such a case, the sudden occurrence of even a light frost during that time is dangerous and could cause frost injuries. The negative effects of a seven-day deacclimation on a decrease in the frost tolerance of winter oilseed rape were characterized in detail in the work [3]. According to [4], deacclimation becomes "a crucial, but widely neglected" part of the problems that are associated with the winter survival of plants.

Cold acclimation triggers many biochemical and physiological changes in plant cells; for example, the most known are changes in the composition of fatty acids, changes in carbohydrate management and in the osmotic potential, the stimulation of the production

of protective proteins and an elevated level of stress hormones [5,6]. Deacclimation, on the other hand, is dangerous because it can reverse these metabolic adjustments. Although climate changes have led to more studies that are devoted to the detailed biochemical changes that accompany deacclimation, knowledge about these changes is still quite scarce.

One of the winter crops that is affected by deacclimation is oilseed rape—a plant that is mainly cultivated as the major source of vegetable oil. Our earlier studies confirmed that during cold hardening of this species, even one week of a warm break at a temperature of 16 °C/9 °C (d/n) had a reverse effect on metabolism. The chemical composition of the leaves, which was measured using FT-Raman spectroscopy, clearly confirmed that there were metabolic differences between the cold-acclimated and deacclimated plants [7]. Deacclimation increased the photosystem II efficiency that was suppressed by cold acclimation [3,7]. The content of soluble sugars was drastically decreased after deacclimation [7], which was accompanied by changes in the osmotic potential in a direction that was not beneficial from the point of view of frost tolerance. The leaf relative water content also increased after deacclimation [7]. Cold hardening also increased the accumulation of proteins BnPIP1 (aquaporin), while deacclimation decreased it [7]. The current work is a continuation of the studies of the deacclimation process of oilseed rape in which, as the next step, we are going to focus on a detailed analysis of hormonal homeostasis and any potential changes in the accumulation of the protective proteins from a group of heat shock proteins (HSP).

All metabolism of plants is controlled by hormones, and hormonal homeostasis is specifically linked to plant growth conditions. External factors such as temperature, light or water availability modify hormonal management, thereby allowing the metabolism to adapt to changing environmental conditions. Hormones, such as cytokinins, gibberellins, auxins, abscisic acid or brassinosteroids (and the interactions among them), play an important role in the growth/development of plants and in plants' reaction to various stressors [8–10]. Hormonal changes that occur during the cold acclimation of plants are important for the survival of plants in low temperatures. A higher level of ABA with a lower level of bioactive cytokinins, auxins and gibberellins was observed in wheat cultivars during cold acclimation [11]. In cold-acclimated oilseed rape leaves discs, the exogenous application of gibberellin GA_3 decreased the frost tolerance, while the application of ABA increased the frost tolerance [12]. The exogenous use of the auxin analogues TA-12 (calcium 4-(2-chloroethoxycarbonylmethyl)-1-naphthalenesulfonate) and TA-14 (ω-trialkylammonioalkyl ester of 1-napthylethanoic acid) on oilseed rape improved the winter hardiness of plants [13]. The exogenous application of jasmonate improved the freezing tolerance of *Arabidopsis thaliana* L. while blocking endogenous biosynthesis. and the signaling pathways of jasmonate caused plants to be hypersensitive to freezing stress [14]. While these are only a few examples, generally, there is a wealth of knowledge about the activity and significance of plant hormones in cold acclimation. However, the hormonal balance during deacclimation and its role in the changes in the frost tolerance of deacclimated plants is quite limited and relatively new. According to the literature, during the deacclimation of *A. thaliana* L., there was an overexpression of the genes related to the metabolism of auxins, gibberellins, brassinosteroids, jasmonate and ethylene [15]. Deacclimated plants of barley (*Hordeum vulgare* L.) were characterized by an increased level of hormones from growth-promoting groups such as indole-3-acetic acid (IAA), IAA methyl ester; the level of some gibberellins was also elevated, i.e., GA_6 or cytokinins (trans-zeatin and cis-zeatin), compared to cold acclimated plants [16]. In our earlier studies, the most abundant brassinosteroid (28-homocastasterone) in oilseed rape was accumulated in higher amounts in the cultivars that had maintained a better frost tolerance after deacclimation [3]. Interestingly, the accumulation of the transcript of BRI1 (which encodes the BR-receptor protein) decreased after cold acclimation, and in the more frost-tolerant cultivars, it remained low even after deacclimation [3].

Although heat shock proteins (HSP) are a group of proteins that are produced in plants especially as a reaction to heat stress [17], changing amounts of HSP are also found in

plants growing at room temperature or even cold-stressed plants [18,19]. There are many types of heat shock proteins that differ in their molecular weight from 10 to 200 kDa and perform various functions [20,21]. Among them, the HSP90 proteins are necessary for the proper functioning of all of the eucaryotic cells and assist other proteins in folding, maintaining and stabilizing the cytosolic proteins, including the proteins that are involved in cell cycle control and signal transduction [22]. Another family is HSP70, which stabilizes the precursor proteins and maintains them in an unfolded form [23]. Specific chloroplastic proteins HSP70 were also identified in plants [23]. For example, HSP70, which is found in the stroma, participates in the photoprotection and reparation of PSII during and after photoinhibition [24]; it is also necessary for heat tolerance [25].

As was mentioned earlier, although the heat shock proteins accumulate in plants that are growing under a high temperature stress, their expression increases under different abiotic stresses as well as during cold acclimation [18]; however, this has been much less studied. In grape plants (*Vitis vinifera* L. cv. Jingxiu), during, among others, cold acclimation stress, an increased level of HSP70 and small HSP17.6 was observed. Moreover, the synthesis of the HSP proteins was parallel to an increase in cold tolerance [18]. In oilseed rape plants, an increased level of HSP90 mRNA was observed in young plant tissues such as the shoot apices, as well as after exposure to high and low temperatures. During an exposure to 5 °C, there was a 15-fold increase in the hsp90 mRNA level, which remained elevated during the entire cold treatment. It decreased again when the temperature increased to 20 °C. This change was observed between the 4th and 5th hours after the transfer to 20 °C [26]. Those results illustrate that HSP90 could be significant in the adaptation of plants to low-temperature stress and in their tolerance to low temperatures. Therefore, the questions of whether the deacclimation process causes the changes in the HSP accumulation or if it correlates with a decrease in frost tolerance of plants arises.

The aim of the current work was to characterize the disturbances in the hormonal balance and changes in the accumulation of protective heat shock proteins as a result of deacclimation in an economically important crop plant—oilseed rape. The results are discussed relative to a deacclimation-induced decrease in the frost tolerance of plants.

2. Materials and Methods

2.1. Plant Material

The experiment was conducted on four cultivars of oilseed rape (*Brassica napus* L. var. *napus* L.): Bojan, President, Rokas (winter cultivars) and Feliks (spring cultivar). These cultivars are available to cultivate in Poland. President is a hybrid cultivar (F1) while Bojan, Rokas and Feliks are population cultivars. The cultivars were selected based on a previous experiment in which their frost tolerance was characterized in controlled conditions [3]. After cold acclimation, the plants were characterized by a higher tolerance (in comparison to non-acclimated plants), while after a period of deacclimation, the frost tolerance decreased, although not to the level that was recorded in the non-acclimated control. The control plants after frost (−5 °C) had one point on a seven-point scale of injuries. The cold acclimated plants were able to survive −15 °C (three to four points on the scale of injuries), while the deacclimated plants were already severely injured by a temperature of −12 °C (one to two points on the scale of injuries). There were differences between cultivars. Rokas and Bojan had the highest basal frost tolerance (noted for non-acclimated plants), President was moderately frost tolerant, while Feliks had the lowest basal frost tolerance [3]. After cold acclimation, Rokas and Bojan were highly frost tolerant, while President and Feliks were characterized by a lower frost tolerance. After deacclimation, Rokas remained the most frost tolerant. Bojan exhibited a moderate tolerance to frost, while President and Feliks had the lowest frost tolerance. Among the tested cultivars, Rokas is a semi-dwarf cultivar. The average height of fully-developed Rokas plants, according to COBORU (Development of Polish Official Variety Testing), is 131 cm. The seeds of cultivars Bojan and Feliks were obtained from The Plant Breeding and Acclimatization Institute (IHAR) the National Research Institute in Strzelce (Radzików, Poland). The seeds of the

President cultivars were obtained from Saatbau (Środa Śląska, Poland), and the seeds of the Rokas cultivar were obtained from Syngenta (Warszawa, Poland).

2.2. Experimental Design and Sampling

The experimental model was similar to an earlier model that was described in detail by [3]. Briefly, the seeds of the oilseed rape were germinated in darkness at 24 °C (two days). Then, the seedlings were transferred into pots (18 plants per pot; details regarding soil are given in [3]). The plants were cultured in a growth chamber (20 °C day/night, 12 h photoperiod, four days; then 17 °C d/n, 12 h photoperiod, three weeks). After that, a group of 15 uniform plants was retained in each pot and the plants were pre-hardened at 14 °C d/n (12 h photoperiod, two days); 12 °C d/n (8 h photoperiod, three days) and 10 °C d/n (8 h photoperiod, two days). Then, for the cold acclimation, the temperature was set at 4 °C (8 h photoperiod, three weeks). Next, for deacclimation, the temperature was set at 16/9 °C d/n (8 h photoperiod, one week). The intensity of light was constant during the experiment (300 µmol m^{-1} s^{-1}; modified LED lamps HORTI A provided by PERFAND LED, Trzebnica, Poland; for details see [3]). The experiment was conducted in the autumn/winter seasons. The scheme of the experiment is visualized in Figure S1 (Supplementary Materials).

For all of the hormonal and HSP analyses, samples of leaves were collected from the non-acclimated plants (NA, control group), cold-acclimated plants (CA) and deacclimated plants (DA). During sampling, all tested plants were in vegetative stage-rosette, but there were some architectural differences between younger (non-acclimated) and older (cold-acclimated and deacclimated); older plants were more compact (pictures available in [3]). The best developed rosette leaves (not too young or senescing) were always selected.

2.3. Measurements

2.3.1. Analysis of the Plant Hormones and Related Metabolites

The collected leaf samples were frozen in liquid N_2 and then stored at −80 °C. Analyses were performed according to [27] with modifications. For the analyses of the hormones and related substances, the material was lyophilized and ground (MM 400, Retsch, Kroll, Germany). Weighed samples (10 mg) were spiked with a stable isotope-labelled internal standard solution (ISTD) in acetonitrile (ACN) and then extracted in 1 mL methanol/water/formic acid (MeOH/H_2O/HCOOH, 15/4/1, *v*/*v*/*v*). The samples were shaken for 10 min, sonicated (5 min) and centrifuged (5 min, 22,000× *g*, 15 °C, Universal R32, Hettich, Haan, Germany). The supernatant was collected and evaporated to dryness under an N_2 stream at 45 °C. The residues were dissolved in 1 mL of 3% MeOH in a 1 M aqueous solution of formic acid, sonicated (5 min), centrifuged (5 min, 22,000× *g*, 15 °C) and purified on cartridges (BondElutPlexa PCX, 30 mg, 1 mL, Agilent Technologies, Santa Clara, CA, USA). The cartridges were activated with 1 mL of methanol and 1 mL of 1 M formic acid. The samples were applied to the cartridges, aspirated slowly and washed with 1 mL of 1 M formic acid. The substances of interest were washed out with 0.5 mL of ACN/MeOH (1/1 *v*/*v*), 0.5 mL of 5% NH_3aq in ACN/MeOH (1/1 *v*/*v*) and 0.5 mL of ACN/MeOH (1/1 *v*/*v*) in succession. The collected eluate was evaporated to dryness under nitrogen and dissolved in 70 µL of ACN prior to the UHPLC analyses. Four or five replicates were analyzed using an UHPLC apparatus (Agilent Infinity 1260, Agilent, Germany) that was coupled to a triple quadruple mass spectrometer MS/MS (6410 Triple Quad LC/MS, Agilent, Savage, MD, USA) with electrospray ionization (ESI). The samples were separated on an Ascentis Express RP-Amide analytical column (2.7 µm, 2.1 mm × 150 mm; Supelco, Bellefonte, PA, USA). Further technical details are given in Table 1 and [27–30]. The following phytohormones and related metabolites were detected: cytokinins: cis-zeatin (cis-ZEA) and cis-zeatin riboside (cis-ZEA-rib); auxins: indole-3-acetic acid (IAA), oxoindole-3-acetic acid (OxIAA), indole-3-acetyl-aspartic acid (IAAsp), indole-3-carboxylic acid (I3CA), indole-3-acetonitril (IAN), indole-3-acetyl-glutamic acid (IAGlu), indole-3-acetamid (IAM); gibberellins: gibberellic acid (GA_3), gibberellin A_6 (GA_6), gibberellin A_{20} (GA_{20}), gibberellin A_{19} (GA_{19}), gibberellin A_{53} (GA_{53}), gibberellin A_7 (GA_7), gibberellin A_4 (GA_4), gibberellin A_{15} (GA_{15})

and gibberellin A_9 (GA_9); stress hormones: benzoic acid (BA), salicylic acid (SA), abscisic acid (ABA), jasmonic acid (JA) and 12-oxo-phytodenoic acid (12-oxo-PDA). The following ratios of hormones were calculated: GA_3/ABA, ratio GA_3 + GA_4 + GA_6 + GA_7/ABA and ratio IAA + cis-ZEA + GA_3 + GA_4 + GA_6 + GA_7/ABA + JA.

Table 1. The optimized mass spectrometry parameters that were used to quantify the phytohormones. The following conditions were optimal for the analyses: capillary voltage 4 kV, gas temperature 350 °C, gas flow 12 L/min and a nebulizer pressure of 35 psi. The measurements were performed using multiple reaction monitoring (MRM) in a positive polarity. MassHunter software was used to control the LC-MS/MS system and data analysis. For the MRM parameters, a MassHunter Optimizer was used. The quantities of the internal standards (ISTD) are given in parenthesis. DHZ-N15 and t-Z-R-D5–internal standards for the cytokinins; GA1-D2 and GA5-D2–internal standard for gibberellins; for the other abbreviations, see Material and Methods Section 2.3.1.

Compound		Type of Ion	Quantifier Transition (Precursor/Product Ions)	Fragmentor Voltage (V)	Collision Energy (V)	MRM Start Time (min.)
DHZ-N15	ISTD (10 pmol)	$[M + H]^+$	226.2/152	124	18	1.5
cis-ZEA		$[M + H]^+$	220.2/136.3	85	9	
oxIAA		$[M + H]^+$	192.2/146.1	54	9	4.0
IAM		$[M + H]^+$	175.1/130	66	17	
t-Z-R-D5	ISTD (10 pmol)	$[M + H]^+$	357.3/225.2	116	17	5.12
cis-ZEA-rib		$[M + H]^+$	352.2/220.3	120	9	
IAAsp		$[M + H]^+$	291.2/130.1	54	25	6.4
BA-D4	ISTD (500 pmol)	$[M + H]^+$	128.1/84.1	61	13	
BA		$[M + H]^+$	123.1/79.1	56	13	
IAGlu		$[M + H]^+$	305.2/130.1	58	29	
GA_3		$[M\text{-}H_2O + H]^+$	329.3/311.3	100	14	8.15
GA1-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	333.3/287.2	58	9	
I3CA		$[M + H]^+$	162.2/118.1	58	9	
IAA-D5	ISTD (100 pmol)	$[M + H]^+$	181.1/135.1	38	14	
IAA		$[M + H]^+$	176.1/130.3	51	9	
SA-D4	ISTD (500 pmol)	$[M + H]^+$	143.2/125.2	80	14	
SA		$[M + H]^+$	139.2/121.2	80	14	
GA6-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	331.3/115.1	96	5	10.4
GA_6		$[M\text{-}H_2O + H]^+$	329.3/283.3	104	14	
IAN-D4	ISTD (100 pmol)	$[M + H]^+$	161.1/134.1	66	13	12.0
IAN		$[M + H]^+$	157.1/130.1	71	13	
ABA-D6	ISTD (30 pmol)	$[M\text{-}H_2O + H]+$	253.4/191.3	80	14	14.6
ABA		$[M\text{-}H_2O + H]^+$	247.4/187.2	80	14	

Table 1. *Cont.*

Compound		Type of Ion	Quantifier Transition (Precursor/Product Ions)	Fragmentor Voltage (V)	Collision Energy (V)	MRM Start Time (min.)
GA5-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	287.3/115.0	96	5	15.45
GA_{20}		$[M\text{-}H_2O + H]^+$	287.3/115.0	96	5	
GA_{19}			345.2/299.1	80	9	16.8
JA-D5	ISTD (100 pmol)	$[M + H]^+$	216.3/153.2	80	5	19.5
JA		$[M + H]^+$	211.3/151.2	80	14	
GA_7		$[M\text{-}H_2O + H]^+$	313.2/223.1	104	14	18.5
GA4-D2	ISTD (10 pmol)	$[M\text{-}H_2O + H]^+$	317.3/271.2	88	9	
GA_4		$[M\text{-}H_2O + H]^+$	315.3/269.3	100	14	
GA_{53}			303.2/285.1	100	9	
GA_9		$[M\text{-}H_2O + H]^+$	271.3/225.2	136	13	22.25
GA_{15}			331.2/285.1	115	9	
dinor-12-oxo-OPDA-D5	ISTD (10 pmol)	$[M + H]^+$	270.3/252.2	84	5	
12-oxo-PDA		$[M + H]^+$	293.3/275.2	68	9	24.54

2.3.2. HSP Analysis

Measurements of the Protein Concentration in the Leaf Extracts. The samples that were obtained from leaves (1 g) were homogenized in liquid N_2 and immediately extracted using a Tricine buffer (100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT, 3 mM EGTA, pH = 8.0 and a protease inhibitor (Protease Inhibitor Cocktail Tablets, Roche, Germany)). The samples were centrifuged for five minutes at 38,030× *g* (MIKRO R, Hettich Centrifugen, Tuttingen, Germany). After the centrifugation, the supernatant was collected and the protein concentration in the obtained extracts was measured according to Bradford [31] using a SynergyTM2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Bovine serum albumin (BSA) (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The analysis was performed in three replications.

Analysis of the Accumulation of HSP90 and HSP70 (Cytosolic and Chloroplastic) in the Leaf Samples Using Immunoblotting. The same amount of protein extracts (selected after being optimized in a range of 2.5 µg to 20 µg for HSP70 cytoplasmic; 5 µg to 30 µg for HSP70 chloroplastic and 5 µg to 30 µg for HSP90), which were isolated from tested samples, were loaded and separated on 12% SDS-PAGE (1 mm polyacrylamide gel) according to the procedure of Laemmli [32]. Based on the testing, we decided to use 3 µg of the protein for the HSP70 cytoplasmic, 5 µg for the HSP70 chloroplastic and 10 µg for the HSP90. The samples were diluted with an SDS loading buffer (0.125 mM TRIS pH 6.8, 4% SDS, 20% glycerol, 5% 2-mercaptoetanol, 0.004% bromophenol blue). The molecular weight standard was Thermo Scientific PageRuler Prestained Protein Ladder (Thermo Scientific, Vilnius, Lithuania). After the proteins were separated, they were blotted to nitrocellulose membranes (0.2 µm, Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a BioRad Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the membranes were blocked with 4% low-fat milk diluted in a Tris-buffered saline/Tween (TSB-T) buffer (containing 0.9% NaCl, 10 mM Tris) overnight. Next, the membranes were washed four times for five minutes with a TBS-T buffer and probed in the appropriate antibodies for 1.5 h (Anti-HSP70 cytoplasmic (AS08 371), 1:3000; Anti-HSP70 chloroplastic (AS08 348), 1:2000; Anti-HSP90-1 (AS08 346), 1:3000 (Agrisera, Vännäs, Sweden)). The membranes were washed four times for five minutes with a

TBS-T buffer and then incubated with the appropriate secondary antibody (Alkaline-Phosphate Conjugated Anti-rabbit, for HSP70 cytoplasmic 1:5000; for HSP70 chloroplastic 1:10,000; for HSP90 1:5000 (Sigma-Aldrich, Poznan, Poland)) for 1.5 h. Dilutions of the antibodies were selected based on the previous optimalization process and the protocol of the manufacturer. Three independent repetitions (both biological and technical) were performed. Densitometric analyses were performed to measure the protein content using ImageJ software (NIH, Bethesda, MD, USA). The averages are expressed as arbitrary units (A.U.) correlated with the area under the densitometric curves. Exemplary blots are presented in Figure S2 (Supplementary Materials).

2.4. Statistical Analyses

All of the statistical analyses (ANOVA, post-hoc Duncan's tests) were conducted using Statistica 13.1 software (StatSoft, Tulsa, OK, USA). For a specific hormone or protein, the non-acclimated, cold-acclimated and deacclimated plants (within each cultivar separately) were compared. The average data are presented as ±SD. Values that are marked with the same letters did not significantly differ according to the Duncan's test ($p < 0.05$).

3. Results

3.1. Hormonal Analyses

Twenty-three hormonal compounds were identified in the group of growth-promoting hormones and stress hormones (active forms, precursors, metabolites, conjugates) in all of the tested cultivars.

Five hormones (more characteristic for the plant stress response) and their precursors were identified: abscisic acid, 12-oxo-phytodenoic acid, jasmonic acid, benzoic acid and salicylic acid (Figure 1A–U). The concentration of abscisic acid (ABA) increased (from 485% in cultivar Bojan to 963% in cultivar Rokas) in all of the cold-acclimated plants (compared to the non-acclimated plants) and then decreased once again in the deacclimated plants. However, the ABA level in the DA plants was still significantly higher than in the NA plants (Figure 1A–D). The abscisic acid (ABA) content had the same, statistically significant pattern of changes in all of the tested cultivars.

Changes in the content of 12-oxo-phytodenoic acid (a precursor of jasmonic acid) were also observed. Lower amounts of this phytohormone were detected after cold acclimation of all of the cultivars from 51% to 77% (Figure 1E–H). After deacclimation, the 12-oxo-PDA content once again increased in three cultivars, the exception was Rokas, in which there were no changes between the CA and DA plants (Figure 1H). As for the active form (jasmonic acid), in the CA plants (Bojan, Feliks), its content was visibly higher by an average of 327% (Figure 1I,J). In President, there was a slight tendency of JA to increase, while in Rokas, no changes were observed between the NA and CA plants. Deacclimation reduced content of JA in Bojan and Feliks to the level that was observed in the NA plants. There was an opposite effect for the deacclimated plants of President and especially Rokas, in which an higher content of JA was observed (Figure 1K,L).

A lower amount of benzoic acid was detected in the cold-acclimated Bojan, Feliks and Rokas plants by an average of 11% compared to the non-acclimated plants. In President, the tendency was similar but statistically insignificant. After the deacclimation process, the effects of cold was generally reversed (Figure 1M–P). There were no differences in the level of salicylic acid in the cold-acclimated cultivars of Bojan, Feliks and President (Figure 1R–T). A decrease in the level of salicylic acid in the cold-acclimated plants was detected only in Rokas (Figure 1U). In all of the cultivars, there were no statistically significant differences in the SA level between the CA and DA plants.

(Figure 1R–T). A decrease in the level of salicylic acid in the cold-acclimated plants was detected only in Rokas (Figure 1U). In all of the cultivars, there were no statistically significant differences in the SA level between the CA and DA plants.

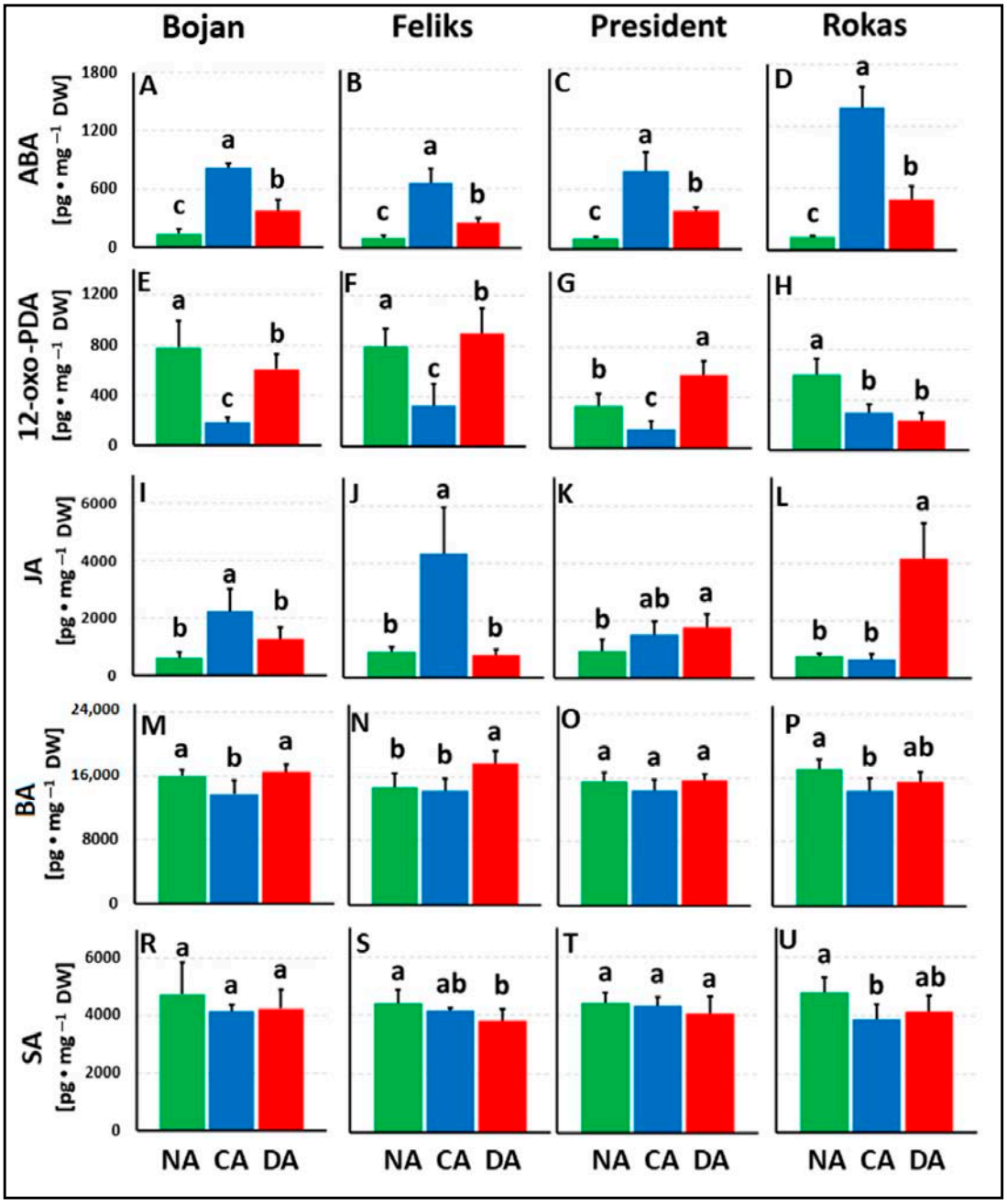


Figure 1. Content of the stress hormones: abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA) and selected precursors (12-oxo-phytodienoic acid (12-oxo-PDA) is a precursor of JA; benzoic acid (BA) is a precursor of SA) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—ABA, (**E**–**H**)—12-oxo-PDA, (**I**–**L**)—JA, (**M**–**P**)—BA, (**R**–**U**)—SA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \geq 0.05$).

The following gibberellins were identified in our studies: GA_3, GA_4, GA_6, GA_7, GA_9, GA_{15}, GA_{19}, GA_{20} and GA_{53}. In the presentation of the results, the gibberellins were divided into two groups according to their biosynthetic pathways [33]. GA_{15}, GA_9, GA_4 and GA_7 are presented in Figure 2A–P, while GA_{53}, GA_{19}, GA_{20}, GA_3 and GA_6 are presented in Figure 3A–U.

In Bojan and President plants, the level of GA_{15} decreased after cold acclimation (54% and 55%, respectively), but after deacclimation, it increased to a level that was similar to the non-acclimated plants (Figure 2A,C). In the Feliks and Rokas plants, there were no

in the tested cultivars between the NA, CA and DA plants (Figure 2F–H); the exception was a slightly, although statistically significant, lower level of this compound in the CA Bojan plants (Figure 2E).

Cold acclimation generally reduced the concentration of GA_4 and GA_7 (Figure 2I–P); deacclimation more or less reversed this effect in almost all of the studied cultivars, which was particularly visible in the case of GA_7 (Bojan, President and Rokas; Figure 2M,O,P).

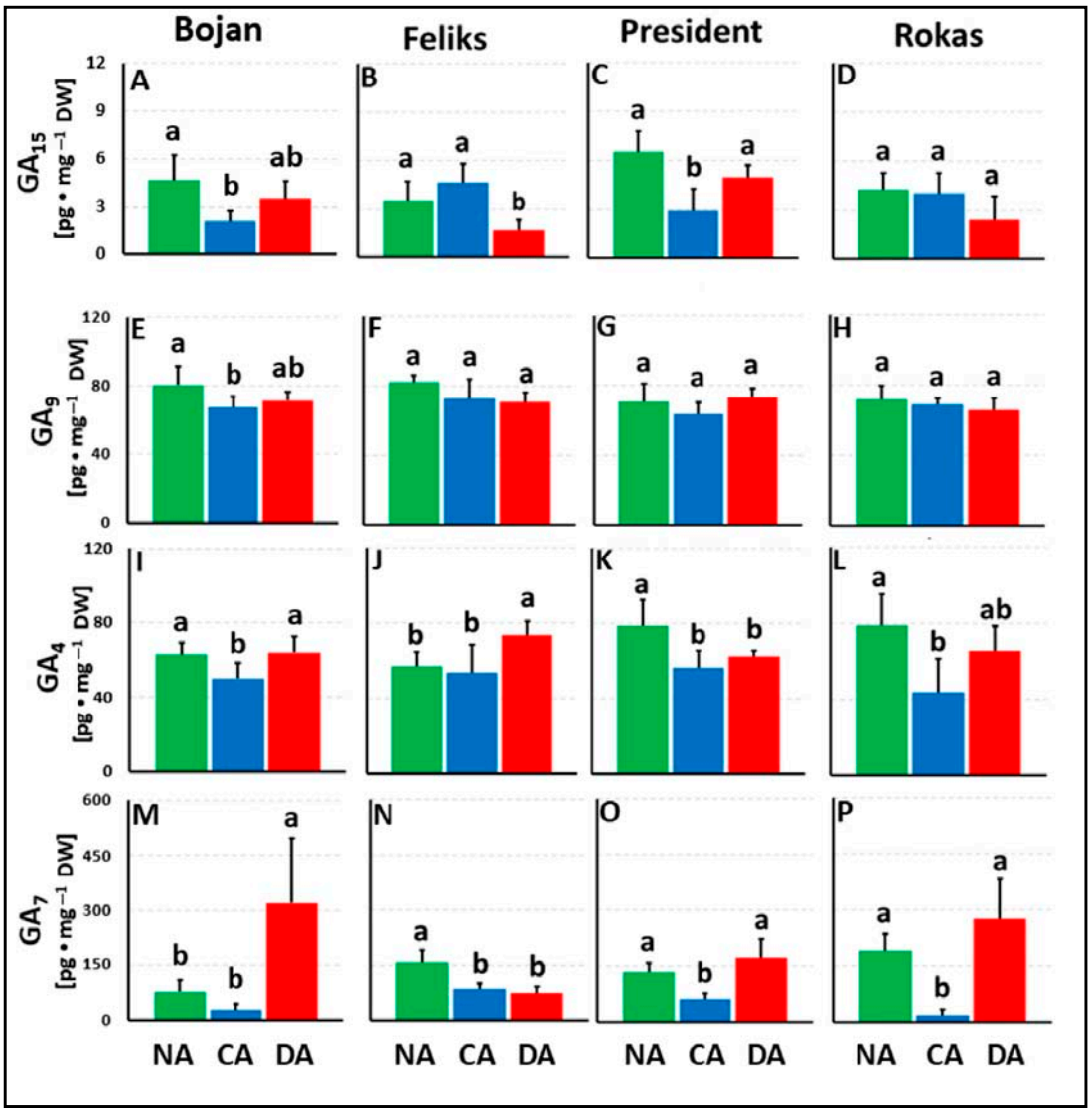


Figure 2. Content of the gibberellins GA_{15}, GA_9, GA_4 and GA_7 in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The gibberellins are presented in order based on the biosynthetic pathway from the precursor GA_{15} via GA_9 to their active forms GA_4 and GA_7 (according to [34]). (**A**–**D**)—GA_{15}; (**E**–**H**)—GA_9; (**I**–**L**)—GA_4; (**M**–**P**)—GA_7. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

Figure 2. Content of the gibberellins GA_{15}, GA_9, GA_4 and GA_7 in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The gibberellins are presented in order based on the biosynthetic pathway from the precursor GA_{15} via GA_9 to their active forms GA_4 and GA_7 (according to [34]). (**A**–**D**)—GA_{15}; (**E**–**H**)—GA_9; (**I**–**L**)—GA_4; (**M**–**P**)—GA_7. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

The tendencies of changes in the concentration of GA_{53} were similar in the Bojan and President cultivars, which accumulated more GA_{53} after cold treatment (compared to the NA plants) by approximately 146% and 534%, respectively (Figure 3A,C). In the Feliks plants, there was no difference in the GA_{53} level between the NA and CA plants, while in the Rokas plants GA_{53} decreased after cold (Figure 3B,D). After deacclimation, the level of this phytohormone was similar to the level in NA plants (Figure 3A–D). The exception was a slightly, although statistically significant, lower level of this compound in the CA Bojan plants (Figure 2E).

Cold acclimation generally reduced the concentration of GA_4 and GA_7 (Figure 2I–P); deacclimation more or less reversed this effect in almost all of the studied cultivars, which was particularly visible in the case of GA_7 (Bojan, President and Rokas; Figure 2M,O,P).

In Bojan and President plants, the level of GA_{15} decreased after cold acclimation (54% and 55%, respectively), but after deacclimation, it increased to a level that was similar to the non-acclimated plants (Figure 2A,C). In the Feliks and Rokas plants, there were no differences in GA_{15} between the NA and CA plants; however, after deacclimation, the GA_{15} content had a tendency to decrease (but statistically significantly only in Feliks) (Figure 2B,D). Generally, we did not detect any significant changes in the amounts of GA_9 in the studied cultivars between the NA, CA and DA plants (Figure 2E–H).

4.5-fold higher than in the NA plants and 2-fold higher than in the CA plants (Figure 3H). The deacclimated President plants accumulated as much as a four-fold higher content of GA_{19} compared to the CA plants (Figure 3G).

In the cold-acclimated plants, the level of GA_{20} was generally similar (Feliks, President, Rokas) or lower (Bojan) than in the non-acclimated plants (Figure 3I–L). The deacclimation increased it only in the case of the President and Rokas plants (Figure 3K,L).

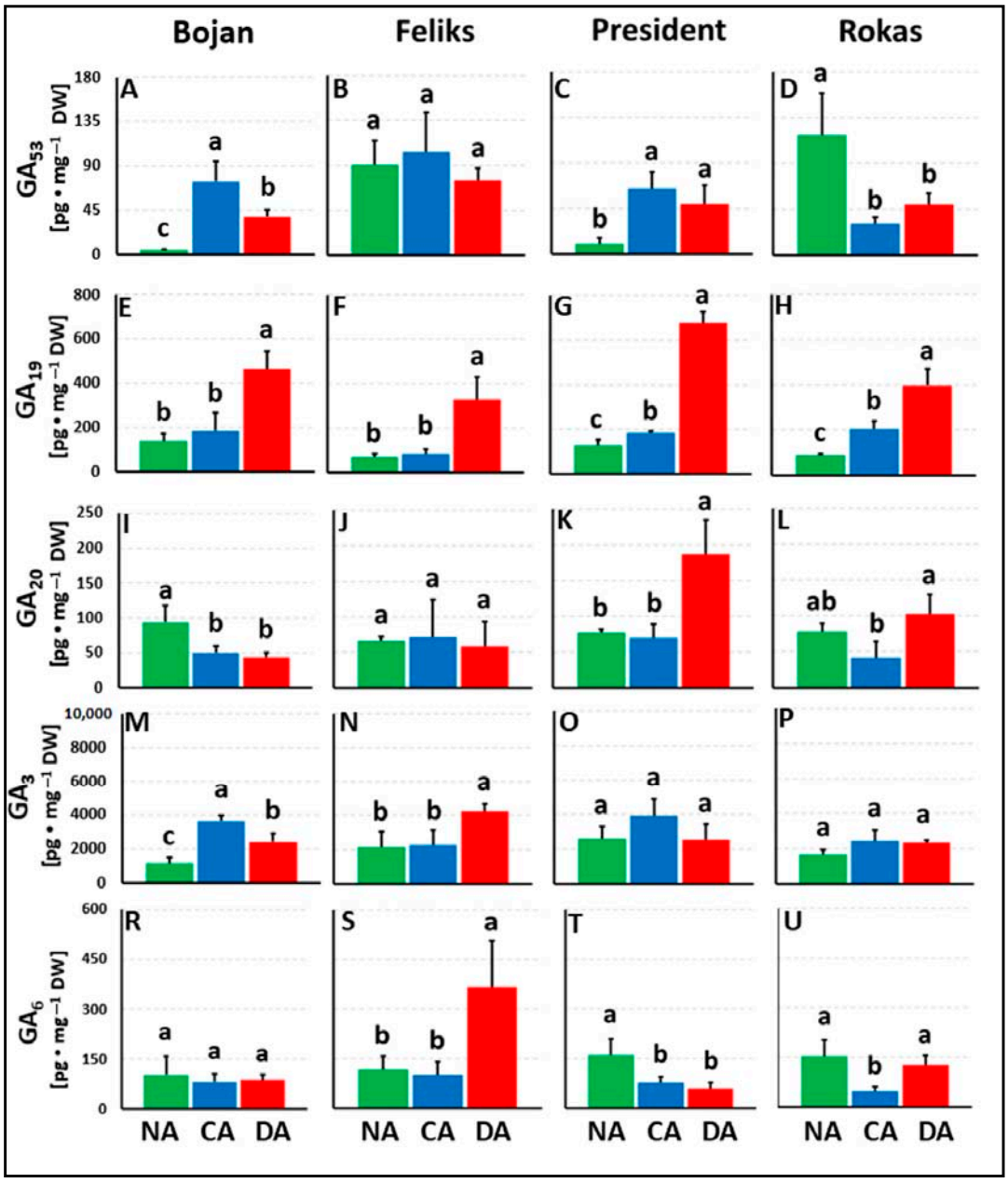


Figure 3. Content of the gibberellins GA_{53}, GA_{19}, GA_{20}, GA_3 and GA_6 in roots of the cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The gibberellins presented in order based on their biosynthetic pathway from precursors to GAs via GA_{53} and GA_{19} to their active forms GA_1 and GA_3 (according to [33]): (**A**–**D**)—GA_{53}; (**E**–**H**)—GA_{19}; (**I**–**L**)—GA_{20}; (**M**–**P**)—GA_3 and (**R**–**U**)—GA_6, which is marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

The level of GA_3 increased in the cold-acclimated Bojan plants and decreased in deacclimated plants (Figure 3M). In the other cultivars, cold had no effect on GA_3 (Figure 3N–P). In the deacclimated plants, the GA_3 level was clearly higher only in the Feliks plants, there was no difference in the GA_{53} level between the NA and CA plants, while in the Rokas plants, GA_{53} decreased after cold (Figure 3B,D). After deacclimation, the level of this phytohormone was similar to the level that was observed in the cold-acclimated plants in three of the four tested cultivars (Figure 3B–D).

The GA_{19} rather showed a tendency to accumulate more in cold acclimated plants (than in NA plants); interestingly, deacclimation further strengthened this effect (Figure 3E–H). For

The tendencies of changes in the concentration of GA_{53} were similar in the Bojan and President cultivars, which accumulated more GA_{53} after cold treatment (compared to the NA plants) by approximately 1461 and 534%, respectively (Figure 3A,C). In the Feliks plants, there was no difference in the GA_{53} level between the NA and CA plants, while in the Rokas plants, GA_{53} decreased after cold (Figure 3B,D). After deacclimation, the level of this phytohormone was similar to the level that was observed in the cold-acclimated plants in three of the four tested cultivars (Figure 3B–D).

The GA_{19} rather showed a tendency to accumulate more in cold acclimated plants (than in NA plants); interestingly, deacclimation further strengthened this effect (Figure 3E–H). For

example, the DA Rokas plants were characterized by a level of GA_{19} that was 4.5-fold higher than in the NA plants and 2-fold higher than in the CA plants (Figure 3H). The deacclimated President plants accumulated as much as a four-fold higher content of GA_{19} compared to the CA plants (Figure 3G).

In the cold-acclimated plants, the level of GA_{20} was generally similar (Feliks, President, Rokas) or lower (Bojan) than in the non-acclimated plants (Figure 3I–L). The deacclimation increased it only in the case of the President and Rokas plants (Figure 3K,L).

The level of GA_3 increased in the cold-acclimated Bojan plants and decreased in deacclimated plants (Figure 3M). In the other cultivars, cold had no effect on GA_3 (Figure 3N–P). In the deacclimated plants, the GA_3 level was clearly higher only in the Feliks plants compared to both the non-acclimated and cold-acclimated plants by approximately 1.9-fold (Figure 3N). In the case of this cultivar, a similar trend was also observed for GA_6 in which the DA plants were characterized by an average 235% higher level of this gibberellin than in the NA and CA plants (Figure 3S). The level of GA_6 after cold acclimation was similar to that after deacclimation in Bojan and President plants (Figure 3R,T). In the Rokas plants, cold decreased the content of GA_6 while deacclimation increased it once again (the accumulation of GA_6 in the non-acclimated and deacclimated plants was at a similar level; Figure 3U).

The following auxins, auxin precursors, metabolites and conjugates were identified in all of the tested cultivars: indole-3-acetamid, indole-3-acetonitril, indole-3-acetic acid, indole-3-acetyl-aspartic acid, oxoindole-3-acetic acid, indole-3-acetyl-glutamic acid and indole-3-carboxylic acid. The auxin precursors (IAM, IAN) and active auxin IAA are presented in Figure 4A–L, while the auxin conjugates (IAAsp, IAGlu), oxIAA and an indole-derivative metabolite-indole-3-carboxylic acid (I3CA) are presented in Figure 5A–P.

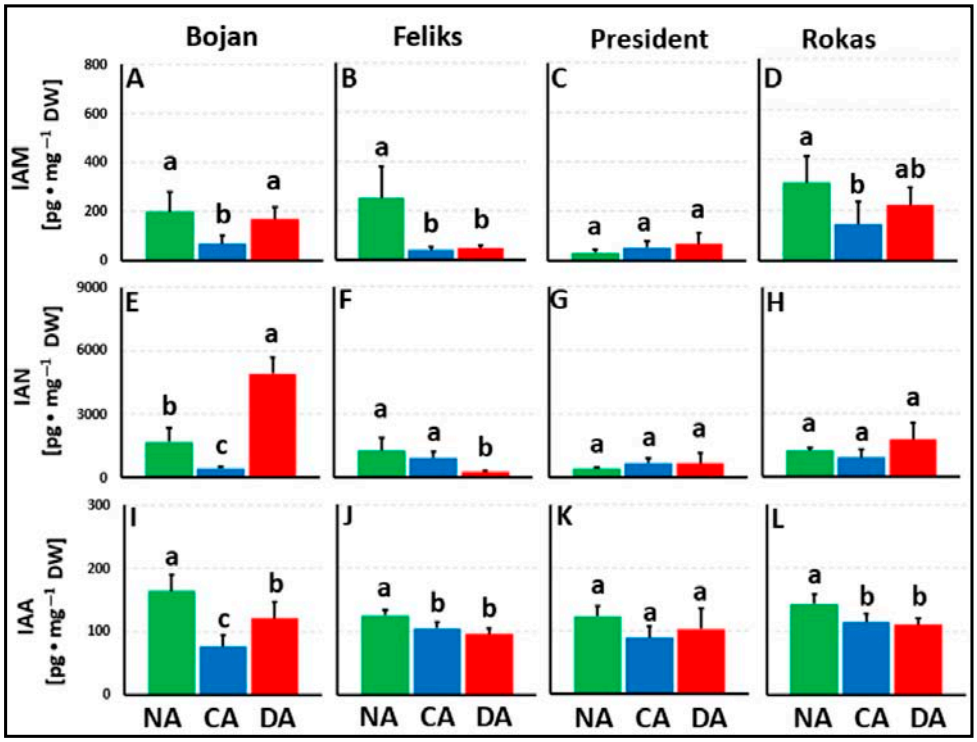
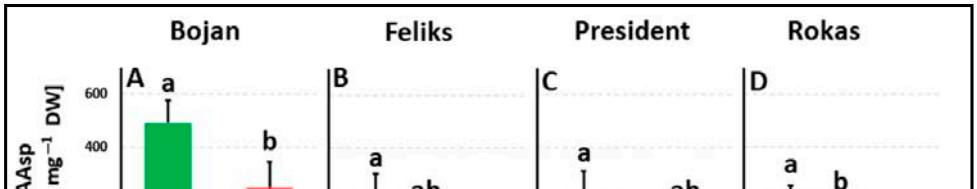


Figure 4. Content of the auxin precursors (indole-3-acetamid (IAM), indole-3-acetonitril (IAN)) and active auxin indole-3-acetic acid (IAA) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—IAM, (**E**–**H**)—IAN, (**I**–**L**)—IAA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).



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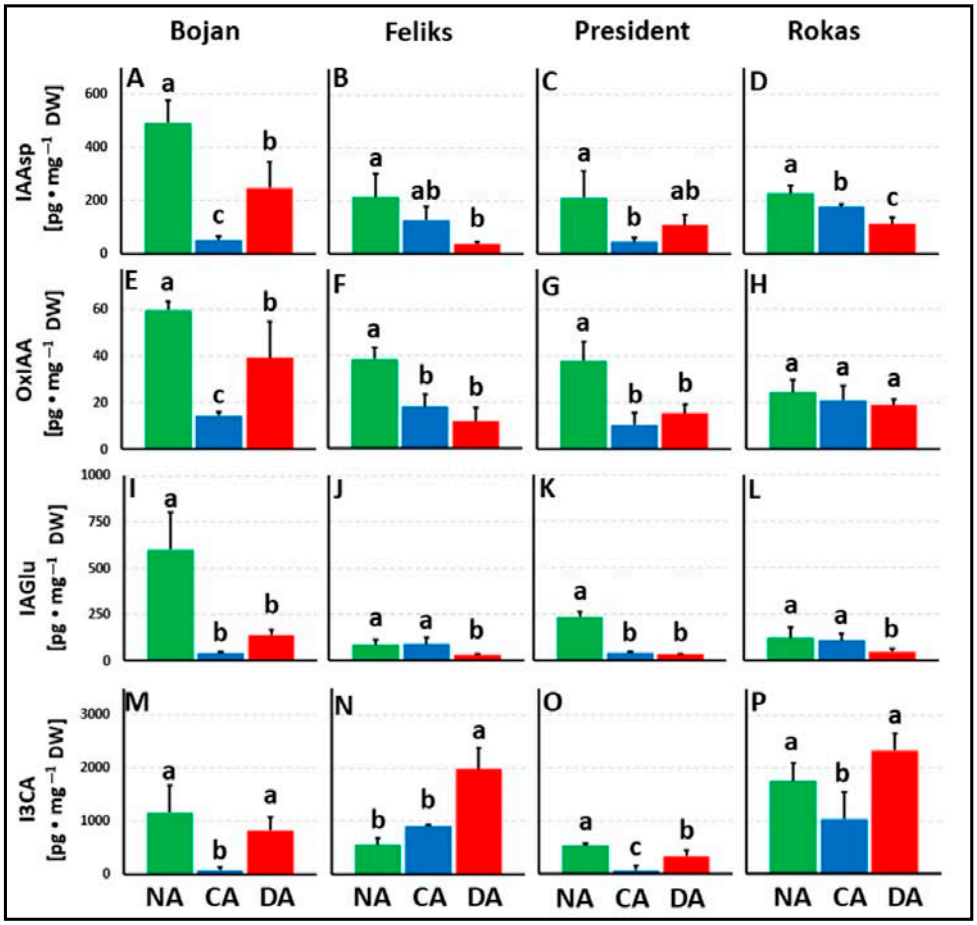

Figure 5. Content of the auxin conjugates (indole-3-acetyl-aspartic acid (IAAsp) and indole-3-a glutamic acid (IAGlu)), the oxidized auxin form (OxIAA) and the indole-derivative metab indole-3-carboxylic acid (I3CA) in four cultivars of the non-acclimated (NA), cold-acclimated and deacclimated (DA) oilseed rape. (**A**–**D**)—IAAsp; (**E**–**H**)—OxIAA; (**I**–**L**)—IAGlu; (**M**–**P**)—I3CA. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

Cold acclimation reduced the level of IAM in three of the four tested cultivars, while in one cultivar (President), IAM remained at a similar level as in the non-acclimated plants (Figure 4A–D). During the deacclimation, there was a significant increase in the level of IAM in the Bojan plants—the accumulation of IAM reached the same level as that in the non-acclimated plants (Figure 4A). There was a similar tendency (statistically insignificant) in the Rokas cultivar (Figure 4D). Deacclimation did not change the content of this hormone in the Feliks and President plants (Figure 4B,C).

The content of the second precursor (IAN) decreased after cold acclimation only in the Bojan plants (there were no changes between the NA and CA plants in the other three cultivars) (Figure 4E–H). After the Bojan plants were deacclimated, IAN was higher once again and reached a level that was 190% higher than in the NA plants and 103% higher than in the CA plants. In the Feliks cultivar (Figure 4F), IAN decreased after deacclimation, while in two of the other cultivars, it remained at the same level as after cold acclimation (Figure 4G,H).

The cold-acclimated Bojan plants were characterized by a decreased level of IAA (by approximately 53% compared to the NA plants) (Figure 4I). The hormone level in this cultivar increased again after deacclimation (although it did not reach the same level as in the NA plants). In the Feliks and Rokas plants, cold acclimation lowered the concentration of IAA slightly, and this level was also maintained after deacclimation (Figure 4J,L). There

were no statistically significant differences in the accumulation of IAA (similar to IAM and IAN) between NA, CA and DA plants in the President cultivar (Figure 4K).

The leaf accumulation of the auxin conjugates IAAsp, IAGlu and the oxidized form OxIAA had the same pattern of changes in the Bojan plants. Their concentrations decreased after cold and increased again after deacclimation, although not to the level that was characteristic for the NA plants (Figure 5A,E,I). In the three other genotypes, cold also decreased (or did not change) the content of these compounds (Figure 5B–D,F–H,J–L), but the accumulation of IAAsp and IAGlu in the leaves of deacclimated Feliks, President and Rokas plants, in most of the cases, reached the lowest values compared to both the NA and CA plants (Figure 5B–D,J–L). The deacclimation of these three cultivars did not change the level of OxIAA (compared to the CA plants) (Figure 5F–H).

The amount of I3CA decreased significantly (from 41% to as much 94%) in the cold-acclimated plants (with the exception of the Feliks plants; Figure 5N). After deacclimation, the content of this hormone returned to a similar level as was detected in the NA plants (Figure 5M,O,P).

Among the cytokinins, cis-zeatin and cis-zeatin riboside were identified (Figure 6A–H). The leaf accumulation of cis-zeatin decreased in the cold-acclimated plants in all of the tested cultivars (from 75% in the Rokas plants to 81% in the President plants) (Figure 6A–D). Deacclimation increased the concentration of cis-zeatin in three of the four cultivars. The exception was the DA Bojan plants in which the cis-zeatin remained unchanged (compared to the CA plants). As for cis-ZEA-rib, there were no differences between the NA, CA and DA plants in the cultivars (the exception was a slight increase in this compound in the CA Feliks plants (Figure 6F) and DA Feliks plants (Figure 6E–H).

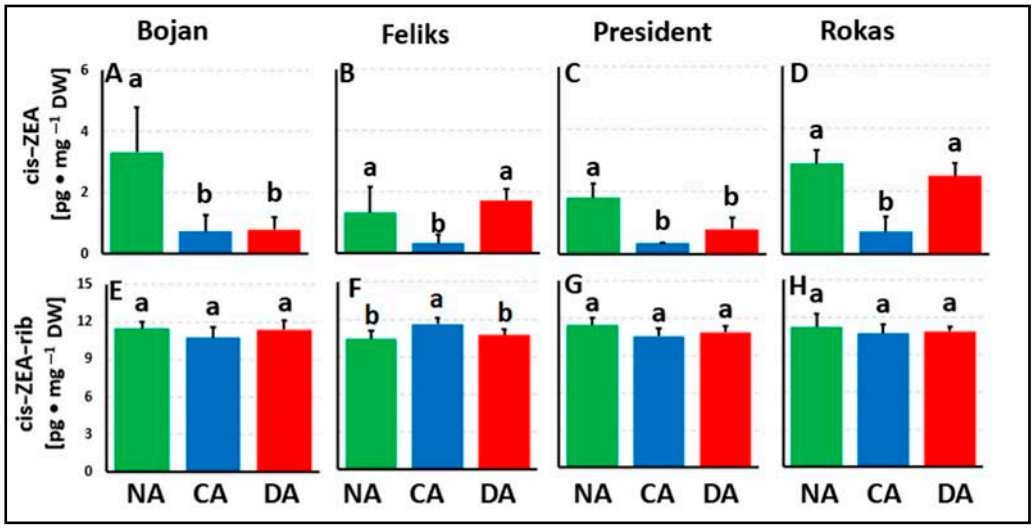

Figure 6. Content of the cytokinins–cis-zeatin (cis-ZEA) and cis-zeatin riboside (cis-ZEA-rib) in four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A–D**)—cis-ZEA; (**E–H**)—cis-ZEA-rib. Values marked with the same letters (within each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

3.2. HSP Analyses

The presence of cytoplasmic HSP70, chloroplastic HSP70 and HSP90 was detected in the leaves of the four cultivars of oilseed rape (Figure 7A–L). The accumulation of cytoplasmic HSP70 increased after the cold acclimation of the Bojan, Feliks, and President cultivars (Figure 7A–C). After deacclimation, the amount of this protein returned to a similar level as was observed in the non-acclimated plants of cv. Feliks and President (Figure 7B,C). In the Bojan plants, the amount of HSP70 cytoplasmic was decreased after deacclimation and finally was lower than in the non-acclimated plants (Figure 7A). The exception from this patterns was changes in cytoplasmic HSP70 in the Rokas plants. This cultivar was characterized by a decrease in the level of the HSP70 cytoplasmic protein after cold acclimation, and a further decrease was also observed after deacclimation (Figure 7D).

As for chloroplastic HSP70, in the Bojan plants, there was an increase in the amount of this protein after cold acclimation. After deacclimation, the level of chloroplastic HSP70

deacclimation more or less decreased it (Figure 7I–K). In contrast to these three cultivars, the Rokas cultivar accumulated the highest amount of HSP90 in the non-acclimated plants, and this amount was surprisingly lower in the cold-acclimated plants and decreased further in the deacclimated plants (Figure 7L).

The common tendency for all of the tested cultivars was a lower accumulation of HSP90 (so as cytoplasmic HSP70) in the DA plants (compared to the CA plants).

cultivar was characterized by a decrease in the level of the HSP70 cytoplasmic protein after cold acclimation, and a further decrease was also observed after deacclimation (Figure 7D).

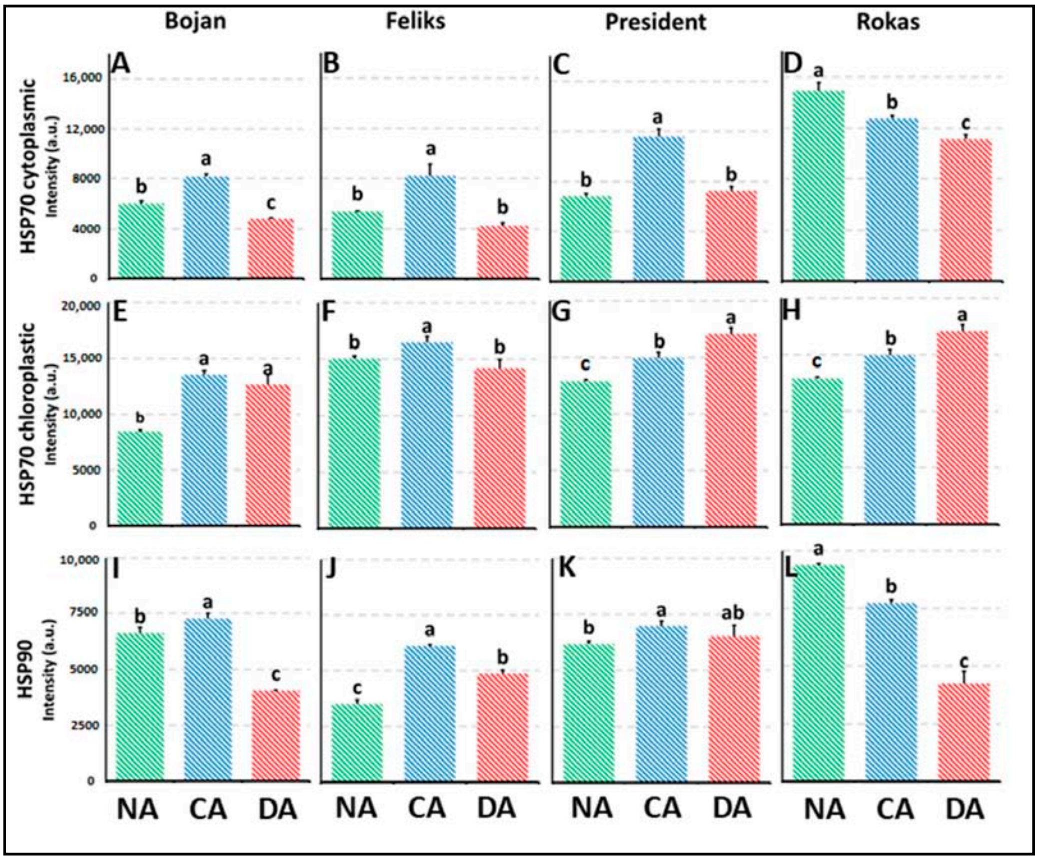

Figure 7. Changes in the accumulation of the HSP proteins in the leaves of four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. (**A**–**D**)—changes in the accumulation of cytoplasmic HSP70; 3 µg of the protein was loaded onto the gel. (**E**–**H**)—changes in the accumulation of chloroplastic HSP70; 5 µg of the protein was loaded onto the gel. (**I**–**L**)—changes in the accumulation of HSP90; 10 µg of the protein was loaded onto the gel. Values marked with the same letters (for each cultivar separately) were not significantly different according to the Duncan test ($p \leq 0.05$).

4. Discussion

4.1. The Impact of Deacclimation on Plant Hormone Management

Generally, the profile of the hormones detected in the oilseed rape was in agreement with the data that are available in the literature. The presence of auxins (for example IAN, IAM, IAA, oxIAA, IAAsp, IAGlu) in oilseed rape was reported by [34], gibberellins (for example GA_1, GA_3, GA_{19}, GA_{20}) by [35] and cytokinins (for example cis-ZEA and cis-ZEA-rib) by [36]. Additionally, stress hormones such as ABA, JA, SA (and its precursor BA) were reported by [37–39]. According to our best knowledge, only 12-oxo-PDA and I3CA were not previously detected in oilseed rape plants; thus, we report it for the first time here.

It is well known that the temperature in which a plant grows modifies the content of hormones in plant tissue. Regarding the cold acclimation (hardening), the changes in ABA are well known. This is related to the fact that the role of ABA in cold hardening in the case of winter plants is to increase their frost tolerance, and therefore, the increase in the level of

As for chloroplastic HSP70, in the Bojan plants, there was an increase in the amount of this protein after cold acclimation. After deacclimation, the level of chloroplastic HSP70 remained similar to what was noted after cold acclimation (Figure 7E). In cultivar Feliks, there was a similar tendency to accumulate more HSP70 chloroplastic in the cold-acclimated plants. The deacclimated Feliks plants were characterized by a lower level of this protein than the cold-acclimated plants. This level was also similar to what was detected in the non-acclimated plants (Figure 7F). Both cultivars President and Rokas had a similar tendency to accumulate HSP70 chloroplastic—the smallest amount was detected in the non-acclimated plants, while it was significantly higher in the cold-acclimated plants; the accumulation was the highest in the deacclimated plants (Figure 7G,H).

In the case of the accumulation of HSP90, a similar pattern of changes was noted for the Bojan, Feliks and President plants—cold increased the content of the protein while deacclimation more or less decreased it (Figure 7I–K). In contrast to these three cultivars, the Rokas cultivar accumulated the highest amount of HSP90 in the non-acclimated plants, and this amount was surprisingly lower in the cold-acclimated plants and decreased further in the deacclimated plants (Figure 7L).

The common tendency for all of the tested cultivars was a lower accumulation of HSP90 (so as cytoplasmic HSP70) in the DA plants (compared to the CA plants).

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It is well known that the temperature in which a plant grows modifies the content of hormones in plant tissue. Regarding the cold acclimation (hardening), the changes in ABA are well known. This is related to the fact that the role of cold hardening in the case of winter plants is to increase their frost tolerance, and therefore, the increase in the level of ABA is important for the development of frost tolerance [40]. This is also confirmed by studies in which this hormone was used exogenously and an increase in frost tolerance of winter plants was noted [41,42]. In our experiment, in winter oilseed rape, after three weeks of cold acclimation, the ABA content increased unequivocally in all four of the tested cultivars (Bojan, Feliks, President, Rokas). An increase in the ABA content (from 300 to almost 600 pg/mg D.W.) in the cold was also observed in the fifth cultivar of winter oilseed rape—Pantheon, which was tested in another experiment (data not shown). These results were expected and are consistent with previous reports on oilseed rape [40]. The rapid increase in the ABA content in the leaf discs was noted within the first three days of cold acclimation at 2 °C. The ABA level remained high during the next 18 days. According to [37], increased levels of ABA were already observed in oilseed rape during early seedling growth in prehardened plants (plants growing at temperature 12 °C vs. plants growing at 20 °C).

Generally, the cold-induced increase in the ABA content in the plants of the cultivars that were studied here corresponded with a significant increase in the frost tolerance of these plants [3]. It was also observed that the most frost-tolerant cultivar, Rokas, accumulated more than 1200 pg of this hormone per mg of D.W. in the leaves after cold acclimation, while the other tested cultivars accumulated approximately two-fold less. This corresponds somewhat to the results of experiments with the exogenous use of ABA, where it was proven that while the supplementation of ABA increased the frost tolerance, the hormone was more effective in cultivars that had a naturally lower content of ABA [43].

In an earlier work [3], deacclimation (7 days 16/9 °C d/n) caused a significant decrease in frost tolerance of the ten tested cultivars of oilseed rape. Considering the significant relationship between ABA and the level of frost tolerance, a decrease in the level of this hormone as a result of deacclimation could be expected. The results obtained in this paper fully support this assumption. In all of the cultivars (Bojan, Feliks, President, Rokas), as well as in the additionally tested cultivar Pantheon (data not shown), this deacclimation caused a decrease in ABA content, although not to the level that was recorded in the control plants (those without cold hardening). This corresponds to the fact that the frost tolerance of the plants after deacclimation was lower, although it remained higher than that of the control plants [3]. Surprisingly, according to [37],the deacclimation of oilseed rape caused a slight increase in the ABA content in both of the tested cultivars of oilseed rape that were tested by the authors.

Although the role of ABA in the cold hardening and in frost tolerance of plants is the most well-known, other typical stress hormones can also contribute to improving the tolerance to temperature stress. Among them, we also studied jasmonic acid and salicylic acid in our experiment. According to the literature, the exogenous application of jasmonic acid improves the freezing tolerance of *A. thaliana* L. with or without the cold acclimation process. In contrast, blocking the endogenous JA biosynthesis resulted in plants that were hypersensitive to freezing [14]. Simultaneously, the content of JA increased also in cold-treated wheat [11]. It is in agreement with our studies, where the leaf content of JA

generally increased after cold acclimation (although an interesting exception was the most frost tolerant Rokas in which there were no changes in NA vs. CA plants). In addition, in some of the cultivars (Bojan and Feliks, partly also President) the relationship between JA and its precursor (12-oxo-PDA) was particularly clear. A higher precursor content in the control plants or deacclimated plants was usually reflected in a lower JA content. A lower content of the precursor in cold-acclimated plants was associated with a higher JA content.

It is also worth emphasizing that, unlike the other three cultivars, after deacclimation, the plants of the Rokas cultivar were characterized by a several times higher content of JA compared to the level of JA in the other cultivars. Because it is believed that JA is important for improving low temperature tolerance ([14,44] and the literature cited there), it is possible that the high levels of this hormone in this cultivar are among the factors that contribute to maintaining a high frost tolerance despite deacclimation.

In the case of the third hormone, which was salicylic acid, the differences between the NA, CA, and DA plants were not too large. Although the exogenous treatment of plants with salicylic acid may induce cold or frost tolerance, i.e., in wheat (not acclimated and cold acclimated plants [45–47]), in our opinion, the significance/importance of SA in the frost tolerance of oilseed rape remains an open question.

Because plant growth is limited during the cold acclimation period, a reduction in the level of the growth-stimulating hormones such as gibberellins, auxins and cytokinins can be expected. In the plants of the same family as oilseed rape-*Arabidopsis*, *GSF (Gibberellin Suppressing Factor)* participates in the response to abiotic stresses such as cold by suppressing the biosynthesis of gibberellins [48]. An increased deactivation of gibberellins (in response to a short period of cold) was reported in winter wheat [11]. According to [37], during the cold acclimation of oilseed rape seedlings (cultivar Górczański), the level of GA_3 decreased slightly. On the other hand, exogenous GA_3 disturbed cold acclimation, thus, increasing the susceptibility of the photosynthetic apparatus to cold-induced photoinactivation [12]. In our studies, the dominating, active gibberellin GA_3 did not change during cold acclimation (or in one cultivar—Bojan—even increased). On the other hand, a cold-induced decrease was observed for the other active gibberellins, mainly for GA_7 and GA_4 (usually accompanied by decrease in the precursor GA_{15}). In two of the four tested cultivars, the content of GA_6 also decreased. Those differences in the contents/changes of specific gibberellins between the cultivars indicates that the effect of temperature on gibberellin biosynthesis in oilseed rape may be (at least partly) cultivar-dependent. This could justify some of the differences between our results and the results in oilseed rape that were obtained by [37]. It is worth mentioning here that the level of gibberellins may dynamically change during the cooling time, which should be taken into account when performing only single/point analyses. For example, in winter wheat, the concentration of GA_3 and GA_6 (gibberellins from the group that is synthetized in the pathway via GA_{53} [33]) began to increase significantly on the 9th day of cold up to the 15th day of cold [49]. On the other hand, GA_4 and GA_7 (gibberellins from group synthetized in pathway via GA_{15}) had only one peak on the 12th day of cold and then their content decreased once again. In barley, there was a tendency to accumulate fewer GA_3 and GA_4 in the cold acclimated plants, while the amount of GA_6 tended to increase [50].

In oilseed rape, however, the gibberellin levels can already begin to increase at the end of the cold period, especially when long (i.e., ten weeks) cooling periods are used [51]. This is connected with the fact that in oilseed rape, gibberellins are engaged in development and in this aspect, cold exposure is crucial to the vernalization process. Winter cultivars require cold for the induction of stem elongation and flowering, and according to [51], vernalization influences GA content and metabolism with GAs serving as probable regulatory intermediaries between the cold treatment and subsequent stem growth. The authors observed a further increase in the level of gibberellins (especially GA_{20}, GA_1 and GA_3) eight days after the plants were moved from cold to 23 °C. In the experiment performed by [37] and in our experiment, the cold period was much shorter (three to four weeks) and there was an increase in the level of gibberellins in the plants, but during the period of deacclimation.

It should be emphasized that in the oilseed rape that we studied, the increase in the level of gibberellins as a result of deacclimation was varied (i.e., depending on the cultivar) but it concerned especially GA_{19} (four cultivars), GA_7 and GA_4 (three cultivars), GA_{20} and GA_6 (two cultivars), GA_3 and GA_{15} (one cultivar). However, unlike the experiment of [51] in which the importance of gibberellins in the context of development was examined, in our experiment, the increase in the level of gibberellins as a result of deacclimation should be interpreted as being unfavorable. Deacclimation in winter could mean a resumption of growth and could be associated with a decrease in the frost tolerance of a plant. It is worth mentioning here that exogenous gibberellins accelerate development ([52] and the literature cited there) and especially at higher concentrations, GAs can decrease the tolerance to low temperature [53,54].

Cytokinins are also engaged in the transition of winter oilseed rape to the generative phase due to vernalization [52]. In our experiment, cold decreased the cis-ZEA levels in oilseed rape (in all four cultivars), while deacclimation caused a statistically significant increase in the level of this hormone in two cultivars; in the third cultivar, a similar trend was observed, but it was not statistically significant. These trends are consistent with those that were observed in previous studies on barley, where it was also shown that cis-ZEA decreased after three weeks of cold, while after deacclimation, the level of cytokinins increased again [16]. A decrease in cis-ZEA was also observed in winter wheat after 2 weeks of cold [49]. However, for oilseed rape [36], reported that in the 21st day of cold, the concentration of cis-ZEA started to increase slightly, while maximal value was reached on the 42nd day of cold. Thus, as in the case of gibberellins, shorter periods of cooling may be associated with a lower level of cytokinins, but after a longer period of cooling, the accumulation of cytokinins can increase due to the progress of the processes of the induction of generative development. However, when it comes to deacclimation (as in the case of gibberellins), a sudden increase in the level of cytokinins as a result of this process, which may occur, e.g., in the middle of the winter, should, in our opinion, be interpreted as unfavorable as it could indicate the resumption of growth related to the lowering of frost-tolerance.

As for the hormones from the third group of growth-promoting substances—auxins, in our studies, the amount of IAA (the main active auxin) generally had a tendency to decrease in the cold acclimated plants, which is in agreement with earlier findings for wheat or barley [11,16,49]. However, the deacclimated plants of barley were characterized by an increased level of hormones such as IAA or IAA methyl ester [16]. In our work, a similar phenomenon was observed for only one of the four tested cultivars—Bojan. In this cultivar, deacclimation very clearly reversed the effect of cold by not only increasing the concentrations of IAA, but it was simultaneously connected to an increase in the production of the precursors in IAA biosynthesis (IAM and IAN). The significant increase was also recorded for IAAsp and oxIAA (and a tendency in IAGlu). OxIAA is characterized by a weak biological activity and is usually irreversibly formed in response to increases in the auxin levels [55]. Both conjugates IAAsp and IAGlu are also a form of deactivation of IAA, and their level usually increases with an increase in the IAA content [56]. The results suggest that generally the entire metabolism of auxins from biosynthesis to conjugation/deactivation in this cultivar was enhanced. In contrast to Bojan, there was a different picture for auxin metabolism in the CA and DA plants for Feliks, President and Rokas. The IAA content generally dropped slightly in these cultivars after cold and this level was maintained after deacclimation. The lower content of the main active auxin was accompanied by a relatively low content of the precursors (mainly IAM in Feliks and Rokas) as well as IAAsp or oxIAA, which reflects a less intense metabolism of auxins than in Bojan, especially after deacclimation.

In the end of this part of the discussion, it is worth emphasizing that the work of [15] provided important genetic support for our studies that were devoted to changes in the content of gibberellins and auxins in the deacclimated plants. According to the authors, during the deacclimation of *A. thaliana* (a plant from the same family as oilseed rape),

there was an overexpression of the genes associated with the metabolism of auxins and gibberellins (but also brassinosteroids, jasmonate and ethylene).

The data regarding the accumulation of I3CA in the aspect of cold acclimation and deacclimation must be taken into account for these very interesting and novel results. I3CA (indole-3-carboxylic acid) is an indolic compound that is mainly recognized as a player in plant pathogen resistance [57,58]. In our studies, its content decreased in cold (in three of the four tested cultivars), while in all four of the tested cultivars, it significantly increased after deacclimation. A simpler, although still theoretical, explanation is that this is connected to the susceptibility of plants to some pathogen infection. Plants growing in higher temperatures (deacclimated and also non-acclimated) are more susceptible to a pathogen infection [59], thus, the involvement of the I3CA accumulation as protective agent is higher (than in cold). There might also be another explanation based on the fact that I3CA is one of the factors that regulate the callose accumulation [58]. Callose is a plant polysaccharide that was mainly studied in the context of plant defense reactions, although it is also engaged in the process of growth and development [60]. During the process of cell division, the cell plate determines the correct composition of the cell wall layer—callose forms a coat-like structure that covers the surface of the cell plates [60]. Limited growth (and cell division) processes in cold might be connected to a lower requirement for callose (thus I3CA as callose synthesis regulator may be lower). After the deacclimation processes, the resumption of plant growth is linked to the higher activity of the growth regulation factors, perhaps also including a higher callose synthesis that is regulated, among others, by I3CA. The temperature-dependent changes in I3CA are a reason for further studies of this matter and the verification presented hypothesis.

To conclude this part of the discussion, a few words need to be devoted to the relationship between ABA and GA, which are hormones with an antagonistic activity [61]. To visualize general changes in the hormonal balance between the active forms of the growth-promoting and stress hormones in non-acclimated, cold-acclimated and deacclimated oilseed rape, the following ratios were calculated: GA_3/ABA, ratio GA_3 + GA_4 + GA_6 + GA_7/ABA and ratio IAA + cis-ZEA+ GA_3 + GA_4 + GA_6 + GA_7/ABA + JA (Figure 8A–F).

As for the ratio of gibberellins to ABA, the model of changes that is presented in Figure 8A–D shows that the value of the ratio decreased in cold but increased again after deacclimation, although (especially in winter cultivars) it did not reach the level that was recorded for the control plants (before acclimation). In the case of the spring cultivar—Feliks (Figure 8B,D), the effect of deacclimation was more pronounced than in the case of the winter cultivars (Figure 8A,C), which (as expected) could indicate a lower tolerance to the deacclimation of the spring cultivar. This lower tolerance to the deacclimation in a spring cultivar is even better expressed in the relationship between all of the studied active forms of the growth-promoting hormones (IAA + cis-ZEA+ GA_3 + GA_4 + GA_6 + GA_7) and stress hormones (ABA + JA) (Figure 8E,F).

Generally, the ratio of gibberellins to ABA corresponded well to the earlier results concerning cold acclimation- and deacclimation-induced changes in frost tolerance [3]. After cold acclimation, the plants were characterized by a higher tolerance, while after a period of deacclimation, the frost tolerance decreased, although not to the level that was recorded in the control plants (basal tolerance). The control plants after frost (−5 °C) had one point on a seven-point scale of injuries. The cold acclimated plants were able to survive −15 °C (three to four points on the scale of injuries), while the deacclimated plants were already severely injured by a temperature of −12 °C (one to two points on the scale of injuries).

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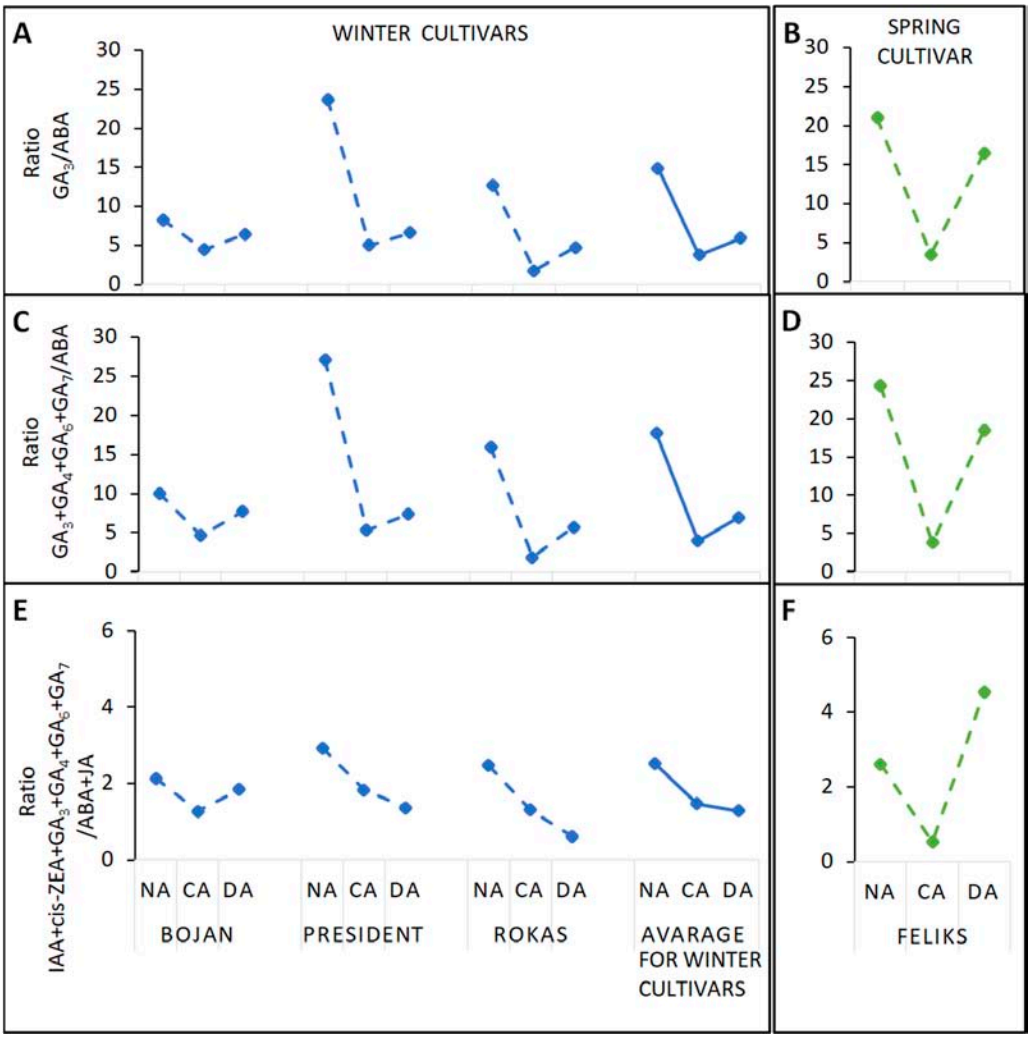

Figure 8. Visualization of the changes in the hormonal balance between the active forms of the growth-promoting and stress hormones in the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) plants of winter and spring cultivars of oilseed rape. Model is based on calculations of ratio GA_3/ABA (**A**,**B**), ratio $GA_3+GA_4+GA_6+GA_7$/ABA (**C**,**D**) and ratio IAA + cis-ZEA + GA_3 + GA_4 + GA_6 + GA_7 / ABA + JA (**E**,**F**).

4.2. The Impact of Deacclimation on Heat Shock Protein Accumulation

As was mentioned in the Introduction, the heat shock proteins play an important role as chaperones and assist other proteins in folding, maintaining and stabilizing [22]. Moreover, HSP90 is an important player that mediates the stress signal transduction [62]. A similar function was also proposed for HSP70 [63]. As for chloroplastic HSP70, this protein contributes to the photoprotection and repair of photosystem II [24]. HSPs are accumulated in higher amounts (by various plants including oilseed rape), particularly during the heat stress [17,26,64], but various classes of HSPs have often also been found elevated in cold treated plants of grapevine [18], winter wheat [65] or barley [19]. That is why the elevated accumulation of HSPs in cold-acclimated oilseed rape, which was observed in our experiment, was an expected phenomenon. On the other hand, in our studies, deacclimation most often resulted in a lower accumulation of HSPs. A higher level of hsp90 mRNA under low temperature was previously described in oilseed rape, after which the accumulation of hsp90 mRNA decreased once again to the level of the control when the cold-treated plants were transferred back to 20 °C [26]. In some way, exposing plants to 20 °C here could reflect deacclimation conditions. Moreover, in *Rhododendron anthopogon* D.Don (an evergreen shrub), the expression of, among others, stromal HSP70 was up-

regulated during the cold-acclimation phases and down-regulated during the transition to the deacclimation phase [66]. Although a decrease (or increase) in gene expression does not always have to correlate with a decrease (or increase) in protein accumulation, in our oilseed rape, the directions of the changes in protein accumulation (an increase after cold acclimation and then a decrease after deacclimation) were generally consistent with the directions of the changes in the HSP gene expression that were previously found in genetic studies on oilseed rape [26] and even in another family plant—*R. anthopogon* [66].

It is worth noting that changes in level of the protective HSP proteins (an increase in the CA plants and usually a decrease in the DA plants) were accompanied by changes in their frost tolerance (at least for three cultivars). As we previously described, deacclimation significantly lowered the cold-induced frost tolerance of the tested cultivars [3]. The exception, however, was a different pattern of changes in the HSP accumulation that was recorded in the most frost tolerant cultivar—Rokas. This cultivar was generally characterized by high basal level of HSP90 (measured in non-acclimated plants) and cytoplasmic HSP70, but accumulation of these HSPs in the CA plants was surprisingly decreased. While deacclimation caused a further decrease in the level of these proteins, the deacclimated Rokas plants maintained the highest frost tolerance compared to the other cultivars [3]. This is difficult to explain, and we can only offer a theory that it had something to do with the complex dependency of HSP with many other factors, such as hormones, various signaling proteins, transcription factors, etc. [62]. We also have to remember that in our experiment, we only tracked the final accumulation of HSP and we did not know anything about the intensity of the complex processes of the synthesis or degradation of the proteins or the balance between them [67].

In the light of all the results presented in this work, it is worth making a brief comment about a potential link between the HSPs, hormone ABA and cytokinins. According to the review of [68], HSP90 may play a role in the signal transduction of ABA and this presumption was made, among others, because of the evidence that a genetic or pharmacological interference with HSP90 could result in disturbances of the ABA-induced stomata closure [69]. HSP90 clients and their potential involvement in the signaling pathways of cytokinins (such as PAS1) were also described [68]). On the other hand, an exogenous ABA treatment of *A. thaliana* and *Festuca arundinacea* Schreb. resulted in a higher level of the expression of various HSPs, together with an improved tolerance to heat stress [70]. The picture from our studies is somewhat similar to the results of [70]. In our work, an increase in the ABA content (not by exogenous treatment but as a natural effect of a low temperature) was usually accompanied by an increase in the HSPs, which was in agreement with an increase in the cold-induced frost tolerance (as was previously described for the tested cultivars [3]). Deacclimation significantly reversed these effects. Against this background, it can be seen that the level of cytokinins decreased in cold and usually increased again after deacclimation, thus, in contrast to the changes in ABA and HSPs. It is commonly known that ABA and cytokinins have antagonistic effects on many physiological processes, however, the dependency of cytokinin level on the HSP expression is more interesting. For example, as was reported by [71], transgenic *A. thaliana* overexpressing *ZmsHSP* was characterized by a lower level of cytokinins (although it was more sensitive to cytokinins). However, on the other hand, according to [67,72], cytokinins up-regulated most of the HSP70s and stimulated protein accumulation. Simultaneously, ABA (and jasmonates) have a negative effect on the HSP70 expression/HSP70 protein accumulation, which only shows that the relationship/interaction between these groups of players (ABA, cytokinin, HSP) is a result of a complex crosstalk on multiple levels that certainly (in the aspect of cold acclimation and deacclimation) requires further studies, probably using plant mutants or inhibitors as well as genetic research. To summarize, a model of the changes in the frost tolerance of cold acclimated and deacclimated oilseed rape against the background of the changes in the concentrations of ABA, cis-ZEA, HSP70 cytoplasmic, HSP70 chloroplastic and HSP90 is visualized in Figure 9.

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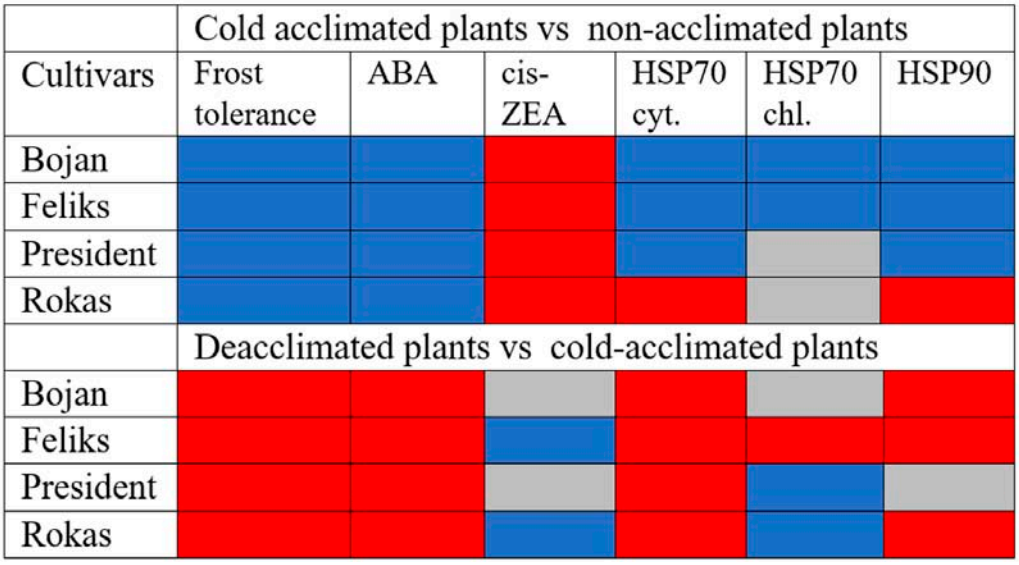

Figure 9. Directions of the changes in the frost tolerance of cold acclimated and deacclimated oilseed rape plants against the background of changes in the concentrations of ABA, cytokinin cis-ZEA, HSP70 cytoplasmic (cyt.), HSP70 chloroplastic (chl.) and HSP90. The visualization is based on the data from [3] (frost tolerance) and on Figure 1A–D, Figure 6A–D and Figure 7A–L. Blue—increase, red—decrease, grey—no changes.

5. Conclusions

In conclusion, in the winter oilseed rape, deacclimation-induced changes in the hormonal balance in the direction of the increased participation of hormones accompanied by a stimulation in plant growth and development (cytokinin, GAs (cultivar-dependent)) while there was a decrease in the concentration of the stress hormone (ABA (cultivar independent)). This is probably one of the factors that is responsible for the growth resumption of deacclimated plants and the lowering of their frost tolerance. The measurements of ABA or the ratio of gibberellins/ABA can be a tool for monitoring the process of deacclimation (and potential changes in frost tolerance) in oilseed rape. In most cases, deacclimation reversed the effect of cold acclimation on the protective heat shock proteins, which could also be responsible for lowering plant frost tolerance. An interrelation between HSP and hormones such as ABA or cytokinins in cold-acclimated and deacclimated plants seems to be an interesting matter for more detailed studies. Similarly, finding an explanation for the reason for changes in the concentration of indolic compound I3CA, which is lower in cold but higher after deacclimation, requires further research. I3CA is an indolic compound that, to date, has mainly been linked with the reaction of plants to pathogens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13030641/s1, Figure S1: The scheme of the experiment and sampling of non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. Figure S2: The accumulation of the HSP proteins in the leaves of four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The visualized bands correspond to the HSP70 cytoplasmic, HSP70 chloroplastic and HSP90 protein identified as described in Section 2.3.2.

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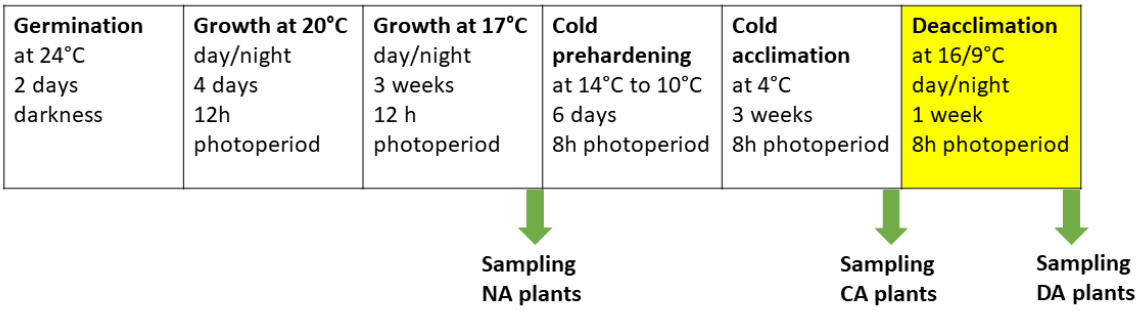

Figure S1. The scheme of the experiment and sampling of non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape.

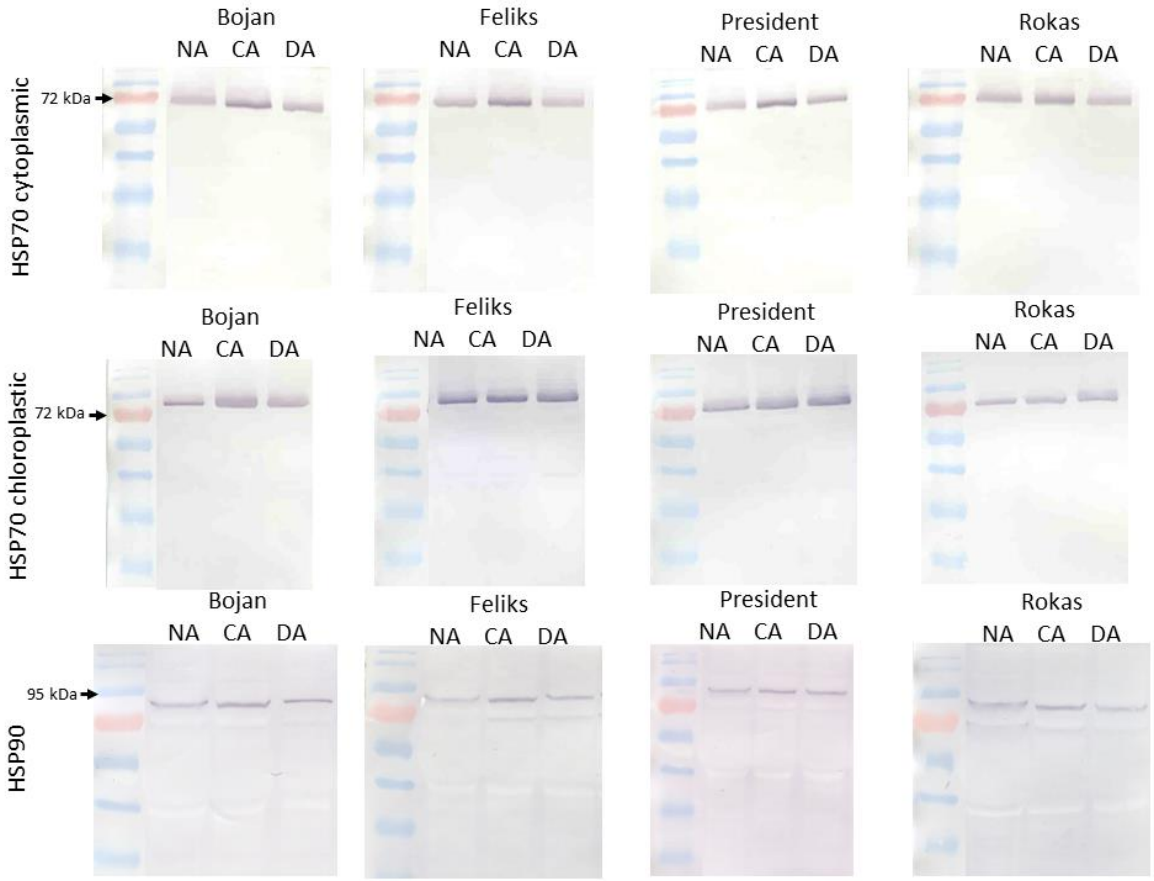

Figure S2. The accumulation of the HSP proteins in the leaves of four cultivars of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape. The visualized bands correspond to the HSP70 cytoplasmic, HSP70 chloroplastic and HSP90 protein identified as described in Section 2.3.2.

OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 10.05.2024 r.

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Oświadczam, że w pracy: Stachurska, J., Sadura, I., Rys, M., Dziurka, M., Janeczko, A. (2023) Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.). Agriculture 13, 641 mój udział polegał na: nauczeniu doktorantki (J. Stachurskiej) techniki Western Blotting i Immunoblotting oraz wykonaniu wraz z doktorantką analizy akumulacji białek HSP (w tym optymalizacji metody).

Iwona Sadura

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(czytelny podpis współautora)

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(czytelny podpis współautora)

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Does deacclimation reverse the changes in structural/physicochemical properties of the chloroplast membranes that are induced by cold acclimation in oilseed rape?

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ABSTRACT

Winter crops acquire frost tolerance during the process of cold acclimation when plants are exposed to low but non-freezing temperatures that is connected to specific metabolic adjustments. Warm breaks during/after cold acclimation disturb the natural process of acclimation, thereby decreasing frost tolerance and can even result in a resumption of growth. This phenomenon is called deacclimation. In the last few years, studies that are devoted to deacclimation have become more important (due to climate changes) and necessary to be able to understand the mechanisms that occur during this phenomenon. In the acclimation of plants to low temperatures, the importance of plant membranes is indisputable; that is why the main aim of our studies was to answer the question of whether (and to what extent) deacclimation alters the physicochemical properties of the plant membranes. The studies were focused on chloroplast membranes from non-acclimated, cold-acclimated and deacclimated cultivars of winter oilseed rape. The analysis of the membranes (formed from chloroplast lipid fractions) using the Langmuir technique revealed that cold acclimation increased membrane fluidity (expressed as the A_{lim} values), while deacclimation generally decreased the values that were induced by cold. Moreover, because the chloroplast membranes were penetrated by lipophilic molecules such as carotenoids or tocopherols, the relationships between the structure of the lipids and the content of these antioxidants in the chloroplast membranes during the process of the cold acclimation and deacclimation of oilseed rape are discussed.

1. Introduction

Oilseed rape (*Brassica napus* L.) is an important crop whose seeds are used in human nutrition due to the high-quality composition of their fatty acids, as well as an ingredient in animal feed. Oilseed rape is also used for biodiesel production (van Duren et al., 2015). Winter cultivars have higher yield potential than spring cultivars but their vegetation (in regions such as Central Europe) must last throughout the winter months when plants are exposed to below zero temperatures. Naturally, cold acclimation (cold hardening) is a process that prepares oilseed rape plants to survive winter conditions. This phenomenon occurs during several weeks of plant growth at a temperature of about 4 °C and is associated with a significant increase in frost tolerance. In such case, plants enter the winter time in stage of hardened leaf rosette. With the warming of the climate, however, the phenomenon of deacclimation (dehardening) is occurring more frequently and is caused by periods of higher temperatures that interrupt the natural processes of cold acclimation in winter crops. Deacclimation makes such plants more susceptible to frost (Rapacz et al., 2017; Stachurska et al., 2022; Vyse et al., 2019). Generally, deacclimation is far more dangerous for the winter cultivars of oilseed rape than for the spring cultivars of oilseed rape, but the spring cultivars can also be exposed to this phenomenon. This is because during the early stages of their growth (spring: March/April) in some regions (like the Central/Eastern EU), temperatures below 0 are nothing abnormal. Simultaneously, temperatures in the range 16–20 °C or even higher are also possible. Oilseed rape that is germinated in cold is hardened and tolerates slight frost well, but if seedlings are exposed to 20 °C and then the weather suddenly changes to frost, the risk of

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frost-induced injuries of leaves increases.

The metabolic changes in leaves during cold acclimation are well understood (Fürtauer et al., 2019). Cold acclimation causes changes, among others, in the membrane lipid composition in the direction of increased fluidity (Uemura et al., 1995), a decreased leaf water content and an increased sugar content (Sasaki et al., 1996), an increased content of the stress hormones, e.g., ABA (Kosová et al., 2012) and an increased accumulation of the protective proteins, e.g., the Heat Shock Proteins (Sadura et al., 2020; Zhang et al., 2008) compared to the non-acclimated plants.

In recent years, after recognising the problem of deacclimation and its impact on economic losses, which are the result of frost injuries of crops (Rapacz et al., 2017; Vyse et al., 2019), there has been an increasing number of papers that describe the metabolic changes during deacclimation and their relationship with the loss of frost tolerance in crops. Studies of a few cultivars of winter oilseed rape have shown that deacclimated plants were characterised by a lower frost tolerance compared to cold-acclimated plants (Rys et al., 2020; Stachurska et al., 2022). It was also well correlated with hormonal changes (Stachurska et al., 2023). A decreased frost tolerance of oilseed rape was also accompanied by a decrease in the content of sugars and an increase in the osmotic potential (Rys et al., 2020). The chemical composition of the leaves, which was measured using FT-Raman spectroscopy, confirmed the metabolic differences between the cold-acclimated and deacclimated plants (Rys et al., 2020). In fact, the metabolic profile of deacclimated oilseed rape was more similar to the metabolic profile of non-acclimated plants.

The reorganisation of the cell membranes (including the chloroplast membranes) is a crucial part of the acclimation of plants to low temperatures (Ogweno et al., 2009). Under stressful conditions, the lipid composition of the chloroplast membranes changes (Filek et al., 2016), as does, as among others, the content of tocopherols and carotenoids, which are localised in the membranes (Munné-Bosch, 2005). The chloroplast membranes are (depending on the plant genotype) composed of about 65–90% of glycolipids such as monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) and also contain a small amount of phospholipids (PL) (Jouhet et al., 2007; Quinn and Williams, 1983). MGDG and DGDG are present in the membrane at about 58% and 29%, respectively, of the total chloroplast lipid content. Unlike DGDG and the majority of phospholipids that create a bilayer lipid structure, MGDG forms hexagonal II or cubic phases (Webb and Green, 1991). Because the non-bilayer lipids are responsible for the permeability and thermal stability of membranes, a high content of the MGDG in the chloroplasts is essential for the proper functioning of these organelles (Dlouhý et al., 2020; Kirchhoff, 2018). In the hydrophobic part, glycolipids are characterised by a high quantity of polyunsaturated fatty acids, especially the linolenic (18:3) and linoleic acids (18:2) (Mackender and Leech, 1974). The presence of unsaturated (at the *cis*-conformation) fatty acids promotes the incorporation of other hydrophilic-hydrophobic molecules into the lipid structure to a greater extent than for the saturated acids (Filek et al., 2017). Some of the molecules that can be incorporated into the membranes are tocopherols. Tocopherols are a family of hydrocarbon compounds with a chromanol ring and a saturated phytol side chain. The main isomer is α-tocopherol (Niki and Abe, 2019). Tocopherols can be involved in the scavenging of ROS, so they are classified as antioxidants (Havaux et al., 2005). Atkinson et al. (2010) hypothesised that α-tocopherol, which is preferentially included in the spaces of membranes that are rich in poly-unsaturated fatty acids, can protect these fatty acids against peroxidation. α-Tocopherol might additionally stabilise the lipid domains similar to what was found for the reaction of cholesterol in rafts. Based on model systems that were mainly built from phospholipids, the specific action of α-tocopherol on the physicochemical properties of membranes such as modifying the phase behaviour and lipid dynamics and decreasing the motional freedom of the lipid fatty acyl chains was confirmed. These responses were particularly observed at a low temperature (Hincha, 2008).

Another group of lipophilic substances that penetrate the chloroplast membranes are carotenoids, which are synthesised in the chloroplasts in the form of isoprenoid metabolites as the structural components of photosystems (Sun et al., 2018). Carotenoids function as antioxidants and photoprotectors. The ones that are found in large amounts in the photosynthetic organs are usually β-carotene, its metabolite zeaxanthin, which is a component of the xanthophyll cycle and also lutein (Dhami and Cazzonelli, 2020; Ponder and Hallmann, 2019). The location of specific carotenoids in the chloroplast membranes is conditioned by their chemical structure (Gruszecki, 2004; Milon et al., 1986). In cold conditions, similar to sterols, carotenoids can modify membrane fluidity by increasing the membrane order and maintaining lateral lipid mobility (Strand et al., 1997; Filek et al., 2016; Seel et al., 2020).

The temperature of plant growth influences the content of carotenoids and tocopherols in plant tissue. An increased level of α-tocopherol was detected during exposure to a low temperature in maize plants (Leipner et al., 1999) and in *A. thaliana* (Bergmüller et al., 2003). The cultivars of winter wheat that accumulated a higher amount of tocopherols had a higher frost tolerance than the cultivar with a lower amount of these compounds in the leaves (Janeczko et al., 2018). The foliar application of α-tocopherol in bell pepper that was then exposed to cold significantly increased the activity of enzymes such as catalase, decreased the amount of H_2O_2 and improved the proline content (Atiq et al., 2021). Carotenoids such as β-carotene are also antioxidants whose biosynthesis and accumulation changes in cold-stressed plants (Sinha et al., 2015). More frost-tolerant cultivars of winter wheat accumulated more β-carotene in their leaves (Janeczko et al., 2018). The exogenous application of carotenoids also helps to protect plants against low temperature stress (Ding et al., 2022).

The current work is a continuation of our earlier studies on the deacclimation process in oilseed rape in which, as the next step, we focused on performing a detailed analysis of the impact of deacclimation on the physicochemical properties of membranes (the chloroplast membranes), which had been isolated from oilseed rape leaves. This permits a better understanding of the mechanisms of the deacclimation process. Our earlier studies showed that cold acclimation reduced intensity of the light reactions of photosynthesis in oilseed rape while deacclimation reversed the changes that had been induced by cold and increased the photosynthetic activity (Rys et al., 2020; Stachurska et al., 2022). The physicochemical properties of membranes may be measured using the Langmuir technique. The Langmuir technique makes it possible to obtain highly ordered, well-defined and controlled monolayers by using the phenomenon of the spontaneous orientation of the hydrophilic-hydrophobic molecules at the water/air interface (Hussain et al., 2018). Because lipids (like long-chain fatty acids) are amphiphilic substances, they are attracted to water molecules with their polar group, while the hydrophobic group is attracted to air. This is a model for the behaviour of lipids in one of the monolayers of the membrane and maps the orientation of the lipids at the border of the cytosol (hydrophilic environment) and the “inside” of the membrane (hydrophobic environment). This enables the creation of molecular architectures that are useful for studying the physical phenomena at the molecular level (Hussain et al., 2018). In the Langmuir method, the tested particles are introduced to the water surface in the area of a special “gutter” at low concentrations. Initially, they are located far from each other and the interactions between them are weak. Therefore, the tested surface tension relative to the pure water (measured as the surface pressure) changes little (initial area of the Langmuir diagram). After a slow compression (through a moving barrier), the lipid molecules come closer to each other reaching the maximum possible orientation in monolayer, which is determined by the properties of attractive-repulsive interactions between the polar groups of the lipids and their hydrophobic groups. In this situation, the surface tension changes rapidly, reaching the maximum of the Langmuir diagram. Further compression would cause the particles to “overlap” (collapse). Calculating the data that

obtained from the Langmuir diagram enables the physicochemical parameters of the monolayer, such as the area that is occupied by a single molecule in a completely "packed" layer and the value of the surface pressure at which the layer collapsed to be obtained. The interpretation of these parameters provides information about the fluidity and elasticity of the monolayer (see section 2.7 for details). Using the Langmuir technique, monolayers can be produced from both pure fatty acids (model monolayers) and also from the fatty acids that have been isolated from plant lipids (native monolayers). In this case, the monolayers serve as a simplified model of natural cell membranes. By examining only the chemical composition of membranes (fatty acids), we obtain general information about the possibility of changes towards more liquid membranes (more unsaturated fatty acids) or more stiffened membranes (more saturated fatty acids). On the other hand, physicochemical studies using a Langmuir trough provide direct information about the actual state of the membrane structure. Moreover, determining of physicochemical parameters of monolayer provides additional information about the possibility of penetration of other molecules (carotene, tocopherols) into the membrane. All modifications occurring within the membranes serve to maintain such structure of the membranes, that allows the proper functioning of the processes occurring in them (e.g. transport, signalling) independently of the growth conditions of plants and the effects of stress factors (including temperature changes).

The aim of the research was to describe the influence of deacclimation on the physicochemical properties of the chloroplast membranes (Langmuir trough studies) as well as any changes in the content of the low-molecular antioxidants – tocopherols and carotenoids in isolated chloroplasts (and in whole leaf tissue) of oilseed rape. Changes in the properties of the membrane structure were investigated by determining the physicochemical parameters of the MGDG (forms hexagonal structure) and PL (bi-layers) chloroplast fractions, which were selected as lipids with a different spatial configuration in the membranes. We verified the hypothesis that cold acclimation causes an increase in the fluidity of the chloroplast membranes while deacclimation can reverse this effect thus decreasing the membrane functionality in a low temperature. The involvement of selected lipophilic molecules (tocopherols and carotenoids) in the stabilisation of the membrane structure during the cold-acclimation and deacclimation processes in oilseed rape is discussed.

2. Material and methods

2.1. Plant material

The experiment was conducted on four cultivars of oilseed rape (*Brassica napus* L. *var. napus* L.). Three were winter cultivars (Bojan, President, Rokas [semi-dwarf]) and one was a spring cultivar (Feliks). The cultivars were selected based on the work of (Stachurska et al., 2022) in which their frost tolerance had been tested. The original results have been recalculated and are presented in Fig. 1 – supplementary data. Moreover, based on the results of the frost testing, the temperature that was required to reduce plant regrowth by 50% (RT50) was calculated for a particular cultivar. The data of RT50 are available in the work (Stachurska et al., 2022).

Briefly, the spring cultivar Feliks had the lowest basal frost tolerance (the frost tolerance of non-acclimated plants); the temperature of frost testing was −3 °C. Non-acclimated winter cultivar Rokas had the highest frost tolerance. After cold acclimation, Feliks (and President) had a lower frost tolerance than Bojan and Rokas (frost test at a temperature of −13 °C). The deacclimated winter cultivars Rokas, Bojan and President were characterised by a similar frost tolerance, but it was clearly a higher frost tolerance than was noted for the spring cultivar Feliks (frost test at a temperature of −12 °C).

The seeds of the cultivars Bojan and Feliks were obtained from The Plant Breeding and Acclimatisation Institute (IHAR), the National Research Institute in Strzelce (Poland). The seeds of the President were obtained from Saatbau (Poland) and the seeds of the Rokas from Syngenta (Poland).

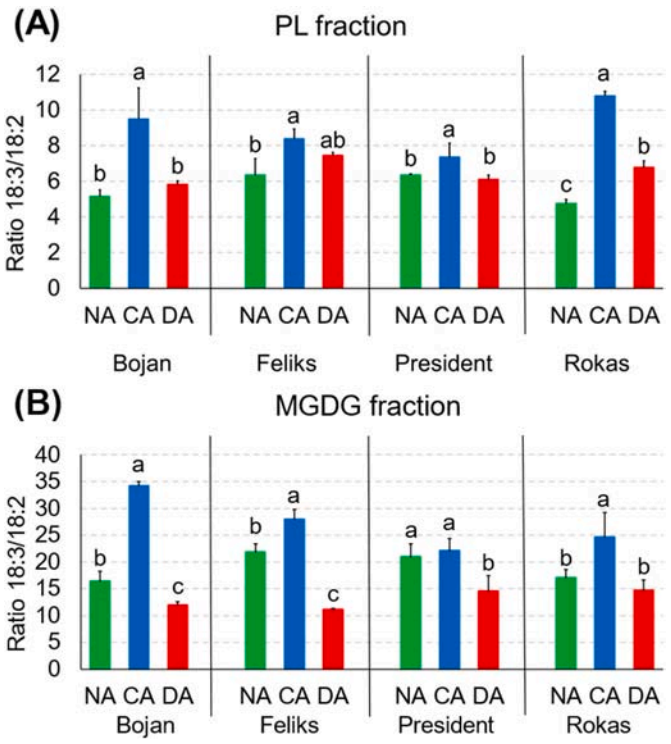

Fig. 1. The values of the ratio between the two most unsaturated fatty acids: α-linolenic acid (18:3) and linoleic acid (18:2) in (A) the phospholipid (PL) fraction and (B) in the monogalactolipid (MGDG) fraction. The lipid fractions that were isolated from the chloroplasts of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) plants of oilseed rape (cultivars Bojan, Feliks, President, Rokas). *Values indicated by the same letters (within each cultivar separately) are not significantly different according to the Duncan test ($p \leq 0.05$).*

2.2. Experimental design and sampling

The experimental model was described in detail by (Stachurska et al., 2022). Briefly, after germination, the seedlings were planted in pots with soil (18 plants per pot) and were cultured for three weeks in a growth chamber (4 day at 20 °C d/n and 17 days at 17 °C d/n; 12h photoperiod). The plants (15) were left in each pot and the plants were pre-hardened for seven days (14 °C d/n, two days – 12h photoperiod; 12 °C d/n, three days – 8h photoperiod; 10 °C d/n, two days – 8h photoperiod). Next, the conditions were changed for the cold acclimation (4 °C d/n, 21 days – 8h photoperiod). Finally, the temperature was increased to 16/9 °C d/n (8h photoperiod, seven days) for the deacclimation of the plants. Light conditions (LED lamps HORTI A were purchased from PERFAND LED, Trzebnica, Poland) were the same during the entire experiment with a light intensity of 300 μmol $m^{-1}s^{-1}$; for details see (Stachurska et al., 2022). The experiment was conducted in the autumn/winter season.

The samples for all of the analyses were always collected from the best-developed leaves in the rosette of the non-acclimated plants (NA, control group), cold-acclimated plants (CA) and deacclimated plants (DA). Pure chloroplasts for further analysis were also isolated from these plant materials. The composition and content of the carotenoids and tocopherols were measured in the leaves and in the chloroplast samples. The composition of selected fatty acids was measured in the chloroplast samples. A Langmuir trough analysis was performed on the lipids that were isolated from the chloroplasts.

2.3. Isolating the chloroplast from the fresh leaves

The chloroplasts were isolated based on the protocol that was described earlier by (Sadura et al., 2021) at 4 °C. Briefly, the samples of fresh leaves were homogenised with a CIB buffer (50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.33 mM sorbitol, pH = 7.5) using a kitchen blender (SM 3735, Severin, Germany)). The samples were filtered through a material filter, collected into tubes, and centrifuged (3 min, 4 °C, 300 *g*). The supernatant was collected into new tubes and centrifuged (10 min, 4 °C, 1200 *g*). On average, it was possible to obtain about 1.22 g of pure chloroplasts from 23 g of fresh leaves. The sediment of the chloroplasts was diluted with a CIB buffer. For the fatty acid analysis, samples of about 580 mg of chloroplasts were diluted with 1.2 mL of a CIB buffer. For the analysis of antioxidants, samples of about 310 mg of chloroplasts were diluted with 1 mL of a CIB buffer. All of the samples were stored at −80 °C for further analyses.

2.4. Extracting and separating the lipids from the chloroplast membranes

2 mL of hot isopropanol (four times 0.5 mL each time) and 0.15 mL BHT (butylated hydroxytoluene) in isopropanol were added to the samples of the chloroplast suspension in the CIB buffer and then shaken for 10 min. Next, the lipids were extracted using a mixture of methanol/chloroform (2:1) according to the method of (Bligh and Dyer, 1959) with the modification of (Janeczko et al., 2009). The obtained extracts of total lipids were dissolved in 1 mL of chloroform and the lipid fractions were separated in a Supleco SPE tube (Discovery DSC-NH_2, Sigma-Aldrich, Poznań, Poland). The fraction of MGDG was eluted from the column with 5 mL (five times 1 mL) of a mixture of acetone/chloroform (1:2) and the fraction of PL with 5 mL (five times 1 mL) of methanol. The fractions were evaporated to dryness under N_2 and dissolved in 1 mL of chloroform. Subsequently, 2 mL of sulfuric acid (95%–97%) in methanol (1:4) was added and the samples were heated in a heating block at 100°C for 1h (shaking every 20 min). After cooling, 1 mL of H_2O was added, and the samples were shaken vigorously. After the phase separation, 1 mL was taken from the chloroform phase and filtered directly into vials through a cotton wool filter. The composition of fatty acids (FA), in particular the isolated fractions, was analysed using gas chromatography (GC). The analyses were performed in three replicates.

2.5. Estimating the FA composition and calculating the FA ratios

The samples were analysed using an Agilent Technologies 7820A (Agilent Technologies, Santa Clara, CA, USA) gas chromatograph equipped with a flame-ionisation detector (FID) and an HP-88 60m × 250 μm x 0.2 μm GC column (Agilent Technologies, Santa Clara, CA, USA). The inlet temperature was set to 280 °C with a split mode (10:1). The mobile phase was hydrogen, gas flow was set to 2 mL/min. The temperature gradient that was used was as follows: initial 120 °C hold 1 min, ramp 10 °C/min to 175 °C, hold 8 min, ramp 5 °C/min to 210 °C, hold 3 min and finally, post time 2 min with 250 °C. The detector temperature was set to 280 °C. The individual fatty acid methyl esters were identified by comparing their retention times with a standard mixture of Supelco 37 component FAME Mix (Merck, Darmstadt, Germany).

The data were expressed as the molar percentage of a specific fatty acid relative to all of the fatty acids that were measured. Due to their importance for the structure of the membranes, the focus was on FA 18:3 and 18:2 and calculating the ratio 18:3/18:2 (Fig. 1). The other fatty acids identified based on Supelco 37 component FAME Mix, were among others: 16:0, 16:1, 18:0, 18:1 and 20:0.

2.6. Analysis of tocopherols and carotenoids

The tocopherols (α-, β-, γ-, δ-) and carotenoids (zeaxanthin, β-carotene) were measured based on the method reported by (Laskoś et al., 2021). The samples were extracted in 1 mL ethanol/acetone/methanol/2-propanol (8/3/3/1 v/v) containing a 1% solution of BHT by shaking in a water bath at 75 °C for 15 min. Then, 250 μL of 80% KOH was added and the extraction was continued for 30 min. Next, the samples were diluted with 3 mL H_2O and cleaned up by a double liquid-liquid extraction with n-hexane. After evaporation under N_2, the combined n-hexane layers were reconstituted in 0.1% BHT in methanol/dichloromethane (3/1 v/v) prior to the HPLC separation. An Agilent 1260 UHPLC binary system with diode array (DAD) and fluorescence (FLD) detectors was used. The separation was performed on an Ascentis Express RP-Amide (3 × 150 mm; 2.7 μm, Supelco, St Louis, MO, USA) column at 0.8 mL/min, 60 °C and a linear gradient of (A) 0.5% formic acid (FA) in acetonitrile (ACN)/H_2O (6/4 v/v) and (B) 0.5 % FA in 2-propanol/ACN (9/1 v/v) from 40 to 100% of B in 15 min. The tocopherols were detected using FLD at an excitation wavelength of 295 nm and an emission wavelength of 330 nm, while the carotenoids were detected using DAD at 450 nm. Calibration was performed based on the data that had been obtained for pure standards in conditions that were identical to those for the samples. Pure α-, β-, γ- and δ-tocopherol were purchased from Sigma-Aldrich (Poznań, Poland). The carotenoids were purchased from DHI Lab Products (Horsholm, Denmark). The analyses were performed in three replicates (chloroplast samples) or five replicates (leaf samples where one replicate = one leaf sample from different plants).

2.7. Forming and measuring the physicochemical parameters of the Langmuir monolayers

The lipid fractions (PL and MGDG) that had been isolated from the chloroplast membranes were used for the experiment using the Langmuir technique (Minitrough, KSV, Finland). The Langmuir monolayers were produced by spreading chloroform solutions of the lipids on the surface of water (as the subphase). The obtained monolayers were compressed at a rate of 3.5–4.6 $Å^2$/molecule x min. A Platinum Wilhelmy plate connected to an electrobalance was used to detect the surface pressure with an accuracy of ±0.1 mN/m. The experiments were conducted at a temperature of 7 °C. The measurements were repeated three to five times in order to prove the high recurrence of the obtained isotherms (±0.1–0.3 $Å^2$). The following parameters, which characterised the physicochemical properties of the lipid monolayers, were calculated based on the dependence of the surface pressure (π) vs the area per lipid molecule (A): A_{lim} – the area that was occupied by a single molecule in a completely packed layer and π_{coll} – the value of the surface pressure at which a layer collapsed. The static compression modulus was calculated as $C_s = -(d\pi/dlnA)$. C_s provides information about the mechanical resistance of the layers and therefore their elasticity.

2.8. Statistical analysis

Statistical analysis was performed in Statistica 13.1 (StatSoft, Tulsa, OK, USA). The average data in the tables/figures are presented ±SD. The Duncan's test was used to compare the averages for each cultivar separately (within a given parameter). Significant differences between the averages are indicated by different letters.

3. Results

3.1. Fatty acid content and composition

The MGDG and PL fractions of the chloroplasts that were isolated from the studied objects were characterised by a composition of fatty acids such as, among others, palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and α-linolenic (18:3) acids (data not shown). Fatty acid 18:3 was presented in the highest molar percentage, reaching a value of about 70% for MGDG and about 50–60% for PL. Because of the

importance of two unsaturated FA (18:3 and 18:2) for the properties of the membranes (particularly in relation to temperature), the ratio of 18:3/18:2 was calculated (Fig. 1). For the MGDG fraction, the values of the 18:3/18:2 ratio were higher than for the PL fraction and reached a value that ranged from 16 to 35 (while in the PL fraction, it was about 5–11). In all of the tested cultivars, cold acclimation increased the ratio 18:3/18:2 compared to the NA plants, while deacclimation reversed the effect of cold – the ratio was lower and was similar to that of the NA plants.

3.2. Physicochemical parameters of the Langmuir monolayers

Examples of the relationship between the surface tension and the area that was occupied by the monolayer that was composed of the PL and MGDG fractions are presented in Figs. 2 and 3. Using these relationships, the physicochemical parameters of the monolayer structure were calculated (Table 1).

For the PL fraction, the area per particle (A_{lim}) for the lipids that were isolated from all of the tested cultivars significantly increased when the temperature decreased (lipids from the CA plants) and decreased once again after deacclimation (lipids from the DA plants). For three of the

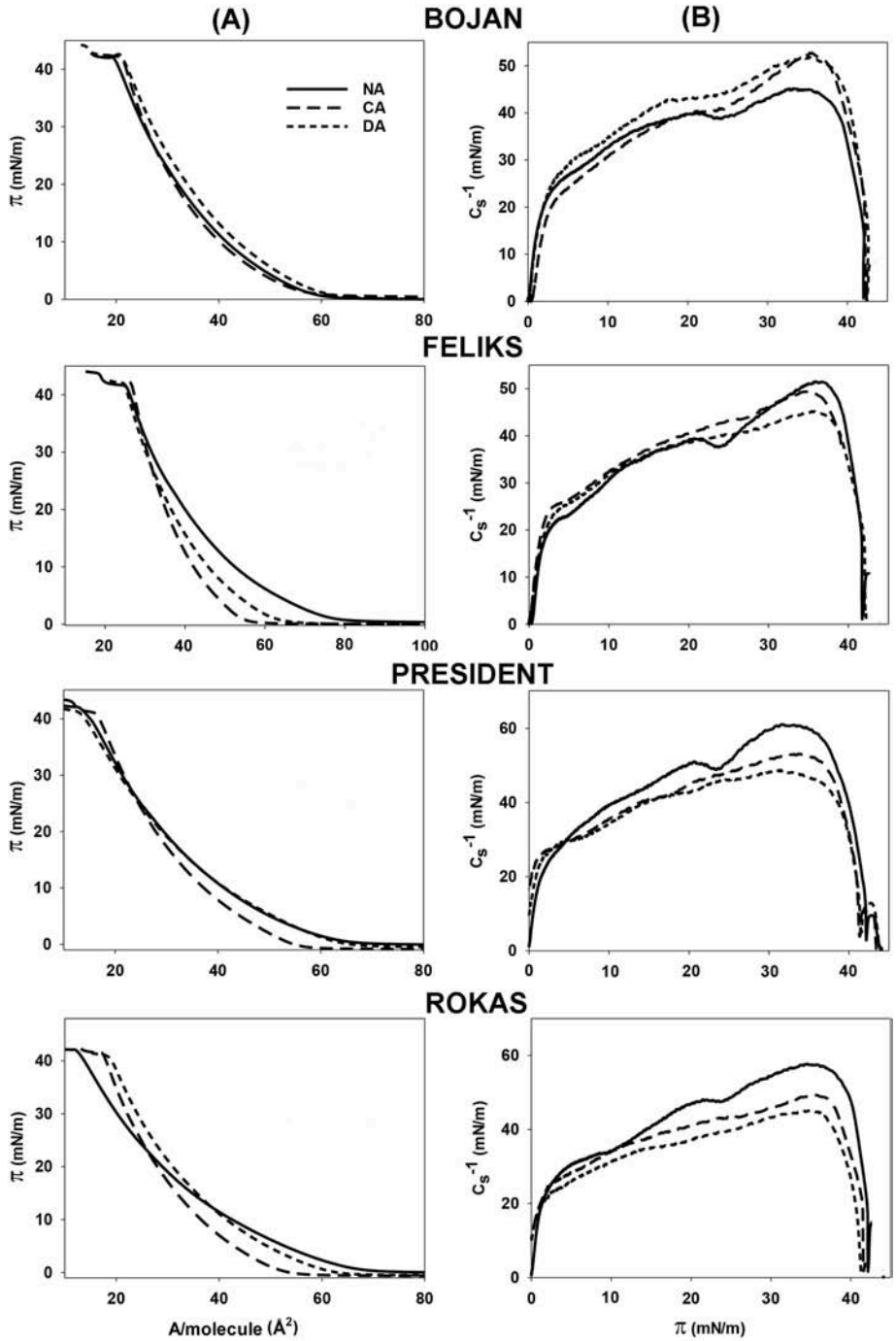

Fig. 2. Exemplary Langmuir isotherms (surface pressure π vs area per molecule A) – column (A) and the dependence of the compression modulus (C_s^{-1}) vs the surface pressure of the analogous systems – column (B) for the phospholipids (PL). The monolayers were prepared from the PL that were isolated from the chloroplasts of four cultivars of winter oilseed rape (Bojan, Feliks, President, Rokas). NA – non-acclimated plants, CA – cold-acclimated plants, DA – deacclimated plants.

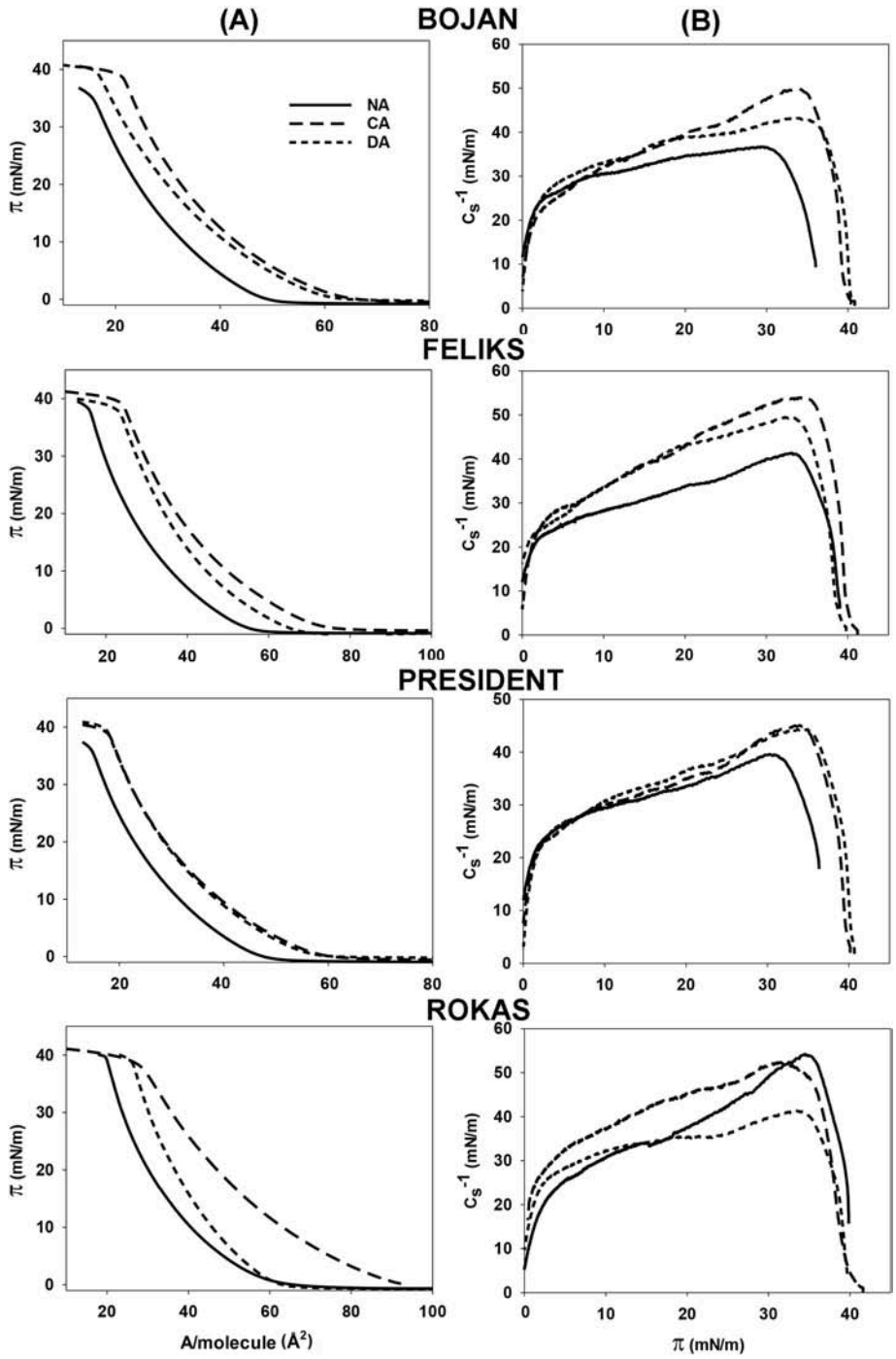

Fig. 3. Exemplary Langmuir isotherms (surface pressure π vs area per molecule A) – column (A) and dependence of the compression modulus (C_s^{-1}) vs the surface pressure of the analogous systems – column (B) for the galactolipids (MGDG). The monolayers were prepared from the MGDG that were isolated from the chloroplasts of four cultivars of winter oilseed rape (Bojan, Feliks, President, Rokas). NA – non-acclimated plants, CA – cold-acclimated plants, DA – deacclimated plants.

four tested cultivars, π_{coll} increased as a result of cold and remained at a similar level in the lipids from the DA plants. The exception here was cultivar Rokas in which cold did not influence π_{coll} (CA vs NA plants) while deacclimation decreased it (DA vs CA plants). For President and Rokas, the C_s^{-1} decreased under the influence of cold acclimation and this effect was stronger in the DA plants. In the case of the lipids that were isolated from cultivar Bojan, cold acclimation and deacclimation increased the value of C_s^{-1} (Table 1 and Fig. 2).

For the MGDG fraction, the parameters that characterised the monolayers was also influenced by the temperature of the growth of the plants from which the lipids were isolated. A_{lim} increased under the influence of cold acclimation for all of the tested cultivars, especially for Rokas and Bojan for which there was close to a two-fold increase compared to the non-acclimated plants (Table 1). For the lipids that were isolated from these two cultivars, after deacclimation, the A_{lim} values decreased once again but did not reach the values that are characteristic for the non-acclimated plants (remained statistically significantly higher). Similar effects were observed also for Feliks although in the CA plants, the increase in A_{lim} was not as high as in the case of Bojan and Rokas. In the case of the cultivar President, deacclimation did not change the values of this parameter (DA vs CA); the values remained at the level that was observed after cold acclimation.

Table 1
The physicochemical parameters: the limiting area per molecule (A_{lim}), collapse pressure (π_{coll}) and compression modulus (C_s^{-1}) of the monolayers that were prepared from the phospholipids (PL) and galactolipids (MGDG) that were isolated from the chloroplasts of four cultivars of winter oilseed rape (Bojan, Feliks, President, Rokas). NA – non-acclimated plants, CA – cold-acclimated plants, DA – deacclimated plants. *Mean values ± SD indicated by the same letters did not significantly differ according to the Duncan test, $p \leq 0.05$.*

System	A_{lim} [Å^2]	π_{coll} [mN/m]	C_{smax}^{-1} [mN/m]
Fraction PL			
BOJAN NA	38.4 ± 0.2^c	41.0 ± 0.3^b	45.2 ± 0.9^b
BOJAN CA	41.4 ± 0.3^a	42.0 ± 0.4^a	52.3 ± 0.8^a
BOJAN DA	40.4 ± 0.5^b	41.8 ± 0.5^a	51.8 ± 1.0^a
FELIKS NA	45.3 ± 0.3^b	40.3 ± 0.6^b	51.4 ± 1.0^a
FELIKS CA	47.0 ± 0.4^a	41.2 ± 0.7^a	49.5 ± 1.1^a
FELIKS DA	45.5 ± 0.4^b	41.7 ± 0.5^a	44.8 ± 0.8^b
PRESIDENT NA	37.2 ± 0.5^b	39.7 ± 0.3^b	60.7 ± 0.5^a
PRESIDENT CA	41.6 ± 0.7^a	40.8 ± 0.8^a	52.9 ± 1.0^b
PRESIDENT DA	35.4 ± 0.6^c	41.1 ± 0.4^a	48.3 ± 1.1^c
ROKAS NA	38.7 ± 0.6^b	42.0 ± 0.6^a	57.3 ± 1.2^a
ROKAS CA	40.1 ± 0.4^a	41.6 ± 0.4^a	48.6 ± 1.3^b
ROKAS DA	38.5 ± 0.3^b	40.8 ± 0.7^b	44.9 ± 0.9^c
Fraction MGDG			
BOJAN NA	33.4 ± 0.4^c	35.2 ± 0.5^b	36.4 ± 0.7^c
BOJAN CA	58.7 ± 0.8^a	39.3 ± 0.3^a	49.6 ± 0.9^a
BOJAN DA	42.2 ± 0.3^b	39.9 ± 0.7^a	42.9 ± 0.9^b
FELIKS NA	32.7 ± 0.6^c	38.3 ± 0.3^b	41.2 ± 1.0^c
FELIKS CA	45.2 ± 0.7^a	39.2 ± 0.7^a	53.9 ± 1.1^a
FELIKS DA	42.9 ± 0.3^b	37.9 ± 0.6^b	49.6 ± 0.9^b
PRESIDENT NA	31.5 ± 0.5^b	35.6 ± 0.7^b	39.1 ± 1.2^b
PRESIDENT CA	36.6 ± 0.6^a	39.4 ± 0.6^a	45.1 ± 1.0^a
PRESIDENT DA	36.5 ± 0.3^a	39.3 ± 0.4^a	44.4 ± 0.9^a
ROKAS NA	35.4 ± 0.7^c	39.9 ± 0.3^b	53.8 ± 0.8^a
ROKAS CA	62.0 ± 0.3^a	41.5 ± 0.8^a	51.9 ± 0.9^b
ROKAS DA	43.7 ± 0.5^b	39.7 ± 0.5^b	41.4 ± 1.1^c

The value of π_{coll} increased after cold in all of the cultivars. In Feliks and Rokas, it decreased after deacclimation once again, while in President and Bojan, there were no differences between the CA and DA plants. The monolayers of the lipids that were isolated from the CA plants were characterised by a higher value of C_s^{-1} (exception Rokas – where C_s^{-1} was lower in the CA plants) and then again decreased in the DA plants (Fig. 3).

3.3. Tocopherol content and composition

The analysis of the content of tocopherols showed that the oilseed rape leaves and isolated chloroplasts primarily contained α-tocopherol (Fig. 4A, Table 2), while the remaining tocopherols were present in amounts that were approximately 100-fold lower; the lowest was for δ-tocopherol (Fig. 4D–Table 2). In the leaves of the non-acclimated plants, α-tocopherol was detected in a range from 141 ng mg^{-1} DW (President) to 177 ng mg^{-1} DW (Feliks). The chloroplasts that were isolated from the non-acclimated plants accumulated α-tocopherol from 102 ng mg^{-1} DW (Bojan) to 190 ng mg^{-1} DW (President). γ-tocopherol was present in the non-acclimated leaves and chloroplasts in a range of 1.8–6.1 ng mg^{-1} DW (Fig. 4C–Table 2). In the leaves and chloroplasts of the non-acclimated plants, β-tocopherol was also detected (2.4–4.7 ng mg^{-1} DW) (Fig. 4B–Table 2). δ-tocopherol was accumulated in all of the cultivars in amounts that averaged 0.5 ng mg^{-1} DW (exception – cultivar Feliks).

Table 2
The content of tocopherols (ng· mg^{-1}) in the leaves of oilseed rape (cultivars: Bojan, Feliks, President and Rokas). NA – the non-acclimated plants, CA – cold-acclimated plants, DA – deacclimated plants. *Mean values ± SD indicated by the same letters (within each cultivar separately) did not significantly differ according to the Duncan test, $p \leq 0.05$.*

cultivar/ treatment	α-tocopherol	β-tocopherol	γ-tocopherol	δ-tocopherol
Bojan NA	143 ± 16^b	2.93 ± 0.26^{ab}	2.58 ± 0.31^b	0.57 ± 0.12^a
Bojan CA	228 ± 14^a	2.28 ± 0.73^b	6.24 ± 1.44^a	0.30 ± 0.09^b
Bojan DA	227 ± 78^a	4.01 ± 1.26^a	3.47 ± 0.88^b	0.43 ± 0.14^{ab}
Feliks NA	177 ± 26^c	4.72 ± 1.47^b	6.10 ± 1.28^b	1.27 ± 0.39^b
Feliks CA	269 ± 52^b	5.25 ± 0.97^b	23.57 ± 5.55^a	4.56 ± 2.16^a
Feliks DA	411 ± 76^a	21.27 ± 2.66^a	10.59 ± 2.69^b	2.08 ± 0.63^b
President NA	141 ± 19^b	3.67 ± 0.83^a	3.08 ± 0.55^b	0.70 ± 0.28^a
President CA	202 ± 24^a	2.28 ± 0.50^b	6.14 ± 1.01^a	0.36 ± 0.09^b
President DA	184 ± 39^a	2.72 ± 0.35^b	3.24 ± 0.95^b	0.36 ± 0.09^b
Rokas NA	150 ± 17^b	2.30 ± 0.77^b	3.34 ± 0.80^b	0.52 ± 0.06^a
Rokas CA	247 ± 46^a	2.80 ± 0.79^{ab}	9.12 ± 3.06^a	0.46 ± 0.13^a
Rokas DA	327 ± 97^a	3.72 ± 1.02^a	4.63 ± 0.56^b	0.38 ± 0.18^a

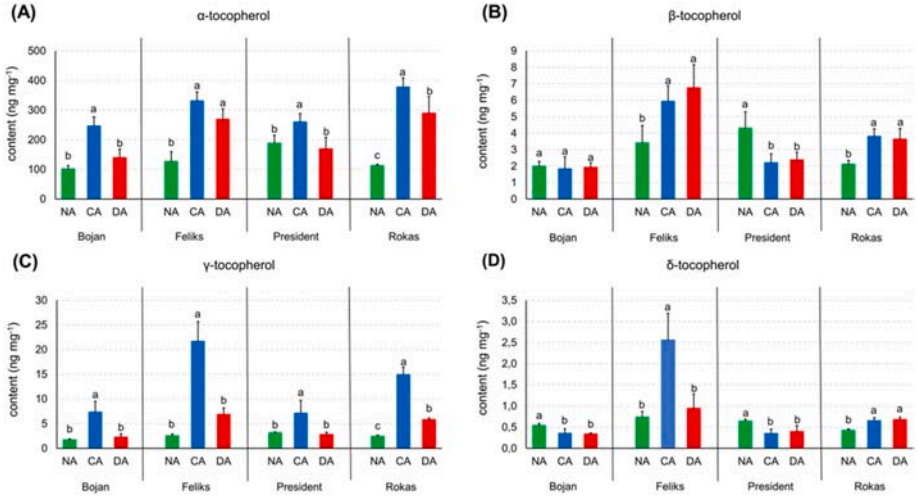

Fig. 4. The content of tocopherols: α – tocopherol (A), β – tocopherol (B), γ–tocopherol (C) and δ – tocopherol (D) in the chloroplasts of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape cultivars (Bojan, Feliks, President, Rokas). *Mean values ± SD indicated by the same letters (within each cultivar separately) did not significantly differ according to the Duncan test, $p \leq 0.05$.*

During cold acclimation, the content of α- and γ-tocopherol increased significantly in the leaves and chloroplasts of all of the cultivars compared to the non-acclimated plants. In some cases, the content of α-tocopherol was doubled (chloroplasts of Bojan) or even tripled (chloroplasts of Rokas). The accumulation of γ-tocopherol was also a few times higher on average after the cold acclimation of the plants. A more differentiated response (increase, decrease or no change) was observed for all of the cultivars in the case of the other two tocopherols. The accumulation of γ-tocopherol in the leaves and chloroplasts of the deacclimated plants decreased and reached a level that was similar to the ones that were observed in the non-acclimated plants, or, in some cases (chloroplasts of Rokas), they were only slightly higher. A similar situation was recorded in the case of the accumulation of α-tocopherol in the chloroplasts. Interestingly, in the deacclimated leaves, the content of α-tocopherol was maintained at the level that was recorded after cold acclimation (Fig. 4, Table 2).

3.4. Carotenoid content and composition

After analysing the content of the carotenoids, it was found that the largest amount was constituted by the β-carotene pool (on average about 3000 ng mg^{-1} in the non-acclimated plants), which was about one-order of magnitude higher than that of zeaxanthin (Fig. 5, Table 3). The content of carotenoids in the leaves decreased after cold acclimation in three of the four tested cultivars. After deacclimation, the leaves of President and Rokas were characterised by a further decrease of the most abandoned carotenoid – β-carotene. In the leaves of Feliks and Bojan, the β-carotene level was maintained at a level that was similar to the one that was observed after cold acclimation. The content of zeaxanthin was similar in the CA and DA plants (in three of the four tested cultivars, there were no statistical differences). The exception was an increase of the zeaxanthin content in the deacclimated President plants

Table 3
The content of carotenoids (ng· mg^{-1}) in the leaves of oilseed rape (cultivars: Bojan, Feliks, President and Rokas). NA – the non-acclimated plants, CA – cold-acclimated plants, DA – deacclimated plants. *Mean values ± SD indicated by the same letters (within each cultivar separately) did not significantly differ according to the Duncan test, $p \leq 0.05$.*

cultivar/treatment	β-carotene	zeaxanthin
Bojan NA	3029 ± 525^{a}	435 ± 48^{a}
Bojan CA	2474 ± 172^{b}	272 ± 88^{b}
Bojan DA	2424 ± 254^{b}	327 ± 52^{b}
Feliks NA	2966 ± 539^{a}	448 ± 55^{a}
Feliks CA	2235 ± 357^{b}	337 ± 44^{b}
Feliks DA	2475 ± 178^{ab}	377 ± 69^{ab}
President NA	2785 ± 287^{a}	454 ± 35^{a}
President CA	2790 ± 231^{a}	293 ± 30^{b}
President DA	2386 ± 104^{b}	410 ± 75^{a}
Rokas NA	3269 ± 284^{a}	412 ± 39^{a}
Rokas CA	2532 ± 267^{b}	368 ± 52^{a}
Rokas DA	2070 ± 171^{c}	387 ± 79^{a}

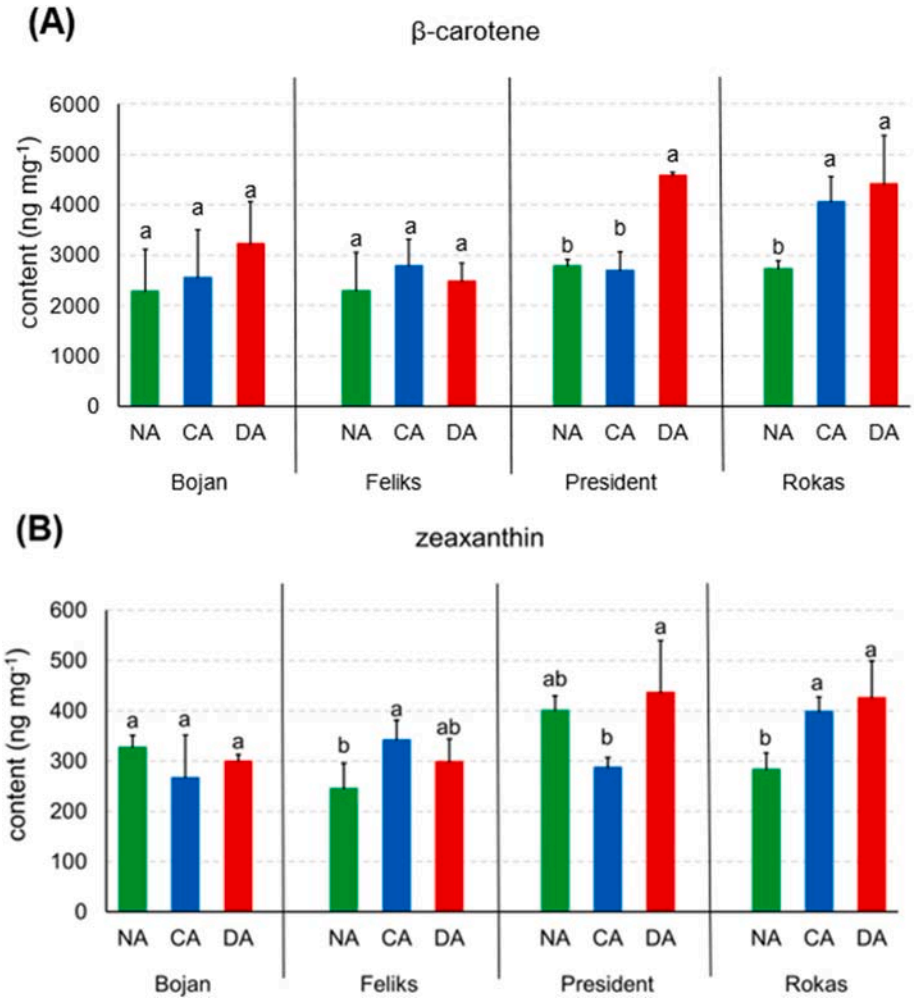

Fig. 5. The content of carotenoids: β-carotene (A) and zeaxanthin (B) in the chloroplasts of the non-acclimated (NA), cold-acclimated (CA) and deacclimated (DA) oilseed rape cultivars. *Mean values ± SD indicated by the same letters (within each cultivar separately) did not significantly differ according to the Duncan test, $p \leq 0.05$.*

(Table 3).

Somewhat less unequivocal results were observed for the isolated chloroplasts. Cold acclimation and deacclimation did not result in any statistically significant changes in any of the carotenoids in Bojan (compared to the non-acclimated plants). In the chloroplasts of the cold-acclimated and deacclimated Feliks, there was a significant increase only for zeaxanthin. The chloroplasts that were isolated from the cold-acclimated plants of President were characterised by a similar content of β-carotene and zeaxanthin as was observed for the chloroplasts from the non-acclimated plants. However, the content of both carotenoids increased after deacclimation. On the other hand, in Rokas, the chloroplasts that were isolated from the cold-acclimated and deacclimated plants accumulated similar amounts of β-carotene and zeaxanthin (but it was more than in the non-acclimated plants) (Fig. 5).

4. Discussion

The fatty acid content and composition of the chloroplast thylakoid membranes is unique to this organelle and differs from the other plant cell compartments thus indicating a major role for the lipids in the structure and function of chloroplasts (Hernández and Cejudo, 2021). In our work, the effect of a low temperature on the FA ratio 18:3/18:2 in the individual lipid fractions was also confirmed (Rudolphi-Szydło et al., 2020).

Changes in the degree of the saturation of the polyunsaturated fatty acid content can regulate the fluidity of the membrane lipids and improve a plant's tolerance to chilling stress (Liu et al., 2018; Zhao et al., 2021). The increase in the ratio of the more unsaturated FA 18:3 to 18:2 that was demonstrated in the presented research under low temperature conditions (CA plants) was expected because such relationships were also demonstrated earlier (Filek et al., 2017). This is generally connected to a higher pool of 18:3 and the simultaneous decrease of 18:2. The cultivars of winter wheat, which were more tolerant to a low temperature, responded (after cold acclimation) with a higher increase in unsaturation than the sensitive ones (Filek et al., 2017). An analysis of the changes in unsaturation that were initiated by a temperature drop (CA plants) showed that the increase in unsaturation in Rokas and Bojan (cultivars with higher frost tolerance) was the highest, particularly in the PL fraction and was also high in MGDG fraction (Fig. 1). An increase in lipid unsaturation is usually considered to be an indicator of augmented membrane fluidity. Hence, it was expected that the increase of the 18:3/18:2 ratio would be correlated with an increase of the A_{lim} values, which characterise a change of the layer structure towards a reduction of the density of its packing compared to the control (the lipid monolayers from non-acclimated plants). Generally, a low temperature resulted in a looseness of the lipid packing in the monolayers for all of the studied cultivars. The fact that the plants of Rokas and Bojan had the biggest changes in the value of the A_{lim} parameter after cold acclimation (MGDG fraction) could confirm the assumption that the more frost-tolerant cultivars of oilseed rape respond with greater changes in membrane fluidity when the temperature decreases. Two other cultivars (President and Feliks; more sensitive to frost) responded with a smaller increase in membrane fluidity after cold.

Deacclimation more or less reversed the influence of cold on the fluidity of the membranes (monolayers, PL fraction), resulting in a state of membrane fluidity that was similar to that of the NA plants. In the case of the MGDG fraction, relatively high monolayer fluidity was maintained after deacclimation. Since MGDGs are the main lipid fraction of chloroplasts, it can be assumed that despite deacclimation, the membranes of these organelles are 'prepared' for another temperature drop.

Another important parameter (C_s^{-1}), which characterises the flexibility of the membrane (resulting from the arrangement of the lipids relative to each other), showed that both cold acclimation and deacclimation modified the elasticity of the membrane of the tested cultivars. In model studies (Brown and Brockman, 2007; Leshem et al., 1988; Rudolphi-Skórska et al., 2014), a higher participation of polyunsaturated FA in the monolayer (or a higher ratio of 18:3/18:2) resulted in a higher fluidity of the monolayer (higher A_{lim}) and simultaneously its increased elasticity (expressed by the lower values of the compression modulus C_s^{-1}). The effect of increased elasticity is connected to the specific organisation of the *cis*-unsaturated fatty acid structures of the adjacent hydrocarbon chains relative to each other. In our studies, the opposite phenomenon was observed especially for the MGDG fraction in which cold treatment increased the A_{lim} values together with an increase of C_s^{-1} (i.e., the density of the molecules in the monolayer decreased with a decrease of its flexibility). Therefore, it can be assumed that in the MGDG fraction, there is a specific spatial fit of the individual molecules in the monolayer. Such interactions of both the hydrophobic part and the polar part (large in the case of this lipid) might result in a lower elasticity of the monolayer. The exception here was the frost-tolerant cultivar Rokas in which a higher elasticity (expressed by a lower C_s^{-1}) characterised the monolayers of the MGDG that had been isolated from the CA plants (compared to NA plants). In the case of the MGDG fraction, the higher temperatures during deacclimation reversed the effects of cold (Bojan, Feliks), but in the case of Rokas, a high elasticity (lower C_s^{-1}) also characterised the monolayers of the DA plants, which could be favourable for a better frost tolerance. As was mentioned above, the effect of increased elasticity is connected to the specific organisation of the *cis*-unsaturated FA. On the other hand, the spatial arrangement of the unsaturated fatty acid groups might depend on the effectiveness of the penetration of such monolayers (and in natural conditions, the membrane of the chloroplast) by the lipophilic molecules, such as, for example, the tocopherols or β-carotene, which are compounds that can modify the physicochemical properties of the membranes (Atkinson et al., 2010).

Since the increase in tocopherol synthesis is believed to be due to the activation of defence mechanisms under stress conditions (Munné-Bosch, 2005), an increase in the amount of tocopherols (mainly α-tocopherol) in the chloroplasts after cold acclimation appears to be justified. A similar effect was also observed in the whole leaves. On the other hand, although deacclimation decreased the content of α-as well as γ-tocopherol in the chloroplasts, interestingly, this did not happen in the leaf tissue. Tocopherols have been proven to be accumulated in the chloroplast membranes (Vidi et al., 2006) and also in the cells in other biological membranes (Atkinson et al., 2010; Maguire et al., 1989). One of theoretical explanations is that after the deacclimation, the rebuilding of the chloroplast membranes causes some amount of tocopherol to be withdrawn from the chloroplasts. Simultaneously, the total cell content of tocopherols remains unchanged or is even additionally increased, possibly even synthetised. Such a situation was observed in the DA Feliks, in which the α- and β-tocopherol content was higher compared to the CA plants (Table 2).

Studies on model membranes have revealed that tocopherols can interact with the polyunsaturated acyl groups of lipids that might stabilise the membrane structure (Wang and Quinn, 2000). Interestingly, α-tocopherol caused a destabilisation of the layers of the lipids that contain only saturated FA (Jurak and Miñones Conde, 2013). On the other hand, (Gzyl-Malcher et al., 2010) conducted Langmuir through studies of the interaction of tocopherols with the monolayers of lipids that had been isolated from the cells of winter wheat. The experiments showed that the mixed lipid/tocopherol monolayers were more stable. This effect was noted, e.g., for MGDG – lipids that are predominant in the chloroplasts. FA unsaturation (and a number of the galactose groups) determined the interactions between the tocopherols and lipids. Tocopherol generally limited membrane destabilisation. According to Gzyl-Malcher et al. (2010), it could be one of the mechanisms of the natural protection of the cells that synthesise this compound under stress. As was shown by (Rudolphi-Skórska et al., 2016), not only tocopherol, but also the products of tocopherol oxidation might stabilise the lipid layers. All of the tested cultivars reacted to cold with an increase in the α- and γ-tocopherol content in the chloroplast membranes,

which combined with the increase in the distance between the lipids (A_{lim}) in the hydrophobic part of the membrane, can favour the formation of sites in which the tocopherol molecules can be located. The formation of tocopherol-containing sites not only stabilises the membrane structure but at the same time, it protects it from oxidation by reactive oxygen species, especially at those sites in the membrane that contain the most unsaturated fatty acids (Rudolphi-Skórska et al., 2016). It is also worth emphasising that various tocopherols are produced in the cells and their slightly different structure can also affect their localisation and, in particular, their interaction with the membranes. To conclude, the accumulation of tocopherols in the chloroplast membranes might be useful for the effectiveness of the cold-acclimation process (as biochemical adjustments to cold). Simultaneously, since deacclimation reversed the metabolic changes that had been generated by cold (and the content of tocopherols in the chloroplast decreased once again), it could be one of many causes of the lower frost tolerance of deacclimated plants.

The second group of low-molecular antioxidants – carotenoids – are compounds that have many functions in plants. One of the most important is the participation of carotenoids in the structure of the photosynthetic antennae, their scavenging effect for reactive oxygen species and in modifying the membrane physicochemical properties (Gruszecki and Strzałka, 2005; Havaux, 1998; Lokstein et al., 2021). A higher accumulation of β-carotene in the membranes provides an additional level of protection of the chloroplasts and might support a higher frost-tolerance in plants. Our earlier studies showed that the more frost-tolerant cultivars of winter wheat accumulated more β-carotene and tocopherols in their leaves (Janeczko et al., 2018). It is also known that carotenoids modulate the stability of the membrane structures (Gruszecki and Strzałka, 2005; Havaux, 1998). β-Carotene increases membrane fluidity while zeaxanthin is considered to be the carotenoid that is responsible for decreasing membrane fluidity (Gabrielska and Gruszecki, 1996; Havaux, 1998). In the studied chloroplasts, the β-carotene content dominated strongly compared to the content of zeaxanthin (and tocopherols), which could be one of factors that create a higher fluidity of the native membranes (beneficial for better frost tolerance). In light of this, it is worth emphasising that a higher content of β-carotene characterised the CA plants of the most frost-tolerant cultivar (Rokas). It is also interesting that after deacclimation, a high accumulation of β-carotene in the chloroplasts was observed not only in Rokas, but also in President (and a slight tendency was observed in Bojan). All three cultivars had a better frost tolerance (after deacclimation) than Feliks (Fig. 1, Supplementary). To summarise, we can offer a theory here that a higher amount of β-carotene in chloroplasts (especially after deacclimation) can be necessary to modulate the membrane fluidity (important for frost tolerance) and may also help to 'prepare' plants (photosynthetic apparatus) for another temperature drop (so-called reacclimation process) and this phenomenon could also be connected to the enhanced photosynthetic efficiency that is characteristic for deacclimation (Rys et al., 2020; Stachurska et al., 2022).

Finally, the results obtained in this work we were trying to discuss/explain in the light of the previously described frost tolerance of the cultivars that are selected for studies. It should be remembered however that acquiring and maintaining different levels of frost tolerance is a complicated and multilevel process, in which the described changes in the chloroplast membranes may only be one of many other changes.

5. Conclusions

Despite the existing some cultivar-dependent differences in response to cold acclimation and deacclimation, the following general conclusions can be drawn regarding oilseed rape:

(1) The cold-induced changes in the unsaturation of fatty acids (expressed as ratio 18:3/18:2) in the chloroplast were associated with changes in the fluidity of the monolayers (A_{lim}). Cold (as was expected) increased the fluidity of the membranes. The seven days of deacclimation partly reverse this effect, which is probably unfavourable from the point of view of frost tolerance.

(2) In the chloroplasts of the cold-acclimated plants, the increase in tocopherols (especially α-tocopherol) and carotenoids (especially β-carotene) can influence membrane fluidity and favour the formation of specific tocopherol-/carotene-lipid "sites" in the membranes (in order to prevent them from the oxidation of the unsaturated fatty acid by reactive oxygen species). The decrease in the accumulation of tocopherols in the chloroplasts as a result of deacclimation might, therefore, contribute to the weakening of the membrane structure and may join with many factors that affect the decrease in plant frost tolerance. On the other hand, the accumulation of larger amounts of carotenoids (β-carotene) in the chloroplast membranes of the deacclimated plants was a more cultivar-dependent phenomenon. Nevertheless, this can be explained by both the enhanced photosynthetic efficiency (that is characteristic for deacclimation) and could also be among the factors that are favourable for a better tolerance of plants to frost, especially after periods of higher temperatures.

Author contributions

J.S. and M.R. – making of plant culture and collecting all samples, M. D.; M.R and J.S. – analysing the tocopherols and carotenoids; P.W., M.R and J.S. – analysing the fatty acids; J.S. and M.R. – isolating the chloroplasts; E.R–S. and M.F. – performing the Langmuir trough studies; A. J., M.F., J.S and M.R. – writing the article [P.W. writing the method for the GC analysis]; J.S., E.R.-S. and M.R. – calculating the results, preparing the figures/tables and performing the statistical analysis under the supervision of A.J.; M.R. – critically reading and correcting the manuscript; J.S. – editing the manuscript for publication; A.J. – creating the idea for the research and acquiring funding. All of the authors have read and agreed to the published version of the manuscript. We thank Dr Iwona Sadura for supervising the chloroplast isolation.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2024.108961.

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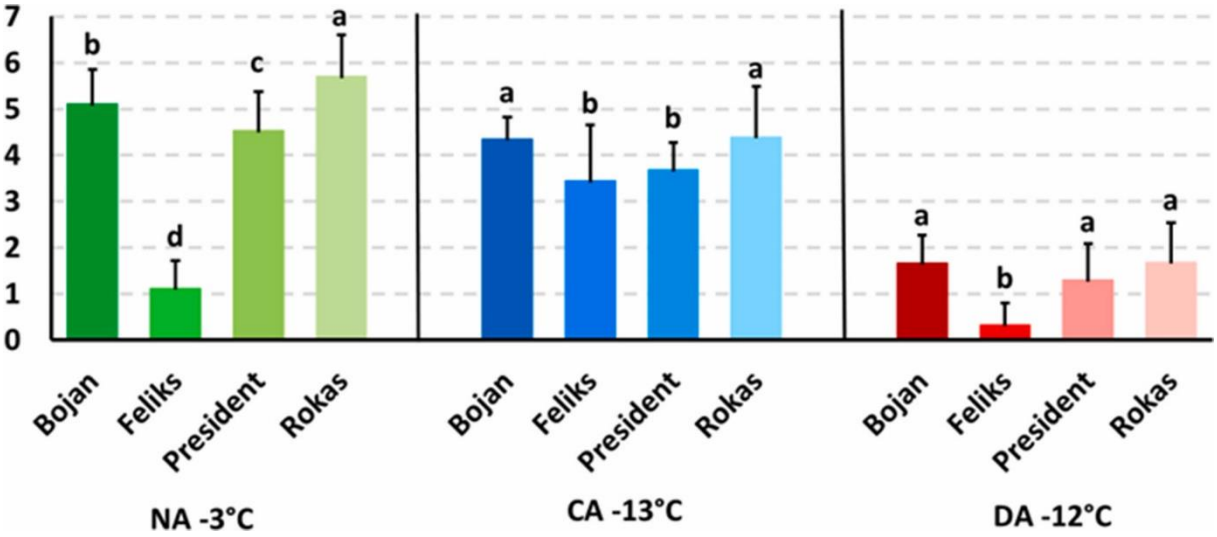
Appendix A. Supplementary data

The following is the Supplementary data to this article:



figs1. The frost tolerance of four cultivars of oilseed rape – Bojan, Feliks, President and Rokas that characterized the non-acclimated plants (NA), cold-acclimated plants (CA) and deacclimated plants (DA). The frost tolerance based on the regrowth scale (0–7 points; where 0 – dead plants, 7 – uninjured plants) after frost treatment (-3, -13 and -12°C, respectively for particular group of plants). Data were originally published in Stachurska et al. 2022, International Journal of Molecular Sciences 23, 5224. Current version is presented with different statistical approach and with permission of publisher MDPI. Mean values ± SD that are marked with the same letters (for each group of plants: NA, CA and DA separately) did not differ significantly at $p < 0.05$ according to Duncan's test, $n = 15$.

OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 29.11.2024 r.

Dr Magdalena Ryś

Zakład Biologii Rozwoju

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Oświadczam, że w pracy: Rys M., Stachurska J., Rudolphi−Szydło E., Dziurka M., Waligórski P., Filek M., Janeczko A. (2024) Does deacclimation reverse the changes in structural/physicochemical properties of the chloroplast membranes that are induced by cold acclimation in oilseed rape? Plant Physiology and Biochemistry, Volume 214, 108961 mój udział polegał na: założeniu i prowadzeniu doświadczenia we współpracy z doktorantką (J. Stachurską), pobieraniu próbek, a także przygotowaniu z pomocą doktorantki próbek do analiz zawartości tokoferoli, karotenoidów i kwasów tłuszczowych - ekstrakcja i oczyszczanie materiału roślinnego do analiz (w tym także wykonanie koniecznych optymalizacji metod i izolacja chloroplastów), współpracy z doktorantką podczas pisania artykułu i opracowywania wyników oraz korekcie artykułu.

.....Magdalena Ryś.....

(czytelny podpis współautora)

OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 03.10.2024

Dr hab. prof. UKEN Elżbieta Rudolphi-Szydło

Katedra Biochemii i Biofizyki

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Uniwersytet Komisji Edukacji Narodowej w Krakowie

Oświadczam, że w pracy: Rys M., Stachurska J., Rudolphi-Szydło E., Dziurka M., Waligórski P., Filek M., Janeczko A. (2024) Does deacclimation reverse the changes in structural/physicochemical properties of the chloroplast membranes that are induced by cold acclimation in oilseed rape? Plant Physiology and Biochemistry, Volume 214, 2024, 108961 mój udział polegał na: przeprowadzeniu we współpracy z doktorantką (J. Stachurską) pomiarów właściwości fizyko-chemicznych membran izolowanych z chloroplastów przy użyciu wagi Langmuira (w tym przeszkoleniu doktorantki z ogólnych zasad działania w/w aparatury).

Elżbieta Rudolphi-Szydło

(czytelny podpis współautora)

OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 09.10.2024

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Cold Acclimation and Deacclimation of Winter Oilseed Rape, with Special Attention Being Paid to the Role of Brassinosteroids

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Abstract: Winter plants acclimate to frost mainly during the autumn months, through the process of cold acclimation. Global climate change is causing changes in weather patterns such as the occurrence of warmer periods during late autumn or in winter. An increase in temperature after cold acclimation can decrease frost tolerance, which is particularly dangerous for winter crops. The aim of this study was to investigate the role of brassinosteroids (BRs) and BR analogues as protective agents against the negative results of deacclimation. Plants were cold-acclimated (3 weeks, 4 °C) and deacclimated (1 week, 16/9 °C d/n). Deacclimation generally reversed the cold-induced changes in the level of the putative brassinosteroid receptor protein (BRI1), the expression of BR-induced *COR*, and the expression of *SERK1*, which is involved in BR signal transduction. The deacclimation-induced decrease in frost tolerance in oilseed rape could to some extent be limited by applying steroid regulators. The deacclimation in plants could be detected using non-invasive measurements such as leaf reflectance, chlorophyll a fluorescence, and gas exchange monitoring.

Keywords: leaf reflectance; BRI1; *COR*; *SERK*; chlorophyll *a* fluorescence; brassinosteroid analogues; 24-epibrassinolide; 28-homocastasterone; CO_2 assimilation; frost tolerance

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1. Introduction

Oilseed rape (*Brassica napus* ssp. *oleifera* L.) is one of the most important oil crops cultivated in the world—it is a major source of vegetable oil for food and various industries. The plants from winter cultivars produce higher yields, and that is why, e.g., in Poland, they are cultivated more often, but this exposes them to growth in conditions of low temperatures (including frost) during winter. However, the winter species have evolved mechanisms of so-called cold acclimation (cold hardening), which enables them to survive temperatures below 0 °C. The biochemical and physiological processes that accompany cold acclimation are generally well known [1,2]. The more important changes include (1) changes in the membrane lipid composition in the direction of formation of more fluidic cell membranes [3,4], (2) a decrease in the leaf water content along with an increase in the soluble sugar content and the contents of other osmoprotectants [5], (3) an increase in the concentrations of stress hormones such as ABA [6], and (4) an increase in the

production of the protective proteins heat shock proteins (HSPs) [7] or cold-responsive proteins (CORs) [8].

Various climate models project that there will be an increase in average global temperatures by at least 2 °C by the end of the 21st century [9]. Global warming in Poland is visible, among other factors, by a rapid increase in the number of days with temperatures higher than 30 °C per year. In the years 1961–90, it was 3.5 days, but between 2011 and 2020 it was already 10.4 days [10]. Global warming is also connected with fluctuations in temperatures in autumn, for example, in regions of the eastern EU. Normally, a systematic drop in temperature to cold (more than zero) is a natural condition for cold acclimation. A period of 3–6 weeks of cold acclimation with a temperature of about 4 °C is usually needed for winter oilseed rape. After such a period, plants become much more frost-tolerant than before the cold acclimation and are able to survive the winter frost [11]. Because of the aforementioned climate changes, the cold acclimation process could be disturbed by sudden episodes of higher temperatures, such as 16 °C or more, e.g., during autumn and early winter, which induces the deacclimation (dehardening) process. This phenomenon decreases the frost tolerance of plants [11,12]. Deacclimation depends on the duration (number of days with higher temperatures) or the range of the higher temperatures [12]. For example, the deacclimation rate is faster at 20 °C than at 12 °C [12]. Longer periods of deacclimation could even lead plants to the resumption of growth and development.

As was mentioned earlier, although the metabolic background of cold acclimation is quite well known, more in-depth studies that are devoted to the mechanisms of the reactionof plants to deacclimation are relatively new and not numerous. To date, research has been conducted on the model plant *Arabidopsis thaliana* (L.) (among others [13,14]) and crop plants such as oilseed rape [12,15], barley [16], and wheat [17]. Regarding oilseed rape, our earlier studies have shown that deacclimation increases the efficiency of the light and dark reactions of photosynthesis, while on the metabolic level it causes changes in hormonal homeostasis, soluble sugars, or the accumulation of HSPs, although there is a revertive effect compared to the effect that is generated by cold [11,15,18]. For example, as a result of deacclimation, there was a decrease in the concentrations of the stress hormones (mainly ABA) and an increase in the growth-promoting hormones [18]. Such a shift in the hormonal balance as a result of deacclimation could be one of the possible causes of the decreased frost tolerance in deacclimated oilseed rape plants. This could happened especially when it is accompanied by a decrease in the concentration of soluble sugars, which have osmoprotective properties, and a decrease in the accumulation of the protective proteins [15].

The current work is a continuation of studies on the metabolic background of deacclimation in this economically important species (oilseed rape). In this study, however, we focused more on the possible role of brassinosteroids in the process of the cold acclimation and deacclimation of oilseed rape, along with the possibilities of using these regulators to counteract the negative effects of frost. It is worth emphasising that although the steroid hormones brassinosteroids (BRs) were first discovered in oilseed rape's pollen [19], their physiological role in this species is still rather poorly explained. The group of brassinosteroids includes 81 compounds. Brassinosteroids are characterised by various numbers of carbon (C) atoms in a molecule. There are three main groups of brassinosteroids: C27, C28, and C29 [20,21]. For example, brassinosteroids include brassinolide, castasterone, 24-epibrassinolide, and 28-homocastasterone. Synthetic analogues of BRs, the physiological activity of which is still being tested [22], are also available. Generally, the role of brassinosteroids in plants is important for regulating their growth and development, while BRs also have a protective effect in plants that are growing under stressful conditions such as drought [23], salinity [24], heavy metal stress [25,26], or low temperatures [27,28]. Regarding low-temperature stress, a limited number of studies have been devoted to the role of BRs as players that prepare the metabolism of plants for subzero temperatures during the cold acclimation/hardening process [20]. The effect of BRs on frost tolerance in cold-acclimated plants has been studied in the monocot plants of a group of cereals [28,29],

as well as in dicot plants (*A. thaliana*, basal frost tolerance) [30]. As for oilseed rape, it is known that cold acclimation reduces the accumulation of the *BRI1* transcript (encoding the BR receptor protein) [11]. After deacclimation, in more frost-susceptible cultivars, the level of the *BRI1* transcript increases once again, but in more frost-tolerant cultivars, the level of *BRI1* remains low, similar to what is observed in the cold-acclimated plants [11]. Interestingly, cold acclimation increased the concentrations of some of the brassinosteroids in oilseed rape, while deacclimation most often decreased their levels [11].

The current article presents the results of a cycle of experiments that were conducted on non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) oilseed rape, in which the aims were as follows:

1. To examine a few brassinosteroids (24-epibrassinolide (EBR) and 28-homocastasterone (HCS)) and brassinosteroid analogues (triolon (TR) and MK-266 (MK)) as potential agents that exhibit a protective activity against frost in NA plants (EBR), CA plants (EBR), and DA plants (EBR, HCS, MK, TR).
2. To describe the impact of steroids on the physicochemical properties of membranes, including describing the interaction of two BR analogues with the defined model lipid monolayers (mimicking cell membranes) in order to assess their influence on the fluidity of the lipid monolayers, which are important from the point of view of frost tolerance.
3. To describe the activity of the abovementioned steroids in regulating photosynthesis and the leaf spectral properties during the cold acclimation and deacclimation of oilseed rape.
4. To characterise any cold-acclimation-induced and deacclimation-induced changes in (a) the accumulation of the protein BRI1 (brassinosteroid membrane receptor), (b) the expression of the genes encoding the proteins that participate in BR signalling (*SERK1* and *SERK2*), and (c) the expression of the BR-dependent genes (*COR*).
5. To verify the possibility of using non-invasive measurement techniques (i.e., leaf reflectance) for the detection of deacclimation in plants.

2. Results

2.1. The Influence of BR and BR Analogues on Frost Tolerance

In the current research, both of the non-acclimated cultivars, Pantheon and President, suffered only minor injuries to their leaves, and on the regrowth scale they reached values close to the maximal seven points (control abs) (Table 1, Figure S1). In both cultivars, application of water with DMSO (control) statistically significantly decreased plant survival by roughly one point. The application of EBR reduced this effect, but only in the cultivar President. There were, however, no statistically significant differences between the plants of the control abs and the EBR-sprayed plants.

The CA plants were able to survive −13 °C with a score that ranged between 3.00 and 4.69 points (Table 1). In both cultivars, spraying the plants with water containing DMSO (control) was not beneficial and lowered frost tolerance (by about 0.7 points compared to the control abs). Interestingly, EBR reduced this effect. However, when the untreated plants (control abs) and the EBR-sprayed plants were compared, EBR improved the frost tolerance in only one cultivar (President). The EBR-treated plants of this cultivar had a score more than 0.8 points higher than the control abs (Table 1, Figure S1).

The oilseed rape plants that had been deacclimated and then exposed to frost of −6 °C were able to survive with a score between 2 and 5 points (Table 1). The DA plants that had been treated with EBR had higher scores compared to both the control abs and control. No cultivar dependency was observed (Figure S1). However, the effect of EBR was, weaker when the plants were exposed to −9 °C, and it disappeared at −12 °C (regrowth between 1 and 2 points) (Table 1, Figure S1).

Table 1. The frost tolerance of the winter oilseed rape cultivars Pantheon and President, which were characteristic for non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) plants based on the regrowth scale. Control abs—untreated plants; control—plants treated with a water solution of DMSO (a solvent of the tested steroids). The other objects represent plants that had been sprayed with brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone), brassinosteroid analogues (MK—MK-266; TR—triolon), and the regulator Asahi SL (AS). NT—not tested. Frost tests at −3 °C and −13 °C (NA and CA plants, respectively); DA plants were exposed to −6 °C, −9 °C, and −12 °C. Mean values ± SD that are indicated by the same letters did not differ significantly according to Duncan's test ($p < 0.05$)—a comparison between plants treated with a different substance that had been grown in the same conditions; statistical analysis was conducted separately for the cultivars Pantheon and President.

Cultivar	Treatment	NA Plants; Frost: −3 °C	CA Plants; Frost: −13 °C	DA Plants; Frost: −6 °C	DA Plants; Frost: −9 °C	DA Plants; Frost: −12 °C
Pantheon	Control abs	6.86 ± 0.53 ^{a}	3.75 ± 0.62 ^{a}	3.14 ± 0.66 ^{d}	2.20 ± 0.42 ^{c}	2.00 ± 0.00 ^{ab}
	Control	5.93 ± 1.10 ^{b}	3.00 ± 0.00 ^{b}	2.20 ± 0.42 ^{e}	2.40 ± 0.55 ^{c}	2.20 ± 0.42 ^{ab}
	EBR	5.80 ± 1.08 ^{b}	3.91 ± 0.94 ^{a}	5.13 ± 0.83 ^{a}	2.29 ± 0.73 ^{c}	2.22 ± 0.44 ^{a}
	HCS	NT	NT	3.53 ± 0.83 ^{cd}	3.17 ± 0.58 ^{b}	1.33 ± 0.78 ^{cd}
	MK	NT	NT	4.21 ± 1.37 ^{bc}	3.92 ± 0.67 ^{a}	0.90 ± 0.57 ^{d}
	TR	NT	NT	5.07 ± 1.27 ^{a}	2.29 ± 0.91 ^{c}	1.90 ± 0.32 ^{ab}
	AS	NT	NT	4.54 ± 1.05 ^{ab}	3.14 ± 0.53 ^{b}	1.75 ± 0.62 ^{bc}
President	Control abs	6.53 ± 0.64 ^{a}	3.92 ± 0.90 ^{b}	3.81 ± 0.98 ^{c}	2.82 ± 0.40 ^{b}	2.20 ± 0.42 ^{a}
	Control	4.87 ± 1.96 ^{b}	3.21 ± 0.43 ^{c}	4.53 ± 1.06 ^{bc}	3.73 ± 0.80 ^{a}	2.00 ± 0.71 ^{ab}
	EBR	7.00 ± 0.00 ^{a}	4.69 ± 0.63 ^{a}	5.60 ± 0.99 ^{a}	3.91 ± 0.83 ^{a}	2.00 ± 0.77 ^{ab}
	HCS	NT	NT	5.47 ± 1.36 ^{a}	3.50 ± 1.02 ^{a}	1.92 ± 0.28 ^{ab}
	MK	NT	NT	3.86 ± 0.95 ^{c}	3.50 ± 0.52 ^{a}	1.44 ± 0.73 ^{b}
	TR	NT	NT	5.33 ± 1.18 ^{ab}	3.83 ± 0.72 ^{a}	1.92 ± 0.29 ^{ab}
	AS	NT	NT	4.87 ± 1.06 ^{ab}	3.33 ± 0.78 ^{ab}	1.64 ± 0.67 ^{b}

Other regulators were also tested on the DA plants: the brassinosteroid HCS, detected earlier in the leaves of oilseed rape [11]; two BR analogues (MK, TR); and the commercial plant growth regulator Asahi (AS). The solvent of steroids (DMSO in the DA control) had some effect compared to the untreated plants (DA control abs), particularly after the frost tests at −6 °C (Pantheon, weakened regrowth) and −9 °C (President, even better regrowth).

Plants of the cultivar Pantheon responded well to the application of the tested regulators in the case of a milder frost. Improved plant regrowth was observed for all of them after the frost test at −6 °C (compared to the control with DMSO), and for MK, TR, and AS compared to the control abs (Table 1). After the frost tests at −9 °C, HCS, MK, and AS were effective. Frost at −12 °C caused severe injuries; however, similar to the case of EBR, the other regulators were no longer effective. Statistically significant negative effects of HCS and MK were even observed.

As for the cultivar President, improved plant regrowth after the frost test at −6 °C was observed for HCS, TR, and AS (compared to the control abs), but only for HCS in comparison to the control with DMSO (Table 1). After the frost tests at −9 °C, compared to the untreated plants, all of the steroid regulators were effective, but due to the relatively good regrowth of the plants of the control with DMSO, there were no statistically significant differences between this control and the plants that had been sprayed. Frost at −12 °C caused severe injuries, and the regulators were no longer effective. Statistically significant negative effects of MK and AS were even observed in comparison to the control abs.

2.2. The Effects of BRs and BR Analogues on Membrane Permeability

After 3 h from the moment of freezing, the electrolyte leakage in the NA control abs leaves reached a value of 53.75 µS (Table 2). After cold acclimation, the electrolyte leakage

in the control abs leaves reached a value of 2.82 µS, while after deacclimation it was at a similar level as in the CA leaves (2.25 µS). Application of DMSO to the NA leaves (control) resulted in a similar value to that in the control abs (57.82 µS). Moreover, in the CA and DA leaves, DMSO did not significantly affect the electrolyte leakage when compared with the control abs. Application of EBR and HCS to the NA and DA plants did not change the electrolyte leakage values compared to the control leaves, but in the CA plants EBR and HCS significantly increased its values. Another regulator, MK, significantly increased the electrolyte leakage in the leaves of the NA, CA, and DA plants compared with control and control abs. TR did not affect the electrolyte leakage in the NA and CA plants, while in the DA plants its application caused the highest electrolyte leakage.

Table 2. Membrane permeability based on electrolyte leakage after 3 h and after 24 h from the moment of freezing in the leaves of non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) oilseed rape (President cultivar). H_2O—redistilled/deionised water (without leaves); NFL—non-frozen leaves; Control abs—frozen leaves of untreated plants; control—frozen leaves of plants treated with a water solution of DMSO (a solvent of the tested steroids). The other objects represent frozen leaves of plants that had been sprayed with brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone) and brassinosteroid analogues (MK—MK-266; TR—triolon). Mean values indicated by the same letters did not differ significantly according to Duncan's test ($p < 0.05$). Comparisons within specific groups of plants (NA, CA, DA).

Growth Conditions and Freezing Time		Electrolyte Leakage after 3 h (µS)	Electrolyte Leakage after 24 h (µS)
NA 1 min 50 s	H_2O	1.4 ± 0.2 ^{c}	1.5 ± 0.2 ^{d}
	NFL	1.7 ± 0.3 ^{c}	2.5 ± 0.7 ^{d}
	Control abs	53.8 ± 27.5 ^{b}	128.2 ± 51.7 ^{c}
	Control	57.8 ± 35.7 ^{b}	149.8 ± 77.8 ^{bc}
	EBR	49.9 ± 17.3 ^{b}	152.3 ± 33.8 ^{bc}
	HCS	58.9 ± 24.9 ^{b}	174.1 ± 61.1 ^{abc}
	MK	108.4 ± 70.1 ^{a}	223.7 ± 96.3 ^{a}
	TR	86.4 ± 28.8 ^{ab}	210.7 ± 36.5 ^{ab}
CA 4 min 30 s	H_2O	1.3 ± 0.1 ^{d}	1.5 ± 0.1 ^{d}
	NFL	2.5 ± 1.2 ^{d}	3.6 ± 2.5 ^{d}
	Control abs	2.8 ± 1.2 ^{d}	5.5 ± 5.2 ^{d}
	Control	17.1 ± 15.8 ^{d}	47.3 ± 48.2 ^{cd}
	EBR	230.6 ± 44.5 ^{a}	459.9 ± 56.0 ^{a}
	HCS	163.5 ± 39.4 ^{b}	417.5 ± 119.0 ^{a}
	MK	77.6 ± 64.2 ^{c}	252.1 ± 155.0 ^{b}
	TR	40.6 ± 35.6 ^{cd}	136.6 ± 109.6 ^{c}
DA 2 min 30 s	H_2O	1.6 ± 0.2 ^{c}	1.8 ± 0.2 ^{c}
	NFL	2.3 ± 0.2 ^{c}	3.0 ± 0.2 ^{c}
	Control abs	2.3 ± 1.0 ^{c}	4.1 ± 3.9 ^{c}
	Control	1.8 ± 0.5 ^{c}	2.4 ± 0.6 ^{c}
	EBR	2.2 ± 0.5 ^{c}	2.7 ± 0.7 ^{c}
	HCS	1.9 ± 0.5 ^{c}	2.4 ± 0.7 ^{c}
	MK	16.6 ± 10.2 ^{b}	80.5 ± 46.7 ^{b}
	TR	59.8 ± 30.4 ^{a}	202.5 ± 93.6 ^{a}

Electrolyte leakage 24 h after freezing in the NA control abs leaves reached a value of 128.24 µS (Table 2). In the control abs plants, after cold acclimation, it was lower than in the NA plants (only 5.54 µS). After deacclimation, the electrolyte leakage in the control abs leaves also was lower than in the leaves of the NA plants. DMSO slightly increased the electrolyte leakage in the NA and CA plants, while it did not affect the DA plants when compared to the control abs. EBR and HCS did not increase the electrolyte leakage in the leaves of the NA plants. In the leaves of the CA plants, those regulators caused a significantly higher electrolyte leakage than in the control and control abs. EBR and HCS

		H_2O	1.6 ± 0.2 ^{c}	1.8 ± 0.2 ^{c}
		NFL	2.3 ± 0.2 ^{c}	3.0 ± 0.2 ^{c}
		Control abs	2.3 ± 1.0 ^{c}	4.1 ± 3.9 ^{c}
	DA	Control	1.8 ± 0.5 ^{c}	2.4 ± 0.6 ^{c}
	2 min 30 s	EBR	2.2 ± 0.5 ^{c}	2.7 ± 0.7 ^{c}
		HCS	1.9 ± 0.5 ^{c}	2.4 ± 0.7 ^{c}
		MK	16.9 ± 10.2 ^{b}	80.5 ± 46.7 ^{b}
		TR	40.6 ± 35.6 ^{a}	156.6 ± 109.6 ^{a}
		H_2O	1.6 ± 0.2 ^{c}	1.8 ± 0.2 ^{c}
		NFL	2.3 ± 0.2 ^{c}	3.0 ± 0.2 ^{c}
		Control abs	2.3 ± 1.0 ^{c}	4.1 ± 3.9 ^{c}
	DA	Control	1.8 ± 0.5 ^{c}	2.4 ± 0.6 ^{c}
	2 min 30 s	EBR	2.2 ± 0.5 ^{c}	2.7 ± 0.7 ^{c}
		HCS	1.9 ± 0.5 ^{c}	2.4 ± 0.7 ^{c}
		MK	16.9 ± 10.2 ^{b}	80.5 ± 46.7 ^{b}
		TR	39.8 ± 30.4 ^{a}	202.5 ± 93.6 ^{a}

did not affect the DA plants. The BR analogue MK significantly increased the electrolyte leakage in the leaves of the NA, CA, and DA plants, compared to the control abs and control. TR increased the electrolyte leakage in DA plants.

2.3. Interaction of BR Analogues (MK, TR) with the Model Membranes

2.3. Interaction of BR Analogues (MK, TR) with the Model Membranes

The exemplary isotherms of surface pressure (π) as a function of surface area per lipid molecule in a monolayer for PC 18:3, PC 16:0, and their mixtures are presented in Figures S2 and S3. Based on these isotherms, the physicochemical parameters such as A_{lim}, π_{coll}, and C_s^{-1} were calculated and are presented in Table S1. The percentage changes in the A_{lim} parameter, which indicates the surface area that is occupied by the lipids in the monolayer, are presented in Figure 1.

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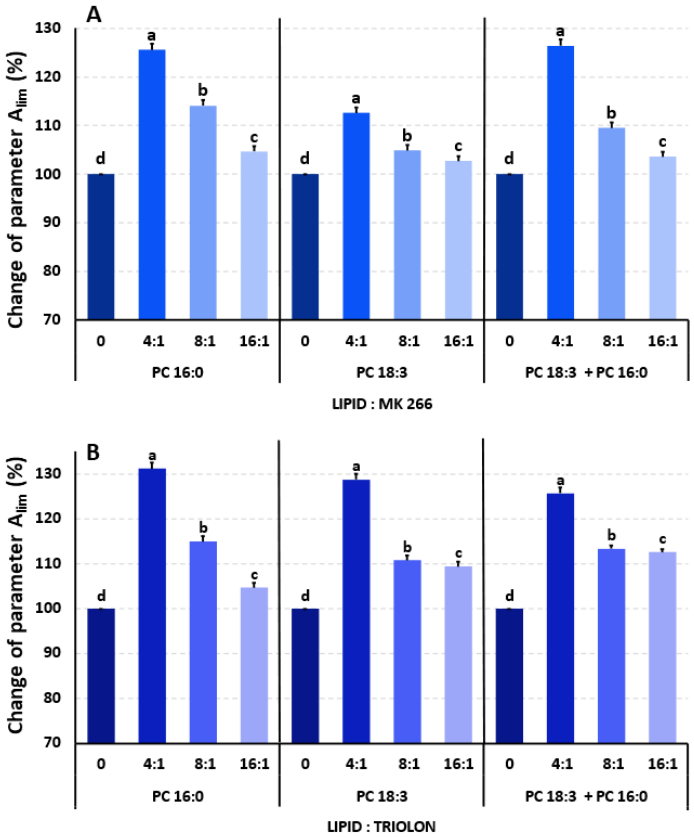

Figure 1. The influence of BR analogues on the limiting area per molecule (parameter A_{lim}) of the model monolayers. The control system (marked 0), considered to be 100%, was represented by the lipid isotherm on the subphase without the addition of hormones. (**A**) Mixture of lipid and MK-266, (**B**) Mixture of lipid and triolon. Percentage values were calculated based on the original data presented in Table S1. Statistically significant differences between systems (Duncan's test, $p \leq 0.05$) are indicated by different letters; statistical analysis was conducted separately for each group: PC 16:0, PC 18:3, and PC 18:3 + PC 16:0.

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Adding the analogues MK-266 and triolon to the single- and double-compound lipid mixtures, consisting of saturated and unsaturated lipids, resulted in an increase in the A_{lim} parameter (Figure 1A,B, Table S1). Higher hormone concentrations were associated with a greater increase in the A_{lim} value, which ranged from approximately 2% to around 31% and was concentration-dependent. The most significant changes in these experimental systems were observed for the 4:1 mixture (M:M).

In the case of the second determined parameter, π_{coll}, the changes that were observed in the studied systems were slight but were statistically significant compared to the control (system without the addition of hormones). Changes in the pressure at which the monolayer collapsed ranged from approximately ±0.5 to 3 mN/m (Table S1).

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For a more detailed analysis of changes occurring under the influence of hormone addition, stability curves of the compression modulus (C_s^{-1}) were plotted against surface pressure, as depicted in Figure S3 (and, as numerical results, in Table S1).

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2.4. Accumulation of the Brassinosteroid Receptor Protein (BRI1)

In the NA Pantheon plants, the accumulation of the putative BRI1 protein was at a level of 3652 (a.u.) (Figure 2A,B). After cold acclimation, the accumulation of this protein decreased significantly by 67%, while after deacclimation the putative BRI1 accumulation increased significantly, by almost 100%; however, it did not reach the level that was observed in the NA plants. In the NA President plants, the accumulation of putative BRI1 reached a level of 2242 (a.u.) (Figure 2B). After cold acclimation, the amount of this protein decreased by 35%. However, after deacclimation, the accumulation of putative BRI1 increased significantly and reached a value that was similar to what was detected in the NA plants. In addition to the cultivars Pantheon and President, BRI1 was also analysed for two other winter cultivars (Bojan, Rokas) and one spring cultivar (Feliks). A similar model of changes in the putative BRI1 accumulation to those that were observed in the NA, CA, and DA plants of Pantheon and President was confirmed in these three additional cultivars (Figure S4).

In the NA Pantheon plants, the accumulation of the putative BRI1 protein was at a level of 3652 (a.u.) (Figure 2A,B). After cold acclimation, the accumulation of this protein decreased significantly by 67%, while after deacclimation the putative BRI1 accumulation increased significantly, by almost 100%; however, it did not reach the level that was observed in the NA plants. In the NA President plants, the accumulation of putative BRI1 reached a level of 2242 (a.u.) (Figure 2B). After cold acclimation, the amount of this protein decreased by 35%. However, after deacclimation, the accumulation of putative BRI1 increased significantly and reached a value that was similar to what was detected in the NA plants. In addition to the cultivars Pantheon and President, BRI1 was also analysed for two other winter cultivars (Bojan, Rokas) and one spring cultivar (Feliks). A similar model of changes in the putative BRI1 accumulation to those that were observed in the NA, CA, and DA plants of Pantheon and President was confirmed in these three additional cultivars (Figure S4).

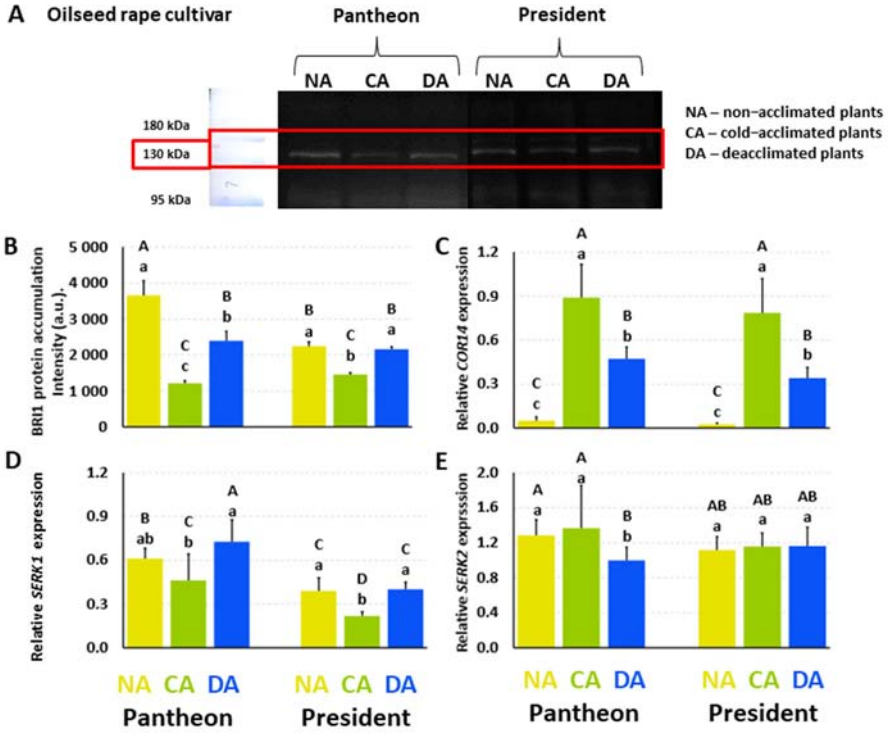

Figure 2. The accumulation of the putative BRI1 protein and relative gene expression (*COR14*, *SERK1*, *SERK2*) levels in the leaves of the non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) oilseed rape plant cultivars Pantheon and President. (**A**) The representative original image, where the visualised bands correspond to the level of putative BRI1 protein; 15 µg of protein was loaded onto the gel. (**B**) The accumulation of the putative BRI1 protein. (**C**) Relative expression of *COR14*. (**D**) Relative expression of *SERK1*. (**E**) Relative expression of *SERK2*. The transcript levels were calculated relative to actin (endogenous reference gene). Mean values ± SE that are indicated by the same letters did not differ according to Duncan's test ($p < 0.05$). Lowercase letters—comparisons between the NA, CA, and DA plants within each cultivar. Capital letters—comparisons between the NA, CA, and DA plants of both cultivars.

2.5. Expression of the Genes Encoding the Proteins That Participate in BR Signalling (SERK1 and SERK2) and the BR-Dependent Gene (COR14)

The relative expression of the *COR14* gene in the NA Pantheon plants reached a value of 0.0494 (Figure 2C). After cold acclimation, there was a significant increase (18-fold) in the *COR14* expression. In the DA plants, the amount of this transcript decreased by almost 50% compared to the CA plants. A similar tendency was observed in the cv. President—in the NA plants, the amount of the *COR14* transcript reached a value of 0.0236, while after cold acclimation there was a significant increase of about 30-fold. In the DA President plants, the expression of *COR14* decreased (about 50%), but it did not reach the level that was characteristic of NA plants.

In the NA Pantheon plants, the *SERK1* expression reached a value of 0.611 (Figure 2D). After cold acclimation, the amount of this transcript decreased significantly, by 25%. However, after deacclimation, the expression of *SERK1* increased to a level that was similar to that of the NA plants. A similar tendency of changes in the *SERK1* expression was observed in the cultivar President. In the NA President plants, the expression of *SERK1* reached a value of 0.3882. After cold acclimation, the expression significantly decreased by 44%, while after deacclimation it increased once again and reached a level similar to that of the NA plants. The expression of *SERK2* in the NA Pantheon plants reached a value of 1.284 (Figure 2E), and after cold acclimation it did not change significantly. In the DA Pantheon plants, the *SERK2* expression decreased significantly, by 27% compared to the CA plants. In the cultivar President, the expression of *SERK2* that was detected in the NA plants reached a value of 1.114, and it did not change significantly in the CA and DA plants.

2.6. The Effects of BRs and BR Analogues on PSII Efficiency

The so-called parameters of yield, or flux ratios (φ_{Po}, ψ_o, and φ_{Eo}), were calculated based on data from fluorescence curves.

The maximum quantum yield of primary photochemistry (φ_{Po}) in the cultivar Pantheon (NA control abs) reached a value of 0.81, and after cold acclimation it significantly decreased by about 9% (Figure 3A). After deacclimation, the φ_{Po} value returned to the level that was characteristic of the NA plants. The application of the working solutions with DMSO (control plants) did not cause any changes in the φ_{Po} value in the NA, CA, and DA plants. The application of the tested regulators did not influence the φ_{Po} values (compared to the control plants).

In the NA President plants (control abs), the value of φ_{Po} reached 0.82 (Figure 3B). After cold acclimation, φ_{Po} significantly decreased by 15%, while after deacclimation φ_{Po} increased and reached a value that was similar to that of the NA plants. The application of DMSO did not affect the φ_{Po} values in the NA and DA plants, but in the CA plants φ_{Po} was increased by about 13% compared to the control abs. No effect of the tested regulators on φ_{Po} was observed.

The probability that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (ψ_o) in the NA Pantheon plants (control abs) reached a value of 0.58 (Figure 3C). After cold acclimation, it did not change significantly, but after deacclimation ψ_o increased to a value that was 22% higher than in the NA plants. There was no effect of the DMSO treatment (control) on the NA, CA, and DA plants. The application of the regulators did not change ψ_o.

In the cultivar President, in the NA control abs plants, the ψ_o value was 0.59 (Figure 3D). After cold acclimation, it decreased significantly by 8%, while after deacclimation it increased to a value of 0.70, which was 17% higher than in the NA plants. The DMSO application did not affect ψ_o in the NA and DA plants, but it significantly increased the ψ_o value in the CA plants compared to the control abs plants (by 11%). The application of the regulators did not change ψ_o significantly.

The quantum yield of electron transport (φ_{Eo}) in the NA Pantheon control abs plants reached a value of 0.47, and it did not change after cold acclimation (Figure 3E). After

reached a value of 0.47, and it did not change after cold acclimation (Figure 3E). After deacclimation, φ_{Eo} increased by 26%. In comparison to the absolute control, there was no effect of DMSO (control) and the applied regulators on φ_{Eo}.

In the NA control abs plants of the cultivar President, the value of φ_{Eo} reached 0.49, and it decreased by 22% after cold acclimation (Figure 3F). After deacclimation, φ_{Eo} increased once again and reached a value 20% higher compared to the NA plants. The DMSO did not affect φ_{Eo} in the NA plants, but in the CA plants it increased φ_{Eo} by 26%. The tested BRs and BR analogues did not induce any significant changes in the values of φ_{Eo}.

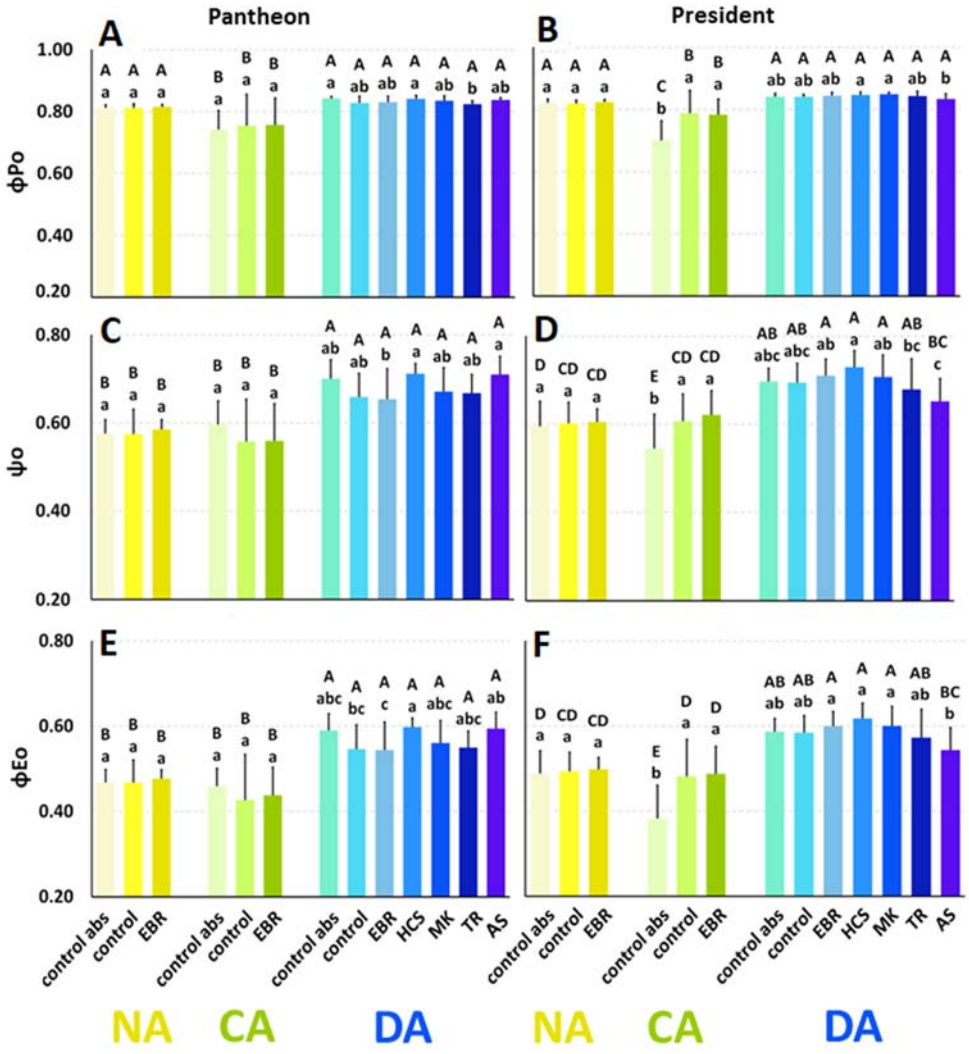

Figure 3. Quantum photosynthetic efficiency described by the chlorophyll *a* fluorescence measurements (yield/flux ratios [31]) of the non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) winter oilseed rape cultivars Pantheon (**A**,**C**,**E**) and President (**B**,**D**,**F**); φ_{Po}—maximum quantum yield of primary photochemistry (at t = 0) (**A**,**B**); ψ_o—probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^- (**C**,**D**); φ_{Eo}—quantum yield of electron transport (at t = 0) (**E**,**F**). Control abs—untreated plants; control—plants treated with a water solution of DMSO (a solvent of the tested steroids). The other objects represent plants that had been sprayed with brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone), brassinosteroid analogues (MK—MK-266; TR—triolon), and the regulator Asahi SL (AS). Mean values indicated by the same letters did not differ significantly according to Duncan's test ($p < 0.05$). Lowercase letters—comparisons between the treatments within a specific group (NA, CA, and DA plants); capital letters—comparisons between the treatments of the plants in all three groups (NA, CA, and DA plants).

In the NA control abs plants of the cultivar President, the value of φ_{Eo} reached 0.49, and it decreased by 22% after cold acclimation (Figure 3F). After deacclimation, φ_{Eo} increased once again and reached a value 20% higher compared to the NA plants. The DMSO did not affect φ_{Eo} in the NA plants, but in the CA plants it increased φ_{Eo} by 26%. The tested BRs and BR analogues did not induce any significant changes in the values of φ_{Eo}.

2.7. The Effects of BRs and BR Analogues on Leaf Gas Exchange

Net photosynthesis intensity (P_N) is a parameter that provides information about the intensity of the CO_2 assimilation. In the cultivar Pantheon, in the NA plants (control abs), the value of P_N reached 11.67 µmol (CO_2) m^{-2} s^{-1} (Figure 4A). After cold acclimation, in the control abs plants, P_N increased by about 36%, while after deacclimation there was a further increase by about onefold in comparison to the NA plants. The application of DMSO did not affect P_N in the NA, CA, or DA plants. The application of EBR significantly increased P_N in the NA, CA, and DA plants. The other regulators that were used in the DA plants, HCS and AS, also caused an increase in P_N, while the application of MK and TR did not affect this parameter.

In the NA President plants (control abs), the value of P_N reached 11.41 µmol (CO_2) m^{-2} s^{-1} (Figure 4B). After cold acclimation, P_N decreased by about 25%. After deacclimation, P_N increased again and reached values of about 25% higher compared to the NA plants. The application of DMSO did not significantly affect the P_N values in the NA, CA, and DA plants. The application of EBR improved P_N in the NA, CA, and DA plants. The application of the other regulators, TR and AS, to the DA plants increased P_N. The application of MK did not affect P_N in the DA President plants.

Transpiration intensity (E) in the NA control abs plants of the Pantheon cultivar reached 2.88 mmol (H_2O) m^{-2} s^{-1}, while after cold acclimation it decreased by 44% (Figure 4C). However, after deacclimation, there was generally about a twofold increase in E when compared to the CA plants. The application of DMSO increased E in the NA plants very slightly, but it did not affect the CA and DA plants. The application of EBR decreased E in the NA plants but did not have a significant effect in the CA and DA plants compared to the controls. Plants sprayed with the other regulators, HCS and AS, had E values similar to the controls. MK and TR even caused a decrease in E.

In the cultivar President, the NA plants were characterised by E reaching 2.92 mmol (H_2O) m^{-2} s^{-1} (Figure 4D). After cold acclimation, E significantly decreased, by about 60%. After deacclimation, in the control abs plants, E increased by about threefold when compared to the CA plants. The application of DMSO did not affect E in the NA and CA plants, but it caused a decrease in E in the DA plants. Although the application of EBR did not affect the NA plants, it caused a significant increase in E in the CA plants. In the DA plants, there was a significant decrease in E after the application of EBR. The application of HCS, TR, and AS caused a decrease in E, while MK caused an increase in E when compared to the control plants.

Stomatal conductance (g_s) in the control abs of NA Pantheon plants reached a value of 0.39 mol (H_2O) m^{-2} s^{-1} (Figure 4E). After cold acclimation, g_s in the control abs plants increased by 78%, while after deacclimation it further increased by 76% compared to the CA plants. The application of DMSO did not affect g_s in the NA and CA plants; however, in the DA plants, it decreased g_s significantly, by about 37%, compared to the control abs. The use of EBR did not cause significant changes in g_s in the NA, CA, and DA Pantheon plants. Among the other regulators that were used in the DA Pantheon plants, HCS, TR, and AS did not change the g_s values, while MK increased g_s.

The value of g_s in the control abs of NA President plants was 0.41 mol (H_2O) m^{-2} s^{-1} (Figure 4F), and after cold acclimation, unlike the Pantheon plants, it did not change significantly. However, after deacclimation, in the control abs plants, g_s increased significantly by about 170% compared to the CA plants. Similar to the Pantheon plants, the application of DMSO did not affect g_s in the NA and CA plants, but it decreased g_s significantly (by about 17%) in the DA plants. In the NA and CA President plants, the application of EBR did not change the values of g_s compared to the control plants. However, in the DA plants, EBR decreased the values of g_s to a level that was similar to those in the NA plants and even in the CA plants. The application of HCS, TR, and AS decreased the g_s value compared to the control abs, and also partially compared to the plants that were treated with DMSO. The plants that were treated with MK were characterised by a level of g_s that was similar to the control abs.

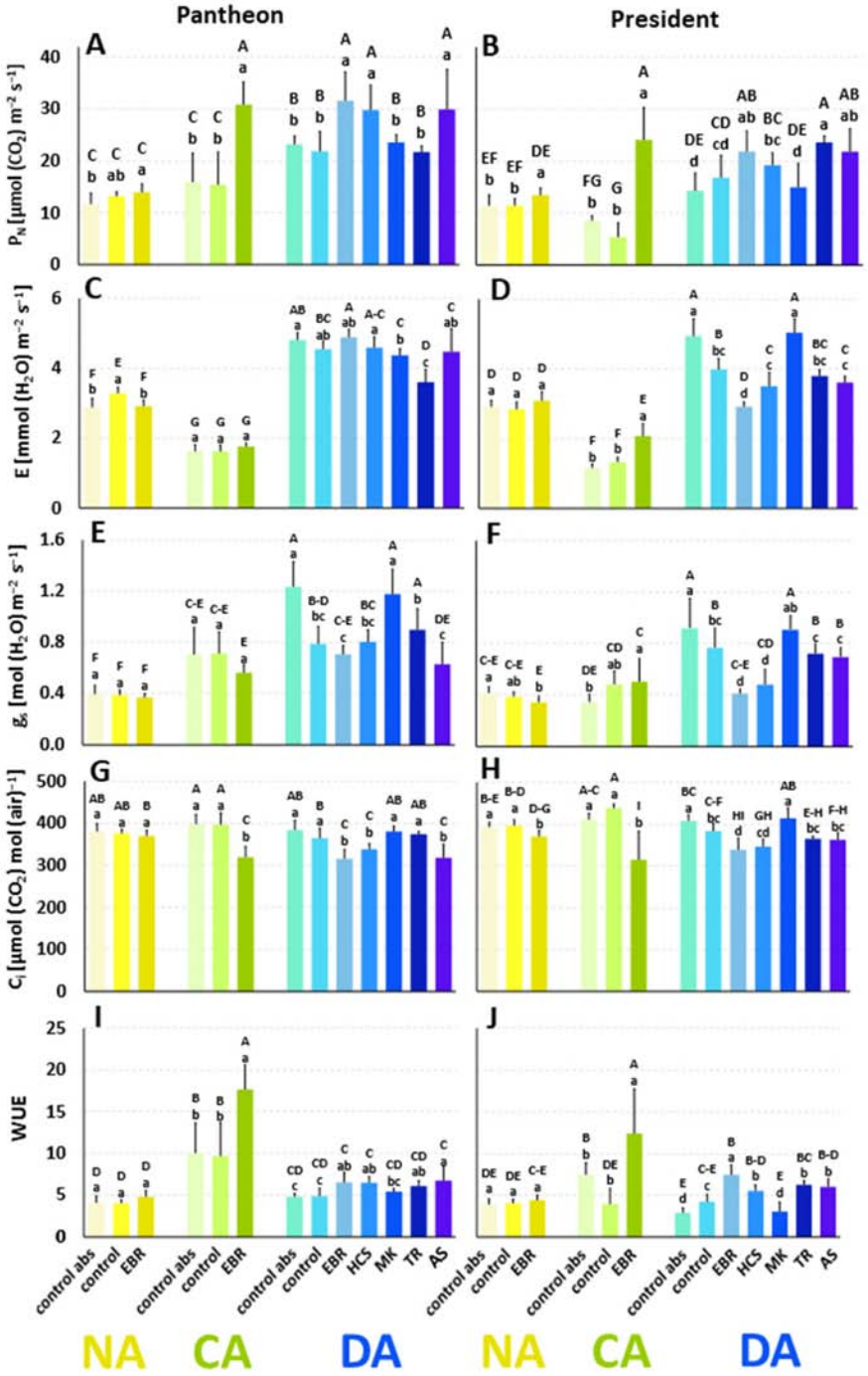

Figure 4. Gas exchange in the non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) leaves of the oilseed rape cultivars Pantheon (**A**,**C**,**E**,**G**,**I**) and President (**B**,**D**,**F**,**H**,**J**). P_N—net photosynthesis intensity (**A**,**B**); E—transpiration (**C**,**D**); g_s—stomatal conductance (**E**,**F**); C_i—intracellular concentration of CO_2 (**G**,**H**); WUE—photosynthetic ratio of water use (**I**,**J**). Control abs—untreated plants; control—plants that were treated with a water solution of DMSO (a solvent of the tested steroids). The other objects represent plants that had been sprayed with brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone), brassinosteroid analogues (MK—MK-266; TR—triolon), and the regulator Asahi SL (AS). Mean values indicated by the same letters did not differ significantly according to Duncan's test ($p < 0.05$). Lowercase letters—comparisons between the treatments within a specific group (NA, CA, and DA plants), capital letters—comparisons between the treatments of plants of all three groups (NA, CA, and DA plants).

The intracellular concentration of CO_2 (C_i) in the plants of the control abs (NA, Pantheon) was 380 µmol (CO_2) mol (air)$^{-1}$ (Figure 4G). In the CA plants, it remained similar (398 µmol (CO_2) mol (air)$^{-1}$), and C_i did not change significantly after deacclimation. There was no effect of DMSO on the values of C_i in the NA, CA, and DA Pantheon plants compared to the control abs. EBR did not affect the values of C_i in the NA plants; however, the steroid did decrease C_i in the CA and DA plants, similar to HCS and AS. There was no effect of MK and TR on the values of C_i in the DA plants compared to both controls.

In the cultivar President, C_i did not vary significantly between the NA, CA, and DA plants of the control abs (Figure 4H). The application of DMSO did not affect the NA and CA plants; however, it caused a slight, but significant decrease in C_i (6%) in the DA plants. The application of EBR affected the NA, CA, and DA plants; the hormone caused significant decreases in C_i. The other BR (HCS) caused a decrease in C_i compared to the control abs. The BR analogue (TR) that was used in the DA plants did not generally change the C_i values. Only MK increased C_i compared to the control.

The value of water-use efficiency (WUE) reached 4.09 in the NA control abs plants of the cultivar Pantheon (Figure 4I). After cold acclimation, there were increases in WUE for the control abs and control (DMSO) plants by about 140%, while for the EBR-treated plants the increase was more than 260%. After deacclimation, WUE generally decreased to values that were similar to those of the NA plants. The application of DMSO did not change the WUE values in the NA, CA, and DA plants when compared to the control abs. In the NA plants, EBR did not affect WUE, while in the CA plants it caused an increase in this parameter. After deacclimation, the application of EBR increased the WUE. The application of all of the regulators (except for MK) slightly increased the values of WUE (by an average of 33%) in the DA plants compared to both of the controls.

In the control abs plants of the President cultivar, the WUE value was 3.91 (Figure 4J). Cold acclimation resulted in an increase in the WUE values; in the control abs plants, there was almost a 90% increase. After deacclimation, the WUE in the control abs plants decreased to a level that was even lower than in the NA plants. The application of DMSO did not affect the NA and CA plants, while it resulted in higher WUE in the DA plants when compared to the control abs plants. No effect of EBR was observed in the NA plants. The application of EBR caused an increase in the WUE value in the CA plants, and a similar effect was observed after deacclimation. Higher WUE was also observed in the DA plants that were treated with HCS, TR, and AS.

2.8. The Effects of BRs and BR Analogues on the Leaf Spectral Properties

The reflectance intensity of the leaves of the cultivars Pantheon and President increased in the range of 500–550 nm for the NA, CA, and DA plants (Figure 5). Then, it decreased in the range of 550–700 nm, increased sharply in the range of 700–750 nm, and reached a plateau at the range of 750–950 nm for both the control abs and control, as well as in EBR-treated plants. Slight visible differences were observed in the reflectance intensity between the NA, CA, and DA plants, especially in the ranges of 500–650 nm and 750–950 nm.

Based on the reflectance curves, the following parameters of reflectance were calculated: the Water Band Index (WBI), Structure Insensitive Pigment Index (SIPI), Red-Edge Normalised Difference Vegetation Index (RENDVI), Anthocyanin Reflectance Index 1 (ARI1), and Anthocyanin Reflectance Index 2 (ARI2) (Figure 6). Additionally, the Triangular Vegetation Index (TVI), Simple Ratio Pigment Index (SRPI), Normalised Difference Vegetation Index (NDVI), Greenness Index (G), and Carotenoid Reflectance Index 1 (CRI1) were also calculated (Figure S5). As for the values of additionally calculated parameters (TVI, SRPI, NDVI, and CRI1), there were no significant changes between NA, CA, and DA plants. There was also no influence of the tested regulators (Figure S5A–F,I,J). An exception was the Greenness Index, which slightly decreased in CA and DA plants. A slight effect of the steroids was observed in the DA group. For example, TR increased the G value in Pantheon plants in comparison to both controls (Figure S5G,H).

decreased in the range of 550–700 nm, increased sharply in the range of 700–750 nm, and reached a plateau at the range of 750–950 nm for both the control abs and control, as well as in EBR-treated plants. Slight visible differences were observed in the reflectance intensity between the NA, CA, and DA plants, especially in the ranges of 500–650 nm and 750–950 nm.

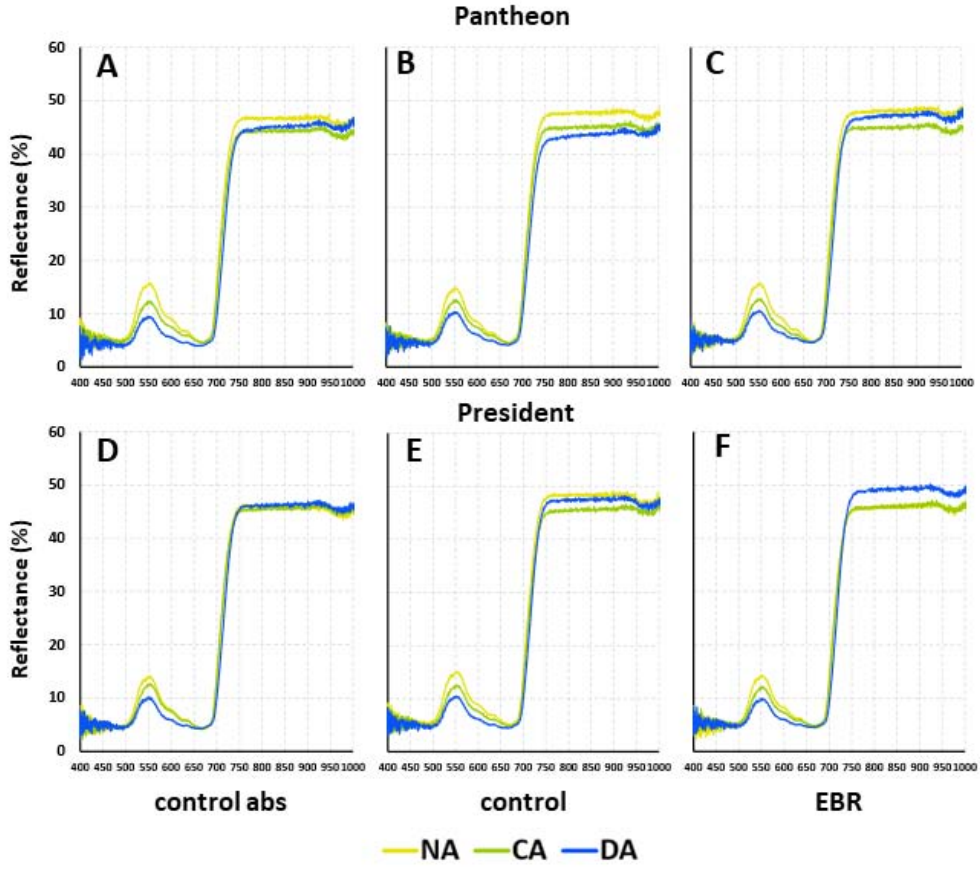

Figure 5. The reflectance curves expressing the reflectance intensity (%) of the leaves of non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) oilseed rape (A–C) Pantheon cultivar, (D–F) President cultivar. X-axis = wave length [nm]. Control abs—untreated plants; control—plants sprayed with a water solution of DMSO (a solvent of the tested steroids); EBR—plants sprayed with the steroid hormone 24-epibrassinolide. Each curve represents the average of the measurements that were taken on ten leaves (each replicate was one leaf from different plants).

Based on the reflectance curves, the following parameters of reflectance were calculated: the Water Band Index (WBI), Structure Insensitive Pigment Index (SIPI), Red-Edge Normalised Difference Vegetation Index (RENDVI), Anthocyanin Reflectance Index 1 (ARI1), and Anthocyanin Reflectance Index 2 (ARI2) (Figure 6). Additionally, the Triangular Vegetation Index (TVI), Simple Ratio Pigment Index (SRPI), Normalised Difference Vegetation Index (NDVI), Greenness Index (G), and Carotenoid Reflectance Index 1 (CRI1) were also calculated (Figure S5). As for the values of additionally calculated parameters (TVI, SRPI, NDVI, and CRI1), there were no significant changes between NA, CA, and DA plants. There was also no influence of the tested regulators (Figure S5A–F,I). An exception was the Greenness Index, which slightly decreased in CA and DA plants. A slight effect of the steroids was observed in the DA group. For example, TR increased the G value in Pantheon plants in comparison to both controls (Figure S5G,H).

The Water Band Index (WBI) provides information about the amount of water in the tested leaf tissue. For the control abs of NA Pantheon plants, the WBI reached a value of 1.023 (Figure 6A). It increased after cold acclimation and remained at the same level after deacclimation. The application of DMSO (control) decreased the WBI only after cold acclimation. EBR increased the values of the WBI only in the NA plants. In the CA and DA plants, EBR had no effect compared to the DMSO-treated control. The other regulators—HC5, MK, TR, and AS, which were applied only to the DA plants—did not affect the WBI.

In the cultivar President, the WBI that was characteristic of the NA (control abs) plants was 1.018, and it remained unchanged in the CA and DA plants (Figure 6B). The application of DMSO did not affect the WBI in the NA and CA plants, however, it significantly decreased the WBI in the DA plants. There was no effect of EBR in the NA and CA plants. In the DA plants, the application of BRs, BR analogues, and AS did not affect the WBI when compared to the control with DMSO.

The Structure Insensitive Pigment Index (SIPI) generally characterises the carotenoids/chlorophyll *a* ratio. In the cultivar Pantheon, there were no significant differences in the SIPI values between the NA, CA, and DA plants (control abs) (Figure 6C). The application of DMSO (control) did not affect the SIPI values in the specific groups of the NA, CA, and DA plants compared to the control abs plants. There were no significant effects of any of the tested regulators on the SIPI values.

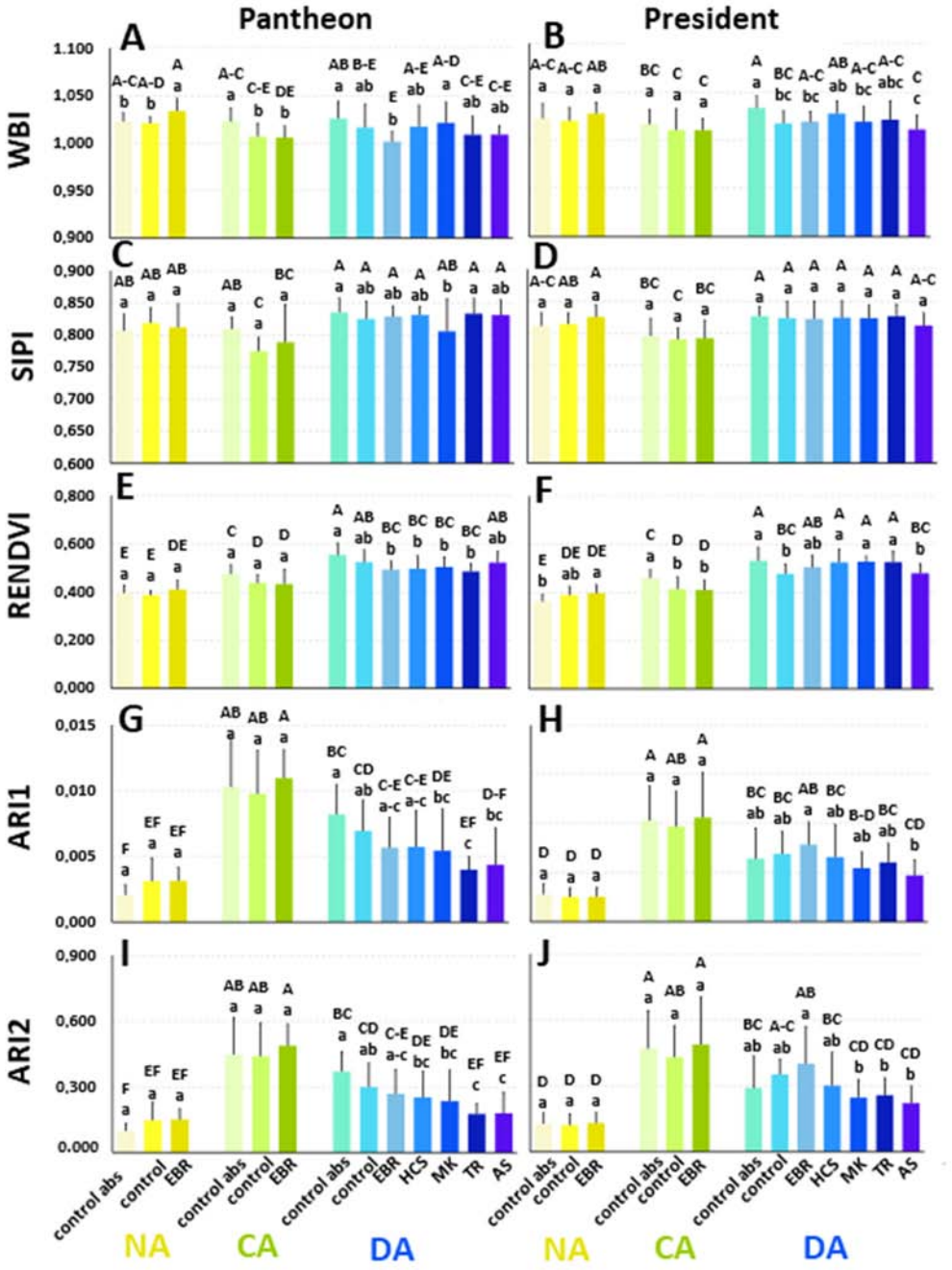

Figure 6. The leaf reflectance parameters of the leaves of the non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) oilseed rape cultivars Pantheon (**A**,**C**,**E**,**G**,**I**) and President (**B**,**D**,**F**,**H**,**J**). WBI—Water Band Index (**A**,**B**); SIPI—Structure Insensitive Pigment Index (**C**,**D**); RENDVI—Red-Edge Normalised Difference Vegetation Index (**E**,**F**); ARI1—Anthocyanin Reflectance Index 1 (**G**,**H**); ARI2—Anthocyanin Reflectance Index 2 (**I**,**J**). Control abs—untreated plants; control—plants treated with DMSO (a solvent of the tested steroids). The other objects represent plants that had been sprayed with brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone), brassinosteroid analogues (MK—MK-266; TR—triolon), and the regulator Asahi SL (AS). Mean values indicated by the same letters did not differ significantly according to Duncan's test ($p < 0.05$). Lowercase letters—comparisons between treatments within a specific group (NA, CA, and DA plants); capital letters—comparisons between the treatments of plants of all three groups (NA, CA, and DA plants).

In the NA President plants, the value of the SIPI was 0.81, and it did not significantly change after cold acclimation and deacclimation in the plants of the control abs (Figure 6D). In the CA plants, the ARI1 value increased significantly, by almost threefold. After deacclimation, ARI1 decreased by about 40% compared to the CA plants; however, it did not

In the NA control abs of President plants, the ARI1 value reached 0.0028 (Figure 6H).

The DMSO in the working solutions did not affect the SIPI values in the sprayed control plants. There was also no effect of the BRs and BR analogues on the SIPI values.

RENDVI is a parameter that is usually used to indirectly estimate the photosynthetic capacity and net primary productivity. In our study, the value measured for the NA control abs (Pantheon plants) reached 0.396 (Figure 6E). After cold acclimation, it increased significantly, by 20%. After deacclimation, there was a further increase of 17% in the value of the RENDVI. The application of the working solutions containing DMSO did not have any effects. EBR did not affect the RENDVI in the NA and CA plants; however, in the DA plants, the use of EBR caused a decrease in the RENDVI value when compared to the control abs. The application of the other regulators did not change the RENDVI values when compared to the control plants.

In the cultivar President, the RENDVI value that was measured for the NA control abs plants reached a value of 0.362. In the CA plants (control abs), the RENDVI value increased significantly, by 27% (Figure 6F). After deacclimation, there was another increase in the RENDVI (16%). The application of a working solution containing DMSO in the NA plants did not affect the RENDVI. In the CA and DA plants, DMSO decreased this index by about 10% compared to the control abs. The application of EBR and the other regulators, except for AS, did not affect the RENDVI value. AS caused a significant decrease in the RENDVI compared to the control abs.

ARI1 and ARI2 are parameters that reflect the presence of anthocyanins in plants. In the NA oilseed rape cv. Pantheon, the value of ARI1 for the control abs plants was 0.0021 (Figure 6G). After cold acclimation, ARI1 increased significantly, by about fourfold, while after deacclimation this value decreased, although it did not reach the value that was characteristic of the NA plants. The same trend was observed for ARI2 (Figure 6I). Spraying with DMSO (control) did not affect the ARI1 and ARI2 values in the NA, CA, and DA plants. EBR also did not affect ARI1 and ARI2 in the NA and CA plants. The application of all of the regulators to the DA plants resulted in a decrease in the ARI1 and ARI2 values, particularly in the cases of TR and AS.

In the NA control abs of President plants, the ARI1 value reached 0.0028 (Figure 6H). In the CA plants, the ARI1 value increased significantly, by almost threefold. After deacclimation, ARI1 decreased by about 40% compared to the CA plants; however, it did not reach the level that was characteristic of the NA plants. An identical trend was observed for ARI2 (Figure 6J). The application of DMSO did not affect ARI1 and ARI2 in the NA, CA, and DA plants. The application of EBR did not change the ARI1 value in the NA and CA plants. In the case of the DA plants, EBR had practically no effect. The use of the other regulators in the DA plants caused only a minor decrease in the values of the calculated parameters.

3. Discussion

3.1. The Frost Tolerance of Plants and the Modulation of the Properties of Their Membranes by BRs and BR Analogues

Basal frost tolerance characterises non-acclimated plants and enables them to survive slight frost, e.g., −1 to −3 °C, usually without injuries or with some small injuries [11]. On the other hand, it is commonly known that, after cold acclimation, winter cultivars become more frost-tolerant. This phenomenon was also observed in our experiment. The attempt to improve the frost tolerance of cold-acclimated plants by applying EBR was only successful for President compared to the absolute control and the DMSO-treated control plants. In Pantheon, the negative effect of DMSO was, in fact, reduced by the application of EBR, but in the end, the frost tolerance of the EBR-treated plants was similar to that of the absolute control. To conclude, in the case of the cold-acclimated oilseed rape plants, the effect of EBR seemed to be cultivar-dependent. This is in agreement with earlier studies on cold-acclimated winter wheat [28] and cold-acclimated perennial ryegrass (*Lolium perenne* L.) [32], where the application of EBR reduced frost injuries in a cultivar-dependent manner. In our experiment, EBR was also applied in order to improve the basal tolerance of oilseed rape (non-acclimated plants); however, it had no

effect. We somehow expected a protective effect of EBR against frost in non-acclimated and cold-acclimated oilseed rape based, among other thing, on earlier studies of [30] devoted to *A. thaliana*—a species of the same family as oilseed rape. Studies on mutants have proposed that brassinosteroids participate in the control of the basal and acquired freezing tolerance of *A. thaliana*. The BR-deficient mutants of *A. thaliana* were hypersensitive to freezing stress, whereas the activation of BR signalling increased their freezing tolerance both before and after cold acclimation [30]. Our results only confirm that the effects that were observed after the exogenous application of the hormone are different from the effects that are observed in mutants with hormonal disturbances. A similar situation was observed in the case of deacclimated plants. The protective effects of selected growth regulators, BRs, or BR analogues against frost in deacclimated plants are reported here for the first time. However, the results are somewhat contradictory to earlier studies that were carried out on deacclimated barley mutants with disturbances of the BR biosynthesis or signalling [16]. Lower contents of endogenous BRs or defects in the BR receptors were accompanied by a better frost tolerance of deacclimated barley mutants. The exogenous application of BRs rather increases the natural concentrations of BRs [33]. The reasons for the contradictions/inconsistencies between the results obtained for mutants and for plants exogenously treated with steroids are complex. Among other factors, they are connected to the additional influence of various factors that will be discussed in a further part of the text.

In addition to the observed cultivar-dependent effects and some structure-dependent activity of the steroids that were used, the intensity of frost was also significant. A positive effect of EBR was observed in the DA plants at a temperature of −6 °C, although that effect disappeared at lower temperatures (−9 and −12 °C), where injuries to the plants were generally too strong to be alleviated by the application of the regulators.

Another factor that seriously disturbed the assessment of the physiological effects of the tested BRs and BR analogues was the presence of the solvent DMSO in the working solutions. It is known that hormone solvents can affect the metabolism of plant cells [34]. This was the situation that most clearly occurred in the DA President plants, where, after the frost test at −9 °C, it was observed that the plants that had been sprayed with DMSO had better frost tolerance than the control abs plants; therefore, the effect of the steroids was not proven. From the point of view of agricultural practice, the effects of the applied regulators should be proven relative to an absolute control with a simultaneous lack of positive effects (or only weak positive effects) being generated by working solutions containing solvents. When it would be recommended that brassinosteroids be used in fields to protect plants against frost, solvents that are less active in plants should probably be sought for commercial preparations.

Finally, the effects of the tested regulators should be considered relative to the extent to which these compounds penetrate the tissue—i.e., pass through the cell walls and membranes—after being sprayed on the leaf surface. The structure of the cell wall and cell membranes depends on the thermal conditions in which plants grow. There are significant differences in the cell wall structure between NA, CA, and DA plants of *A. thaliana* [35]. On the other hand, the fluidity of membranes is different in plants that are growing at higher temperatures [36,37] than in plants that are growing at lower temperatures [38–41]. Because the biological membranes of cells alters their lipid composition in order to acclimate the plant to lower/higher temperatures, this "rearrangement" of the composition results in a different membrane fluidity, which consequently causes a varied localisation and interaction of different compounds. According to [4], steroids that have different chemical structures interact differently with lipid monolayers with a different degree of fluidity and different lipid components, such as phospholipids and galactolipids.

Because EBR and HCS have already been proven to be modulators of the physicochemical properties of membranes [4,42], in the current work, we only conducted supplementary studies for two BR analogues. The analysed MK-266 and triolon have basic chemical structures that are typical for steroids. The strongly hydrophobic character of the molecular skeleton favours localisation within membranes [43]. Indeed, studies that have been

conducted in model systems that differ in the degree of lipid saturation have revealed changes that occur at the physicochemical and structural levels of membranes due to treatment with MK-266 and triolon. Specifically, an increase in the A_{lim} parameter with a rising concentration of the hormone in the examined monolayers might indicate their interaction/localisation within the model membrane. An analogous trend in membrane modifications had previously been observed for other steroids [42,44]. An increase in the values of the A_{lim} parameter, which provides information about the fluidity of the monolayer and was induced by BR analogues in the current work and by EBR in earlier studies, could be one of the mechanisms responsible for improving the frost tolerance of the tested plants. Because of these mechanisms, the proteins that are present in the membranes can function properly despite any changes in temperature and alterations in the mechanical properties of the membranes. However, it should be remembered that, in our Langmuir studies, only defined (and somewhat higher) concentrations of steroids were active. In conclusion, the fluidity of the membranes, which was increased by the analogues, would be important for frost tolerance, but this is only one of the possible mechanisms. Generally, the weaker effects (on frost tolerance) that were obtained for the plants that had been sprayed with MK rather than with TR or EBR could be a result of differences in chemical structure between these steroids. In MK, an additional ring is attached to a carbon chain at position C17 of the sterane skeleton. This could be a reason for the weakened physiological activity of MK (see also: net photosynthesis in Figure 4A). However, this hypothesis should be confirmed in further research.

In the context of the importance of membrane fluidity for the effects that are caused by steroids, it is worth commenting on the results of experiment 2, which included a simplified laboratory test that was performed on cut leaves, and in which the effects of steroids were investigated by determining the electrolyte leakage after the leaf tissue was frozen. Measuring the electrolyte leakage is a method that enables the condition of cellular membranes—specifically, their permeability—to be assessed [45]. In cold-acclimated *A. thaliana*, when the freezing tolerance increased, the electrolyte leakage decreased compared to the non-acclimated plants [46]. In our studies, this simple phenomenon was confirmed in the control abs of the non-acclimated and cold-acclimated leaves. On the other hand, the decreased electrolyte leakage that was observed here in the deacclimated and frozen leaves (vs. the cold-acclimated leaves) was surely connected to the shorter duration of freezing that was used for the leaves of the DA plants. However, the most important observation in experiment 2 was that the application of steroids increased the membrane permeability of the detached leaves. This effect was particularly visible for all of the steroids that were applied to the cold-acclimated plants (Table 2). As for the leaves of the non-acclimated and deacclimated plants, when the steroids were applied to the plants that were growing at a higher temperatures, the effect was only statistically proven for MK and TR. In this context, earlier studies of the interactions of HCS and castasterone with model membranes, which also showed a dependency of the effects caused by these steroids on the temperature at which they were applied to the lipid monolayer, are also interesting [42]. Specifically, these natural brassinosteroids increased the fluidity of the model membranes when they were applied at 20 °C, but they decreased it when they were applied at cold temperatures (10 °C). Thus, theoretically, in experiment 2, the application of natural BRs (such as EBR or HCS) in the final week of cold acclimation could "stiffen" the membranes, thus making the leaves more susceptible to freezing temperatures. In contrast to the natural BRs, the BR analogues increased the membrane permeability on a more or less similar level in the leaves of the NA, CA, and DA plants (Table 2). Therefore, it is possible that this effect is also structure-dependent. The explanation of the observed phenomenon requires further studies, but the preliminary conclusions that can be drawn here could have a practical aspect. The moment that a steroid is applied, particularly natural BRs, and the specific composition/fluidity of the cell membranes at that time should not be neglected. It seems that in order to improve the frost tolerance of plants that have been subjected to cold acclimation, the steroid (EBR, HCS) should instead be applied before the

cold acclimation, as was the case in experiment 1. In turn, in order to improve the frost tolerance of plants that are at risk of deacclimation, natural steroids could be administered during deacclimation, but detailed systematic applied studies are required.

3.2. Model of the Changes in the Accumulation of the Putative Brassinosteroid Receptor (BRI1) and the Accumulation of the Transcripts of the COR and SERK Genes

To the best of our knowledge, the presence of the putative BRI1 protein (brassinosteroid receptor) in oilseed rape is reported for the first time in this work. The accumulation of this protein decreased after cold acclimation, which is consistent with a decrease in the accumulation of the *BRI1* transcript [11]. It is worth emphasising that this phenomenon was characteristic of almost all of the studied cultivars ([11]; Figures 2 and S4). The increased content of putative BRI1 that was observed in tested cultivars after deacclimation was accompanied by a higher abundance of the *BRI1* transcript in only two of them—President and Feliks [11]. An earlier analysis of endogenous BRs revealed significant differences in the contents of specific BRs between the four tested cultivars, and the pattern of changes was not very clear during cold acclimation or after deacclimation [11]. Generally, cold acclimation rather increased the concentrations of some of the BRs in oilseed rape. According to the literature, at higher concentrations, the BR content has stress-protective functions while at lower concentrations it has growth-promoting functions [47]. Thus, the increase in the concentrations of some of the BRs in cold could be expected and seemed to be justified. After deacclimation, a decrease can be observed in some of the BRs [11]. A lower content of BRs, which have growth-promoting activity [47], could then be somehow accompanied by a resumption of growth after deacclimation. The higher abundance of the receptor protein (despite a decrease in some of the BRs after deacclimation) could mean a strong signal transduction towards the resumption of growth that is induced by these growth-promoting steroids, which is unfavourable from the point of view of maintaining frost tolerance. Additionally, this would be consistent with the hypothesis of [47] that, at higher concentrations, BRs have stress-protective functions, and that the growth-promoting effects of BRs occur at lower concentrations because, as our results indicate, this might result from an increased level of the receptor protein.

In terms of stress-protective functions, it is known that BRs can affect, e.g., the COR protein expression [30]. In our study, we observed some agreement between the changes in the BR contents [11] and *COR* expression—an increase in those parameters during cold acclimation and a decrease after deacclimation.

The increased expression of the *SERK* genes, including *SERK1* and *SERK2*, was previously observed under abiotic stress conditions such as salinity stress in barley [48]. Moreover, an increased expression of *AcSERK2* was observed at a low temperature (4 °C) in *Ananas comosus* [49]. However, our results are more in line with the findings of [50], who observed a decreased expression of *DlSERK1* in *Diospyros lotus* under low-temperature treatment, as we also observed in CA oilseed rape. Generally, SERK1 is involved in BR signal transduction [51]; in our studies, the lower level of the *SERK1* transcript (but not *SERK2*) in the CA plants was accompanied by a decrease in the accumulation of the putative BR receptor (BRI1). The opposite effect was observed in the DA plants, which might confirm the role of BRI1 together with the SERK1 protein in the resumption of growth as an effect of a longer exposure of plants to higher temperatures (during deacclimation).

3.3. The Effects of BRs and BR Analogues on Photosynthesis

Regarding the light reactions of photosynthesis, PSII efficiency was assessed based on the chlorophyll *a* fluorescence measurements. Similar to our previous studies [11], there were some slight differences in the values of the parameters that describe PSII efficiency in both cultivars, and there were more significant changes between the groups of NA, CA, and DA plants. Generally, PSII efficiency, which is expressed by yield/flux ratios (φ_{Po}, ψ_o, φ_{Eo}), was lower in the cold-acclimated plants. This effect was more visible in the cultivar President but was weaker in Pantheon, where only one of the three calculated

parameters was lower in cold. The fact that cold acclimation decreases the efficiency of PSII has been well studied. The cold acclimation of barley at 5 °C (three weeks) resulted in a decrease in the maximum quantum efficiency of the PSII photochemistry (F_v/F_m parameter), as well as a decrease in the general PSII efficiency, described as the $P.I._{ABS}$ index [52]. In cold-acclimated *Avena sativa* L. (4/2 °C d/n, four weeks), the values of the maximum quantum efficiency of the PSII photochemistry (F_v/F_m) decreased [53]. The cold acclimation of oilseed rape also decreased the maximal fluorescence (F_m) [11] and F_v/F_m values [15]. After a period of cold, an increase in the temperature (during deacclimation) increases PSII efficiency. This effect was observed for both cultivars in our experiment, and it is in agreement with previous studies [11].

In our studies, calculation of yield/flux ratios showed that BRs had no effect on PSII efficiency in the NA, CA, or DA plants of oilseed rape. However, some effect of BRs was expected because, according to the literature, in cereals, BRs can limit the effects of low temperatures on the light reactions of photosynthesis, and especially on the energy flow in PSII [29]. In a cold-acclimated, EBR-treated, frost-tolerant cultivar of winter rye, the energy flow from the photosynthetic antennas to the electron transport chain was more effective than in the untreated control plants, and the energy that was lost as heat was lower. Even though a moderately frost-tolerant cultivar presented slightly different patterns of changes, EBR still decreased the energy lost as heat [29].

In contrast to the light reactions of photosynthesis, the measurements of gas exchange clearly indicated that, in both cultivars, BRs had an effect on some of the parameters associated with the dark reactions of photosynthesis. The values of the most important parameter (P_N), which provides information about the CO_2 assimilation, were on a similar level in the NA and CA plants of both controls, which is in agreement with the earlier findings of [15]. On the other hand, ref. [29] observed that six weeks of cold acclimation of winter rye resulted in a higher activity of Rubisco (a CO_2-binding enzyme), which might be associated with the increased need for sugar accumulation. Typically, during cold acclimation, the accumulation of soluble sugars increases in order to protect the cell sap from freezing [5]. This process is favourable for increasing frost tolerance, and it was also observed earlier by [15] for two cultivars of oilseed rape. In our experiment 1, in both cultivars of oilseed rape, EBR strongly increased the CO_2 assimilation in cold, which was clearly accompanied by a lower intracellular concentration of CO_2. According to the literature, the exogenous application of EBR on cold-acclimated winter rye also increased the activity of Rubisco and increased the accumulation of soluble sugars [29]. The application of EBR on cold-stressed maize seedlings resulted in increased amounts of sugars: glucose, starch, and sucrose [54]. Taken together, higher CO_2 assimilation in EBR-treated oilseed rape growing in cold conditions could be accompanied by higher sugar production and could be responsible, as one of several possible mechanisms, for the better frost tolerance of cold-acclimated plants, especially in the cultivar President. A barely provable effect of EBR on the CO_2 assimilation (P_N) was visible in the non-acclimated plants, and the frost tolerance was also not affected by this hormone in these plants. In the deacclimated plants that had been treated with EBR and HCS, P_N increased, and here the explanation could still be the same as for CA plants. Better assimilation of CO_2 may favour sugar production and could be one of many mechanisms that are beneficial for a better frost tolerance of steroid-treated plants. Regarding the BR analogues and their influence on the assimilation of CO_2 in connection with the effects of these steroids on frost tolerance, some cultivar dependency was observed. In the DA President plants that had been treated with TR, higher assimilation of CO_2 was still accompanied by increased frost tolerance. In the case of the MK-treated plants, there was no effect on the assimilation of CO_2, and there was also no effect on their frost tolerance. In Pantheon, this connection was not so clear. As mentioned earlier, weaker activity of MK could be a result of differences in its chemical structure (Figure S7) in comparison to TR or to natural BRs. At position C17 of the sterane skeleton, MK has an additional ring attached through a carbon chain.

Generally, in our experiments, higher values of P_N were observed in the deacclimated plants than in the NA and, particularly, the CA plants. In our opinion, this is an effect that could be expected for photosynthesis at higher air temperatures, and which could be accompanied by a later resumption of growth (due to the need for the higher synthesis of the assimilates). In the literature, however, the opposite effects have been observed. In the studies of [55] in deacclimated oilseed rape plants of the winter cultivar Górczanski, there was decreased activity of the CO_2-binding enzyme (Rubisco) compared to the cold-acclimated plants. The authors of [15] also reported a decrease in the P_N values in the DA cultivars of winter oilseed rape compared to the CA plants. The matter of regulation of photosynthesis in deacclimated plants could thus be more complex and requires further studies.

The other parameters of gas exchange—transpiration [E] and WUE—also exhibited specific dynamics and were different in the NA, CA, and DA plants of both cultivars. Transpiration was predictably lower in cold and increased at higher temperatures in NA and DA plants. The photosynthetic ratio of WUE, here understood as the ratio P_N/E, is a parameter that generally provides information about the amount of carbon that is assimilated as biomass [56]. Because the values of transpiration were low in cold, while P_N was similar in NA and CA plants, the WUE ratio was also increased in cold in both cultivars. WUE decreased again after deacclimation relative to the intensified transpiration processes. Due to the especially high values of P_N in the CA plants that had been treated with EBR, the WUE was additionally increased in these plants compared to the CA control abs and the control.

To conclude, from a practical point of view, monitoring the gaseous exchange—especially changes in E and WUE (also in P_N)–can provide good indicators that enable the moment that plants become deacclimated be recognised.

3.4. Leaf Spectral Properties and Their Usefulness for Detecting Deacclimation

No significant effects of the tested regulators on leaf spectral properties were observed. Measurements of leaf reflectance, however, appear to be a good non-invasive method that could enable the early detection of deacclimation in plants. According to the literature, the spectral properties of leaves might be an indicator of the stresses that affect plants [57]. Of particular interest is the reflectance at visible wavelengths of 400–720 nm, and in our study most of the changes were detectable within this range. From the point of view of the early detection of deacclimation, attention was paid to the reflection at 550 nm, which gave the first pick on the reflectance curve (Figure 5). This phenomenon was present independent of the cultivar or treatment (hormone EBR, control with DMSO). The NA plants were characterised by the highest values of reflectance at 550 nm, while lower values were observed for CA and the lowest for DA. Currently, in agriculture satellite imagery, the so-called "red-edge" region of reflectance (670–760 nm) is promoted for detecting plants' stress [58]. The shift in the reflectance curve (red-edge spectral region) on the left side is interpreted as characterising stressed plants [58]. In our experiment, the curves that characterised the NA, CA, and DA plants were in the same place within the red-edge spectral region (Figure 5A–F). For this reason, we suggest that attention should be paid to the range of 520–650 nm, with particular emphasis on the peak at 550 nm, to assess the occurrence of deacclimation.

Based on the reflectance curve, various parameters and ratios can be calculated, e.g., WBI, SIPI, RENDVI, ARI1, and ARI2.

The common range of WBI for green vegetation is from 0.80 to 1.20 [59]. The results that were obtained for the Pantheon and President plants from all of the treatments had a WBI that ranged between 1.00 and 1.03. Those values are in agreement with [59] and did not mean a water deficit in the CA and DA plants, even though there were some significant changes between the BR-treated and control plants. The patterns of the changes in the values of WBI for the NA, CA, and DA plants of Pantheon and President were slightly different than the results that were obtained by [15] for different cultivars of oilseed rape. However, despite this, those values did not indicate a water deficit in the plant tissues.

From a practical point of view of the non-invasive detection of deacclimation in plants, WBI does not seem to be useful.

The Structure Insensitive Pigment Index (SIPI) is a function of chlorophyll *a* and carotenoids [60]. In addition, the SIPI is an approach that minimises the confounding effects of the leaf surface and mesophyll structure in estimating the carotenoid/chlorophyll *a* ratio [60]. However, the SIPI is sensitive to the leaf water status, among a few other factors [61]. The values for the SIPI range from 0 to 2. The typical range for green vegetation is between 0.8 and 1.8 [60,61]. In our studies, the values ranged from 0.774 to 0.835. Only minor changes, which were mostly insignificant, were observed between the NA, CA, and DA plants, as well as after the steroid treatments. Similar to the WBI, from the practical point of view, the SIPI does not seem to be useful for the non-invasive detection of deacclimation in plants.

The Red-Edge Normalised Difference Vegetation Index (RENDVI) is a modification of the Normalised Difference Vegetation Index (NDVI), which is a broadband index that is associated with green biomass and has been used to indirectly estimate the photosynthetic capacity and net primary productivity using the reflectance measurements along the red-edge [62]. In our experiment, this index increased with the duration of vegetation, independent of the cultivar.

In contrast to the RENDVI, the values of the Greenness Index (G) decreased during plant growth, and they were highest in the NA plants and lowest after deacclimation. G is slightly correlated with the contents of chlorophyll and other photosynthetic pigments [63]. From a practical point of view, for the non-invasive detection of deacclimation in plants, both the RENDVI and G could be useful. However, due to the changes in the values of the RENDVI and G over time, we cannot exclude the possibility that these values change as a result of the progress of plant growth/development. Hence, this issue requires further studies, where plants grown in parallel are not acclimated throughout the entire experiment or are cold-acclimated for a longer period than in the current study (i.e., six weeks).

The Anthocyanin Reflectance Index 1 (ARI1) enables the accumulation of anthocyanins to be estimated even in small amounts in intact senescing and stressed leaves [64]. The common range of this index is from 0.001 to 0.1. Another index that reflects the anthocyanin content is the Anthocyanin Reflectance Index 2 (ARI2), which is a modified anthocyanin reflectance index that is less dependent on leaf thickness and density, and is able to detect higher concentrations of anthocyanins in vegetation [64]. In our study, both of those indices exhibited a tendency to increase in the CA plants and decrease in the DA plants. This is a similar tendency to the one that was observed for ARI, calculated according to the equation for ARI2, in the earlier studies of [15] on two other cultivars. Changes in the values of the parameters from the ARI group are also supported by fact that, under abiotic stresses such as a low temperatures, plants accumulate an increasing number of anthocyanins, and this phenomenon has been observed in many plant species—for example, *A. thaliana* and apple (*Malus domestica*) [65,66]. Thus, an increase in the ARI values in cold, along with their later decrease at higher temperatures (deacclimation), is in agreement with these data. Therefore, it can be concluded that the ARI parameters (1 and 2) could be a useful indicator for assessing a plant's deacclimation using non-invasive measurements.

To summarise, attempting to find methods that could enable us to detect deacclimation seems to be increasingly important today. Due to climate change and the more frequent changes in weather patterns, the phenomenon of deacclimation threatens many winter crop plant species, especially when sudden frost occurs after a warm period of deacclimation. Earlier detection of deacclimation using non-invasive techniques, such as measuring photosynthesis or leaf reflectance properties, could enable some of the regulators that at least limit the possibility of frost injury after deacclimation to be used. In our research, the results were obtained in control conditions; thus, it is advisable that the pattern of the changes of specific parameters be confirmed in open-field conditions. However, the positive information is that non-invasive methods for the early detection of deacclimation

could also be useful on a large scale by using drones or satellites to detect any changes in reflectance [58,67] or changes in chlorophyll *a* fluorescence [68].

Concluding remarks are given in Section 5.

4. Materials and Methods

4.1. Plant Material

Experiment 1 was conducted on two winter cultivars of oilseed rape (*Brassica napus* L. var. *napus* L.): President and Pantheon. Both cultivars are hybrid cultivars (F1), and the seeds were obtained from Saatbau, Środa Śląska, Poland. The plant material in the experiments were analysed on these cultivars, while exceptionally, for the analysis of the accumulation of the protein BRI1, three additional cultivars of oilseed rape were included in the experiment—the winter cultivars Rokas and Bojan, and the spring cultivar Feliks. In experiment 2, only the President cultivar was chosen.

4.2. Experimental Design

The experimental design consisted of a few separate experiments (1, 2, and 3), which are a continuation of previous research devoted to the deacclimation-induced biochemical/physiological changes in oilseed rape [11,15,18].

4.2.1. Experiment 1

In experiment 1, the main aim was to verify the hypothesis that the selected BRs and BR analogues might improve the frost tolerance of oilseed rape, especially the frost tolerance after deacclimation.

The experimental design was similar to the model that was described in detail in our earlier article [11]. The detailed number of plants used in the experiment, i.e., the number of plants in the pods/treatments, is given there [11]. Growth conditions such as light source, light spectrum, and intensity are also given in [11]. The experimental design is presented in Figure S6. Briefly, the seeds of oilseed rape were germinated on Petri dishes at 24 °C in darkness for three days. The seedlings were transferred into pots with soil and cultured at 20 °C for four days, and then at 17 °C for 17 days. Earlier, at day 21 of vegetation, the pots with plants were divided into three groups.

In the first group, referred to as non-acclimated plants (NA), the 21-day-old plants were divided into the next three groups: NA plants that had been sprayed with 24-epibrassinolide (EBR), NA plants that had been sprayed with DMSO—a solvent of EBR (control)—and NA plants that had not been sprayed and served as an absolute control (control abs). Three days after spraying, the plants were exposed to frost (−3 °C), and the frost tolerance of the NA plants—so-called basal frost tolerance—was estimated based on the regrowth of the plants (for details, see Section 4.3.1).

The second group of 21-day-old plants continued growing at 17 °C for three days. Then plants were pre-hardened for six days and then cold-acclimated at 4 °C for three weeks (days 31–51 of vegetation). This group was referred to as cold-acclimated plants (CA). One day before beginning the process of cold acclimation, the plants were sprayed with EBR or DMSO, and a group of unsprayed plants was left as an absolute control. All of the 51-day-old CA plants, after pre-hardening and cold acclimation, were exposed to frost (−13 °C), and then their frost tolerance was estimated based on the regrowth of the plants (for details, see Section 4.3.1).

The third group of 21-day-old plants continued growing at 17 °C for three days. Next, the plants were pre-hardened, cold-acclimated as described above, and then were also deacclimated at 16/9 °C between days 52 and 58 of vegetation. This group was referred to as deacclimated plants (DA). On day 55 of vegetation, the plants were divided and then sprayed with the following compounds: brassinosteroids—EBR and 28-homocastasterone (HCS); and brassinosteroid analogues—triolon (TR) and MK-266 (MK). The plants that had been sprayed with DMSO served as controls. Moreover, the group of plants in this group was also sprayed with a commercial preparation, Asahi SL, (Agrecol, Wieruszów,

Poland) (AS). The plants that had not been sprayed served as absolute controls. At day 58 of vegetation (the seventh day of deacclimation), the DA plants had been exposed to frost at −6, −9, and −12 °C, and then their frost tolerance was estimated based on the regrowth of the plants (for details, see Section 4.3.1).

In all of the cases, the concentration of the tested steroids, BRs, and their analogues was 0.5 mg/L. The working solutions were prepared based on stock solutions of these steroids that had been dissolved in DMSO as a solvent (2 mg/0.5 mL of DMSO). This is why the control plants were sprayed with solutions that contained an adequate concentration of DMSO in water. In the case of Asahi SL, its concentration was adjusted based on the manufacturer's protocol. In all of the cases, about 10 mL of the working solution was spread out over all of the plants growing in one pot. The BR analogues were generously provided by the Laboratory of Growth Regulators, Faculty of Science, and Institute of Experimental Botany of the Czech Academy of Sciences (Olomouc, Czech Republic). The EBR and HCS were purchased from Sigma Aldrich, Poznań, Poland.

The effects of BRs and BR analogues on photosynthesis were studied in this experiment. Non-invasive measurements of photosynthesis (PSII efficiency, gas exchange) and the leaf spectral properties (leaf reflectance) were always taken for the leaves of the plants from all of the groups (NA, CA, and DA). In NA and DA plants, measurements were taken one day before the frost test. In CA plants, measurements were taken two days before the frost test. The non-invasive measurements were always taken from the best-developed leaf in the leaf rosette of the plants. Moreover, before the frost tests, samples were taken for analyses of the accumulation of the protein BRI1 (brassinosteroid membrane receptor), the accumulation of transcripts of genes encoding the proteins that participate in BR signalling (*SERK1* and *SERK2*), and the accumulation of the transcript of *COR*. *COR* is a BR-regulated gene that is connected to the acclimation of plants to low temperatures. Samples were also taken from the best-developed leaves and immediately frozen in liquid nitrogen. The exact times of the measurements and sampling are given in Figure S6.

4.2.2. Experiment 2

In experiment 2, the aim was to answer the question of whether/how BRs and their analogues modify the membrane permeability that was measured for the leaves of NA, CA, and DA plants that had been exposed to frost.

Briefly, this experimental design was similar to the one described in [11] and Section 4.2.1, with modifications (see also: Figure S6). In comparison with experiment 1, the spraying of plants with steroids (EBR, HCS, MK, TR) was carried out in each group of plants (NA, CA, and DA). Plants that had been sprayed with a solution containing DMSO (a solvent for steroids) served as controls, while plants that had not been sprayed served as absolute controls. Further, in comparison with experiment 1, the moment of spraying of a group of CA plants was changed, and spraying took place seven days before the end of a period of cold acclimation. The regulator Asahi was not tested in this experiment.

Two days after being sprayed, the best-developed leaves were cut off from the NA and DA plants. Next, the leaves were placed in Petri dishes (one leaf/one dish) and exposed to frost. In the case of CA plants, the leaves were cut off three days after spraying. In all three groups (NA, CA, and DA), after frost exposure, the electrolyte leakage was measured. Electrolyte leakage provides information about any changes in membrane permeability. A detailed description of the frost test and the measurements of electrolyte leakage is presented in Section 4.3.2.

The preparation of the solutions for spraying was the same as those described in experiment 1, and the concentrations that were used were also the same. The temperature of the frost test and the duration of keeping the leaves in the frost chamber were established based on preliminary trials.

4.2.3. Experiment 3

The main aim of experiment 3 was to describe the interaction of two BR analogues, triolon and MK-266, with defined model lipid systems that have been described previously. The chemical structure of those analogues is given in Figure S7. Similar to the study of [42], in order to assess the impact of the tested MK-266 and triolon, lipids with varying saturation levels but with unchanged polar components were selected for the Langmuir monolayer model studies. 1,2-Dilinolenoyl-sn-glycero-3-phosphocholine (PC 18:3) was selected to represent unsaturated lipids, while 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC 16:0) was selected as a lipid that only contains saturated fatty acids. The mixed system of PC (18:3) + PC (16:0) at a 1:1 molar ratio was selected as being equivalent to natural membrane systems, in which saturation is regulated by the proportions of the saturated and unsaturated acids. The research system that was used, in which the polar parts remained constant, permitted a clear analysis of any modifications in the impact of the tested compounds on the membranes, which resulted solely from changes in the fatty acid saturation.

The lipid solutions were prepared by dissolving synthetic PC 16:0 and PC 18:3 (Avanti Polar Lipids, Alabaster, Alabama, USA) in chloroform (Avantor, Gliwice, Poland) to achieve a final concentration of 1 mg/mL. Then, the two-compound lipid solution, which was represented by the saturated (PC 16:0) and unsaturated (PC 18:3) hydrophobic parts, was mixed at a molar ratio of 1:1 (PC 16:0 to PC 18:3). The triolon and MK-266 hormones were dissolved in chloroform. The final concentration was 1 mg/mL, with a small volume of methanol added to preserve solubility. Next, the solutions were mixed with the lipids at the following molar ratios (lipid-to-hormone)—4:1, 8:1, and 16:1.

4.3. Measurements

4.3.1. Estimation of the Freezing Tolerance of Whole Plants in Pods

The temperatures for the freezing tolerance testing in experiment 1 were selected based on the authors' previous experience [11] and were matched to the predicted frost tolerance of particular plant groups (NA, CA, and DA). The detached plants were tested at −13 °C, and the deacclimated plants were tested at −6 °C, −9 °C, and −12 °C. Technical details of reaching given temperatures below 0 °C are given in [11]. After the freezing test, the plants were transferred to a greenhouse with the temperature set at about 12 °C (natural light, November/December, Poland—eastern EU region). Two weeks later, the plant survival rate was estimated based on the visual score. The detailed description of the visual score is provided in our earlier article [11]. Briefly, the notes ranged between 0 and 7 points, where a 0–1 point score indicates a dead plant with no signs of leaf regrowth, and a 6–7 point score indicates a plant with few or no symptoms of injury visible on leaves. The frost test was carried out on 15 plants from each group: NA, CA, and DA plants.

4.3.2. Measurements of Conductivity (Electrolyte Leakage) of the Detached Leaves

The leaves were cut, placed in sterile Petri dishes (⌀ 85 mm; one leaf/dish), and frozen at −18 °C in a freezing chamber for a duration that was selected based on previous testing and optimisation: 1 min 50 s, 4 min 30 s, and 2 min 30 s for NA, CA, and DA plants, respectively. Attempts were made to select the freezing time so that the leaves were only slightly injured. The analysis of electrolyte leakage, providing information about changes in the cell membrane permeability, was performed as described in [27,69], with necessary modifications. After the leaves were frozen in open Petri dishes, they were covered with redistilled and deionised water (20 mL per dish). The water that was used was produced using an Elgastat Maxima purification system (Elga, High Wycombe, UK). The Petri dishes with leaves in the water were covered with lids and then left at a temperature of 20 °C. Conductivity was measured 3 h and 24 h after the moment of freezing. Electrolyte leakage measurements were taken using a pH/conductivity meter (CPC-502, Elmetron, Zabrze, Poland). For each group of plants (NA, CA, and DA), and for each treatment (with BRs and BR analogues), ten replicates were made (10 different leaves).

4.3.3. Langmuir trough Studies

The systems were prepared based on the method described in [42]. Single-compound solutions (PC 18:3 and PC 16:0) as well as mixed systems (PC 18:3 + PC 16:0 M:M molar

4.3.3. Langmuir trough Studies

The systems were prepared based on the method described in [42]. Single-compound solutions (PC 18:3 and PC 16:0) as well as mixed systems (PC 18:3 + PC 16:0 M:M molar ratio) were spread on an ultrapure deionised water subphase (HLP 5 apparatus "Hydrolab" (Poland)) to obtain the surface pressure isotherms. All of the systems were tested using a Langmuir trough (Minitrough, KSV, Finland) with a Pt-Wilhelmy plate. Each experiment was repeated three to five times to maintain a high level of repeatability and accuracy in the surface tension measurements (+/−0.1 mN/m). During the experiments, a constant temperature (20 °C) was thermostatically maintained.

Based on the Langmuir isotherms, three physicochemical parameters were calculated and determined (1) A_{lim}—the area occupied by a single molecule in a maximum-packed layer, (2) π_{coll}—the value of surface pressure at which the layer collapsed, and (3) the static compression modulus, which is defined as $C_s^{-1} = -(d\pi/dlnA)$ and represents the mechanical resistance during mechanical compression and provides information about the layer stiffness.

4.3.4. Analysis of the BRI1 Protein Accumulation

Measurements of the Protein Concentration in the Leaf Extracts

The samples that had been obtained from the leaves (1 g) were homogenised in liquid nitrogen and immediately extracted using 2.5 mL of extraction buffer containing 250 mM sucrose, 50 mM HEPES-KOH pH 7.5, 5% glycerol, 0.5% Triton X-100, 50 mM $Na_4P_2O_7$, 1 mM Na_2MoO_4, 25 mM NaF, 2 mM DTT, and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The samples were centrifuged for five minutes at 38,030× *g* (MIKRO R, Hettich Centrifugen, Tuttingen, Germany). After centrifugation, the supernatant was collected, and the protein concentration was measured using 2-D Quant Kit (Cytiva, Marlborough, MA, USA) according to the manufacturer's protocol, using a SynergyTM2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Bovine serum albumin (BSA) (2-D Quant Kit, Cytiva, Marlborough, MA, USA) was used as the calibration standard. The analysis within experiment 1 was performed in three replications.

Analysis of the Accumulation of the BRI1 Protein in the Leaf Samples Using Immunoblotting

The samples were diluted in an SDS loading buffer at a ratio of 3:1. The SDS loading buffer contained 200 mM Tris pH 6.8, 400 mM DTT, 8% SDS, 40% glycerol, and 0.1% bromophenol blue. Protein denaturation was performed at 90 °C for 5 min. The same amounts (15 µg) of protein extracts were loaded and separated on 10% SDS-PAGE (1 mm polyacrylamide gel) according to the procedure described in [70]. After the proteins were separated, they were blotted onto low-fluorescence PVDF membranes (0.45 µm, Bio-Rad Laboratories, Inc., Hercules, CA, USA) (30 min, 25V/1A) using a semi-dry transfer (Bio-Rad Trans-Blot Turbo Transfer System, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Then, the membranes were blocked with 5% low-fat milk diluted in a Tris-buffered saline/Tween (TSB-T) buffer (containing 0.9% NaCl, 10 mM Tris, and 0.5% Tween 20) for 1 h at room temperature (RT) with agitation. Next, the membranes were washed four times for five minutes with a TBS-T buffer at RT with agitation, and then they were incubated in the primary antibody (dilution: 1:2000; Anti-BRI1 (AS12 1859), Agrisera, Sweden) at 4 °C overnight. Next, the membranes were washed four times for five minutes with a TBS-T buffer, and then they were incubated with the secondary antibody (HRP-conjugated Goat anti-Rabbit IgG (H&L (AS09 602), Agrisera, Sweden) diluted with TBS-T buffer (1:5000) for 2 h at RT with agitation. The membranes were then washed three times for five minutes with a TBS-T buffer at RT with agitation, and the protein was visualised using the chemiluminescence method (AgriseraECL SuperBright (AS16 ECL-S), Agrisera, Sweden), with three-minute exposure duration of the membrane.

Dilutions of the antibodies were selected based on the previous optimisation process and the protocol of the manufacturer.

The optimisation of the analysis method included a trial on *A. thaliana* as a positive control in which the BRI1 accumulation was confirmed (AS12 1859 antibody manufacturer's product information, Agrisera, Sweden). Simultaneously, on the same gel, samples of oilseed rape leaves were loaded. The obtained bands from samples of *A. thaliana* and oilseed rape were observed at the same level and were located above 130 kDa, as expected by the antibody producer (Agrisera). Thus, we claim that putative BRI1 protein was found in samples of oilseed rape. The results presented in this article were obtained using the same method.

Three independent replicates were performed. The densitometric analyses of the staining intensity of the visualised bands were performed to quantify the BRI1 protein content using ImageJ software version 1.53k (NIH, Bethesda, MD, USA). The averages are expressed as arbitrary units (A.U.) correlated with the area under the densitometric curves.

4.3.5. Analysis of SERK1, SERK2, and COR14 Gene Expression

The quantitative real-time PCR analysis for the *COR14*, *SERK1*, and *SERK2* expression was performed using QuantStudio 3 (Thermo Fisher Scientific, Waltham, MA, USA). RNA extraction was carried out according to the manufacturer's protocol using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) from 50 mg of leaf tissue. The concentration and quality of each RNA sample were determined spectrophotometrically (Quawell, San Jose, CA, USA). Approximately 700 ng of RNA was subjected to a genomic DNA elimination, and a reverse-transcription reaction was performed immediately (QuantiTect Reverse Transcription Kit, Qiagen, Hilden, Germany) according to the manufacturer's protocol.

The PCR primers for the *COR14*, *SERK1*, *SERK2*, and actin *Brassica napus* genes (Table 3) were designed using Primer Express Software v.3.0.1 (Applied Biosystems by Life Technologies, Foster City, CA, USA). The PCR amplifications were conducted in triplicate as described in [71], with a dissociation step to confirm the specificity of the reactions. The PCR data analysis was performed using QuantStudio Design and Analysis Software v.1.5.0. The relative standard curve method (Applied Biosystems) was used to calculate the relative gene expression. The *COR14*, *SERK1*, and *SERK2* expression levels were calculated relative to that of actin. The analyses were performed within experiment 1 in five biological repetitions.

Table 3. Genes, sequence origins, and designed primers used in the study.

Gene Name	GenBank ID	Forward Primer	Reverse Primer
COR14	AY456378.1	GTCAGATTTGGCCGGAAAAC	CTCGGCGTAGATCAACGACTT
SERK1	KT281978.1	CGACCACTGCGACCCTAAGAT	CCCTTTCACCCAGTCAAGCA
SERK2	KR869962.1	GAGCCTCATCAGCTTGGATCT	GAAGTGTTCTTACAAGGTCACCCC
actin	AF111812.1	TCAGTGGTGGTTCGACCATGT	CCGTGATCTCTTTGCTCATACG

4.3.6. Chlorophyll A Fluorescence Measurements

To describe the efficiency of PSII, chlorophyll *a* fluorescence measurements were taken using a Plant Efficiency Analyser (PEA, Hansatech, King's Lynn, UK). The leaves were covered with special clips for 30 min in order to adapt them to darkness. The following parameters were calculated based on the fluorescence (OJIP) curve as described in [31]: (1) φ_{Po}—maximum quantum yield of the primary photochemistry (at t = 0); $\varphi_{Po} = TR_0/ABS = [1 - (F_o/F_m)]$; (2) ψ_o—probability (at t = 0) that a trapped exciton moves an electron into the electron transport chain beyond Q_A^-; $\psi_o = ET_0/TR_0 = (1 - V_J)$; and (3) φ_{Eo}—the quantum yield of electron transport (at t = 0); $\varphi_{Eo} = ET_0/ABS = [1 - (F_o/F_m)]\psi$). The measurements were taken within experiment 1. The measurements were always taken on the middle part of the best-developed leaf that was selected from the leaf rosette, and they were taken in ten replicates (each replicate was one leaf from different plants).

4.3.7. Leaf Gas Exchange Measurements

Gas exchange was measured using an LCpro-SD infrared gas analyser (ADC BioScientific Ltd., Hoddesdon, UK), which automatically controlled the measurement conditions. The following parameters were measured: photosynthetic rate (P_N), transpiration (E), stomatal conductance (g_s), and intercellular concentration of CO_2 (C_i). The instantaneous water-use efficiency (WUE) was determined based on the quotient of the photosynthetic rate and transpiration (P_N/E). The conditions in the measurement chamber were as follows: carbon dioxide concentration 470 µmol mol^{-1} air; temperature, air humidity, and PAR intensity equal to ambient. The measurements were taken in experiment 1 on the middle part of the first leaf from the top, in seven replicates.

4.3.8. Leaf Spectral Properties (Leaf Reflectance Measurements)

To analyse the leaf reflectance in experiment 1, a CI−710S SpectraVue Leaf Spectrometer (CID–BioScience, Camas, WA, USA) was used. The following parameters of leaf reflectance were measured/calculated: (1) Water Band Index; WBI = (R900/R970) [59]. (2) Structure Insensitive Pigment Index; SIPI = (R800 − R445)/(R800 + R680) [60]. (3) Red-Edge Normalised Difference Vegetation Index; RENDVI = (R750 − R705)/(R750 + R705) [62]. (4) Anthocyanin Reflectance Index 1; ARI1 = (1/R550.8839) − (1/R700.9216). (5) Anthocyanin Reflectance Index 2; ARI2 = R801.1251·((1/R550.8839) − (1/R700.9216)) [64] modified. Additionally, the following parameters were measured/calculated: (1) Triangular Vegetation Index; TVI = 0.5·(120·(R750 − R550)–200·(R670 − R550) [72]. (2) Simple Ratio Pigment Index; SRPI = R430/R680 [60]. (3) Normalised Difference Vegetation Index; NDVI = (R800 − R680)/(R800 + R680) [73]. (4) Greenness Index; G = R554/R677 [63]. (5) Carotenoid Reflectance Index 1; CRI1 = (1/R510)–(1/R550) [74]. The measurements were always taken on the middle part of the best-developed leaf that was selected from the leaf rosette. Measurements were taken in experiment 1 in ten replicates, and each replicate was one leaf from the different plants.

4.4. Statistical Analyses

All of the statistical analyses were performed using Statistica 13.1. software (StatSoft, Tulsa, OK, USA). The results were analysed with ANOVA and Duncan's post hoc test. Values that are marked with the same letters do not differ significantly ($p < 0.05$).

5. Conclusions

From the point of view of climate change, studies that are devoted to mechanisms of deacclimation are important. The knowledge that is acquired could help to counteract the adverse effects of warm breaks, e.g., in autumn/early winter, on winter crops that require the cold acclimation (hardening) process to survive temperatures below 0 °C in winter. Deacclimation generally induces a reversal of the cold-induced changes in the level of the putative brassinosteroid receptor protein (BRI1), the expression of BR-induced *COR*, and the expression of *SERK1*, which is involved in BR signal transduction. The dynamics of the changes in the accumulation of putative BRI1 and *SERK1* indicate that BRs might participate in the regulatory mechanisms that adapt plants to growth in different temperature conditions. The deacclimation-induced decrease in frost tolerance in oilseed rape could to some extent be limited by applying steroid regulators. The deacclimation in plants could be detected using non-invasive measurements such as leaf reflectance measurements (recommended parameters: ARI1 and ARI2). Characteristic changes in the leaf reflectance spectrum in the range 500–650 nm, could also be useful for the satellite monitoring of deacclimation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25116010/s1. References [75,76] are cited in the Supplementary Materials.

Author Contributions: J.S. and M.R. cultured the plants; J.S. sprayed the plants, collected the samples, and took the chlorophyll *a* fluorescence and leaf reflectance measurements; J.S. performed

the conductivity measurements (after optimising the method); J.S. calculated the results, performed the statistical analyses, and prepared the figures, tables, and the draft of the manuscript and literature search under the supervision of A.J.; J.S. and I.S. analysed the accumulation of the BRI1 protein (including the optimisation of the method); B.J., M.R. and J.S. analysed the *SERK1*, *SERK2*, and *COR14* transcript accumulation (including the optimisation of the method); J.S. and E.P. performed the frost tolerance tests and took the photos; A.O. and J.S. measured the leaf gas exchange; E.R.-S., B.D. and J.S. performed the Langmuir trough studies; J.O. and M.K. prepared and delivered the brassinosteroid analogues; M.R. critically read the manuscript; A.J. conceived the idea for the research and handled the funding acquisition, along with critically reading and correcting the manuscript; J.S. carried out the final editing of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Supplementary-Figures

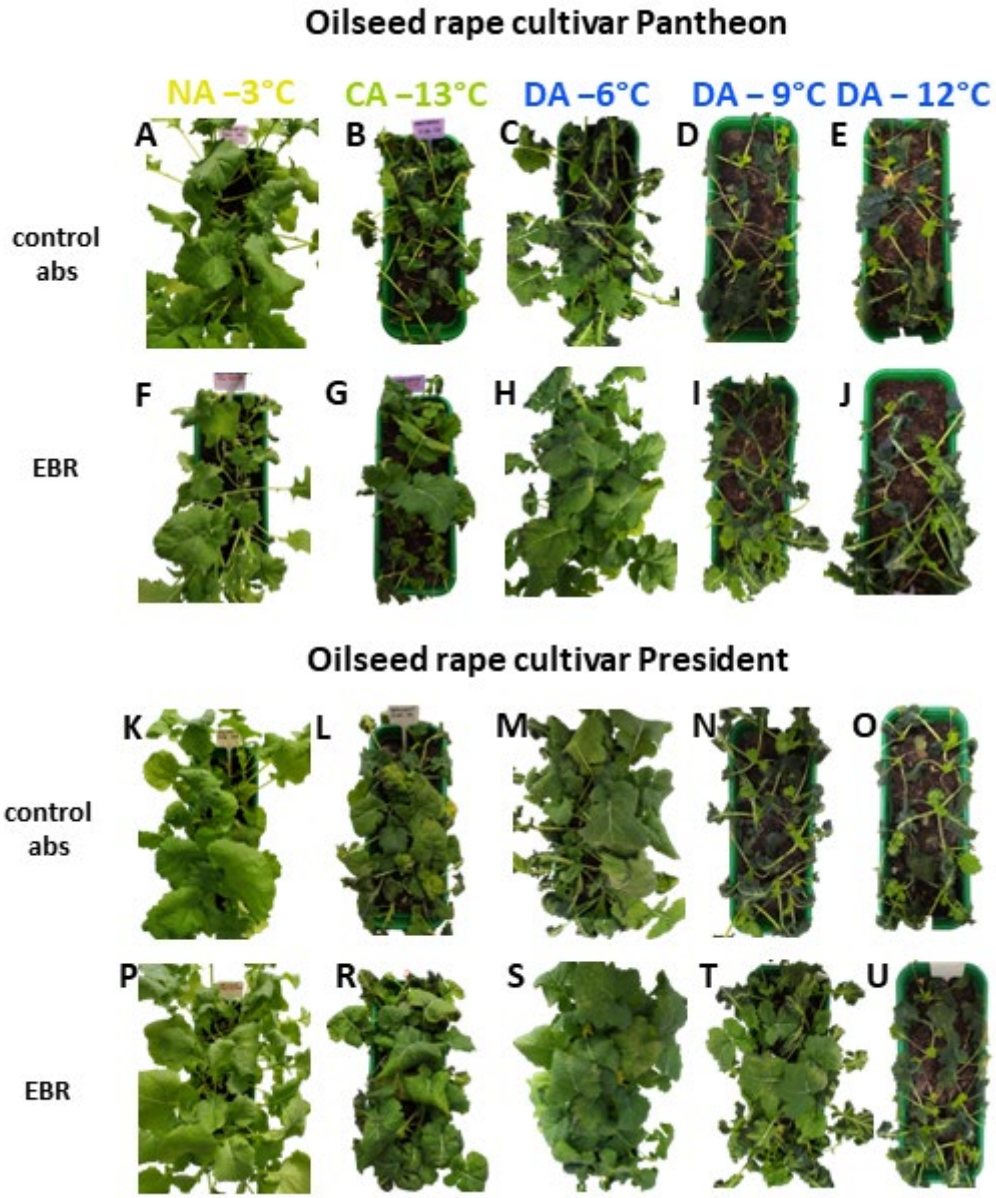

Figure S1. Exemplary photographs of non-acclimated (NA), cold-acclimated (CA), and deacclimated (DA) plants of the oilseed rape cultivars Pantheon and President after the frost tests and a period (two weeks) of regrowth at 12 °C. (A–E) unsprayed Pantheon plants of the absolute control; (F–J) Pantheon plants sprayed with brassinosteroid (EBR—24-epibrassinolide); (K–O) unsprayed President plants of the absolute control; (P–U) President plants sprayed with EBR.

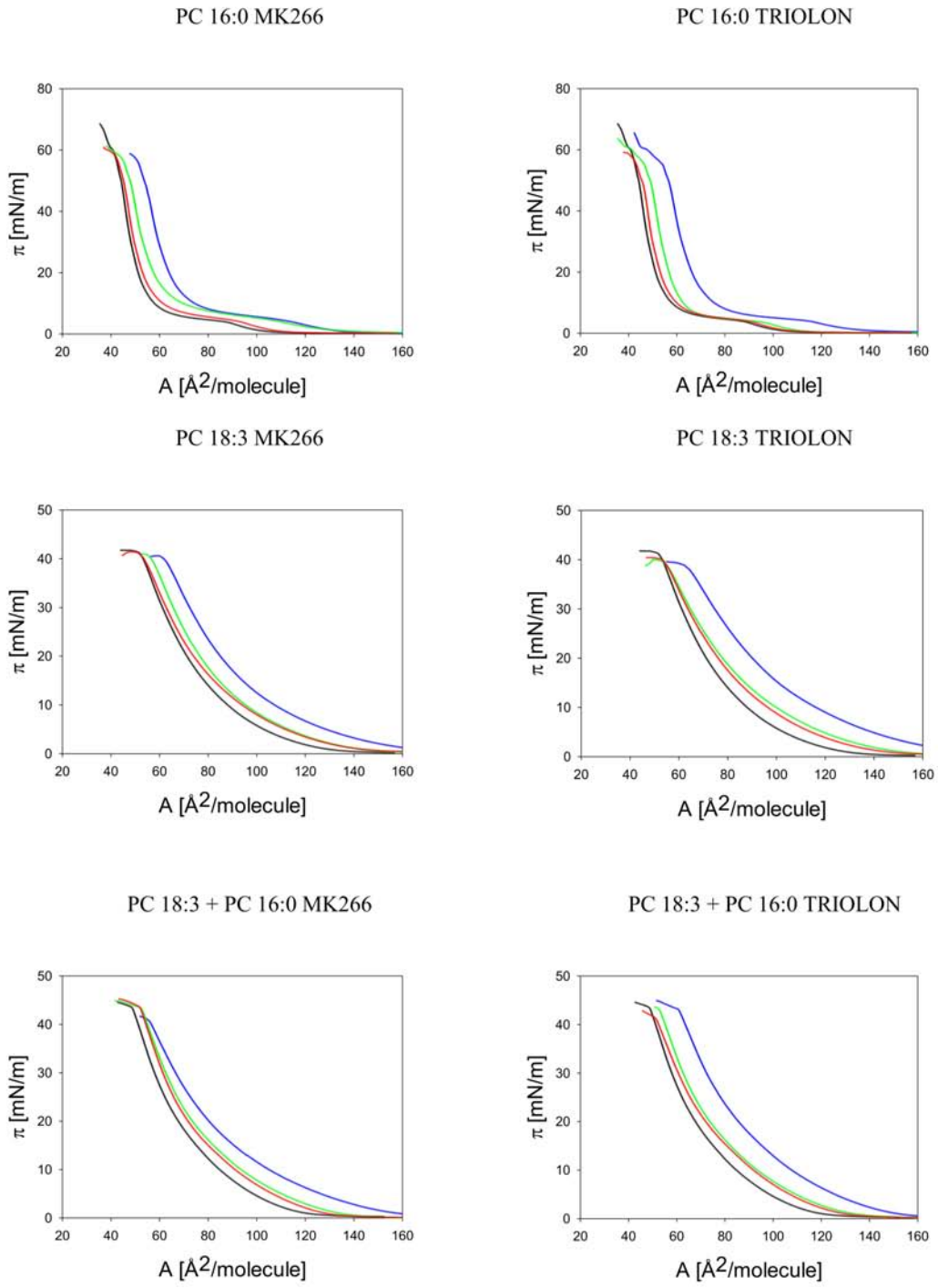

Figure S2. Langmuir isotherms (surface pressure (π) vs. molecular area occupied by lipid) for monolayers of saturated phosphocholine (PC 16:0), unsaturated phosphocholine (PC 18:3), and a lipid mixture (PC 16:0: PC 18:3; 1:1 M:M) without hormones (black line), and with the mixtures of triolon and MK-266 at different molar ratios (lipid–hormone): 16:1 (red line), 8:1 (green line), 4:1 (blue line).

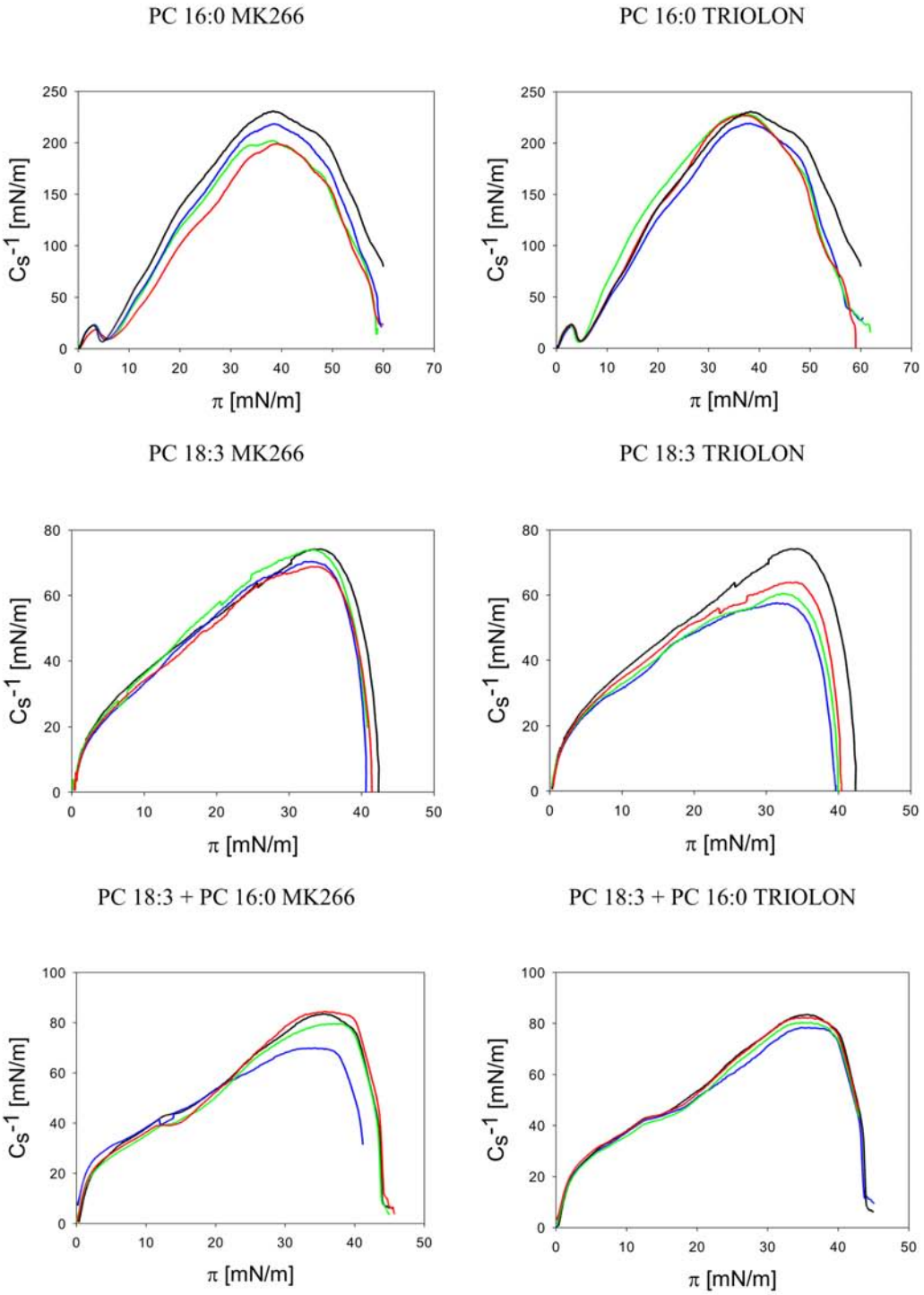

Figure S3. The static compression modulus (C_s^{-1}) as a function of surface pressure (π) for monolayers of saturated phosphocholine (PC 16:0), unsaturated phosphocholine (PC 18:3), and a lipid mixture (PC 16:0: PC 18:3; 1:1 M:M) without hormones (black line), and with the mixtures of triolon and MK-266 at different molar ratios (lipid–hormone): 16:1 (red line), 8:1 (green line), 4:1 (blue line).

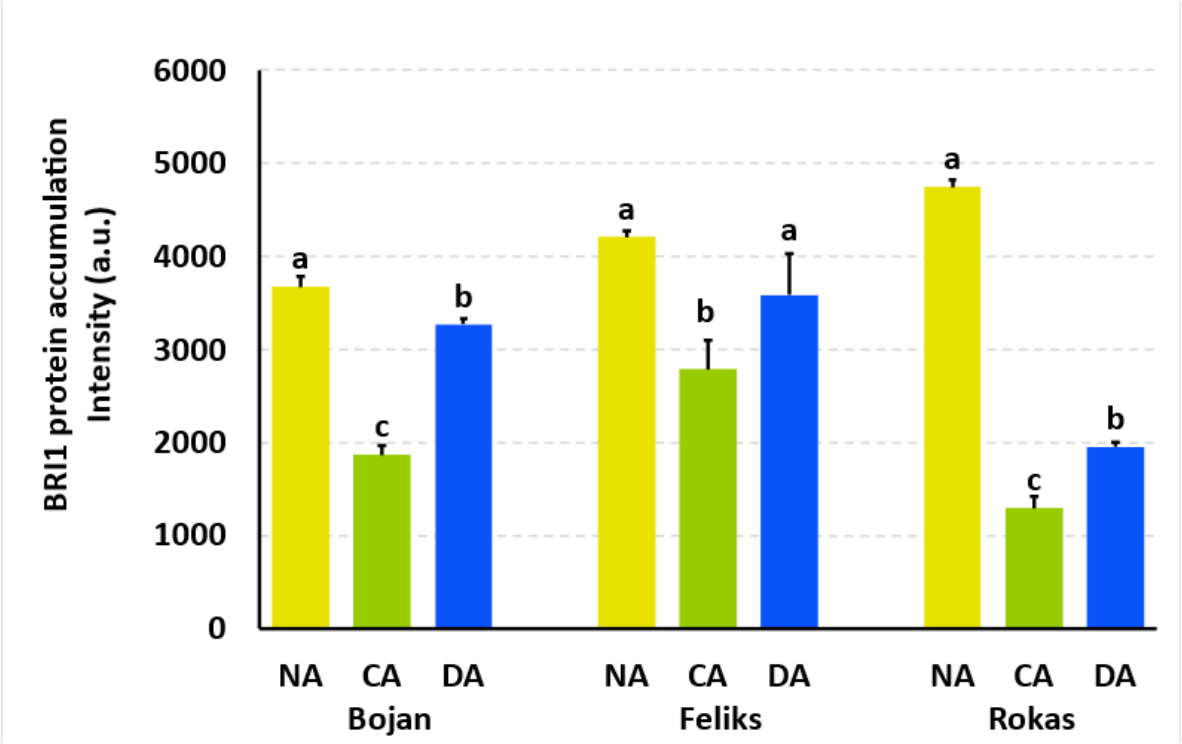

Figure S4. The accumulation of putative BRI1 protein in the leaves of non–acclimated (NA), cold–acclimated (CA) and deacclimated (DA) oilseed rape cultivars Bojan, Feliks and Rokas. The visualised bands corresponding to the level of putative BRI1 protein. 15 μg of protein was loaded onto gel. *Mean values ± SE marked with the same letters do not differ according to Duncan's test ($p<0.05$). Comparison between NA, CA and DA plants within each cultivar.*

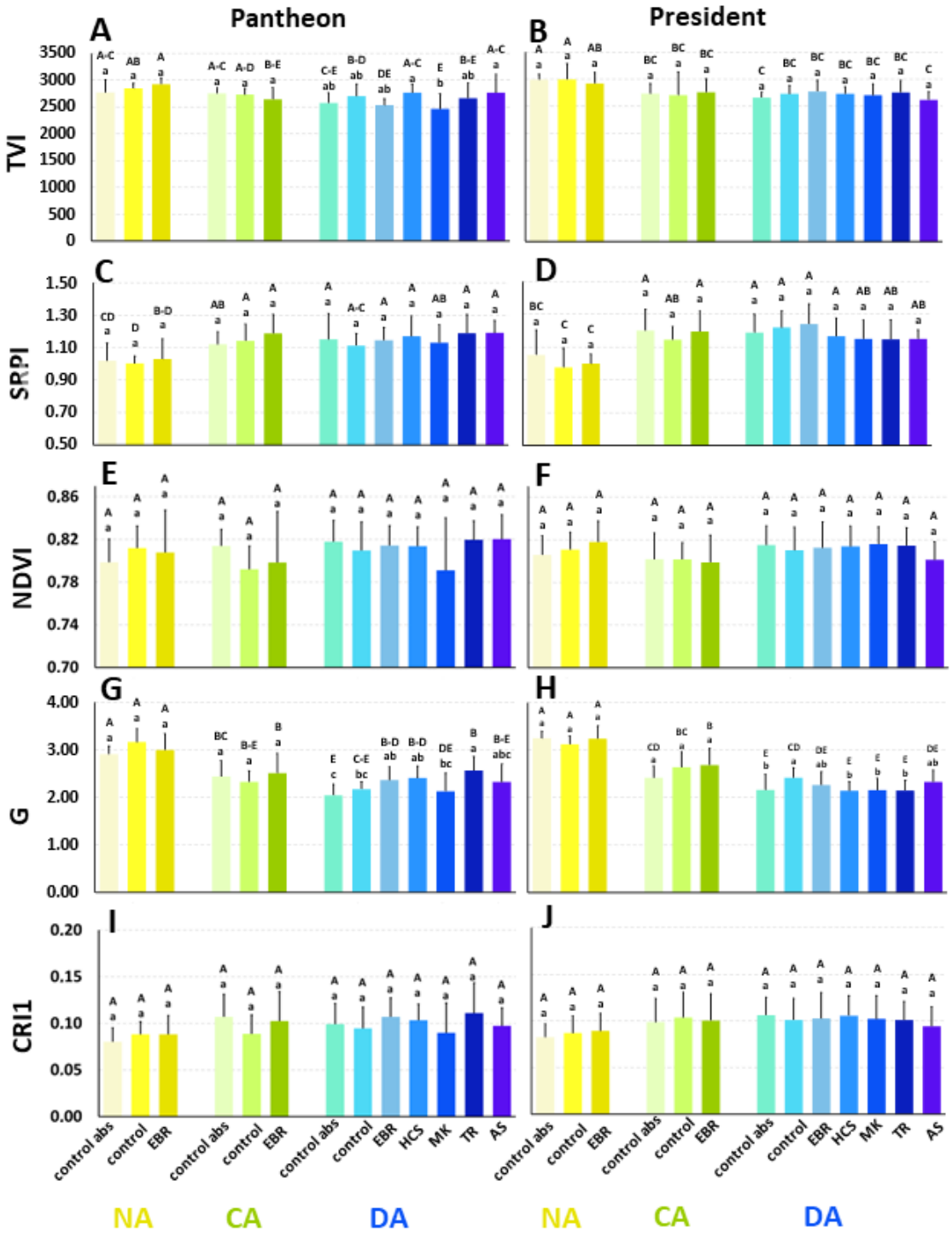

Figure S5. The leaf reflectance parameters of leaves of non-acclimated (NA), cold-acclimated, (CA) and deacclimated (DA) oilseed rape cultivars Pantheon (A,C,E,G,I) and President (B,D,F,H,J). TVI—Triangular Vegetation Index (A,B); SRPI—Simple Ratio Pigment Index (C,D); NDVI—Normalised Difference Vegetation Index (E,F); G—Greenness Index (G,H); CRI1—Carotenoid Reflectance Index 1 (I,J). Control abs—untreated plants; control—plants treated with DMSO (solvent of tested steroids); The other objects represent plants sprayed by brassinosteroids (EBR—24-epibrassinolide; HCS—28-homocastasterone), brassinosteroid analogues (MK—MK-266; TR—triolon), and the regulator Asahi SL (AS). *Mean values marked with the same letters did not differ significantly according to Duncan's test ($p < 0.05$). Lowercase letters—comparisons between treatments within a particular group (NA, CA, and DA plants);*

capital letters—comparisons between treatments of plants of all three groups (NA, CA, and DA plants) together.

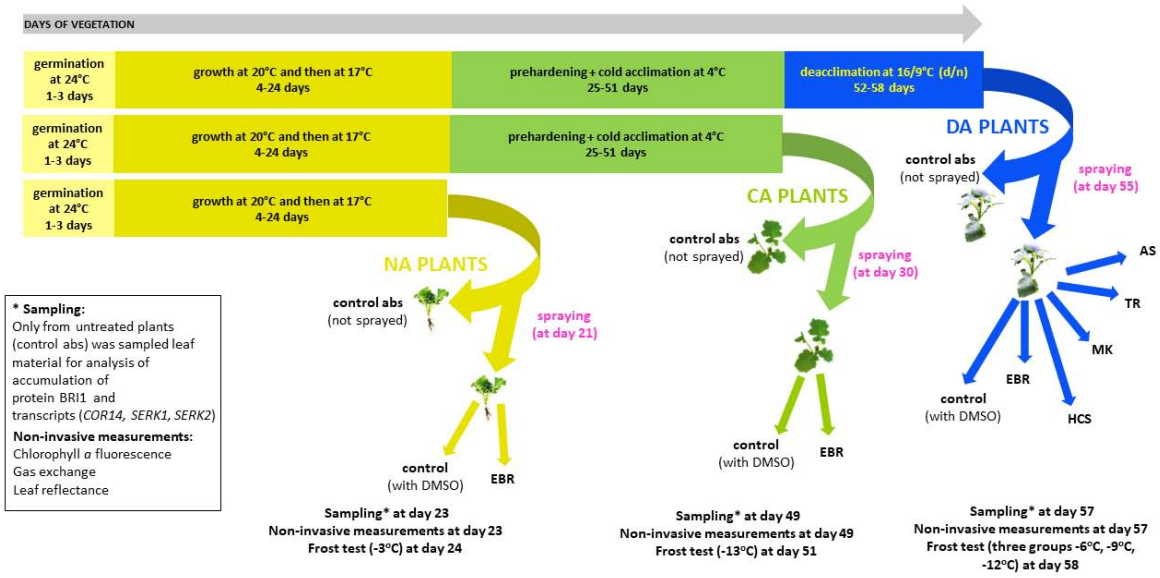

Figure S6. Simplified model of experiment 1. NA plants—non-acclimated plants, CA plants—cold-acclimated plants, DA plants—deacclimated plants; EBR—24-epibrassinolide, HCS—28-homocastasterone, brassinosteroid analogues (MK—MK-266; TR—triolon), AS—regulator Asahi SL, DMSO—solvent of steroids, control abs—absolute control (untreated plants). Non-invasive measurements and frost tests were carried out on all plants. Samples for protein and transcript analysis were collected only from untreated plants of the absolute control (control abs).

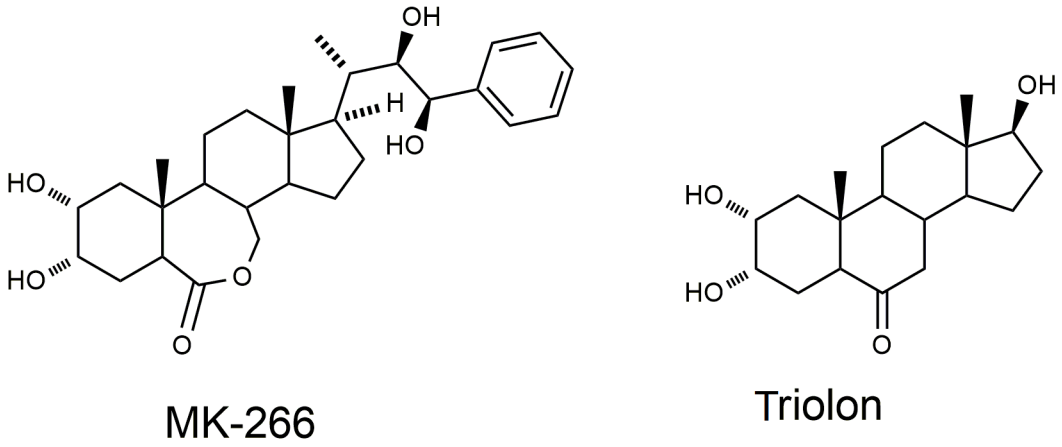

Figure S7. Structure of brassinosteroid analogues MK-266 and triolon. These compounds were synthesised as described in [75,76].

Table S1. Physicochemical parameters of the Langmuir monolayers: the limiting area per lipid molecule (A_{lim} [Å^2]), collapse pressure (π_{coll} [mN/m]) and compression modulus (C_s^{-1} [mN/m]). Statistically significant changes for systems with different molar concentrations (lipid:hormone 4:1 vs. 8:1 vs. 16:1) are noted by uppercase letters for MK−266 and lowercase letters for triolon.

Lipids and BR analogues	A_{lim} [Å^2/molecule]	π_{coll} [mN/m]	C_s^{-1} $_{max}$ [mN/m]
PC 16:0	57.3$^{D/d}$	59.2±0.1$^{A/c}$	229.3±0.1$^{A/a}$
PC 16:0 + MK 4:1	72.0^{A}	57.0±0.1^{C}	218.2±0.1^{B}
PC 16:0 + MK 8:1	65.4^{B}	58.3±0.2^{B}	202.0±0.1^{C}
PC 16:0 + MK 16:1	60.0^{C}	57.1±0.1^{C}	199.9±0.1^{D}
PC 16:0 + TR 4:1	75.2^{a}	60.9±0.1^{a}	220.0±0.2^{d}
PC 16:0 + TR 8:1	65.9^{b}	60.4±0.1^{b}	226.5±0.1^{c}
PC 16:0 + TR 16:1	60.3^{c}	59.0±0.2^{c}	227.0±0.1^{b}
PC 18:3	93.2$^{D/d}$	41.8±0.1$^{A/a}$	73.8±0.3$^{A/a}$
PC 18:3 + MK 4:1	105.0^{A}	40.2±0.2^{B}	70.5±0.1^{B}
PC 18:3 + MK 8:1	97.8^{B}	40.5±0.2^{B}	74.0±0.1^{A}
PC 18:3 + MK 16:1	95.7^{C}	40.1±0.2^{B}	68.8±0.2^{C}
PC 18:3 + TR 4:1	120.0^{a}	39.4±0.1^{b}	58.1±0.1^{d}
PC 18:3 + TR 8:1	103.3^{b}	38.6±0.1^{c}	60.9±0.1^{c}
PC 18:3 + TR 16:1	102.0^{c}	39.6±0.1^{b}	63.2±0.1^{b}
PC 18:3 + 16:0	82.8$^{D/d}$	43.6±0.2$^{A/a}$	83.5±0.1$^{A/a}$
PC 18:3 +16:0 MK 4:1	104.7^{A}	40.9±0.1^{B}	69.7±0.1^{D}
PC 18:3 +16:0 MK 8:1	90.7^{B}	43.2±0.3^{A}	79.3±0.2^{C}
PC 18:3 +16:0 MK 16:1	85.8^{C}	43.3±0.2^{A}	84.4±0.2^{B}
PC 18:3 +16:0 TR 4:1	104.1^{a}	42.9±0.2^{b}	79.0±0.1^{d}
PC 18:3 +16:0 TR 8:1	93.9^{b}	43.1±0.1^{b}	80.9±0.2^{b}

PC 18:3 +16:0 TR 16:1	93.3[c]	41.3±0.1[c]	82.7±0.1[c]

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Oświadczam, że w pracy: Stachurska, J.; Sadura, I.; Jurczyk, B.; Rudolphi-Szydło, E.; Dyba, B.; Pociecha, E.; Ostrowska, A.; Rys, M.; Kvasnica, M.; Oklestkova, J.; Janeczko, A. Cold Acclimation and Deacclimation of Winter Oilseed Rape, with Special Attention Being Paid to the Role of Brassinosteroids. International Journal of Molecular Sciences 2024, 25, 6010 mój udział polegał na: wykonaniu pomiarów wymiany gazowej roślin we współpracy z doktorantką J. Stachurską (po jej przeszkoleniu w zakresie prowadzenia w/w pomiarów).

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Olomouc, 3.6.2024

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I declare that in work: Stachurska, J.; Sadura, I.; Jurczyk, B.; Rudolphi-Szydło, E.; Dyba, B.; Pociecha, E.; Ostrowska, A.; Rys, M.; Kvasnica, M.; Oklestkova, J.; Janeczko, A. Cold Acclimation and Deacclimation of Winter Oilseed Rape, with Special Attention Being Paid to the Role of Brassinosteroids. International Journal of Molecular Sciences 2024, 25, 6010 my participation was: to synthetize and deliver the brassinosteroid analogues (MK−266 and Triolon) for studies of their biological activity.

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OŚWIADCZENIE WSPÓŁAUTORA

Kraków, 03.10.2024 r.

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Anna Janeczko

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(czytelny podpis współautora)

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Review

Physiological and Biochemical Background of Deacclimation in Plants, with Special Attention Being Paid to Crops: A Minireview

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Abstract: Global climate change, which is connected to global warming and changes in weather patterns, affects various parts of the environment, including the growth/development of plants. Generally, a number of plant species are capable of acquiring tolerance to frost after exposure to cold (in the cold-acclimation/cold-hardening process). In the last few decades, there have been more and more frequent periods of higher temperatures—warm periods that, e.g., break down the process of cold acclimation. This generates deacclimation, which could stimulate growth and lower frost tolerance in plants. Generally, deacclimation causes the reversal of changes induced by cold acclimation (i.e., in concentration of sugars, accumulation of protective proteins, or hormonal homeostasis). Unlike cold acclimation, the phenomenon of deacclimation has been less studied. The aim of this article was (1) to briefly describe the problem of deacclimation, with more attention being paid to its significance for economically important winter crop species, (2) to review and characterize the physiological-biochemical changes that are induced in plants by deacclimation, and (3) to discuss the possibilities of detecting deacclimation earlier in order to counteract its effects on crops.

Keywords: cold acclimation; deacclimation; frost tolerance; climate change; crop plants; plant metabolism

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1. Introduction—Significance of Cold Acclimation and Deacclimation for Crops

Global climate change (regardless of its causes) is accompanied by global warming. In the first two decades of the 21st century, the global surface temperature has increased by 0.99 °C when compared with the years 1850–1900 [1]. In countries located in the temperate climate zone, the average air temperature has changed in the past sixty years. In Poland, the last decade was exceptionally warm compared to previous periods [2]. The average annual temperature reached as high as 9.1 °C. For comparison, in the reference period 1961–1990, it was 7.5 °C. Global climate change has a variety of effects on nature and the environment, including on the growth of plants. This may be important, especially for cultivated species, for example, from the group of winter plants. Because the temperature in the world is generally rising, the episodes of higher temperatures in autumn and winter are happening more frequently, i.e., in countries where winter cultivars of crops are cultivated. To winter cultivars belong many economically important species, such as oilseed rape or cereals. Normally, winter cultivars require exposure to cold in order to complete their growth cycle and induce generative development (in the vernalization process) [3]. Simultaneously, growth in cold conditions results in the acclimation of plants to lower temperatures (below zero), which increases their frost tolerance. Cold acclimation, for example in eastern EU, takes place in late autumn at a temperature usually ranging from between +2 to +4 °C, 3–6 weeks. Well-cold-acclimated (cold-hardened) crops in autumn can survive freezing temperatures that reach approximately −20 °C [4]. Cold acclimation is accompanied by, among other things, the following more important biochemical and physiological changes: (1) an increased accumulation of sugars, which thicken the cell sap and decrease sap freezing temperature [5]; (2) an increase in membrane fluidity due to an increased

under cold temperatures [6,7]; (3) an increased concentration of stress hormones, scisic acid (ABA), which regulates, e.g., water management [8]; (4) an increased a lation of the heat shock proteins (HSP), which play protective functions for other p [9,10]; (5) an accumulation of the cold-responsive proteins (COR) [11]; and (6) a l intensity of photosynthesis [12,13]. Cold generally affects all parts of a cell as we signaling pathways. For example, microtubules, which are generally considered t scaffolds of cells, are involved in sensing physical stimuli such as cold and they di ble as a result of cold exposure [14]. On a molecular level, cold acclimation gene duces the expression of the cold-induced genes that function against freezing injur and literature cited there).

Winter cultivars (cereals, oilseed rape) are threatened by more frequent epis higher temperatures, e.g., 9–16 °C or more in late autumn or early winter, becaus periods might stimulate growth and lower their frost tolerance (Figures 1 and 2). of higher temperatures induce deacclimation, which is accompanied by the revers metabolic adaptations that occur during cold acclimation. In such cases, a sudd after the deacclimation period significantly increases the risk of frost injuries deacclimated plants are more susceptible to frost [13,16,17]. In winter crops such as rape of the cereals that are cultivated in regions of the EU, deacclimation can occ warm periods, e.g., 9–16 °C or more in autumn (in late November and December), ter (in warm periods in January and February), and even in early spring. The pr deacclimation in winter crops can usually be reversed when the temperature de once again after warm periods (this is called the reacclimation/rehardening proce

amount of unsaturated fatty acids, which improves membrane functioning under cold temperatures [6,7]; (3) an increased concentration of stress hormones, e.g., abscisic acid (ABA), which regulates, e.g., water management [8]; (4) an increased accumulation of the heat shock proteins (HSP), which play protective functions for other proteins [9,10]; (5) an accumulation of the cold-responsive proteins (COR) [11]; and (6) a lowered intensity of photosynthesis [12,13]. Cold generally affects all parts of a cell as well as its signaling pathways. For example, microtubules, which are generally considered to be the scaffolds of cells, are involved in sensing physical stimuli such as cold and they disassemble as a result of cold exposure [14]. On a molecular level, cold acclimation generally induces the expression of the cold-induced genes that function against freezing injuries ([15] and literature cited there).

Winter cultivars (cereals, oilseed rape) are threatened by more frequent episodes of higher temperatures (e.g., 9–16 °C or more in late autumn or early winter, because warm periods might stimulate growth and lower their frost tolerance (Figures 1 and 2). Periods of higher temperatures induce deacclimation, which is accompanied by the reversal of the metabolic adaptations that occur during cold acclimation. In such cases, a sudden frost after the deacclimation period significantly increases the risk of frost injuries because deacclimated plants are more susceptible to frost [13,16,17]. In winter crops such as oilseed rape of the cereals that are cultivated in regions of the EU, deacclimation can occur after warm periods, e.g., 9–16 °C or more in autumn (in late November and December), in winter (in warm periods in January and February), and even in early spring. The process of deacclimation in winter crops can usually be reversed when the temperature decreases once again after warm periods (this is called the reacclimation/rehardening process).

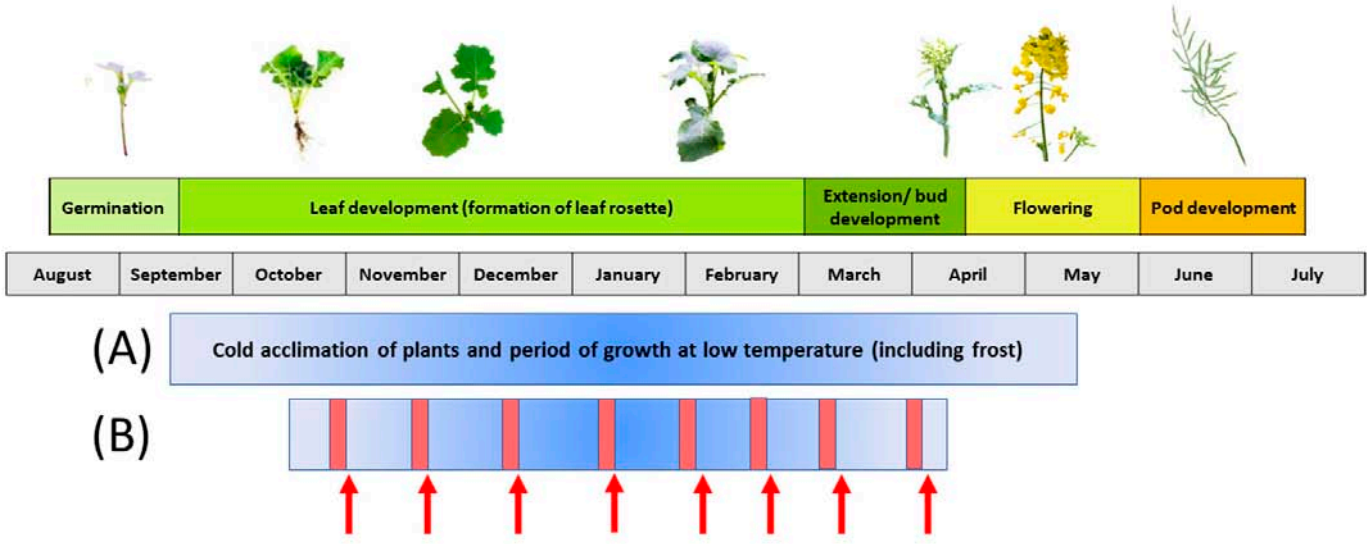

Figure 1. Simplified growth cycle of winter oilseed rape. The times of the year for particular stages of development are characteristic, for example, for regions of central/eastern EU. Panel (**A**) shows the approximated time frames for the period during which cold acclimation occurs and period of growth of plants at a low temperature (including frost episodes). Panel (**B**) shows exemplary occurrences of warm breaks (dark pink rectangle) that deacclimate plants, thus increasing the risk of frost injuries in the event of a sudden frost (red arrows).

Figure 1. Simplified growth cycle of winter oilseed rape. The times of the year for particul of development are characteristic, for example, for regions of central/eastern EU. Panel (A the approximated time frames for the period during which cold acclimation occurs and period of growth of plants at a low temperature (including frost episodes). Panel (B) show plary occurrences of warm breaks (dark pink rectangle) that deacclimate plants, thus increa risk of frost injuries in the event of a sudden frost (red arrows).

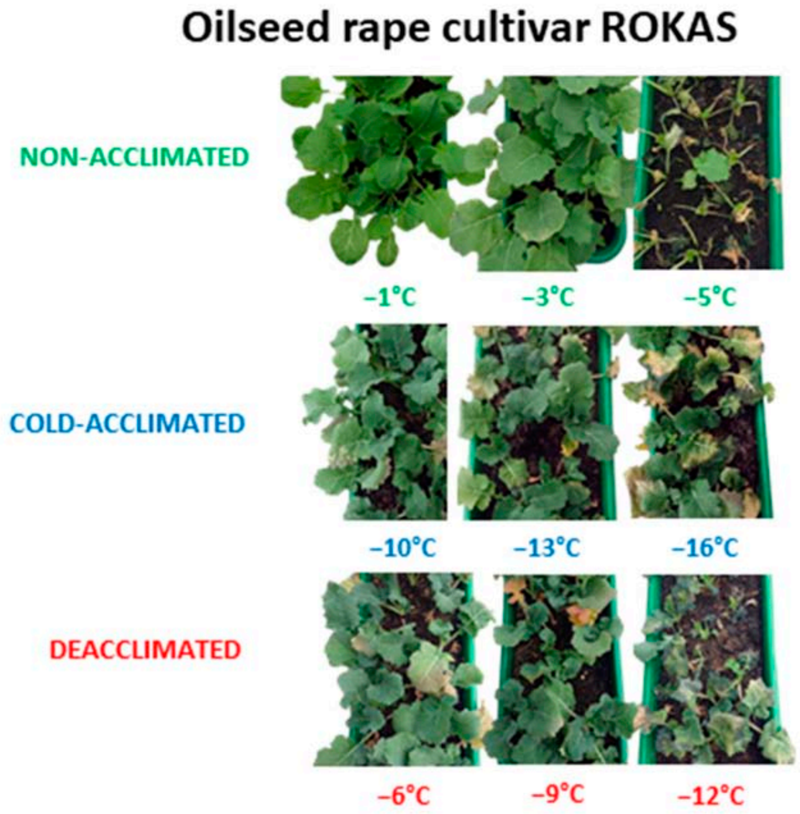

Figure 2. Changes in the frost tolerance of the non-acclimated, cold-acclimated, and deacclimated winter oilseed rape cultivar Rokas. Frost tests were performed at temperatures ranging between −1 °C and −16 °C. The estimation of frost injuries was based on observations of the regrowth of plants (previously exposed to frost) after growing for two weeks at 12 °C (for detailed data, see [13]). Photographs—J. Stachurska.

The phenomenon of cold acclimation and deacclimation is therefore important from the point of view of the cultivation of winter crops. However, it also concerns other species that grow in more extreme temperature conditions, including some trees (e.g., birch, mulberry, pine trees), grasses (e.g., Antarctic hair grass, creeping bentgrass), etc. [18–22]. However, for each of these species the temperature conditions and the periods needed for cold acclimation/deacclimation or reacclimation can differ. Generally, however, deacclimation requires a shorter time frame than cold acclimation. At the transcriptome level, the effects of deacclimation are very rapid—changes can be observed within 24 h [23,24]. Physiological/metabolomic changes (and a decrease in frost tolerance as a result) are observed later (usually in a matter of days), although there can be differences between species. In oilseed rape, a significant reverse of the metabolic changes that are induced by cold acclimation was observed after seven days of a higher temperature (16 °C/9 °C d/n), while cold acclimation in this case took three weeks [13,25]. On the other hand, in *Arabidopsis thaliana* L. the deacclimation-induced changes (in the plasma membrane proteins) were observed even faster—after two days of a warm temperature [26]. In the studies of *Solanum commersoni* (Poir.) it was shown that a few hours (2–3 h) at 20 °C was enough to initiate the deacclimation process [27]. One day was sufficient to deacclimate the tested plants while, in this case, the cold acclimation took 15 days at 2 °C [27].

Contrary to the well-studied process of the acclimation of plants to low temperatures [28], especially the metabolic changes that occur during deacclimation, are still less known [17]. In the presented article, we aim to focus on summarizing and updating the knowledge concerning the characteristics of the physiological-biochemical changes that are induced in plants by deacclimation, with special attention being paid to crop plants. We

also discuss the possibilities of the earlier detection of deacclimation in crops in order to counteract its effects in winter crops.

2. Factors That Influence the Phenomenon of Deacclimation

The kinetics of deacclimation depend on a few factors. Among them, the most important are temperature and the length of the warm period. Usually, higher temperatures and longer periods of warmth result in more effective deacclimation. Cold-acclimated cabbage (*Brassica oleracea* (L.)) seedlings that had been exposed to deacclimation at different temperatures (15 °C, 20 °C, and 25 °C) showed that the higher the deacclimation temperature was, the more accelerated was the loss of freezing tolerance [29]. Studies on oilseed rape revealed that deacclimation was more effective at 20 °C than at 12 °C [30]. Furthermore, when deacclimation lasted no longer than five days, reacclimated oilseed rape plants were able to recover their tolerance to a low temperature at a level that was close to (or even higher) than before the deacclimation process [31]. Moreover, in this species, the changes that are induced by deacclimation might be reversible when it is not accompanied by elongational growth [30] and when there are temperature conditions that allow plants to reacclimate once again. The deacclimation temperature can differ between day and night, but a higher temperature during the night is also important for this process. Studies conducted on Antarctic hair grass (*Deschampsia antarctica* (E. Desv.)) revealed that the deacclimation process had a greater effect in nocturnal-warmed plants [21].

In addition to the temperature and the length of the warm period, another important factor is also the species/cultivars. Specific species, and even cultivars, can be more or less susceptible to deacclimation. In this context, we can use the term "tolerance for deacclimation". Tolerance to deacclimation can be understood as the capacity of a plant to maintain the highest possible frost tolerance despite deacclimation [13]. A comparison of two species of *Solanum* (*S. multidissectum* and *S. megistacrolobum* subsp. *toralapanum*) that had been exposed to deacclimation (at 18 °C for 12 h) showed that the rate of deacclimation depends on the cultivar [32]. In oilseed rape, the tolerance for deacclimation also differs depending on the cultivar [13]. According to Pagter et al. [33], hardier species (which was defined based on their winter freezing tolerance) of *Hydrangea*, *H. paniculata* (Siebold) deacclimated more quickly than *H. macrophylla* (Thunb.), a less hardy species. In *H. macrophylla*, the deacclimation kinetics showed a sigmoid course with a short lag-phase that was followed by a rapid deacclimation rate.

Tolerance to deacclimation can also depend on the genotype of a plant. Pociecha et al. [34] tested the frost tolerance of the deacclimated barley cultivar Bowman and its near isogenic lines with disturbances in the biosynthesis and the signaling of brassinosteroids (plant steroid hormones). The authors found that both lines tolerated frost better after deacclimation compared to the wild-type cultivar (Bowman). Wójcik-Jagła and Rapacz [35] also demonstrated that some lines of barley were characterized as being more tolerant to deacclimation than others. According to the authors, the results indicated that the freezing tolerance and the tolerance to deacclimation might be independent traits, whereas cold-acclimated plants with a high freezing tolerance can be sensitive to deacclimation [35].

Finally, Takeuchi and Kasuga [18] discovered an interesting phenomenon that different tissues of one plant might deacclimate in a different manner. In birch (*Betula platyphylla* var. *japonica* (Hara)), bark cells required much higher temperature (10–20 °C) to decrease their freezing tolerance than xylem cells (deacclimated at subfreezing temperature −2 °C).

The physiological and biochemical basics of the tolerance to deacclimation require further studies. Based on the current knowledge, we can suspect that the decreases in frost tolerance after deacclimation might be lower in those plants/cultivars/lines (or even parts of plants) in which the metabolic changes that are induced by cold acclimation are sufficiently maintained after deacclimation.

3. The Physiological-Biochemical Changes That Occur in Plants during Deacclimation

As mentioned, compared to the process of the acclimation of plants to low temperatures, the metabolic changes that occur during deacclimation are still less well known and have been under more detailed research relatively recently, partly due to the intensification of studies that have been devoted to global climate change. The metabolic changes that accompany the process of deacclimation have been studied in woody plants [36], herbaceous plants [4], and grasses [22]. Much deacclimation research has been done in the model plant *Arabidopsis thaliana* ((L.) Heynh), among others, [24,37–39]. In the group of crop plants, the deacclimation process has been studied in oilseed rape [13,25,30,40], barley [34], rice [41], wheat [42], and triticale [43], among others.

3.1. The Cell Walls and Cell Membranes

The structural and compositional changes in the cell wall during the process of cold acclimation and deacclimation are important for the acquisition or loss of freezing tolerance as well as in the growth response [39]. In earlier studies, Solecka et al. [44] observed that the deacclimation of oilseed rape resulted in decreased content of pectin to a level similar to control plants. During the deacclimation of *Arabidopsis* (a plant of the same family as oilseed rape), the genes encoding cell-wall-related proteins such as xyloglucan endotransglycosylase, xylosidase, xylose isomerase, pectinesterase, and the arabinogalactan proteins were up-regulated [23]. The components of the cell wall of *Arabidopsis*, such as the arabinogalactan proteins and pectic galactan, changed along with the changes in frost tolerance and growth during cold acclimation and deacclimation [39]. Interestingly, although some reversible tendencies were induced in the cell wall by deacclimation (versus cold acclimation), arabinan and xyloglucan did not return to the level observed in the non-acclimated control. According to Kutsuno et al. [39], deacclimation rather initiates a specific novel composition of the cell wall. Cell wall polysaccharides could probably work to achieve the regulation that is necessary to balance the trade-off between freezing tolerance and growth in plants, and also prepare for, for example, reacclimation.

The changes in the lipid part of cell membranes that are induced by cold acclimation are well known and they usually go in the direction of an increase in membrane fluidity, which improves membrane functioning at low temperatures [6,7]. The fluidity of the cell membrane is significantly associated with larger amounts of the unsaturated fatty acids that are incorporated into membranes. It can be additionally modified via incorporation into the membranes, including the chloroplast membranes, of compounds such as sterols or tocopherols and carotenoids [45,46].

Deacclimation can modify lipid composition, which was observed in trees, mulberry bark (*Morus bombysis* (Koidz.)), and Scots pine roots (*P. sylvestris* (L.)) [19,47]. The ratio of unsaturated to saturated fatty acids of the phospholipids decreased, and that was a reversal effect compared to the changes that were induced by cold acclimation. These findings are in agreement with the results of our last studies, which were conducted on lipids that had been isolated from the chloroplasts of four cultivars of non-acclimated, cold-acclimated and deacclimated oilseed rape. Deacclimation (7 days, 9°/16 °C d/n) changed the molar percentage of the fatty acids of lipids; however, not in all of the fatty acids [48]. The reversal effect of deacclimation compared to cold acclimation was visible the best after the calculation of the ratio of the two most unsaturated fatty acids 18:3/18:2 (linoleic [18:2] and α-linolenic [18:3]) in the fractions of the monogalactolipids and phospholipids. The ratio increased after cold, while after deacclimation it decreased again. In some cultivars, the ratio decreased even to the level that had been observed in non-acclimated plants.

Under cold conditions, simultaneously with the alterations of the membrane lipid composition, there are changes in the content of low-molecular antioxidants such as the tocopherols and carotenoids [46]. α-Tocopherol, the tocopherol that occurs most often [49], is involved in the scavenging of ROS [50]. The carotenoid (β-carotene) also acts as a ROS scavenger [51]. Therefore, β-carotene and α-tocopherol might limit the membrane lipid peroxidation. β-Carotene and α-tocopherol, after incorporation into the membranes,

also modify their physicochemical properties [52,53]. β-Carotene acts as membrane stabilizer [53]. The studies of Hincha [52] showed the influence of the tocopherols on the stability and lipid dynamics of model membranes (mimicking the lipid composition of the plant chloroplast membranes) in cold conditions. Based on model systems that were mainly built from the phospholipids, the specific action of α-tocopherol on the physicochemical properties of the membranes, such as modifying the phase behavior and lipid dynamics and decreasing the motional freedom of the lipid fatty acyl chains, was confirmed. These responses were particularly observed at a low temperature. Regarding deacclimation, our studies on oilseed rape revealed that the content of tocopherols after deacclimation generally decreased compared to the cold-acclimated plants [48]. α-Tocopherol and γ-tocopherol were the most abandoned in the chloroplast membranes [48]. In some of the tested cultivars, the content of α-tocopherol in the chloroplasts after deacclimation even reached the level that had been observed in the non-acclimated control. In the case of β-carotene, the changes after deacclimation were more cultivar-dependent.

The reverse effect of deacclimation on membrane components such as the fatty acids or the content of low-molecular antioxidants might be one of the more important reasons for the deacclimation-induced lower tolerance of plants to frost. The cold-acclimated (7 days) and then frost-exposed seedlings of cabbage (*B. oleracea* (L.)) were characterized by decreased electrolyte leakage (less membrane injuries) than the seedlings that had been exposed to frost after only one day of deacclimation [5]. Electrolyte leakage (membrane permeability), which was measured in *Arabidopsis* leaves, was clearly lower in the cold-acclimated and frost-exposed plants compared to non-acclimated plants or deacclimated plants that were then exposed to frost [26]. Lower electrolyte leakage (membrane permeability) in cold-acclimated/frost-exposed plants informs about the lower injuries of membranes. Higher values of electrolyte leakage in non-acclimated, deacclimated/frost-exposed plants informs about the more severe injuries of membranes. One of the causes of membrane injuries as a result of exposition to low temperature (frost) may be lipid peroxidation. It is usually measured by MDA (malondialdehyde) content [54]. MDA is considered to be the final product of lipid peroxidation in the plant cell membrane. Finally, it is also worth mentioning that the cold-acclimated winter wheat cultivars that had accumulated a higher amount of tocopherols and β-carotene had a higher frost tolerance than the cultivars with a lower amount of these compounds in the leaves [55].

3.2. Soluble Sugar Concentration and Water Management

Cold acclimation generally increases the accumulation of sugars, which thickens the cell sap and lowers its freezing temperature [5]. It is usually accompanied by decreased osmotic potential and an increase in frost tolerance. Studies on many species have confirmed a reverse tendency in sugar accumulation after deacclimation compared to cold acclimation. In cabbage seedlings (*B. oleracea* L.), after only one day of deacclimation, the amounts of sugars such as sucrose, glucose, and fructose decreased significantly [5]. A decrease in the content of soluble sugars was also observed in deacclimated cultivars of white clover stolons (*Trifolium repens* L.) [56], the stems of *Hydrangea* plants (*H. macrophylla* ssp. *macrophylla* Thunb.) [33], seedlings of Aleppo pine (*Pinus halepensis* Mill.) and radiata pine (*Pinus radiata* D. Don) [20], crowns of grasses (*Agrostis stolonifera* L. and *Poa annua* L.) [22], and the shoot tissues of peach (*Prunus persica* L.) [57]. In deacclimated (at temperature 16/11 °C d/n, 2 weeks) blackcurrant (*Ribes nigrum* L.), although the concentration of a primary soluble carbohydrate (sucrose) decreased significantly in the buds of all of the tested plants, the rate of the decrease depended on the cultivar [58]. Additionally, the concentration of raffinose decreased in these species as a result of deacclimation [58]. Rys et al. [25] described a lower accumulation of soluble sugars in the leaves of deacclimated (7 days, 9°/16 °C d/n) oilseed rape. The content of these sugars decreased in this species to a level that was observed in the non-acclimated control. The authors also noted that cold acclimation reduced the osmotic potential of oilseed rape (by approximately 20–25%), while deacclimation increased it again by approximately 23–45%. The changes were accompanied

by a decrease in the frost tolerance of the plants [25]. Finally, a deacclimation-induced lower content of sugars was also described by Kutsuno et al. [39] in *A. thaliana.*

In addition to lowered content of sugars in plant tissues, deacclimation causes the up-regulation of some genes associated with carbohydrate metabolism, such as β-galactosidase, sucrose synthase, and β-fructosidase [23]. β-Galactosidase is involved in lactose catabolism, while sucrose synthase and β-fructosidase are involved in sucrose metabolism [23].

A high content of water in tissues is unfavorable for freezing tolerance, because temperatures below 0 may lead, e.g., to membrane injuries by forming ice outside or inside a cell. Deacclimation generally results in an increase in the osmotic potential and rehydration of tissues [25,58]. Ögren [59], in experiments made in field conditions on *Vaccinium myrtillus*, observed a significant dependency between sugar content and rehydration of tissues and susceptibility of deacclimated plants to frost. In the buds of deacclimated blackcurrant (*Ribes nigrum* (L.)), a higher water content was observed (calculated as [Fresh Weight-Dry Weight]/Dry Weight) [58]. In oilseed rape (cultivar Kuga and Thure), cold acclimation generally decreased the value of the relative water content (RWC). The RWC increased significantly in the deacclimated plants of one cultivar [25].

The dehydration of plant tissues is controlled by proteins such as aquaporins, which are involved in water transport in cells [60]. In cold-acclimated oilseed rape cultivars, the accumulation of protein BnPIP1 (plasma membrane intrinsic protein) was generally higher, probably for the purpose of better dehydrating the cells [25]. Deacclimation decreased BnPIP1 to a level that was also observed in non-acclimated plants, or even lower. Interestingly, the cold acclimation of oilseed rape drastically reduced the accumulation of the transcript aquaporin *BnPIP1*, but its level remained unchanged after deacclimation [25]. It did not correlate with changes in the protein accumulation, which could suggest a role of some of the posttranscriptional mechanisms in controlling aquaporin production differently in cold-acclimated and deacclimated plants; however, this requires further studies.

To conclude, the reverse effect of deacclimation (compared to cold acclimation) on the sugar metabolism and water management is surely one of the more important factors that are responsible for lowering the tolerance of plants to frost.

3.3. Accumulation of Selected Proteins

Generally, proteomic analyses that have investigated the influence of deacclimation on plasma membrane proteins have shown that an increase or a decrease in particular proteins during cold acclimation has the opposite tendency during deacclimation [26]. In deacclimated *A. thaliana*, the majority of the membrane proteins whose accumulation is stimulated by cold returned after deacclimation, even to a level that was similar to the non-acclimated plants [26]. In deacclimated tea (*Camellia sinensis* (L.) Kuntze), comparative proteomic studies also revealed differences in the accumulation of many proteins from the groups that are involved in, among other things, the cell wall, photosynthesis, protein synthesis, antioxidation, or sugar metabolism when compared with cold-acclimated plants [61]. In deacclimated plants compared to cold-acclimated plants, 115 up-accumulated and 136 down-accumulated proteins were detected. Compared to the non-acclimated plants, 477 differentially accumulated proteins, including 253 up-accumulated proteins and 224 down-accumulated proteins, were observed in deacclimated plants [61].

Based on the aforementioned works, it can be said that, similar to the research of the cell wall conducted by Kutsuno et al. [39], deacclimation initiates a novel composition of many proteins from different tested groups. However, focusing our review on three groups of proteins with particular significance for cold acclimation (COR, heat shock proteins (HSP), and dehydrins (DHN)), it can be seen that most often deacclimation has the reverse effect on them compared to cold acclimation. For example, in deacclimated wheat and oilseed rape, the COR78 protein decreased to an undetectable level, which was similarly observed in non-acclimated plants compared to the increased accumulation in cold-acclimated plants [62]. Our studies on oilseed rape showed that during cold acclimation the accumulation of the protective proteins from a group of HSP is enhanced [63]. The main role of HSP is to act

as molecular chaperones. They are responsible for regulating protein folding as well as its accumulation, location, translocation, and degradation [64,65]. After the deacclimation of oilseed rape, the amount of cytoplasmic HSP70 and HSP90 most frequently decreased compared to cold-acclimated plants [63]. Deacclimation also results in a decrease in the accumulation of DHN in Scots Pine (*Pinus sylvestris*) [66]. DHN are protective proteins that are produced in dehydrated tissues [67], which is observed in cold [68]. The role of DHN is to protect the other proteins and membranes against negative structural changes that are induced by the dehydration of tissues [67]. In deacclimated wheat and oilseed rape, the accumulation of dehydrin (47-kD) decreased to a level that was observed in the non-acclimated plants [62]. The relative expression of a gene that is related to dehydrins (*PpDhn1*) revealed a decrease in its amount during the deacclimation of peach (*Prunus persica* (L.) Batsch) [57].

To summarize, in deacclimated plants there is usually a decreased accumulation of stress-related and protective proteins [62,63], along with a simultaneous increase in the number of proteins associated with the metabolic processes [26,61]. This seems to be an important reason for the deacclimation-induced lowering of frost tolerance.

3.4. Hormonal Balance

Plant hormones and the interactions between them play an important role in a plant's stress responses, including low-temperature stress. The interplay between growth-promoting hormones such as gibberellins, auxins, and cytokinins and stress hormones such as abscisic acid (ABA) seem to be particularly important in the context of the balance between growth and the frost tolerance of cold-acclimated plants. The deacclimation process can disturb this balance. The research that was conducted on deacclimated *A. thaliana* revealed an increased expression of the genes that are associated with growth-promoting hormones biosynthesis, for example, gibberellins and auxins [24]. In deacclimated *Vitis* plants, the downregulation of ABA synthesis might play an important role in the loss of cold hardiness and budbreak [69]. In deacclimated barley (*Hordeum vulgare* L.) plants, there was an increase in the growth-promoting hormones, including indole-3-acid (IAA), some gibberellins, and cytokinins [34]. Similar phenomena were observed in deacclimated bermudagrasses (*Cynodon* spp.)—the amount of cytokinin was higher while the amount of ABA was lower [70]. Stachurska et al. [63] conducted detailed analysis of the hormonal homeostasis in cold-acclimated and deacclimated oilseed rape (four cultivars). In the tested cultivars, the concentration of ABA increased (in a cultivar-independent manner) in the cold-acclimated plants, while it decreased significantly as a result of seven days of deacclimation [63]. Changes in the growth-promoting hormones (their precursors and inactivated forms) were more cultivar-dependent, but the general tendency was a decrease in their concentrations, i.e., cytokinin: cis-zeatin, active auxin (IAA) and its precursors or some gibberellins (GA_7) during cold acclimation that was followed by an increase after deacclimation. Studies allowed the preparation of a model of hormonal changes characterizing the winter and spring cultivars of cold-acclimated and deacclimated oilseed rape [63].

Another group of hormones that have recently been studied in the aspect of deacclimation are brassinosteroids (BR). BR are plant steroids that are involved in plant growth and stress response. Pagter et al. [24] found that, in deacclimated *A. thaliana*, the expression of the genes related to brassinosteroid biosynthesis was higher. Our studies on cereals [71] and oilseed rape [72] showed that endogenous BR generally increased in leaves, but only after cold acclimation. In the case of oilseed rape, there was, however, a dependency on the cultivar and on the type of analyzed BR. Deacclimation (in oilseed rape) rather decreased some of the BR [72]. In the winter wheat cultivar Grana, long periods of cold acclimation (43 days) finally resulted in a high accumulation of brassinosteroid (28-homocastasterone) in crowns (Figure 3). Deacclimation (7 days) reversed this effect (Figure 3).

Interestingly, in contrast to the BR level, the accumulation of the BR receptor (BRI1) clearly decreased in cold but increased after deacclimation [72]. This higher accumulation of the BR receptor protein (despite the fluctuations in the BR levels in deacclimated plants)

Agronomy **2024**, *14*, x FOR PEER REVIEW 9 of 17

of the BR receptor protein (despite the fluctuations in the BR levels in deacclimated plants) could mean that there is a strong signal transduction towards the resumption of growth that is induced by these growth-promoting steroids, which can be unfavorable from the point of view of frost tolerance.

Shifting the hormonal balance (of which a model is presented in the work [63]) in the direction of increasing the concentrations of the growth-promoting hormones and decreasing the amount of the stress hormone (ABA) might be one of the possible causes of the decrease in plant frost tolerance after deacclimation in oilseed rape and other plants. On the other hand, the example of brassinosteroids shows that not only the concentration of hormones may be important. Attention should also be paid to the elements of signal transduction, including the appearance/concentration of receptor proteins that receive hormonal signals. These issues, however, will require further research.

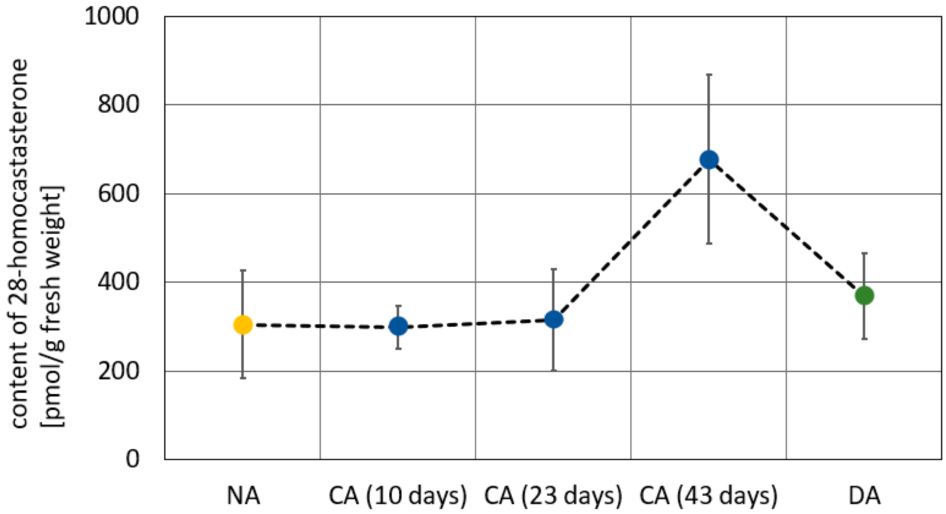

Figure 3. Changes of content of brassinosteroid (28-homocastasterone) in the crowns of winter wheat cultivar Grana in NA, CA, and DA plants. NA—control plants (non-acclimated plants) growing at 20 °C (11 days), CA—cold-acclimated plants (10, 23, 43 days at 5 °C), DA—deacclimated plants (after cold acclimation exposed to 20 °C (7 days)). Data represent mean values ± SE (n = 3), [73].

3.5. Photosynthesis

The process of photosynthesis is a marker of the different stresses that affect plants, including low-temperature stress. Despite its importance for plants, little is known about its changes in deacclimated plants. Regarding the efficiency of the light reactions during photosynthesis, Pukacki et al. [74] observed that after the deacclimation of the needles (and during release of deep rest) in *Picea schrenkiana* there was an increase in the intensity of the delayed fluorescence emissions. The effect was stronger in the frost-susceptible *P. schrenkiana*, which reacted to the deacclimation more strongly. Research on wheat revealed that the differences between cold-acclimated and deacclimated plants were changes in the values of the parameters that described the phenomenological and specific energy fluxes (expressed, among other things, by the parameters ABS, TRo, DIo, or φo [16]). Studies on oilseed rape also revealed that, after deacclimation, the values of the Fv/Fm parameter, which is the maximum quantum yield of PSII photochemistry, was significantly higher compared to the values that were observed in the cold-acclimated plants, even compared to the values that are characteristic for non-acclimated plants [25]. Similarly, later studies by Stachurska et al. [13] showed that deacclimation reversed the cold-induced changes in the photosystem efficiency and resulted in an intensification of the light reactions of photosynthesis in oilseed rape. The changes in PSII efficiency appeared to be related with changes in the chlorophyll content. According to Rys et al. [25], the cold acclimation of oilseed rape decreased the chlorophyll content, while deacclimation increased it. In Scots pine (*Pinus sylvestris* L.), cold acclimation and deacclimation were associated with the reduction and recovery of Amax (light-saturated assimilation rate) and α (quantum

yield) [75]. Further, regarding the dark reactions of photosynthesis, studies by Rapacz and Hura [76] showed that deacclimated oilseed rape plants had, conversely to cold-acclimated plants, a decreased activity of Rubisco and sucrose-phosphate synthase (SPS), which is in agreement with the later studies of Rys et al. [25], where the authors showed that, after deacclimation, the intensity of photosynthesis (net photosynthesis, parameter P_N) was lower in both of the tested cultivars of oilseed rape (Kuga and Thure). On the other hand, this seems to be a slight contradiction to our last observations [77] of two cultivars of oilseed rape (Pantheon and President), where the deacclimated plants were characterized by an increased intensity of the CO_2 assimilation. In our opinion, an increase in P_N is more likely after deacclimation because it is accompanied by a further resumption of growth. This is why the issue may require more studies, especially because P_N is sensitive to the soil water content, and therefore even a slight deficit of water in a controlled pot culture is mirrored in photosynthetic activity due to the effect on the stomata closure.

As a result of the deacclimation of *A. thaliana*, the following genes that are connected to photosynthesis, chlorophyll a/b-binding protein; ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit; photosystem I reaction center subunit II, and photosystem II 5 KD protein, were up-regulated [23]. Other studies on *A. thaliana* revealed that, although they were suppressed during cold acclimation, the genes associated with photosynthesis, which encode the D2 subunits of the photosystem II complex, were reactivated during deacclimation [37]. Research on the gene expression of cold-acclimated and deacclimated *B. napus* revealed differences in the genes that are responsible for, among other things, photosynthesis and light-regulated diurnal responses, whose expression was reversed within one week of deacclimation [78].

3.6. Other Changes

Antioxidants. Research conducted on the needles of Norway spruce (*Picea abies* L.) revealed that the concentration of the $O^{\cdot -}{}_2$ radical and H_2O_2 did not vary significantly between the tree populations during the deacclimation period [79]. In contrast, the concentrations of the low-molecular-weight antioxidants such as flavonoids, ascorbic acid, and glutathione were lower, while similarly a decrease was also observed in the activity of superoxide dismutase (SOD) and guaiacol peroxidase (PO). The activity of catalase (CAT) did not change between the tree populations [79]. In winter wheat plants, the concentrations of antioxidants such as ascorbate and glutathione generally tended to decrease after deacclimation, while they were higher in the cold-acclimated plants [80]. In olive leaves (*Olea europea* L.), antioxidant activity such as ascorbate peroxidase, CAT, peroxidase, and SOD increased during cold acclimation and decreased during deacclimation [81]. It is known that low-temperature stress is accompanied by oxidative stress (increasing the accumulation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, and hydroxyradicals). A too-high concentration of ROS is harmful for cellular structures and macromolecules. Plants developed a defense system against oxidative stress, which is among others connected with increased activity of the aforementioned ROS-scavenging enzymes. By lowering the activity of ROS-scavenging enzymes or lower accumulations of other antioxidative compounds, deacclimation weakens plant antioxidative capacities, thus making them more susceptible to frost-induced damages.

Under abiotic stress such as low temperature, plants accumulate an increasing amount of anthocyanins [82], and this phenomenon was observed in many plant species, e.g., maize, grape, apple [83–85]. The expression of the genes responsible for the biosynthesis of anthocyanins was enhanced under low-temperature treatment [86]. In oilseed rape, cold acclimation significantly increased the content of anthocyanins, while deacclimation caused the decrease in anthocyanin content [25].

Amino acids (proline). Proline is accumulated by plants under stress and is involved in, among other things, antioxidative protection [87]. In *A. thaliana*, the concentration of proline decreased gradually during the three days of deacclimation, compared to the high levels of this amino acid in cold-acclimated plants [38]. In oilseed rape, the level of proline

decreased significantly after the exposure of plants to elevated temperature (18/16 °C) [88]. A lower concentration of proline and amino acids was also detected in deacclimated winter wheat when compared with cold-acclimated plants [80].

General metabolic profile. The FT-Raman technique, which provides general information about the changes in the metabolic profile (the content and composition of carotenoids, chlorophylls, flavonoids, fatty acids, or polysaccharides, etc.), has been implemented to compare non-acclimated, cold-acclimated, and deacclimated oilseed rape [25]. A cluster analysis (chemometric method) revealed clear differences in the FT-Raman spectra. The non-acclimated and deacclimated plants were in one group, while the cold-acclimated plants were in another group. This indicates that seven days of the deacclimation of oilseed rape is generally the period that significantly reverses the cold-induced metabolic changes, thus making the plant metabolism more similar rather to non-acclimated plants than to cold-acclimated plants.

Physiological and biochemical changes that occur in plants during cold acclimation and deacclimation have been summarized in Table 1, where we compared two species from the same family, Brassicaceae, a model plant *Arabidopsis thaliana*, and a crop plant oilseed rape.

Table 1. The effects of cold acclimation and deacclimation on the physiological, biochemical, and genetical changes in two plants of the *Brassicaceae* family—the model plant *A. thaliana* and the crop plant oilseed rape. Cold-acclimated plants were compared to non-acclimated plants. Deacclimated plants were compared to cold-acclimated plants.

	Arabidopsis thaliana		**Oilseed Rape**	
	Cold-Acclimation	**Deacclimation**	**Cold-Acclimation**	**Deacclimation**
Cell walls	Down-regulation of the genes encoding cell-wall-related proteins such as putative xyloglucan endotransglycosylase, xylosidase arabinogalactan protein, and xylosidase [23]	Up-regulation of the genes encoding cell-wall-related proteins such as putative xyloglucan endotransglycosylase, xylosidase, xylose isomerase, pectinesterase, and arabinogalactan protein [23]	The content of pectin in the cell walls increased [44] Higher levels of non-covalently bound pectins and an increased content of galactose, arabinose, and glucose in the pectins and of galactose and arabinose in the hemicelluloses [89]	The content of pectin in the cell walls decreased to a level similar to that in the control plants [44]
	Suppression of the cell wall-related genes [37]	Reactivation of the cell-wall-related genes [37]		
	Increase in the arabinogalactan protein content [39]	Decrease in the arabinogalactan protein content [39]		
Cell membranes	Lower electrolyte leakage (membrane permeability) after the frost test [26]	Higher electrolyte leakage (membrane permeability) after the frost tests [26]	Increased ratio of the unsaturated to saturated fatty acids [48]	Decreased ratio in the unsaturated to saturated fatty acids [48]
	Increase in membrane fluidity [6]			
Sugars	Increased sugar content [38]	Decreased sugar content [38]	Increased sugar content [25]	Decreased sugar content [25]
	Increased starch content [39]	Decreased starch content [39]		
	Downregulation of the genes associated with carbohydrate metabolism (e.g., β-galactosidase, sucrose synthase) [23]	Upregulation of the genes associated with carbohydrate metabolism (e.g., β-galactosidase, sucrose synthase) [23]		
Water management	Down-regulation of the genes encoding the water channel proteins (such as tonoplast intrinsic protein gamma (TIP)) [23]	Up-regulation of the genes encoding water channel proteins (such as tonoplast intrinsic protein gamma (TIP)) [23]	Decreased osmotic potential, decreased relative water content (RWC), increased accumulation of aquaporin (BnPIP1) [25]	Increased osmotic potential, increased relative water content (RWC) in one cultivar, decreased accumulation of aquaporin BnPIP1 [25]
Proteins	Increased *COR* expression [12]	Reduced amount of *COR* transcripts [38]	Increased accumulation of heat shock proteins (HSP) [63]	Decreased accumulation in the heat shock proteins (HSP) [63]
	Increased accumulation of the cell structure-related proteins and decrease in protein synthesis, destination and storage-related proteins [26]	Reversal of the changes induced by cold in the majority of the protein accumulations (e.g., in the protein synthesis, destination and storage-related proteins, decrease in cell structure-related proteins [26]	Increased accumulation of dehydrins (DHNs) [62]	Decreased accumulation in dehydrins (DHNs) [62]
			Increased accumulation of the cold-regulated protein (COR78) [62]	Decreased accumulation in cold-regulated protein (COR78) [62]

Table 1. *Cont.*

	Arabidopsis thaliana		Oilseed Rape	
	Cold-Acclimation	**Deacclimation**	**Cold-Acclimation**	**Deacclimation**
Photosynthesis	Suppression of the photosynthesis-related genes encoding the D2 subunit of the PSII complex [37]	Reactivation of the photosynthesis-related genes encoding the D2 subunit of the PSII complex [37]	Decreased maximum quantum yield of the PSII photochemistry (Fv/Fm) [25]	Increased maximum quantum yield of the PSII photochemistry (Fv/Fm) [25]
	Inhibition of photosynthesis (reactions of the dark phase) and increase in sucrose-phosphate synthase (SPS) activity [12]	Increased expression of the genes involved in the light reactions of photosynthesis [37]	Limited intensity of the light reactions of photosynthesis [13]	Intensification of the light reactions of photosynthesis [13]
	Down-regulation of the photosynthesis-related protein genes, e.g., encoding a small subunit of RuBisCO [23]	Up-regulation of the photosynthesis-related protein genes, e.g., encoding a small subunit of RuBisCO [23]	Increased activity of RuBPCO and SPS [76]	Decreased activity of RuBPCO and SPS [76]
			Unchanged or decreased intensity of CO_2 assimilation (P_N) (cultivar-dependency) [25,77]	Decreased or increased intensity of photosynthesis (CO_2 assimilation, P_N) (probably dependent by additional factors) [25,77]
Hormonal homeostasis	Down-regulation of the genes involved in auxin and gibberellin metabolism, down-regulation of the BR biosynthesis pathway [90]	Increased expression of the genes associated with growth-promoting hormones (auxins and gibberellins) and an increased expression of the genes associated with BRs [24]	Increased ABA content and a decrease in growth-promoting hormones [63]	Decreased ABA content and an increase in growth-promoting hormones [63]
			Tendency to increase the content of brassinosteroids (dependent on the cultivar and on the type of analyzed steroid) [13]	Tendency to decrease the content of brassinosteroids (dependent on the cultivar and on the type of analyzed steroid) [13]
			Decrease in the accumulation of the brassinosteroid receptor (BRI1) [72]	Increase in the accumulation of the brassinosteroid receptor (BRI1) [72]
Other effects	Increased level of proline [38]	Decreased level of proline [38]	Increased level of proline [88]	Decreased level of proline [88]
			Increased anthocyanin content [25]	Decreased anthocyanin content [25]

4. How Could the Results of Deacclimation Studies Be Used in Practice?

Summarizing the results presented in this work, it should be emphasized that the degree of the reversal of the cold-induced changes via deacclimation is dependent on the species, cultivar, and above all, on the duration and the temperature of the deacclimation. Moreover, some physiological or biochemical parameters return to the values that are observed before cold acclimation more easily, while others are more stable. The research on the physiological-biochemical changes that accompany deacclimation will require further research. Nevertheless, the described physiological and biochemical changes that are induced by deacclimation can help to explain the basics of a lower frost tolerance in deacclimated plants. Deacclimation is a problem, especially in the case of winter crop plants. This is because the frost tolerance decreased by the deacclimation generates specific economic problems, as it may be connected to the loss of yields.

Today, we are dealing with the problem of global climate change and the challenge of feeding an increasing number of people in the world. Because the problem of deacclimation is occurring more frequently, we will have to consider the following: (1) cultivating crops that are characterized by a higher frost tolerance after deacclimation (deacclimation tolerant), (2) choosing cultivars that are characterized by a slower/later deacclimation process [91], or even (3) applying special preparations to protect deacclimated plants from frost injuries. It is also important to extend the current knowledge about the timeframes of deacclimation in order to predict more precisely how fast particular changes occur (in given species/cultivars).

Tracking the metabolic changes induced by deacclimation in field conditions is complicated because it is difficult to evaluate those changes visually, while complex laboratory analyses require the invasive collection of samples and are time-consuming. Fortunately, there are a few non-invasive measurements, such as measuring the chlorophyll *a* fluorescence, whose effectiveness has been proven for detecting deacclimation-induced changes in crop plants [13,16,25]. Measurements of the efficiency of photosystems based on chloro-

phyll *a* fluorescence can be used as a sensitive tool for monitoring the deacclimation process. What is even more important is that this method could also be used on large-scale cultivations in fields using drones or satellites to detect any changes in the fluorescence. Another method that could be used to evaluate the rate of deacclimation could be measuring the spectral properties of leaves (leaf reflectance measurements). Selected parameters such as ARI1 and ARI2, which define the level of anthocyanins, correspond well with the changes that are induced by deacclimation [25,77].

Another technique that could be used in assessing plant freezing tolerance during deacclimation could be electrical impedance tomography (EIT). Even though this technique is not yet popular in plant science, it could be used to examine the tolerance of plants to various stressors [92]. EIT is a rapid, nondestructive measurement, and the values of EIT images can be useful in a quantitative evaluation of changes induced during deacclimation and estimation of frost tolerance [92].

The results of laboratory research on deacclimation and non-invasive measurements taken together indicate that the deacclimation process (as mentioned above) generally partly or fully reverses the changes that are induced by acclimation in cold. However, many of the results were obtained in a controlled environment (plants grown in growth chambers with controlled light, temperature, humidity, etc.). It seems reasonable to conduct further similar studies in field conditions, which are more unpredictable. Already, in 1976, Gusta and Fowler [93] conducted research on winter wheat crowns artificially cold-acclimated and field-cold-acclimated. The results clearly indicated that the field-cold-acclimated crowns deacclimated faster and had a higher water content than the artificially acclimated plants [93].

Finally, it is worth emphasizing once again that the phenomenon of deacclimation is dangerous (e.g., for winter crops) when frost occurs after a period of warming. Deacclimated plants have the ability to reacclimate again, which may even result in acquiring higher frost resistance [31]. The mechanisms of this phenomenon are also worth deeper studies.

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Zjawisko hartowania i rozhartowania roślin w kontekście zmian klimatu

Cold hardening and dehardening of plants in the light of climate change

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Abstrakt

Zmiany klimatu prowadzą do wzrostu temperatur na świecie oraz występowania częstszych okresów z podwyższoną temperaturą w okresie jesienno-zimowym. Rośliny ozime, w tym ważne rośliny uprawne, których okres wegetacyjny przypada na ten czas, są szczególnie narażone na niekorzystny wpływ podwyższonej temperatury, która może zaburzać naturalny proces hartowania do niskich temperatur. Zjawisko to określane jest mianem rozhartowania i powoduje najczęściej odwrócenie zmian fizjologiczno-biochemicznych zaindukowanych hartowaniem. Celem niniejszej pracy jest (1) przybliżenie zagadnienia wpływu rozhartowania na mrozoodporność ozimin, (2) analiza zmian fizjologiczno – biochemicznych zachodzących w komórkach w czasie procesu hartowania i rozhartowania, (3) krótka dyskusja nieinwazyjnych metod umożliwiających ocenę stanu rozhartowania upraw dla celów praktyki rolniczej.

Słowa kluczowe: rozhartowanie, hartowanie, zmiany klimatu, mrozoodporność

Abstract

Climate change leads to an increase in global temperatures and the more frequent occurrence of periods that are characterised by higher temperatures in autumn and winter. Winter plants, including important crops whose growing season is during these periods, are particularly exposed to the adverse effects of higher temperatures, which could disturb the natural process of hardening to low temperatures. This phenomenon is called dehardening and most often results in the reversal of the physiological and biochemical changes that are induced by cold hardening. The aim of this work is (1) to describe the problem of the influence of dehardening on the frost tolerance of winter crops, (2) to analyse the physiological and biochemical changes that occur in cells during the cold hardening and dehardening process and (3) to briefly discuss non-invasive methods that will enable the dehardening state of crops to be assessed for the purposes of agricultural practice.

Key words: dehardening, cold-hardening, climate change, frost tolerance

WPROWADZENIE

Zmiany klimatu, bez względu na ich przyczyny (okresowe zmiany w aktywności Słońca, erupcje wulkanów, aktywność człowieka czy inne), związane są z globalnym ociepleniem. W Polsce ostatnie dekady były szczególnie ciepłe – w ciągu ostatnich sześćdziesięciu lat średnia temperatura powietrza zimą wzrosła z -1,9° do -0,2°C (Nauka o klimacie, https://naukaoklimacie.pl/aktualnosci/zmiana-klimatu-w-polsce-na-mapkach-468/). Co więcej, średnia roczna temperatura powietrza też osiągnęła wyższą wartość w ostatnich latach, a liczba ciepłych dni (z temperaturą powyżej +30°C) ciągle się zwiększa (Nauka o klimacie, https://naukaoklimacie.pl/aktualnosci/zmiana-klimatu-w-polsce-na-mapkach-468/). W pierwszych dniach stycznia 2023 roku można było zaobserwować kilka dni z temperaturą sięgającą nawet +17°C (Archiwalne wykresy meteo, http://meteo2.ftj.agh.edu.pl/meteo/archiwalnewykresymeteo), a wrzesień tego roku był cieplejszym miesiącem niż czerwiec (IMGW, 2023). Takie zmiany mogą szczególnie niekorzystnie wpływać na wegetację roślin ozimych. Nasiona roślin ozimych wysiewane są jesienią, ponieważ w fazie siewki rośliny te wymagają okresu chłodu (tzw. wernalizacji – zwykle w +1 do +6°C; Chouard 1960) do indukcji rozwoju generatywnego i do ukończenia cyklu rozwojowego. Wyróżnia się m.in. ozime odmiany rzepaku (*Brassica napus* L.), pszenicy (*Triticum aestivum* L.), jęczmienia (*Hordeum vulgare* L.) oraz żyta (*Secale cereale* L.). Ponieważ plon roślin ozimych jest wyższy niż jarych, są one częściej uprawiane w Polsce, mimo że są bardziej narażone na niekorzystne działanie mrozu. Rośliny te rozwinęły jednak naturalne mechanizmy umożliwiające im przetrwanie niekorzystnych warunków atmosferycznych, takich jak ujemne temperatury zimą. Dobrze zahartowane (zaaklimowane), zwykle w temperaturze rzędu +2° do +4°C, rośliny mogą przetrwać mrozy sięgające nawet ok. -20°C (Rapacz i Janowiak 1998). Jesienne hartowanie roślin powoduje między innymi: (1) zwiększoną akumulację cukrów zagęszczających sok komórkowy i obniżających temperaturę jego zamarzania (Sasaki i współaut. 1996) oraz obniżenie zawartości wody w liściach (Charest i Ton Phan 1990; Rys i współaut. 2020), (2) wzrost płynności błon komórkowych poprzez zwiększenie udziału nienasyconych kwasów tłuszczowych w ich strukturze, co ułatwia funkcjonowanie błon komórkowych w chłodzie (Uemura i współaut. 1995; Filek i współaut. 2017), (3) zwiększoną akumulację białek ochronnych (np. HSP, ang. Heat Shock Proteins), które m.in. chronią inne białka przed uszkodzeniem (Zhang i współaut. 2008; Sadura i wspólaut. 2020), (4) zwiększenie poziomu hormonów stresu, np. kwasu abscysynowego (ABA), który m.in. reguluje gospodarkę wodną (Kosová i współaut. 2012).

Występujące obecnie w wielu rejonach zmiany klimatyczne powodują zaburzenia naturalnego procesu hartowania oraz mogą prowadzić do rozhartowania (deaklimacji, dehartowania) roślin. Zjawisko to najczęściej powoduje odwrócenie powstałych na skutek hartowania zmian metabolicznych (Rapacz i współaut. 2017; Rys i współaut. 2020; Stachurska i współaut. 2023).

Odwrócenie zaindukowanych hartowaniem w +4°C zmian może zachodzić już po kilku dniach ekspozycji roślin na podwyższoną temperaturę (np. 7 dni z temperaturą +16°C w dzień i +9°C w nocy) (Rys i współaut. 2020; Stachurska i współaut. 2022). Z kolei w temperaturze +20°C rozhartowanie zachodzi już po 2 dniach (Rapacz, informacja ustna). Rozhartowanie może wystąpić po okresach podwyższonej temperatury jesienią (np. na przełomie listopada i grudnia), zimą (ciepłe okresy w styczniu i lutym) a nawet wczesną wiosną. Nagłe pojawienie się mrozu po okresie rozhartowania znacząco zwiększa ryzyko uszkodzeń mrozowych (Rapacz i współaut. 2017; Rys i współaut. 2020; Stachurska i współaut. 2022). W niektórych latach (np. w roku 2012) uszkodzenia spowodowane niską temperaturą wymusiły konieczność zaorania 32% upraw rzepaku ozimego (Wałkowski 2016), co wiąże się ze stratami ekonomicznymi.

Stopień rozhartowania roślin zależny jest od różnych czynników, m.in. od wysokości temperatury oraz czasu trwania tzw. ciepłej przerwy. Szybkość rozhartowania była większa w temperaturze 20° niż w 12°C (Rapacz 2002). Podatność na rozhartowanie może różnić się w zależności od gatunku (Byun i współaut. 2014). Badania prowadzone na różnych odmianach rzepaku wykazały także, że tolerancja warunków rozhartowujących w pewnym stopniu zależna jest od odmiany rośliny (Stachurska i współaut. 2022). Z kolei u jęczmienia zaobserwowano nawet różnice między genotypami/liniami (Pociecha i współaut. 2020, Wójcik-Jagła i Rapacz, 2023). Podłoże fizjologiczno-biochemiczne tej tolerancji wymaga jeszcze badań. Najprawdopodobniej spadki mrozoodporności będą mniejsze po działaniu temperatur rozhartowujących u tych roślin/odmian, u których zmiany metaboliczne zaindukowane wcześniej chłodem utrzymały się na zadowalającym poziomie przez okres rozhartowywania.

Jeśli po okresie rozhartowania nastanie ponownie okres chłodu oraz nie doszło do silnego wzrostu elongacyjnego, a w szczególności wybicia pędu kwiatowego (np. u roślin rzepaku), możliwe jest odwrócenie zmian spowodowanych rozhartowaniem (tzw. rehartowanie) oraz ponowny wzrost mrozoodporności (Rapacz 2002).

CHARAKTERYSTYKA ZMIAN FIZJOLOGICZNO-BIOCHEMICZNYCH ZACHODZĄCYCH W CZASIE ROZHARTOWANIA

W przeciwieństwie do zmian fizjologiczno – biochemicznych zachodzących w roślinach na skutek hartowania do niskich temperatur, zmiany zachodzące w tkankach roślin w czasie rozhartowania są relatywnie słabo poznane i stały się przedmiotem bardziej szczegółowych badań dopiero w ostatnich latach (Vyse i współaut. 2019; Fürtauer i współaut. 2019). Badania wykonywano dotychczas m.in. na modelowej roślinie *Arabidopsis thaliana* (L.) Heynh. (m.in. Pagter i współaut. 2017; Byun i współaut. 2014) oraz na roślinach uprawnych takich jak: rzepak (m.in. Rapacz 2002; Rys i współaut. 2020), jęczmień (Pociecha i współaut. 2020) oraz pszenica (Vaitkevičiūtė i współaut. 2022). Działanie podwyższonych temperatur powodujące rozhartowanie stanowi dla roślin impuls do wznowienia wzrostu i rozwoju. Dochodzi bowiem najczęściej do odwrócenia kierunku zmian indukowanych działaniem chłodu i pobudzenia metabolizmu.

Ściany i błony komórkowe. Pod wpływem rozhartowania następują zmiany w obrębie elementów składowych ścian komórkowych *Arabidopsis thaliana* (L.) Heynh., takich jak zmiany ilościowe białek arabinogalaktanowych (AGPs) i pektynowego galaktanu. Co ciekawe, arabinian i ksyloglukan po rozhartowaniu nie wracają *stricte* do poziomu obserwowanego w roślinach niehartowanych. Wynika z tego, że polisacharydy ścian komórkowych zmieniają się w czasie hartowania (w porównaniu do

kompozycji charakteryzującej rośliny niehartowane), ale rozhartowanie indukuje powstanie specyficznej, nowej kompozycji i struktury ściany, która nie była obserwowana ani w roślinach niehartowanych ani hartowanych (Kutsuno i współaut. 2023). Nieco inaczej wygląda sprawa błon komórkowych (w tym błon chloroplastów). Ich reorganizacja (najczęściej w kierunku zwiększenia płynności) jest niezwykle ważnym elementem hartowania roślin do niskiej temperatury (Ogweno i współaut. 2009). Zmiany dotyczą głównie składu lipidowego (m.in. udziału poszczególnych kwasów tłuszczowych) w błonach (Filek i współaut. 2016), zawartości tokoferoli i karotenoidów (Munné-Bosch 2005) oraz steroli (Willemot 1980). Badanie zmian właściwości membran przy użyciu wagi Langmuira wykazało, że rozhartowanie generalnie powoduje odwrócenie zmian powstałych na skutek działania chłodu. Zmiany dotyczą kompozycji kwasów tłuszczowych zlokalizowanych w membranach chloroplastowych. Przykładowo stosunek zawartości kwasów 18:3/18:2 [kwas α-linolenowy (18:3) i linolowy (18:2)] we frakcjach monogalaktolipidów i fosfolipidów w badanych odmianach rzepaku wzrósł w czasie hartowania, ale rozhartowanie odwróciło tę zmianę (Rys i współaut. 2024).

Gospodarka węglowodanowa i wodna. Istotna część zmian metabolicznych zachodzących w czasie rozhartowania dotyczy gospodarki węglowodanowej. Podczas rozhartowania dochodzi bowiem do spadku zawartości cukrów rozpuszczalnych, niezbędnych do zagęszczenia soku komórkowego, co zwiększa podatność komórek na uszkodzenia mrozowe (Pagter i współaut. 2008; Rys i współaut. 2020). Jest to potęgowane dodatkowo zwiększaniem się zawartości wody w komórkach. Znaczący wzrost względnej zawartości wody (wyrażony parametrem RWC, ang. Relative Water Content) zaobserwowano w liściach rzepaku poddanych rozhartowaniu i był to trend odwrotny do obserwowanego w czasie hartowania (Rys i współaut. 2020). Podobnie jak w rzepaku, w rozhartowanych roślinach *Arabidopsis thaliana* (L.) Heynh. i kapusty (*Brassica oleracea* L.) zaobserwowano zmniejszoną akumulację cukrów rozpuszczalnych (Sasaki i współaut. 1996; Kutsuno i współaut. 2023). Na poziomie molekularnym po rozhartowaniu zmianie uległa ekspresja genów związanych z gospodarką węglowodanową – w rozhartowanym *Arabidopsis thaliana* (L.) Heynh., stwierdzono zwiększoną ekspresję genów β-galaktozydazy i syntazy sacharozy (Oono i współaut. 2006).

Białka. U rzepaku w czasie rozhartowania obserwowany jest spadek zawartości białek ochronnych z grupy HSP, których akumulacja wcześniej wzrastała w warunkach chłodu (Stachurska i współaut. 2023). Z kolei w przypadku *Arabidopsis thaliana* (L.) Heynh. poddanego rozhartowaniu, większość białek błonowych akumulowanych na skutek hartowania powróciła w czasie rozhartowania do poziomu sprzed hartowania, choć część białek związanych ze stresem pozostała jednak na wyższym poziomie (Miki i współaut. 2019). Rozhartowanie powoduje spadek akumulacji dehydryn, które są białkami produkowanymi w celu ochrony membran i innych białek przed niekorzystnymi zmianami wywołanymi dehydratacją tkanek (Kosová i współaut. 2007). Do dehydratacji tkanek dochodzi m.in. w czasie hartowania roślin do niskich temperatur (Kalberer i współaut. 2006). Badania prowadzone na białkach błony komórkowej wykazały, że generalnie białka, których akumulacja zwiększa się lub zmniejsza w czasie hartowania, na ogół wykazują odwrotną tendencję w czasie rozhartowania (Miki i współaut. 2019). W czasie rozhartowania szczególnie obniża się akumulacja białek odpowiedzi na stres abiotyczny oraz kinaz/fosfataz (Miki i współaut. 2019). Badania proteomiczne rozhartowanych roślin herbaty chińskiej (*Cammelia sinensis* (L.) Kuntze) wykazały różnice w akumulacji białek z różnych grup, w tym białek ściany komórkowej, białek zaangażowanych w fotosyntezę, syntezę białek, antyoksydację i metabolizm cukrów w porównaniu do roślin hartowanych (Ding i współaut. 2023).

Homeostaza hormonalna. Nieco późniejszym efektem rozhartowania może być wykształcenie się pędu kwiatowego, a nawet rozwój pąków. Hartowane chłodem rośliny np. rzepaku znajdują się bowiem w stadium rozety

liściowej, w której najlepiej znoszą mróz. Jedną z przyczyn tego zjawiska może być spadek stężenia hormonów stresu (głównie ABA) i zwiększenia się udziału hormonów odpowiedzialnych za wzrost i rozwój (Stachurska i współaut. 2023). Takie przesunięcie równowagi hormonalnej w wyniku rozhartowania to także jedna z możliwych przyczyn obniżenia mrozoodporności roślin rzepaku po rozhartowaniu (Stachurska i współaut. 2023). Badania prowadzone na *Arabidopsis thaliana* (L.) Heynh. potwierdzają, że po rozhartowaniu następuje wzrost ekspresji genów związanych z biosyntezą hormonów wzrostu i rozwoju, m.in. giberelin i auksyn (Pagter i współaut. 2017). Podobnie, rozhartowane rośliny jęczmienia (*Hordeum vulgare* (L.)) charakteryzowały się zwiększeniem koncentracji hormonów wzrostowych, w tym auksyny (kwasu indolilo-3-octowego, IAA) oraz podwyższonym poziomem giberelin i cytokinin (Pociecha i współaut. 2020). Rozhartowanie skutkuje też zmianami ekspresji wielu genów zaangażowanych między innymi w tzw. sygnalizację hormonalną (takich jak receptor brasinosteroidów BRI1). W bardziej mrozoodpornych odmianach rzepaku, po rozhartowaniu ekspresja BRI1 pozostała na poziomie podobnym jak u roślin hartowanych (Stachurska i współaut. 2022).

NIEINWAZYJNE METODY OKREŚLANIA STOPNIA ROZHARTOWANIA ROŚLIN

Nasilanie się zmian klimatycznych i zwiększanie częstotliwości zjawisk powodujących rozhartowanie roślin skłania do zastanowienia się, czy i jakie środki zaradcze można/trzeba będzie podjąć w przyszłości w uprawach ozimin. Jedną z opcji może być dobór i uprawa odmian charakteryzujących się mniejszą podatnością na rozhartowanie. Inną alternatywą może być zastosowanie preparatów zapobiegających uszkodzeniom mrozowym roślin ozimych w trakcie wegetacji po wystąpieniu ciepłych przerw. W takim przypadku stan upraw ozimin należałoby monitorować przy pomocy nieinwazyjnych metod umożliwiających szybką ocenę stopnia rozhartowania roślin, bowiem ocena szczegółowych zmian metabolicznych wymagająca zebrania liści i wykonania złożonych analiz laboratoryjnych, może być niepraktyczna. Istnieje jednak kilka nieinwazyjnych metod pomiarowych, które umożliwiają dostrzeżenie nawet wczesnych etapów rozhartowania roślin. Obiecujące są metody pomiaru własności spektralnych liści (tzw. refleksji liści), dostarczających informacji o strukturze, uwodnieniu i zmianach w obrębie koncentracji barwników w liściach. Przykładem może być parametr ARI opisujący poziom barwników antocyjanów, którego wartości dobrze korelują ze zmianami powodowanymi rozhartowaniem – u hartowanych roślin rzepaku ARI osiąga wysokie wartości, które – niezależnie od odmiany – spadają kilkakrotnie po rozhartowaniu (Rys i współaut. 2020; Stachurska i współaut. 2024). Ponieważ fotosynteza jest dobrym wskaźnikiem odzwierciedlającym wpływ różnych czynników na rośliny, to analiza fluorescencji chlorofilu *a* (i efektywności działania fotosystemów) jest jedną ze skutecznych metod oceny rozhartowania upraw, co wykazano w przypadku zbóż (Rapacz i współaut. 2017) oraz potwierdzono u rzepaku (Rys i współaut. 2020; Stachurska i współaut. 2022). Można tego dokonać wykorzystując drony, bezzałogowe statki powietrzne czy satelity do monitorowania zmian fluorescencji pól uprawnych.

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Oświadczam, że w pracy: Stachurska J., Janeczko, A. (2024) Zjawisko hartowania i rozhartowania roślin w kontekście zmian klimatu (Kosmos, tom 73, nr 1 (341), str. 37-46 mój udział polegał na: ukierunkowaniu pracy doktorantki (J. Stachurskiej) w trakcie pisania manuskryptu oraz wykonaniu niezbędnej korekty manuskryptu.

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Udział w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej

A. **Stachurska, J.**, Rys, M., Pociecha, E., Kalaji, H.M., Dąbrowski, P., Oklestkova, J., Jurczyk, B., Janeczko, A. (2022) Deacclimation-Induced Changes of Photosynthetic Efficiency, Brassinosteroid Homeostasis and *BRI1* Expression in Winter Oilseed Rape (*Brassica napus* L.)—Relation to Frost Tolerance. International Journal of Molecular Sciences, 2022, 23, 5224

Mój udział w tworzeniu publikacji polegał na: założeniu doświadczenia i uprawie roślin (w tym zbiorze próbek), wykonaniu pomiarów fluorescencji chlorofilu *a* pod nadzorem dr M. Ryś, wykonaniu testu mrozowego i dokumentacji fotograficznej roślin pod nadzorem dr hab. inż. E. Pociechy, współpracy z dr hab. inż. B. Jurczyk przy wykonywaniu analiz akumulacji transkryptu *BRI1*, przygotowaniu/pisaniu manuskryptu (w tym wykonaniu przeglądu literatury, wykonaniu analiz statystycznych i opisu uzyskanych wyników, przygotowaniu rycin i tabel,) pod kierunkiem prof. dr hab. inż. A. Janeczko oraz edycji końcowej manuskryptu wg wytycznych czasopisma.

B. **Stachurska, J.**, Sadura, I., Rys, M., Dziurka, M., Janeczko, A. (2023) Insight into Hormonal Homeostasis and the Accumulation of Selected Heat Shock Proteins in Cold Acclimated and Deacclimated Winter Oilseed Rape (*Brassica napus* L.). Agriculture 13, 641.

Mój udział w tworzeniu publikacji polegał na: założeniu doświadczenia, uprawie roślin i zebraniu próbek we współpracy z dr M. Ryś, wykonaniu analiz akumulacji białek z grupy HSP (w tym HSP70 cytoplazmatycznego, HSP70 chloroplastowego i HSP90) wraz z optymalizacją

metody pod nadzorem dr I. Sadury-Berg, wykonaniu ekstrakcji, oczyszczania materiału roślinnego i przygotowania próbek oraz analizie zawartości fitohormonów pod nadzorem dr M. Dziurki i dr M. Ryś, przygotowaniu manuskryptu (w tym wykonaniu przeglądu literatury, analizy densytometrycznej wyników akumulacji białek programem ImageJ, wizualizacji, opisu i interpretacji uzyskanych wyników, wykonaniu analiz statystycznych, przygotowaniu rycin i tabel) pod kierunkiem prof. dr hab. inż. A. Janeczko, edycji końcowej manuskryptu wg wytycznych czasopisma. Jako autor korespondencyjny, byłam dodatkowo odpowiedzialna za wysłanie manuskryptu i uczestniczyłam w dyskusji z recenzentami.

C. Rys M., **Stachurska J.**, Rudolphi-Szydło E., Dziurka M., Waligórski P., Filek M., Janeczko A. (2024) Does deacclimation reverse the changes in structural/physicochemical properties of the chloroplast membranes that are induced by cold acclimation in oilseed rape? Plant Physiology and Biochemistry, Volume 214, 108961

Mój udział w tworzeniu publikacji polegał na: założeniu doświadczenia, uprawie roślin i zebraniu próbek we współpracy z dr M. Ryś, wykonaniu ekstrakcji i oczyszczenia próbek do analizy zawartości tokoferoli (w tym α-, β-, γ-, δ-tokoferolu) i karotenoidów (β-karotenu i zeaksantyny) pod nadzorem dr M. Dziurki i dr M. Ryś, wykonaniu ekstrakcji i oczyszczenia próbek do analizy zawartości kwasów tłuszczowych we współpracy z dr hab. P. Waligórskim i dr M. Ryś, izolacji chloroplastów pod nadzorem dr M. Ryś, przygotowaniu/napisaniu manuskryptu (w tym wykonaniu przeglądu literatury, wykonaniu analiz statystycznych i opisu uzyskanych wyników, przygotowaniu rycin i tabel) pod nadzorem dr M. Ryś i prof. dr hab. inż. A. Janeczko, edycji końcowej manuskryptu wg wytycznych czasopisma.

D. **Stachurska J.**, Sadura I., Jurczyk B., Rudolphi−Szydło E., Dyba B., Pociecha E., Ostrowska A., Rys M., Kvasnica M., Oklestkova J., Janeczko A. (2024) Cold acclimation and deacclimation of winter oilseed rape − special attention being paid to role of brassinosteroids. International Journal of Molecular Sciences 25, 6010.

Mój udział w tworzeniu publikacji polegał na: założeniu doświadczenia i uprawie roślin we współpracy z dr M. Ryś, wykonaniu oprysków roślin roztworami brasinosteroidów, wykonaniu pomiarów fluorescencji chlorofilu *a*, wykonaniu pomiarów własności spektralnych

(refleksji) liści, przeprowadzeniu pomiarów konduktometrycznych (wraz z optymalizacją metody), przygotowaniu/napisaniu manuskryptu (w tym wykonaniu przeglądu literatury, wykonaniu analiz statystycznych i opisu uzyskanych wyników, przygotowaniu rycin i tabel), współpracy z dr hab. inż. B. Jurczyk przy wykonywaniu analizy akumulacji transkryptów genów *SERK1, SERK2, COR14*, wykonaniu analizy akumulacji białka BRI1 wraz z optymalizacją metody z dr Iwoną Sadurą-Berg, wykonaniu testu mrozowego pod nadzorem dr hab. inż. E. Pociechy, pomocy dr inż. A. Ostrowskiej w wykonaniu pomiarów wymiany gazowej, pomocy dr B. Dybie i dr hab. E. Rudolphi-Szydło w wykonaniu analiz membran na wadze Langmuira, edycji końcowej manuskryptu wg wytycznych czasopisma. Jako autor korespondencyjny, byłam dodatkowo odpowiedzialna za wysłanie manuskryptu i uczestniczyłam w dyskusji z recenzentami.

E. **Stachurska, J.**; Janeczko, A. (2024) Physiological and Biochemical Background of Deacclimation in Plants, with Special Attention Being Paid to Crops: A Minireview. Agronomy 14, 419.

Mój udział w tworzeniu publikacji polegał na: wykonaniu przeglądu literatury dotyczącej zjawiska hartowania oraz rozhartowania roślin, napisaniu manuskryptu i przygotowaniu rycin pod kierunkiem prof. dr hab. inż. A. Janeczko, edycji końcowej manuskryptu wg wytycznych czasopisma, wysyłce przygotowanego manuskryptu oraz poprawieniu manuskryptu wg wytycznych recenzentów.

F. **Stachurska J.**, Janeczko, A. (2024) Zjawisko hartowania i rozhartowania roślin w kontekście zmian klimatu. Kosmos, tom 73, nr 1 (341), str. 37-46

Mój udział w tworzeniu publikacji polegał na: wykonaniu przeglądu literatury dotyczącej zjawiska hartowania i rozhartowania roślin, napisaniu manuskryptu pod nadzorem prof. dr hab. inż. A. Janeczko, edycji końcowej manuskryptu wg wytycznych czasopisma, wysyłce przygotowanego manuskryptu oraz poprawieniu manuskryptu wg wytycznych recenzentów.

Julia Stachurska

(podpis)