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Zakład Biologii Rozwoju

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Rola brasinosteroidów w procesach aklimatyzacyjnych roślin jęczmienia do niskiej i wysokiej temperatury

Rozprawa doktorska

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Spis treści

1. Wykaz prac naukowych wchodzących w skład cyklu publikacji do rozprawy doktorskiej

Praca stanowi podsumowanie badań przeprowadzonych w latach 2016 – 2020, a niniejsze opracowanie przygotowane zostało na podstawie wyników opisanych w sześciu poniższych publikacjach (zestawionych chronologicznie).

A. **Sadura Iwona**, Janeczko Anna (2018) Physiological and molecular mechanisms of brassinosteroid-induced tolerance to high and low temperature in plants. Biologia Plantarum 62: 601-616; IF (2019): 1,601; 5-letni IF (2019): 1,898; punktacja MEiN: 70.

B. **Sadura Iwona**, Pociecha Ewa, Dziurka Michał, Oklestkova Jana, Novak Ondrej, Gruszka Damian, Janeczko Anna (2019) Mutations in the *HvDWARF*, *HvCPD* and *HvBRI1* genes – involved in brassinosteroid biosynthesis/signalling: altered photosynthetic efficiency, hormonal homeostasis and tolerance to high/low temperatures in barley. Journal of Plant Growth Regulation 38: 1062-1081; IF (2019): 2,672; 5-letni IF (2019): 2,962; punktacja MEiN: 70.

C. **Sadura Iwona**, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020a) The HSP transcript and protein accumulation in brassinosteroid barley mutants acclimated to low and high temperature. International Journal of Molecular Sciences 21:1889; IF (2019): 4,556; 5-letni IF (2019): 4,653; punktacja MEiN: 140.

D. **Sadura Iwona**, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020b) Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature. Journal of Plant Physiology 244:153090; IF (2019): 3,013; 5-letni IF (2019): 3,615; punktacja MEiN: 100.

E. Rudolphi-Szydło Elżbieta, **Sadura Iwona**, Filek Maria, Gruszka Damian, Janeczko Anna (2020) The impact of mutations in the *HvCPD* and *HvBRI1* genes on the physicochemical properties of membranes from barley acclimated to low/high temperatures. Cells 9:1125; IF (2019): 4,366; 5-letni IF (2019): 5,276; punktacja MEiN: 140.

F. **Sadura Iwona**, Latowski Dariusz, Oklestkova Jana, Gruszka Damian, Chyc Marek, Janeczko Anna (2021) Molecular dynamics of chloroplast membranes isolated from wild-type barley and a brassinosteroid-deficient mutant acclimated to low and high temperatures. Biomolecules 11: 27; IF (2019): 4,082; 5-letni IF (2019): bd; punktacja MEiN:100.

Suma IF (2019) publikacji 20,29
Średni IF (2019) publikacji 3,38

Suma punktów MEiN 620
Średnia punktów MEiN 103,33

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2. Wprowadzenie

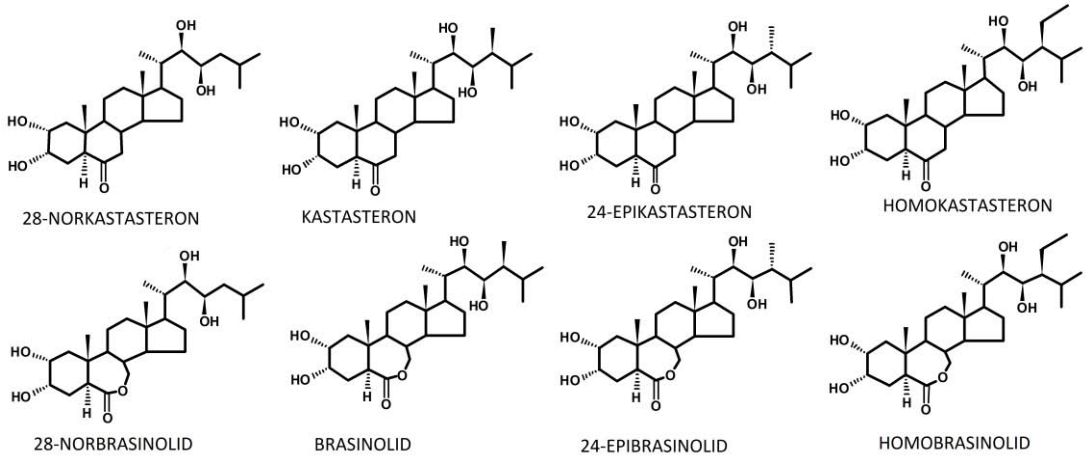
2.1. Brasinosteroidy

Brasinosteroidy (BR) są to steroidowe hormony roślinne, które pod względem struktury wykazują podobieństwo do hormonów steroidowych występujących u zwierząt (kortykosteroidów, estrogenów, androgenów, progesteronu) i owadów (ekdysteroidów). Wszystkie te związki ogrywają podstawową rolę w regulacji metabolizmu wymienionych organizmów. BR zostały wyizolowane po raz pierwszy w latach 70 XX w. z pyłku rzepaku *Brassica napus*. Zidentyfikowano wtedy pierwszy BR zwany brasinolidem (Grove i wsp. 1979). Obecnie grupę tę stanowi 81 związków (Liu i wsp. 2017) różniących się strukturą oraz związaną z nią aktywnością fizjologiczną (Bajguz i Tretyn 2003). Ze względu na obecność wielu grup hydroksylowych w strukturze, związki te można określić polihydroksysteroidami. W roślinach wyższych i glonach BR występują zarówno w formie wolnej jak i związanej (koniugaty z kwasami tłuszczowymi lub cukrami). BR obecne są w komórkach wszystkich tkanek roślin wyższych w ilościach rzędu nano- i pikogramów na gram świeżej masy. Omawiane związki chemiczne charakteryzują się wielopierścieniowym szkieletem węglowym 5α-cholestanu, a różnice w ich budowie wynikają z obecności różnych grup funkcyjnych i ich położenia w obrębie pierścieni cyklicznych A i B oraz łańcucha węglowego (Bishop 2003; Bajguz i Tretyn 2003). Ze względu na liczbę atomów węgla w cząsteczce wyróżnia się trzy główne grupy BR: C_{27}, C_{28} oraz C_{29}. Prekursorami w biosyntezie wszystkich BR są sterole. W przypadku BR C_{27} prekursorem jest cholesterol, dla BR C_{28} kampesterol i 24-β-metylocholesterol, a dla BR C_{29} – sitosterol (**Sadura i Janeczko, 2018 – Publikacja A**). Wzory strukturalne wybranych BR przedstawiono na Rycinie 1.

Pierwszy szlak biosyntezy BR wykryto u *Arabidopsis thaliana* oraz w hodowli komórkowej *Catharanthus roseus* dla BR typu C_{28} brasinolidu (Yokota i wsp. 1990; Fujioka i Yokota 2003; Clouse 2015). Przyjęto, że metabolitami pośrednimi w tym szlaku są: katasteron, teasteron, tyfasterol i kastasteron w szlaku tzw. wczesnego utleniania węgla C6 oraz 6-deoksokatasteron, 6-deoksoteasteron, 6-deoksotyfasterol i 6-deoksokastasteron w szlaku tzw. późnego utlenienia węgla C6 (Fujioka i Yokota 2003). W przypadku BR o innej budowie, szlaki ich biosyntezy mogą być zmodyfikowane, a ich uproszczone wersje przedstawiono w publikacji **Sadura i Janeczko (2018) – Publikacja A**. Poznane są mutanty z zaburzeniami w przemianach BR głównie na szlaku biosyntezy C_{28}. Mutanty te charakteryzują się deficytem BR, w szczególności kastasteronu i brasinolidu (Bishop 2003; Gruszka i wsp. 2011; Dockter i wsp. 2014). Enzymy odpowiedzialne za biosyntezę BR,

należące do rodziny cytochromu P450, są zlokalizowane w błonie retikulum endoplazmatycznego (Jørgensen i wsp. 2005; Gruszka i Małuszyński 2010).

Przyjmuje się, że szlak transdukcji sygnału BR przebiega następująco: BR są wiązane przez transbłonowy receptor BRI1 (ang. *Brassinosteroid-Insensitive1*), co wywołuje



Ryc. 1. Wzory strukturalne wybranych BR: z grupy C_{27} (28-norkastasteron, 28-norbrasinolid), C_{28} (kastasteron, brasinolid i 24-epikastasteron, 24-epibrasinolid) oraz C_{29} (homokastasteron, homobrasinolid).

utworzenie heterodimeru z jego koreceptorem BAK1 (ang. *BRI1-Associated Receptor Kinase1*). Auto- i transfosforylacja między BRI1 i BAK1 powoduje oddzielenie się zlokalizowanego w membranie komórkowej inhibitora BKI1 od receptora BRI1 (BKI1 jest zatrzymywany przez białka 14-3-3). Następnie w wyniku fosforylacji dwóch zakotwiczonych w błonie komórkowej kinaz BSK1 (ang. *Brassinosteroid-Signalling Kinase1*) i CDG1 (ang. *Constitutive Differential Growth1*), dochodzi do ich uwolnienia od receptora BRI1, przekazania przez nie sygnału BR i aktywacji fosfatazy białkowej BSU1 (ang. *BRI1 Suppressor*), która następnie defosforyluje i dezaktywuje kinazę BIN2 (ang. *Brassinosteroid-Insensitive2*) (negatywnie regulującą przekazywanie sygnału BR). Gdy zawartość BR jest niska, BIN2 fosforyluje dwa homologiczne czynniki transkrypcyjne BZR1 (ang. *Brassinazole Resistant1*) i BES1 (ang. *BRI1-EMS-Suppressor1*), po czym są one wychwytywane przez białka 14-3-3, w celu zahamowania ich gromadzenia się w jądrze. Natomiast, gdy zawartość BR jest wysoka, BIN2 jest dezaktywowany, a BZR1 i BES1 są defosforylowane przez PP2A (ang. *Protein Phosphatase 2A*). Niesfosforylowane BZR1 i BES1 gromadzą się w jądrze i bezpośrednio kontrolują transkrypcję genów wrażliwych na BR (ang. *BR-responsive genes*), a tym samym regulują wiele procesów rozwojowych w roślinie (Wang i wsp. 2006, 2014; Li i Jin 2007; Chung i Choe 2013; Planas-Riverola i wsp. 2019). W kontekście niniejszej pracy doktorskiej warto zaznaczyć, iż na działanie BIN2 mogą także oddziaływać białka szoku cieplnego HSP90. Badania na *Arabidopsis thaliana* pokazały, że zlokalizowane w jądrze

komórkowym HSP90 oddziałują z BIN2, odgrywając kluczową rolę w podtrzymywaniu jego funkcji w jądrze. Przy zwiększonej zawartości BR dowiedziono, że do oddziaływania HSP90 z BIN2 dochodzi w cytoplazmie (Samakovli i wsp. 2014).

Pomimo, że już w latach 70-tych ubiegłego wieku stwierdzono, iż egzogenne podawanie BR stymuluje wydłużanie łodyg i podziały komórkowe w roślinach (Mitchell i wsp. 1970; Grove i wsp. 1979), związki te uznano za nową klasę fitohormonów dopiero w latach 90-tych XX wieku. Wtedy odkryto kilka genów odpowiadających m.in. za biosyntezę i percepcję BR, ale co ważniejsze stwierdzono, że mutacja związana z utratą funkcji tych genów, prowadzi zazwyczaj do znacznych zaburzeń wzrostu rośliny (m.in. krótki hypokotyl, karłowatość siewek i dojrzałych roślin, ciemnozielone skrócone liście) (Yang i wsp. 2011). Jednak dziś już wiadomo, że działanie BR jest wielokierunkowe także jako regulatorów koniecznych do prawidłowego rozwoju roślin. Badania przeprowadzone na roślinach wyższych sugerują, że BR regulują proces kwitnienia (Yang i wsp. 2011; Janeczko i wsp. 2015), rozwój kwiatów i owoców (Castle i wsp. 2003; Symons i wsp. 2006; Chai i wsp. 2013; Castorina i Consonni 2020; Hu i wsp. 2020; Lin 2020). Jednak za szczególnie interesującą właściwość BR należy uznać ich zdolność do przeciwdziałania negatywnym skutkom stresów środowiskowych u roślin (Castle i wsp. 2003; Krishna 2003; Bajguz i Hayat 2009; Vardhini i Anjum 2015; Anwar i wsp. 2018; Ahammed i wsp. 2020; Bartwal i Arora 2020). W pracy przeglądowej włączonej w cykl publikacji (**Sadura i Janeczko 2018 – Publikaca A**) przedyskutowano szczegółowo (na podstawie dostępnej literatury) temat roli BR w odpowiedzi roślin na stres temperaturowy.

2.2. Stres temperaturowy i zjawisko aklimacji

Rośliny są narażone na działanie różnorodnych czynników środowiskowych – biotycznych (patogeny) i abiotycznych – zbyt wysoka lub zbyt niska temperatura, niedobór lub nadmiar wody i składników mineralnych, zasolenie podłoża, zbyt słabe lub zbyt intensywne światło, zanieczyszczenia środowiska wynikające z działalności człowieka, promieniowanie jonizujące, itp. (Koyro i wsp. 2012). Spośród stresów abiotycznych stresy temperaturowe są szczególnie poważnym problemem w rolnictwie i ogrodnictwie (Hasanuzzaman i wsp. 2012; Koyro i wsp. 2012). Takie gatunki jak ogórki, pomidory czy kukurydza są bardzo wrażliwe na chłód. Z kolei mróz, zwłaszcza przy niewystarczającej pokrywie śnieżnej na polach (oraz poprzedzony dodatkowo okresem podwyższonej temperatury o działaniu rozhartowującym) może być powodem znacznych strat plonów gatunków ozimych. Z kolei stres

wysokotemperaturowy jest szczególnie niebezpieczny dla roślin, jeśli występuje w okresie wegetacji w miesiącach letnich w połączeniu z suszą.

Tolerancja na mróz u roślin ozimych wzrasta w wyniku zmian metabolicznych zachodzących w okresie hartowania chłodem (aklimacji). Jesienne okresy chłodu (zwłaszcza temperatury 2 – 5°C) hartują zboża ozime i rzepak ozimy zwiększając ich tolerancję na mróz w czasie zimy. Tolerancja na mróz wzrasta wraz z czasem aklimacji (Fowler i wsp. 1996), a za wystarczający uważa się okres chłodu 3 – 6 tygodni. Zdolność aklimacji w chłodzie wykazują także zboża jare, co jest przydatne w sytuacji wystąpienia anomalii temperaturowych (zwłaszcza przymrozków) w okresie wiosennym. Na Rycinie 2 przedstawiono przykład uszkodzeń (żółknięcie liści) roślin jęczmienia w wyniku wiosennego spadku temperatury. Warto także wspomnieć, że niektóre gatunki (zwłaszcza pochodzące z ciepłych rejonów świata) mają słabe zdolności zahartowania się w chłodzie i nabycia tolerancji na jeszcze niższe temperatury. Takie rośliny, których dobrym przykładem jest kukurydza, są bardzo wrażliwe nawet na chłód, który przyczynia się do uszkodzenia tych roślin.

Ryc. 2. Uszkodzenia wiosenne jęczmienia jarego na polach w wyniku przymrozków. (https://golden-seeds.pl/jeczmien-jary-a-przymrozki/)

Zmiany metaboliczne w czasie procesu hartowania chłodem są dość dobrze poznane, wśród nich można wymienić: zahamowanie wzrostu roślin, spadek uwodnienia tkanek, zmianę składu lipidowego i struktury membran (wzrost zawartości nienasyconych kwasów tłuszczowych lipidów membranowych), zmiany w zawartości białek m.in. wzrost akumulacji białek późnej embriogenezy (ang. *Late Embryogenesis Abundant proteins*, LEA), białek chroniących przed zamarzaniem (ang. *Anti-Freezing Proteins*, AFP), białek opiekuńczych (ang. *Cold Shock Proteins*, CSP) (Ding i wsp. 2019). Wśród innych zmian wymienić można wzrost poziomu/aktywności enzymów np. związanych z biosyntezą osmoprotektanów i przeciwutleniaczy (Seki i wsp. 2003) oraz wzrost stężenia rozpuszczalnych cukrów (Guy

1990; Uemura i wsp. 2006; Yamori i wsp. 2014). Podczas hartowania, pod wpływem niskiej temperatury aktywowane są geny pełniące różne funkcje, miedzy innymi geny odpowiedzialne za transdukcję sygnału wywołanego działaniem niskiej temperatury. Najważniejszy jest gen czynnika transkrypcyjnego kluczowego dla hartowania, czyli CBF (ang. *C-repeat Binding Factor*). Indukowana chłodem lub konstytutywna ekspresja *CBF1* zmniejsza poziom bioaktywnych giberelin (czemu towarzyszy wzrost transkryptów *GA2ox3* i *GA2ox6*), co skutkuje zmianą stabilności białek DELLA i ich akumulacją. Ponadto, chłód i CBF zwiększają poziomy transkrypcji *RGL3* (ang. *Repressor of GA3*). Niska temperatura zwiększa również poziom ekspresji *GA2ox1* za pomocą mechanizmu niezależnego od *CBF1*. W wyniku akumulacji DELLA następuje spowolnienie wzrostu roślin, natomiast zwiększenie poziomu aktywnych giberelin wiosną odpowiada za ich wzrost (Achard i wsp. 2008; Zhou i wsp. 2017). CBF indukuje także ekspresję genów *COR* (ang. *Cold-Responsive Genes*), które są istotne w procesach aklimacji roślin do niskiej temperatury (Thomashow 1998; Yadav 2010). Za fundamentalną rolę COR uważa się ochronę komórek roślinnych przed utratą wody (Yadav 2010). Zarówno czynniki transkrypcyjne CBF jak i *COR* pomagają w zapewnieniu roślinom tolerancji na działanie mrozu poprzez m.in. przyspieszenie syntezy osmolitów, czy przebudowanie błon komórkowych (Yadav 2010; Nievola i wsp. 2017).

Z kolei wzrost roślin w podwyższonej temperaturze (w tym aklimacja np. w 27°C) może przyczyniać się do zwiększonej ich tolerancji na działanie bardziej ekstremalnej – wysokiej temperatury (np. 40 – 45°C). W odróżnieniu jednak od hartowania do niskich temperatur zwiększona tolerancja na wysoką temperaturę nabywana jest przez rośliny (zależnie od gatunku) po krótszym okresie już nawet od kilku godzin (Qin i wsp. 2008) po 7 – 14 dni (Thussagunpanit i wsp. 2015; Végh i wsp. 2018) ekspozycji na podwyższoną temperaturę. Mechanizm aklimacji roślin do wysokiej temperatury obejmuje m.in. zmiany w składzie i integralności membran, w tym zwiększenie zawartości nasyconych kwasów tłuszczowych w celu ograniczenia nadmiernego wzrostu płynności membran. Spośród innych zmian wymienić należy uruchomienie ścieżek sygnałowych z udziałem cząsteczek sygnałowych (np. jonów wapnia, tlenku azotu, nadtlenku wodoru) lub ścieżek sygnałowych zależnych od fitohormonów (kwas abscysynowy, kwas salicylowy, etylen). Wymienione cząsteczki sygnałowe/fitohormony przyczyniają się do aktywacji czynników transkrypcyjnych szoku cieplnego HSF (ang. *heat shock transcription factors*) indukujących zmiany w ekspresji genów HR (ang. *heat responsive genes*) zależnych od wysokiej temperatury (Nievola i wsp. 2017).

W załączonej pracy przeglądowej (**Sadura i Janeczko 2018** – **Publikacja A**) przeprowadzono analizę literatury dotyczącej wpływu BR i stresu temperaturowego na zmiany metaboliczne i rozwój roślin. Na podstawie przeanalizowanej literatury omówiono wpływ BR na strukturę i właściwości membran komórkowych, funkcjonowanie komórkowego układu antyoksydacyjnego, fotosyntezę, gospodarkę hormonalną, zawartość aminokwasów i białek (w tym ekspresję białek szoku cieplnego). Przeprowadzona analiza pozwoliła stwierdzić, że BR podawane egzogennie ograniczają negatywny wpływ stresu temperaturowego (w przypadku działania niskiej i wysokiej temperatury) poprzez wielokierunkowe działanie na różne elementy strukturalne i funkcjonalne komórki, kluczowe dla rozwoju roślin i ich odpowiedzi na warunki stresowe (**Sadura i Janeczko 2018** – **Publikacja A**). Wykazano, że BR mogą zmieniać właściwości fizykochemiczne membran, regulować ekspresję niektórych genów (np. *COR*) i regulować metabolizm poprzez interakcje z innymi hormonami i cząsteczkami sygnałowymi (ABA, H_2O_2). Przeprowadzony przegląd literatury dotyczącej stresu temperaturowego wskazuje jednak na istnienie niewielkiej ilości prac, w których do badań wykorzystano mutanty z zaburzeniami biosyntezy lub percepcji BR (Mazorra i wsp. 2011; Setsungnern i wsp. 2020; Eremina i wsp. 2016; Fang i wsp. 2019). Oznacza to, że wiele wniosków na temat roli BR w reakcji roślin na stres niskiej/wysokiej temperatury wyciągnięto na podstawie modelu badawczego z zastosowaniem egzogennych BR. Taki stan wiedzy może wymagać pewnej weryfikacji. Z tego też względu obiektem badawczym w badaniach przeprowadzonych w ramach przedłożonej rozprawy doktorskiej były rośliny jęczmienia z zaburzoną biosyntezą BR (zmniejszona zawartość BR z grupy C_{28} oraz C_{29}) oraz z zaburzoną percepcją BR (defekt receptora BRI1 i zwiększona zawartość BR z grupy C_{28}). Wybrany do doświadczeń mutant 522DK oraz linie bliskoizogeniczne BW084 i BW312 zostały ogólnie omówione w części „Materiał roślinny i schemat eksperymentu”, a bardziej szczegółowo scharakteryzowane w publikacjach wchodzących w skład cyklu publikacji.

W badaniach prowadzonych na omawianym materiale roślinnym koncentrowano się w głównej mierze na zmianach zachodzących pod wpływem temperatury w obrębie lipidów i białek membranowych, bowiem zmiany struktury i właściwości membran są szczególnie ważne w przystosowaniu roślin do niskich/wysokich temperatur. Membrany komórkowe to półprzepuszczalne błony otaczające cytoplazmę komórki. Zbudowane są one z dwuwarstwy lipidowej zawierającej białka i inne cząsteczki. Membrany komórkowe charakteryzują się dynamiką molekularną oraz asymetrią i odgrywają bardzo ważną rolę w funkcjonowaniu komórek. Ze względu na to, że membrany komórkowe w całym wnętrzu komórki są

połączone na kilka sposobów poprzez układy cytoplazmatyczne w tym sieci cytoszkieletowe, systemy transdukcji sygnału oraz inne sieci strukturalne, enzymatyczne i komunikacyjne, nie są one samodzielnymi/autonomicznymi strukturami komórkowymi. W tkankach, membrany są także połączone poza komórką z macierzą zewnątrzkomórkową/ścianą komórkową, innymi komórkami i śródmiąższowymi/międzywarstwowymi strukturami białkowymi. Zatem membrany komórkowe są w pełni zintegrowanymi strukturami, które jednocześnie są czułe na wszelkie sygnały oraz stresy środowiskowe, dodatkowo szybko i selektywnie na nie reagującymi (Nicolson 2014).

Membrany komórkowe odgrywają istotną rolę w wykształconych przez rośliny strategiach radzenia sobie z negatywnymi skutkami działania stresów abiotycznych, w tym działania stresu temperaturowego (Prasad i Rengel 2006). Według Horváth i wsp. (2012) membrany komórkowe można uznać za „czujniki termiczne", a zachodzące w nich zmiany są preludium do wielu metabolicznych przemian zachodzących w komórkach, w tym do ekspresji określonych genów. Podczas aklimacji do niższej lub wyższej temperatury, zmiany w płynności membran komórkowych wiążą się z modyfikacją w proporcji nienasyconych kwasów tłuszczowych, co skutkuje przegrupowaniem struktury membrany i zmianą jej właściwości. Właściwości membran mogą także ulec modyfikacji w wyniku wbudowania w nie różnych komponentów np. steroli, tokoferoli, steroidów (Gzyl-Malcher i wsp. 2010) czy węglowodanów (Nievola i wsp. 2017).

Na skutek zadziałania na roślinę niskiej temperatury następuje usztywnianie membrany (Los i Murata 2004; Nievola i wsp. 2017; Niu i Xiang 2018). Spadek temperatury generalnie powoduje zmniejszenie ruchliwości cząsteczek lipidów i zmniejszenie płynności, co może przyczyniać się do dysfunkcji skutkujących zwiększeniem przepuszczalności membran, a następnie wiązać się z utratą wody i wewnątrzkomórkowych substancji rozpuszczonych oraz z inaktywacją kanałów transportowych (Nievola i wsp. 2017; Ramirez i Poppenberger 2020). Aby temu przeciwdziałać w czasie aklimacji (hartowania) w chłodzie do membrany włączane są w większym stopniu nienasycone kwasy tłuszczowe (Uemura i wsp. 2006; Penfield 2008). Taka modyfikacja skutkuje utrzymaniem właściwej płynności w chłodzie, dzięki czemu membrana nadal posiada odpowiednie parametry do pełnienia swoich funkcji. Warto także zauważyć, że wzrost sztywności membran na skutek zadziałania niskiej temperatury może przyczynić się do wzrostu wewnątrzkomórkowego stężenia jonów Ca^{2+}, które jest spowodowane napływem tego jonu przez kanały wapniowe zlokalizowane w membranie i/lub uwolnieniem Ca^{2+} zmagazynowanego w wakuoli. Według Thomashow (2010) indukcja genów CBF (ang. *C-repeat binding factor*) wywołana jonami Ca^{2+} następuje

już po 15 minutach od umieszczenia roślin *A. thaliana* w 4°C. Te aktywowane geny *CBF* generują czynniki transkrypcyjne, które z kolei aktywują geny *COR* (ang. *cold-responsive genes*), co także jest jednym z czynników przyczyniających się do aklimacji roślin do chłodu (Nievola i wsp. 2017). Funkcję ochronną u roślin poddanych aklimacji do chłodu mogą także pełnić białka szoku cieplnego (HSP) (Krishna i wsp. 1995; Horváth i wsp. 2012).

Z kolei zadziałanie stresu wysokiej temperatury powoduje nadmierny wzrost płynności membran (Penfield 2008; Niu i Xiang 2018) i przyczynia się do wypływu jonów, czyniąc membrany bardziej podatnymi na uszkodzenia, co ostatecznie może prowadzić do zaburzeń przebiegu różnych procesów komórkowych (w tym transportu jonów i metabolitów) i fizjologicznych jak fotosynteza, a szczególnie jej faza jasna zlokalizowana w membranach (Los i Murata 2004; Nievola i wsp. 2017). W celu przeciwdziałania negatywnym skutkom wywołanym działaniem stresu wysokiej temperatury, w czasie aklimacji roślin w podwyższonej temperaturze następuje wzrost udziału nasyconych kwasów tłuszczowych w membranach. Pozwala to zwiększyć temperaturę przejścia fazowego (topnienia) lipidów i zapobiega fluidyzacji membran w warunkach podwyższonej temperatury, przyczyniając się do zwiększenia tolerancji rośliny na działanie stresu wysokiej temperatury (Źróbek-Sokolnik 2012; Bita i Gerats 2013; Nievola i wsp. 2017).

Jak wspomniano wcześniej, w odróżnieniu od aklimacji do niskiej temperatury (wymagającej zwykle np. u zbóż okresu kilku tygodni), aklimacja roślin do warunków podwyższonej temperatury przebiega znacznie szybciej, w wielu wypadkach wystarczające do przystosowania się rośliny do takich warunków może być kilka dni, a nawet krótszy okres czasu. Według Nievola i wsp. (2017), Niu i Xiang (2018) oraz Kothari i Lachowiec (2021), wywołany działaniem wysokiej temperatury wzrost płynności membran, aktywuje szlaki sygnałowe obejmujące m.in. aktywację kanałów wapniowych zlokalizowanych w membranach. Jony Ca^{2+} wnikające do komórki przyczyniają się przede wszystkim do aktywacji czynników transkrypcyjnych szoku cieplnego (HSF) i białek szoku cieplnego (HSP). Udowodniono, że również rola BR w regulacji ruchu wtórnych przekaźników – Ca^{2+}, może pośredniczyć w odpowiedzi roślin na wysoką temperaturę (Kothari i Lachowiec 2021). Związane jest to z przyłączaniem kalmoduliny (jednego z głównych białek sensorowych Ca^{2+}, mogących zmieniać sygnały Ca^{2+} w odpowiedź komórkową) do cytoplazmatycznej domeny receptora BRI1 (Oh i wsp. 2012). Według Zhao i wsp. (2013) wiązanie BR do receptora BRI1 powoduje otwieranie kanałów Ca^{2+} w membranach, zwiększając tym samym zawartość cytozolowego Ca^{2+}.

Produkcja białek szoku cieplnego (HSP) jest bardzo ważnym mechanizmem zachodzącym w membranach i związanym z aklimacją roślin do wysokiej temperatury. Uważa się nawet, że HSP są kluczowe dla nabycia tolerancji na działanie wysokiej temperatury, a indukcja ich ekspresji może nastąpić już po kilku sekundach od wzrostu temperatury. Maksymalny poziom transkryptów *HSP* może być obserwowany w ciągu jednej do dwóch godzin po ekspozycji na wysoką temperaturę (Nievola i wsp. 2017).

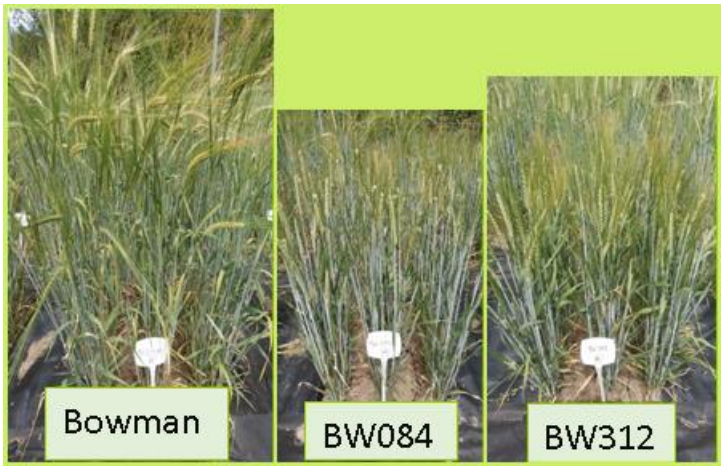
Z racji roli membran w procesie przystosowania roślin do różnych temperatur, w doświadczeniach przeprowadzonych w ramach przedłożonej pracy doktorskiej starano się określić udział BR w zmianach powiązanych z membranami i zachodzących w czasie aklimacji jęczmienia w chłodzie (5°C) i w podwyższonej temperaturze (27°C). Oceniano wpływ BR na strukturę (skład lipidowy) i własności fizykochemiczne membran jak i ekspresję wybranych białek membranowych o funkcjach ochronnych (HSP) oraz transportujących wodę (akwaporyny), a także ekspresję H^+-ATPazy (membranowej pompy protonowej). Badania dotyczące zmian zachodzących w membranach wzbogacono analizami gospodarki hormonalnej oraz aktywności fotosyntetycznej roślin w czasie aklimacji w 5°C i 27°C oraz starano się powiązać te zmiany z tolerancją, odpowiednio na mróz (-6°C i -8°C) i wysoką temperaturę (38°C i 45°C). Dotychczasowy szczegółowy stan wiedzy z tego zakresu odnoszący się do BR i ich aktywności jest przytoczony i skomentowany we wstępach oraz dyskusjach w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej.

3. Cel pracy

Celem pracy doktorskiej jest przybliżenie roli brasinosteroidów w kształtowaniu tolerancji roślin jęczmienia na mróz i wysoką temperaturę. W cyklu eksperymentów poszukiwano odpowiedzi na następujące pytania: (1) Czy u jęczmienia poddanego aklimacji w 5°C oraz 27°C deficyt brasinosteroidów i zaburzenia percepcji tych związków zmniejszają tolerancję, odpowiednio – na mróz i wysoką temperaturę (38°C i 45°C)? (2) Czy/jak deficyt brasinosteroidów i zaburzenia percepcji tych związków modyfikują gospodarkę hormonalną oraz wydajność fotosyntetyczną roślin w czasie procesu aklimacji w 5°C i 27°C? (3) Jak deficyt brasinosteroidów i zaburzenia percepcji tych związków wpływają na strukturę i funkcjonowanie membran komórkowych (skład lipidowy, właściwości fizyko-chemiczne membran oraz ekspresję białek błonowych: H^+ATPazy i akwaporyny HvPIP, a także białek szoku cieplnego (HSP90, HSP70, HSP18 i HSP17)) roślin poddanych aklimacji w 5°C oraz 27°C?

4. Materiał roślinny i schemat eksperymentu

Materiał badawczy stanowiły rośliny jęczmienia odmiany Bowman (odmiana referencyjna tzw. typ dziki) wraz z liniami bliskoizogenicznymi (NIL, ang. *Near-Isogenic Lines*) charakteryzującymi się zaburzeniami w biosyntezie BR, a także percepcji BR (rośliny z defektem receptora BR) (Ryc. 3):



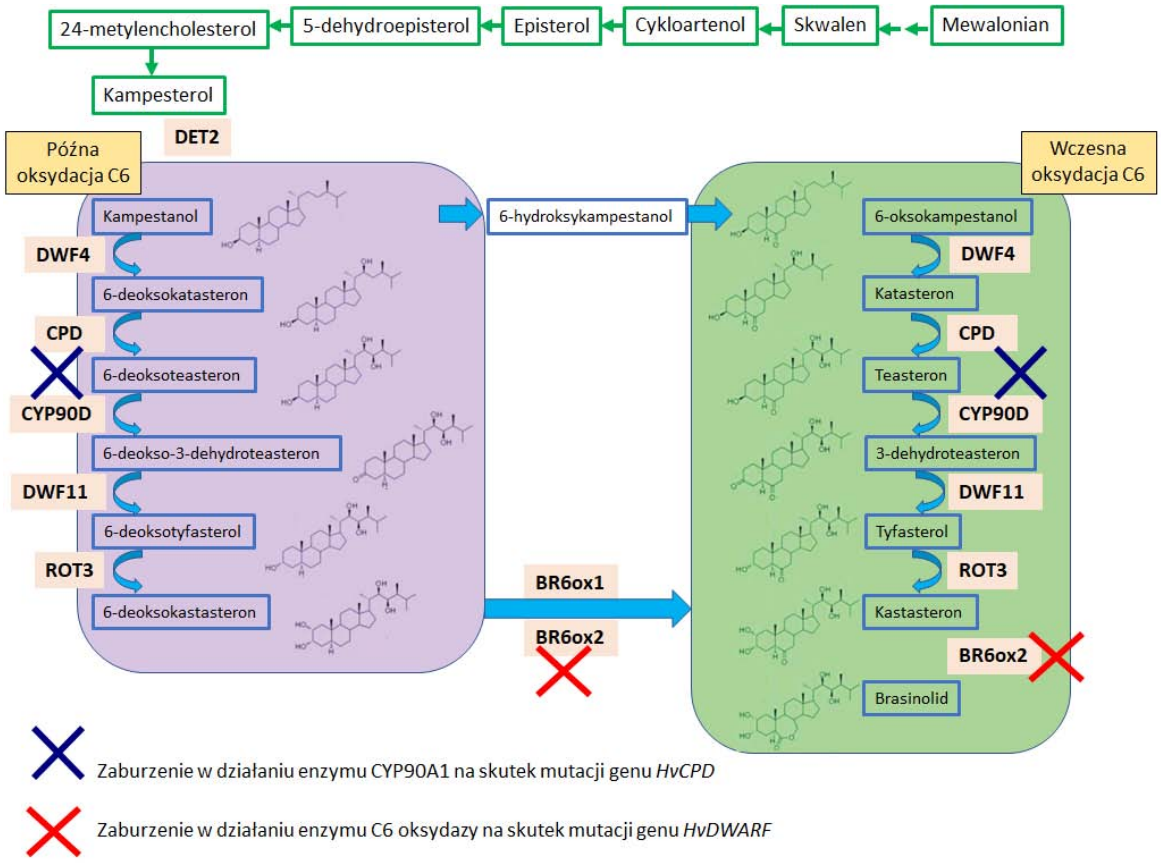
Ryc. 3. Odmiana jęczmienia Bowman i jej półkarłowe linie bliskoizogeniczne z zaburzoną biosyntezą BR (BW084) i z zaburzoną percepcją BR (BW312).

- BW084 - linia z zaburzoną biosyntezą BR. Zaburzenia te występują na wczesnym etapie szlaku biosyntezy BR i wynikają z wadliwego działania enzymu CYP90A1. Enzym ten uczestniczy w szlaku biosyntezy BR w przemianach tak wczesnych intermediatów jak 6-deoksoteasteron i 6-deokso-3-dehydroteasteron (Chung i Choe 2013). Wadliwe działanie enzymu CYP90A1 wynika z mutacji genu *HvCPD*. Liście roślin charakteryzują się znacznie zredukowaną ilością BR przede wszystkim z grupy C_{28} (kastasteron, brasinolid) oraz zmniejszoną ilością BR z grupy C_{29} (28-homokastasteron). Szczegółowe informacje na temat mutacji oraz szerszą charakterystykę profilu BR linii BW084 można znaleźć w pracy Dockter i wsp. (2014) oraz **Sadura i wsp. (2019) – Publikacja B**.

- BW312 – linia z zaburzoną percepcją BR. Na skutek mutacji *HvBRI1* w kinazie receptora wiążącego BR – BRI1, dochodzi do dysfunkcji domeny receptora wiążącej steroidy i blokowane jest wiązanie BR. Rośliny akumulują zwiększone ilości BR z grupy C_{28} (kastasteron, brasinolid) (Dockter i wsp. 2014; **Sadura i wsp. 2019 – Publikacja B**), spośród których brasinolid jest uważany za główny ligand receptora BRI1.

Rośliny linii BW084 i BW312 charakteryzują się niższym wzrostem (są półkarłowe) w porównaniu do roślin odmiany Bowman (Ryc. 3).

W publikacjach składających się na rozprawę doktorską jako obiekt badawczy występuje także odmiana Delisa i jej mutant 522DK uzyskany na drodze mutagenezy chemicznej. 522DK jest mutantem z zaburzoną biosyntezą BR. W odróżnieniu od BW084 zaburzenia występują na późnym etapie szlaku biosyntezy BR i wynikają z mutacji genu *HvDWARF*, który koduje enzym C6-oksydazę. Enzym ten jest odpowiedzialny m.in. za końcowe przemiany z kastasteronu do brasinolidu w szlaku biosyntezy BR. Mutant charakteryzuje się nieco niższą zawartością BR, niż jego odmiana referencyjna (Gruszka i wsp. 2011; **Sadura i wsp. 2019 – Publikacja B**). Jednak w związku ze sposobem uzyskania tego mutanta



Ryc. 4. Schemat biosyntezy BR przedstawiający lokalizację zaburzeń przemian poszczególnych intermediatów w związku z mutacjami genów *HvCPD* i *HvDWARF* kodujących enzymy – odpowiednio CYP90A1 (X) i C6-oksydazę (X) (Gruszka i Małuszyński 2010; Chung i Choe 2013). DWF4 – hydroksylaza C-22α kodowana przez gen *DWF4* (*DWARF4*); CPD – hydroksylaza C-23 kodowana przez gen *CPD* (ang. *CONSTITUTIVE MORFOGENESIS AND DWARFISM*); CYP90D – oksydaza C-3, kodowana przez gen CYP90D (ang. *CYTOCHROME P450 90D*); DWF11 - enzym CYP724B1 kodowany przez gen *DWF11* (*DWARF11*); ROT3 – hydroksylaza C-2α kodowana przez gen *ROT3* (*ROTUNDIFOLIA3*); BR6ox1 i BR6ox2 – oksydazy C-6 kodowane przez geny *BR6ox1* (*BR-6-oxidase1*) i *BR6ox2* (*BR-6-oxidase2*) (Gruszka i Małuszyński 2010).

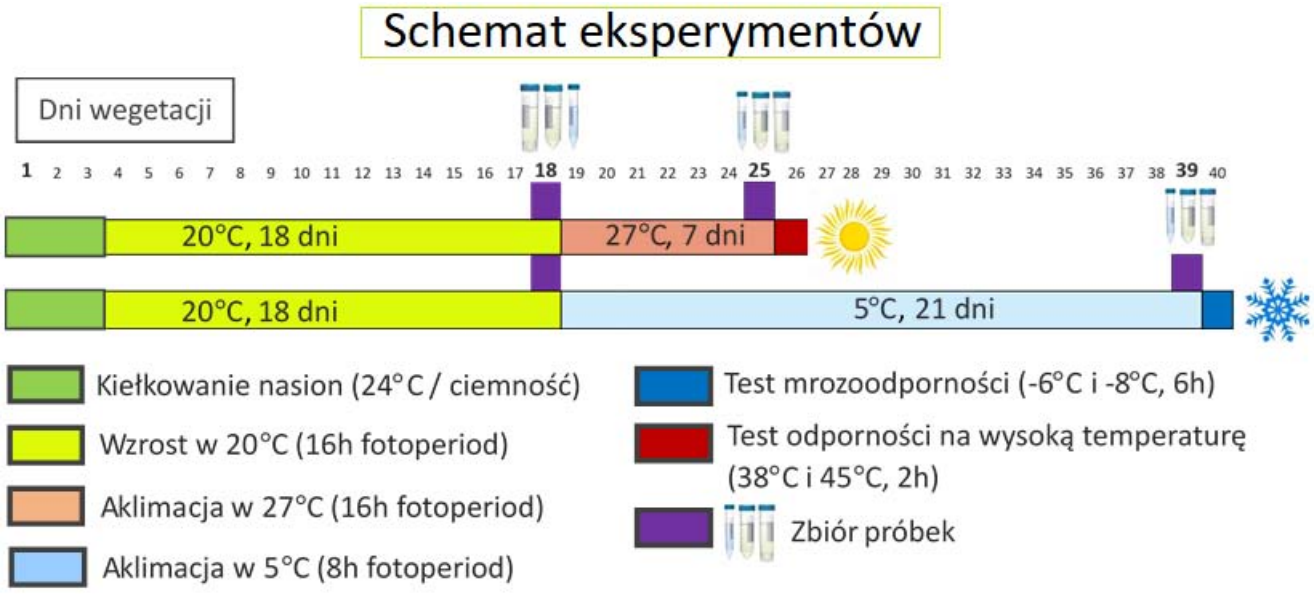
(niespecyficzna mutageneza chemiczna), jego tło genetyczne może być bogate w dodatkowe mutacje, które mogą mieć przełożenie na przebieg/intensywność procesów fizjologicznych. Z tego względu do wyników uzyskanych dla mutanta 522DK, należy się odnosić z pewną

ostrożnością i będą one w mniejszym stopniu dyskutowane w niniejszej rozprawie (zwłaszcza w kontekście ekspresji białek).

Na Rycinie 4 zaprezentowano schemat biosyntezy BR przedstawiający lokalizację zaburzeń wywołanych omawianymi wyżej mutacjami genów *HvCPD* i *HvDWARF*.

Bardziej szczegółową genetyczną charakterystykę badanych roślin z zaburzeniami w biosyntezie i percepcji BR, przedstawiono w publikacji Gruszka i wsp. 2011, a także w publikacjach wchodzących w skład niniejszej rozprawy doktorskiej: **Sadura i wsp. (2019) – Publikacja B; Sadura i wsp. (2020a) – Publikacja C; Sadura i wsp. (2020b) – Publikacja D; Rudolphi-Szydło i wsp. (2020) – Publikacja E; Sadura i wsp. (2021) – Publikacja F.**

Poniżej przedstawiono ogólny model eksperymentu, który obowiązywał w publikacjach wchodzących w skład rozprawy doktorskiej. W skrócie rośliny po wzroście w 20°C dzielono na dwie grupy, z których jedną poddano aklimacji w 5°C, a drugą w 27°C. Próbki zbierano z roślin rosnących w 20°C, a następnie w ostatnim dniu aklimacji (a na potrzeby niektórych analiz dodatkowo w połowie czasu aklimacji) (**Sadura i wsp. (2019) – Publikacja B; Sadura i wsp. (2020a) – Publikacja C; Sadura i wsp. (2020b) – Publikacja D; Rudolphi-Szydło i wsp. (2020) – Publikacja E; Sadura i wsp. (2021) – Publikacja F)**. W przypadku doświadczenia opisanego w publikacji **Sadura i wsp. (2019) - Publikacja B** rośliny po aklimacji w 5°C poddano działaniu stresu mrozu (-6°C i -8°C), a rośliny po aklimacji w 27°C poddano działaniu wysokiej temperatury (38°C i 45°C).



5. Metody badawcze

Wszystkie metody analityczne, pomiary i obserwacje omówiono szczegółowo w poszczególnych publikacjach składających się na rozprawę doktorską. Poniżej przedstawiony jest jedynie spis stosowanych metod i analiz:

	Metoda	Analizy	Publikacja
1.	Wysokosprawna chromatografia cieczowa sprzężona ze spektrometrią masową	(1) analiza zawartości hormonów z grupy auksyn, cytokinin, giberelin, a także kwasu abscysynowego i kwasu salicylowego w materiale roślinnym (UHPLC-MS) (2) analiza zawartości hormonów z grupy BR w materiale roślinnym (UHPLC-MS/MS)	**Sadura i wsp. (2019) – Publikacja B;** **Sadura i wsp. (2021) – Publikacja F**
2.	Wirowania różnicowe (ultrawirówka Beckman L-30)	Izolacja frakcji membran komórkowych z roślin jęczmienia	**Sadura i wsp. (2020a) – Publikacja C;** **Sadura i wsp. (2020b) – Publikacja D**
3.	(1) Real-Time PCR (2) Western blot i Immunoblot	(1) analiza akumulacji transkryptów (*HSP – HSP90, HSP70, HSP17, HSP18;* ATPazy – *ATPase1*; akwaporyny – *HvPIP1;1*) (2) ilościowa analiza akumulacji białek (HSP – HSP90, HSP70, HSP17, HSP18; ATPazy – H^+-ATPaza; akwaporyny – PIP1)	**Sadura i wsp. (2020a) – Publikacja C;** **Sadura i wsp. (2020b) – Publikacja D**
4.	Chromatografia gazowa	Analiza zawartości kwasów tłuszczowych	**Rudolphi-Szydło i wsp. (2020) – Publikacja E**
5.	Waga powierzchniowa Langmuira	Analiza właściwości fizyko-chemicznych membran (monowarstw lipidowych)	**Rudolphi-Szydło i wsp. (2020) – Publikacja E**
6.	Elektronowy Rezonans Paramagnetyczny	Analiza dynamiki molekularnej membran chloroplastów	**Sadura i wsp. (2021) – Publikacja F**
7.	Konduktometria oraz metody fluorescencyjne wzbogacone przez obserwacje wizualne w celu charakterystyki uszkodzeń liści	Ocena przeżywalności roślin w wysokich i niskich temperaturach	**Sadura i wsp. (2019) – Publikacja B**

6. Ważniejsze wyniki

6.1. Charakterystyka tolerancji jęczmienia na mróz i wysoką temperaturę po aklimacji roślin w 5°C i 27°C – PUBLIKACJA B

(1) Rośliny odmiany Bowman, linii BW084, BW312, a także odmiany Delisa i jej mutanta 522DK po aklimacji w 5°C charakteryzują się zbliżoną, wysoką przeżywalnością w temperaturze -6°C (8.5 – 9.0 pkt. w 9 pkt. skali Larsena; **Sadura i wsp. 2019 – Publikacja B**). W temperaturze -8°C odmiana Bowman utrzymuje dość wysoką przeżywalność (7.5 pkt.), natomiast odmiana Delisa jest znacznie mniej tolerancyjna na mróz (3 pkt.). W przypadku odmiany o wyższej tolerancji na mróz (Bowman) deficyt BR, czy zaburzenia percepcji BR występujące u linii BW084 i BW312 wiążą się z obniżoną przeżywalnością w mrozie (do ok. 4.5 – 6.0 pkt.; **Sadura i wsp. 2019 – Publikacja B**). W przypadku wrażliwej na mróz odmiany Delisa, deficyt BR nie ma wpływu na dalsze obniżenie tolerancji roślin na mróz.

(2) Po aklimacji w 27°C wszystkie badane rośliny z deficytem BR lub zaburzeniami w ich percepcji charakteryzują się lepszą tolerancją na stres wysokiej temperatury (38°C i 45°C), niż ich odmiany referencyjne Bowman i Delisa (**Sadura i wsp. 2019 – Publikacja B**).

6.2. Występowanie brasinosteroidów w tkance liściowej i izolowanych chloroplastach jęczmienia oraz zmiany poziomu brasinosteroidów podczas aklimacji (5°C i 27°C) – PUBLIKACJA B i F

(1) **W liściach jęczmienia oznaczono trzy BR: brasinolid** (obecny tylko w roślinach z 20°C) **oraz kastasteron i homokastasteron** obecne w tkance niezależnie od temperatury wzrostu/aklimacji roślin (**Sadura i wsp. 2019 – Publikacja B**). **W chloroplastach izolowanych z liści stwierdzono osiem BR: brasinolid, kastasteron, homokastasteron, 28-norkastasteron, 24-epibrasinolid, dolicholid, homodolicholid i homodolichosteron** (**Sadura i wsp. 2021 – Publikacja F**). Zarówno w liściach jak i w chloroplastach **homokastasteron i kastasteron występowały w największej ilości, a zatem u jęczmienia stanowią one dominujące BR.** Wykrycie większej liczby BR w chloroplastach, niż w liściach prawdopodobnie było możliwe z racji użycia znacznie większej ilości materiału liściowego potrzebnego do uzyskania chloroplastów, w porównaniu do ilości tkanki wykorzystanej do analiz zawartości BR w liściach. Warto podkreślić, że umożliwiło to stwierdzenie występowania **w chloroplastach BR z rzadziej spotykanych szlaków biosyntezy** C_{29} (homodolichosteron → homodolicholid oraz dolicholid), a także C_{27} (28-norkastasteron).

(2) W liściach mutanta 522DK potwierdzono występowanie mniejszej ilości BR ze szlaku C_{28} (kastasteron → brasinolid), w porównaniu do odmiany Delisa (Ryc. 5B; **Sadura i wsp. 2019 – Publikacja B**), jednak w chloroplastach ta prawidłowość nie występowała, a zawartość kastasteronu u odmiany Delisa i mutanta była porównywalna (Ryc. 5C; **Sadura i wsp. 2021 – Publikacja F**). Można zatem przypuszczać, że **w chloroplastach występują niepoznane mechanizmy akumulacji BR, pozwalające na utrzymanie ich niezbędnego poziomu niezależnie od poziomu ogólnego w tkance (i zaburzeń biosyntezy).** W tym miejscu warto dodatkowo podkreślić, że w porównaniu do odmiany Delisa, mutant 522DK akumulował wyraźnie podwyższone ilości dolicholidu w chloroplastach niezależnie od temperatury wzrostu roślin (**Sadura i wsp. 2021 – Publikacja F**). **Występowanie BR w chloroplastach w pewien sposób potwierdza możliwość pełnienia przez te związki funkcji fizjologicznych, takich jak np. regulacja transkrypcji genów chloroplastowych, co sugerowały wcześniejsze prace** (Efimova i wsp. 2012). Zagadnienie regulacyjnej roli BR w procesie fotosyntezy, w aspekcie tolerancji roślin na wysoką i niską temperaturę, omówiono na podstawie literatury w publikacji przeglądowej (**Sadura i Janeczko 2018 – Publikacja A**).

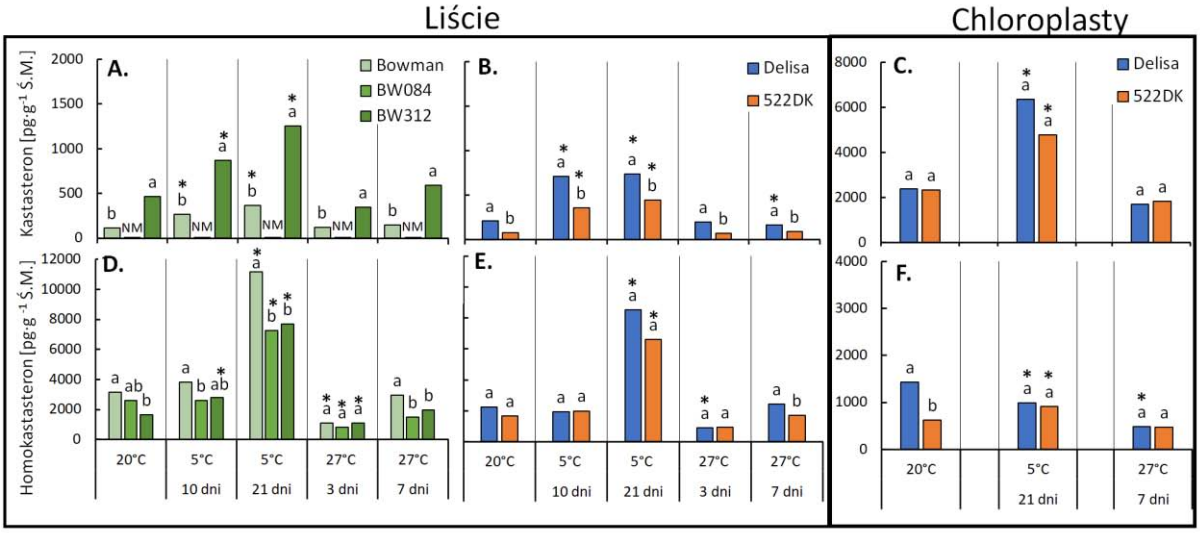
(3) W liściach linii BW312 (z dysfunkcją receptora BRI1) potwierdzono charakterystyczną dla tego typu mutacji zwiększoną, w porównaniu do odmiany referencyjnej, akumulację BR z grupy C_{28} takich jak kastasteron – prekursor brasinolidu oraz stanowiący główny ligand BRI1, sam brasinolid, który jednak występuje w granicach detekcji jedynie w 20°C (**Sadura i wsp. 2019 – Publikacja B**). Prawidłowość ta nie dotyczyła jednak homokastasteronu (obecnego w mniejszej ilości u linii BW312, niż u odmiany Bowman). Nie można wykluczyć, że w komórkach BW312 homokastatasteron był przekształcany w kastasteron (stąd też niższy poziom homokastasteronu u BW312, w porównaniu do odmiany referencyjnej). Wobec dysfunkcji receptora jest bowiem prawdopodobne, że na zasadzie sprzężenia zwrotnego w komórce syntetyzowane były duże ilości kastasteronu (przekształcanego następnie w akumulowany w dużej ilości w 20°C brasinolid), a do tego celu zużywane były także szkielety węglowe z innych BR, w tym przypadku homokastasteronu. Takie przemiany BR opisano wcześniej u ryżu (Joo i wsp. 2015). **Jednocześnie fakt, że BW312 nie akumuluje zwiększonych ilości homokastasteronu sugeruje, że hormon ten może być ligandem innego białka wiążącego BR niż receptor BRI1.** Byłoby to zgodne z wcześniejszymi badaniami Xu i wsp. (1994), w których stwierdzono wiązanie różnych BR zarówno we frakcjach membranowych jak i cytoplazmatycznych, a zarazem możliwość występowania różnych białek wiążących BR.

Z kolei obniżony względem odmiany referencyjnej poziom homokastasteronu u linii BW084 z deficytem BR z grupy C_{28} oraz podobna tendencja u 522DK prawdopodobnie mogą wynikać z faktu, że w szlaku biosyntezy homokastasteronu biorą udział te same enzymy, co w przypadku szlaku biosyntezy kastasteronu. Pozwala to przypuszczać, że zaburzenia w działaniu enzymów CYP90A1 oraz C6-oksydazy wywołane mutacją genów *HvCPD* i *HvDWARF* mogą się przyczyniać nie tylko do spadku zawartości kastasteronu, ale także do spadku zawartości homokastasteronu odpowiednio u linii BW084 oraz mutanta 522DK, w porównaniu do odmian referencyjnych.

(4) **Aklimacja roślin obu odmian Bowman i Delisa w 5°C powodowała wzrost akumulacji BR w liściach – w przypadku kastasteronu bardziej stopniowy, widoczny już od 10 dnia działania chłodu (Ryc. 5A, B), a w przypadku homokastasteronu bardziej skokowy (i widoczny dopiero po 21 dniach aklimacji) (Ryc. 5D, E)** (**Sadura i wsp. 2019 – Publikacja B**). Podobną tendencję w odniesieniu do kastasteronu zaobserwowano także u mutanta 522DK i linii BW312 (Ryc. 5A, B) oraz w odniesieniu do homokastasteronu (522DK, linia BW084 i BW312) (Ryc. 5D, E). U wszystkich roślin rosnących w chłodzie nie stwierdzono występowania brasinolidu. Warto także podkreślić, że podczas gdy w liściach mutanta 522DK zaobserwowano wyraźnie większą zawartość homokastasteronu (nawet ok. 7 000 $pg \cdot g^{-1}$ ś.m. po aklimacji w 5°C) niż kastasteronu (ok. 500 $pg \cdot g^{-1}$ ś.m. po aklimacji w 5°C) (Ryc. 5B, E), w chloroplastach zaobserwowano odwrotną tendencję, tj. zawartość kastasteronu znacznie wzrastała (ok. 5 000 $pg \cdot g^{-1}$ ś.m. po aklimacji w 5°C) i przewyższała zawartość homokastasteronu (ok. 1 000 $pg \cdot g^{-1}$ ś.m. po aklimacji w 5°C) (Ryc. 5C, F) tak jak i pozostałych wykrytych BR (**Sadura i wsp. 2021 – Publikacja F**). Można zatem podejrzewać, że większa akumulacja kastasteronu w chloroplastach jest bardziej pożądana i może się wiązać z funkcją fizjologiczną, jaką ten BR pełni w tych organellach w czasie aklimacji w chłodzie, jednak jej poznanie wymaga dalszych badań.

(5) Generalnie **zmiany zawartości BR w wyniku aklimacji w 27°C były znacznie mniej intensywne niż te obserwowane w 5°C, co szczególnie dotyczy tkanki liści.** Aklimacja w 27°C nie powodowała znaczących zmian w zawartości kastasteronu w liściach jęczmienia. Wyjątek stanowiły rośliny odmiany Delisa (Ryc. 5B), dla których zaobserwowano spadek akumulacji tego BR w porównaniu do roślin z 20°C (**Sadura i wsp. 2019 – Publikacja B**). W przypadku homokastasteronu, 3-dniowa aklimacja w 27°C przyczyniła się do przejściowego spadku jego zawartości u obu odmian (Delisa i Bowman), ale po 7 dniach aklimacji nastąpiło wyrównanie jego zawartości do poziomu notowanego u roślin z 20°C

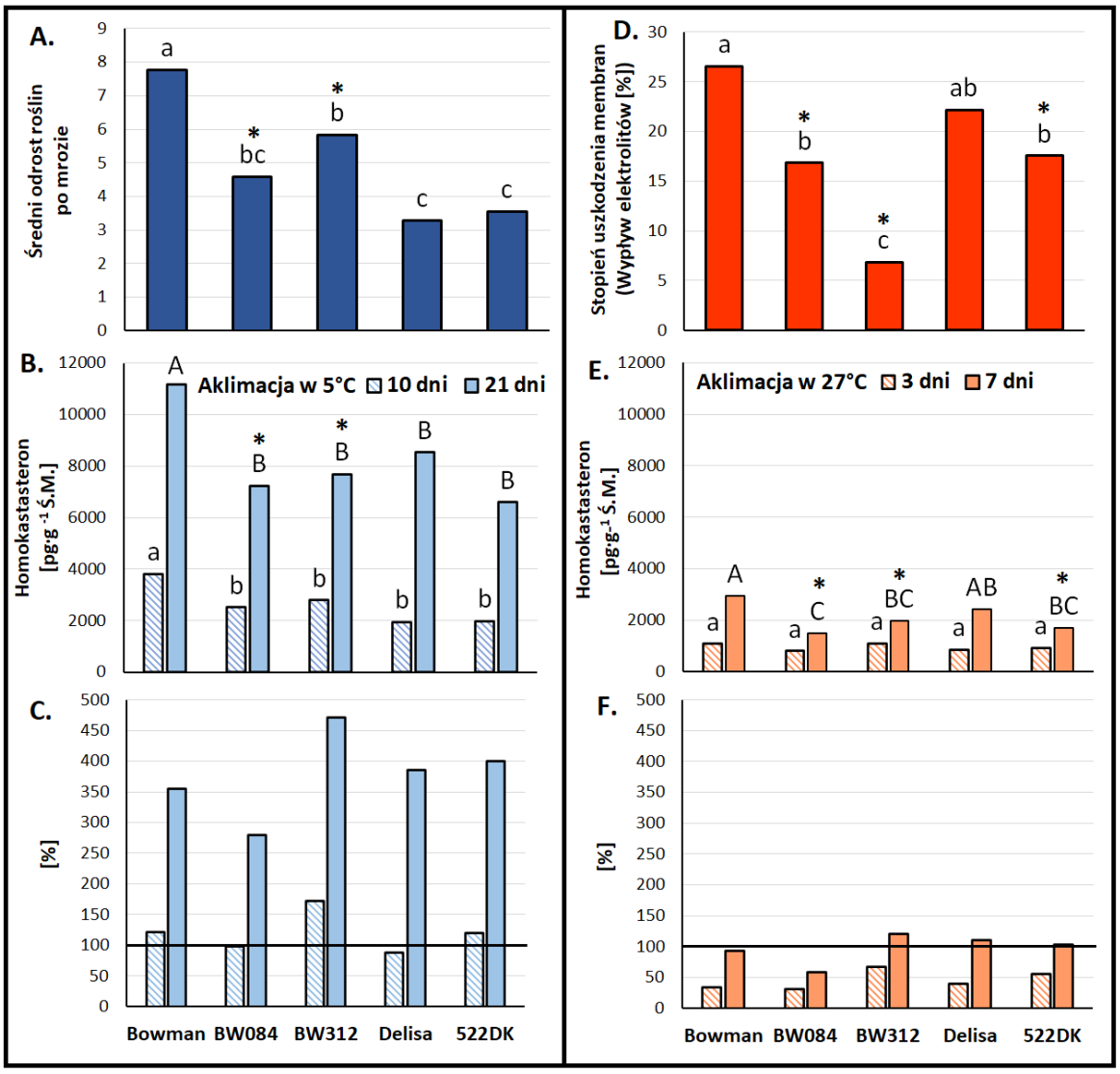
(Ryc. 5D, E). Podobne zjawisko zaobserwowano u obu linii bliskoizogenicznych (Ryc. 5D). Mutant 522DK poddany aklimacji w 27°C nie różnił się zawartością obu BR względem roślin z 20°C. W chloroplastach akumulacja BR pozostawała na zbliżonym poziomie dla roślin z 20°C i 27°C lub też spadała (homokastasteron, 24-epibrasinolid i dolicholid u odmiany Delisa; homodolicholid u mutanta 522DK) (**Sadura i wsp. 2021 – Publikacja F**).



Ryc. 5. Zawartość kastasteronu i homokastasteronu u jęczmienia odmiany Bowman i jej linii bliskoizogenicznych BW084 i BW312 (A, D) oraz odmiany Delisa i jej mutanta 522DK (B, C, E, F) rosnących w 20°C i poddanych aklimacji w 5°C i 27°C. *Istotne statystycznie różnice między odmianami i ich liniami/mutantem w obrębie poszczególnych temperatur są zaznaczone różnymi literami (dla odmiany Bowman i jej linii zastosowano test Duncan'a, p≤ 0,05; dla odmiany Delisa i jej mutanta wykorzystano test t'Studenta, p≤ 0,05)). W celu wykazania różnic wywołanych wpływem temperatury na zawartość kastasteronu i homokastasteronu u badanych odmian, linii i mutanta, dokonano porównania w parach testem t'Studenta, p≤ 0,05 (za temperaturę odniesienia przyjęto 20°C) według przykładu: Bowman w 20°C i 5°C (10 dni); BW084 w 20°C i 5°C (10 dni); BW312 w 20°C i 5°C (10 dni); itd. Różnice istotne statystycznie oznaczono gwiazdką „*". NM – wartości nie mierzalne.*

(6) Analizując **tolerancję jęczmienia na mróz** (po aklimacji w 5°C i dla temperatury różnicującej (-8°C)) obie odmiany, linie BW084 i BW312 oraz mutant 522DK można uszeregować od najbardziej do najmniej tolerancyjnej następująco: Bowman, BW312, BW084, Delisa oraz 522DK. Odmiana Bowman zawierająca **w liściach najwięcej homokastasteronu, była zarazem najbardziej tolerancyjna na mróz** (Ryc. 6A, B). Jej linie BW zawierające mniej homokastasteronu były od niej mniej tolerancyjne. Odmiana Delisa i mutant 522DK były najmniej tolerancyjne i zawierały najmniej homokastasteronu (Ryc. 6A, B). Zważywszy, że wcześniejsze badania na pszenicy ozimej (Janeczko i wsp. 2019) wykazały związek pomiędzy poziomem homokastasteronu a poziomem tolerancji odmian na mróz (odmiany tolerancyjne na mróz akumulowały więcej tego związku niż odmiany wrażliwe), można sądzić, że **homokastasteron może być szczególnie istotny w aklimacji**

chłodowej oraz w kształtowaniu tolerancji mrozowej roślin. Warto odnotować także zjawisko odwrotne – u roślin linii BW084 i BW312 oraz 522DK poddanych aklimacji w 27°C



Ryc. 6. Tolerancja roślin jęczmienia (odmiany Bowman i jej linii bliskoizogenicznych BW084 i BW312 oraz odmiany Delisa i jej mutanta 522DK) na mróz i wysoką temperaturę a poziom homokastasteronu po aklimacji w 5°C i 27°C. (A) Odrost roślin po działaniu mrozu (-8°C) według 9-stopniowej skali Larsena; (D) stopień uszkodzenia membran (wypływ elektrolitów) u roślin poddanych działaniu stresu wysokiej temperatury (45°C). Zawartość homokastasteronu u badanych roślin poddanych aklimacji w 5°C trwającej 10 i 21 dni (B) oraz aklimacji w 27°C trwającej 3 i 7 dni (E). Procentowe zmiany zawartości homokastasteronu u roślin jęczmienia poddanych aklimacji w 5°C (C) i 27°C (F) w odniesieniu do 20°C (wyniki uzyskane dla roślin rosnących w 20°C przyjęto za 100%). *Różnice statystyczne pomiędzy wszystkimi 5 obiektami (odmiany, linie, mutant) wykazano za pomocą testu Duncana ($p \leq 0.05$). Obiekty oznaczone tymi samymi literami nie różnią się istotnie. W przypadku rycin A i D (tolerancja na mróz i wysoką temperaturę) różnice oznaczono małymi literami. W przypadku rycin B,C, E,F (analizy zawartości homokastasteronu) różnice statystyczne przedstawiono osobno dla krótszego czasu trwania aklimacji (małe litery) i osobno dla dłuższego czasu aklimacji (wielkie litery). Dodatkowo gwiazdką oznaczono różnice w parach: Bowman vs BW084, Bowman vs BW312 i Delisa vs 522DK.*

niższemu poziomowi homokastasteronu (w porównaniu do odmian referencyjnych) **towarzyszyła jednocześnie wyższa tolerancja na wysoką temperaturę** (Ryc. 6D, E). Procentowe zmiany zawartości homokastasteronu w roślinach po aklimacji w 5°C i 27°C względem roślin rosnących w 20°C (wartość przyjęta za 100%) przedstawiono na Rycinie 6C, F. Chociaż mechanizm działania homokastasteronu w aspekcie tolerancji na stres temperaturowy będzie przedmiotem dalszych badań, to obecnie można przykładowo założyć, że związek ten modyfikuje własności membran komórkowych, a jego większa w nich akumulacja powoduje zwiększenie ich płynności (oraz większą tolerancję na niską temperaturę). Mniejsza akumulacja homokastasteronu w membranach wywoływałaby efekt odwrotny. Jednocześnie nie można wykluczyć, że związek ten moduluje ekspresję genów odpowiedzialnych za przystosowanie roślin do różnych warunków termicznych lub też wywołuje efekty tzw. niegenomowe (dobrze poznane w przypadku hormonów steroidowych ssaków) związane np. z regulacją przepuszczalności membran komórkowych.

6.3. Gospodarka hormonalna jęczmienia poddanego aklimacji (5°C, 27°C) a zaburzenia biosyntezy i percepcji brasinosteroidów – PUBLIKACJA B

W badanych roślinach jęczmienia zidentyfikowano auksyny (IAA – kwas indolilooctowy, IBA – kwas indolilo-3-masłowy, oxIAA – kwas 2-oksindolo-3-octowy oraz IAAMe – ester metylowy kwasu indolilooctowego), cytokininy (t-Z – trans-zeatyna, c-Z – cis-zeatyna, DHZ – dihydrozeatyna, t-ZOG – O-glukozyd trans-zeatyny), gibereliny (GA_1, GA_3, GA_4, GA_5, GA_6) oraz kwas abscysynowy (ABA) i kwas salicylowy (SA) (**Sadura i wsp. 2019 – Publikacja B**). **Zaburzenia biosyntezy BR lub dysfunkcja receptora BRI1 skutkowały zmianami zawartości wielu spośród badanych fitohormonów, co świadczy o udziale BR w skomplikowanej sieci połączeń hormonalnych i powiązaniu BR ze szlakami biosyntezy/sygnalingu auksyn, cytokinin, giberelin, ABA i SA.** Wyniki syntetycznie ujęto w Tabeli 1. Na intensywność zmian hormonalnych w liniach bliskoizogenicznych z mutacjami *HvCPD* i *HvBRI1* miała wpływ dodatkowo temperatura wzrostu/aklimacji roślin. Najwięcej zmian hormonalnych występowało u roślin z 20°C, a także z 5°C. **W liniach bliskoizogenicznych mutacji *HvCPD* i *HvBRI1* towarzyszył spadek zawartości cytokinin i wzrost poziomu giberelin GA_3, GA_4 i GA_5 (spadek GA_6).** Na uwagę w aspekcie tolerancji na mróz zasługuje w szczególności podwyższony poziom niektórych giberelin, uważa się bowiem, że jest to zjawisko raczej niekorzystne dla tolerancji mrozowej i kojarzone bardziej z indukcją rozwoju generatywnego. Jeśli chodzi o pozostałe fitohormony to słabszy efekt obserwowano w przypadku auksyn, a zmiany dotyczyły jedynie prekursora IAAMe oraz mało

aktywnej formy degradacji auksyn – oxIAA. Jednak w tym przypadku podobne zmiany w odniesieniu do tych dwóch związków potwierdzono także u roślin mutanta 522DK z mutacją *HvDWARF* (**Sadura i wsp. 2019 – Publikacja B**). Warto podkreślić także **obniżenie zawartości ABA (hormonu stresu) u roślin obu linii – BW084 i BW312 rosnących w 5°C. Obok zwiększonego poziomu niektórych giberelin, może to bowiem być jedną z przyczyn obniżonej tolerancji na mróz tych linii w stosunku do odmiany Bowman.** W przypadku aklimacji w 27°C, gospodarka hormonalna linii BW084 i BW312 była w zasadzie niezmieniona na tle odmiany referencyjnej (poza spadkiem poziomu GA_6). Eliminuje to możliwość tłumaczenia podwyższonej tolerancji obu linii na wysoką temperaturę zmianami w gospodarce hormonalnej.

Tabela 1. Zmiany w zawartości poszczególnych fitohormonów u jęczmienia z mutacjami powodującymi zaburzenia biosyntezy BR (mutacja *HvCPD)* lub dysfunkcję receptora BR (mutacja *HvBRI1*) przedstawione w relacji do poziomu tych fitohormonów w odmianie referencyjnej Bowman. Kolorem zielonym oznaczono wzrost poziomu fitohormonu w porównaniu do odmiany referencyjnej, kolorem czerwonym spadek w porównaniu do odmiany referencyjnej, a brak zmian w porównaniu do odmiany referencyjnej kolorem niebieskim. *IAA – kwas indolilooctowy; oxIAA – kwas 2-oksindolo-3-octowy; IAAMe – ester metylowy kwasu indolilooctowego; IBA – kwas indolilo-3-masłowy; t-Z – trans-zeatyna; c-Z – cis-zeatyna; DHZ – dihydrozeatyna; t-ZOG – O-glukozyd trans-zeatyny; GA_1 – giberelina A_1; GA_3 – kwas giberelinowy; GA_4 – giberelina A_4; GA_5 – giberelina A_5; GA_6 – giberelina A_6; ABA – kwas abscysynowy; ABAGlc – ester glukozylowy kwasu abscysynowego; SA – kwas salicylowy.*

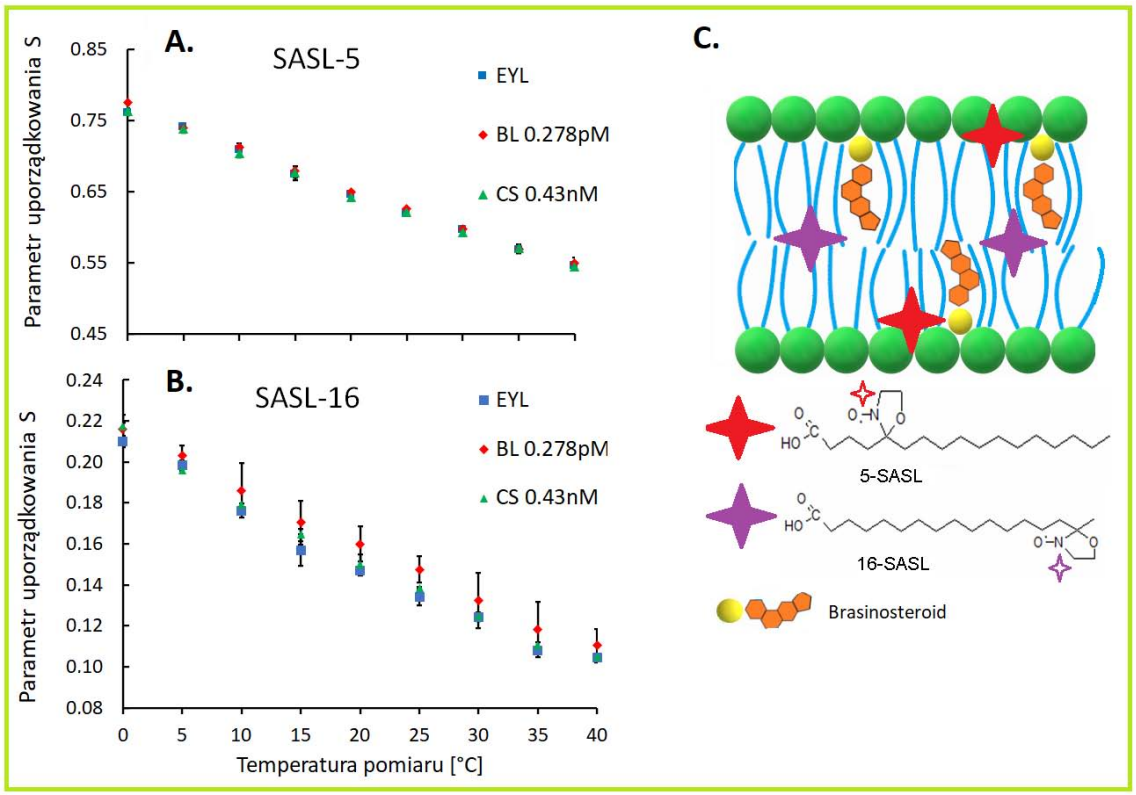
Fitohormon	Rodzaj mutacji					
	HvCPD			*HvBRI1*		
	20ºC	5ºC	27ºC	20ºC	5ºC	27ºC
Auksyny						
IAA						
oxIAA						
IAAMe						
IBA						
Cytokininy						
t-Z						
c-Z						
DHZ						
t-ZOG						
Giberelliny						
GA_1						
GA_3						
GA_4						
GA_5						
GA_6						
ABA						
ABAGlc						
SA						

6.4. Brasinosteroidy a zmiany w membranach komórkowych jęczmienia poddanego aklimacji w 5°C i 27°C

6.4.1. Wpływ deficytu i zaburzeń percepcji brasinosteroidów na skład lipidowy oraz właściwości fizykochemiczne membran – PUBLIKACJA E i F

Linie jęczmienia z deficytem BR oraz defektem receptora BRI1 charakteryzowały się zależnym od temperatury zmienionym procentem molowym kwasów tłuszczowych (KT, od 14:0 do 20:1) we frakcjach galaktolipidowych i fosfolipidowych izolowanych z membran komórkowych, w porównaniu do odmiany referencyjnej (**Rudolphi-Szydło i wsp. 2020 – Publikacja E**). Szczególnie dotyczy to KT 18:3n-3, który stanowi największy % udział całej puli KT (ok. 80% w galaktolipidach i 50% w fosfolipidach). U linii bliskoizogenicznych we frakcjach galaktolipidów % udział tego KT po aklimacji roślin w 5°C był podwyższony, a w przypadku 27°C obniżony, zwłaszcza u BW312. Odwrotne zjawisko obserwowano we frakcji fosfolipidów. Jednocześnie u linii bliskoizogenicznych we frakcjach galaktolipidów % udział KT 16:0 po aklimacji roślin w 5°C był obniżony, a w przypadku 27°C podwyższony, w stosunku do odmiany Bowman. W przypadku frakcji fosfolipidów występowała odwrotna tendencja. Na tej podstawie można przypuszczać, że **BR uczestniczą w zależnej od temperatury regulacji biosyntezy KT lub też sterują procesami odpowiedzialnymi za transport/wbudowywanie KT w membrany,** jednak mechanizm tej regulacji wymaga dalszych badań. Zmiana proporcji KT w badanych frakcjach lipidowych galaktolipidów i fosfolipidów znajduje odzwierciedlenie w płynności membran (wyrażonych zmianami wartości parametru A_{lim} (pole powierzchni przypadające na cząsteczkę lipidu)) (**Rudolphi-Szydło i wsp. 2020 – Publikacja E**). Wykazana **niższa płynność membran (monowarstw zbudowanych z frakcji digalaktolipidów i fosfolipidów) obu linii BW084 i BW312 poddanych aklimacji w 5°C, w porównaniu do odmiany referencyjnej, może być jednym z czynników odpowiedzialnych za ich obniżoną tolerancję na mróz** (-8°C). Z drugiej strony **obniżony stopień płynności membran (monowarstw zbudowanych z frakcji mono- i digalaktolipidów) linii poddanych aklimacji w 27°C, może zostać powiązany ze zwiększoną ich tolerancją na wysoką temperaturę** (38°C i 45°C) na tle odmiany Bowman. Warto podkreślić, że obserwowane zmiany w parametrach opisujących właściwości fizykochemiczne membran u badanych linii pozwalają przypuszczać, że **poprzez modyfikację struktury, a co za tym idzie właściwości membran komórkowych, BR mogą wywierać także pośrednio wielokierunkowy wpływ na wiele procesów fizjologicznych zależnych od membran, w tym na ekspresję wielu genów.**

Metodą Elektronowego Rezonansu Paramagnetycznego (EPR, ang. *Electron Paramagnetic Resonance*) badano także wpływ BR na dynamikę molekularną membran chloroplastowych izolowanych z odmiany Delisa i mutanta 522DK (**Sadura i wsp. 2021 – Publikacja F**) oraz membran modelowych (z lecytyny; dane niepublikowane), weryfikując hipotezę, że BR oddziałują z membranami i je stabilizują. Do badań wybrano właściwy obiekt (mutant z deficytem BR oraz jego odmianę referencyjną), jednakże w trakcie prowadzenia eksperymentu ustalono, że w izolowanych chloroplastach (w odróżnieniu od liści) całkowita zawartość BR jest porównywalna zwłaszcza w temperaturze 5°C i 27°C. Należy to uznać za przyczynę stosunkowo niedużych różnic (dotyczących głównie roślin poddanych aklimacji w 5°C) w dynamice molekularnej membran chloroplastów pomiędzy Delisą i 522DK. Równolegle w przypadku membran modelowych (z lecytyny), w które wbudowywano BR (kastasteron i brasinolid) w stężeniach fizjologicznych oraz w stężeniach od nich wyższych, nie udało się udowodnić wpływu BR na dynamikę molekularną badanych membran (Ryc. 7). Zagadnienie oddziaływania BR na dynamikę molekularną membran będzie wymagało dalszych pogłębionych badań, prawdopodobnie z użyciem membran modelowych o składzie bardziej zbliżonym do naturalnych membran obecnych w komórkach roślinnych.



Ryc. 7. Zależność parametru uporządkowania S membran modelowych zbudowanych z lecytyny jaja kurzego (EYL – ang. *Egg Yolk Lecithin*) z wbudowanym znacznikiem SASL-5 (A) lub SASL-16 (B) oraz brasinosteroidami (BL – brasinolid; CS – kastasteron w przykładowych stężeniach odpowiadających stężeniom w chloroplastach), od temperatury w jakiej prowadzono pomiary widm metodą EPR. (C) Model przygotowywanej membrany

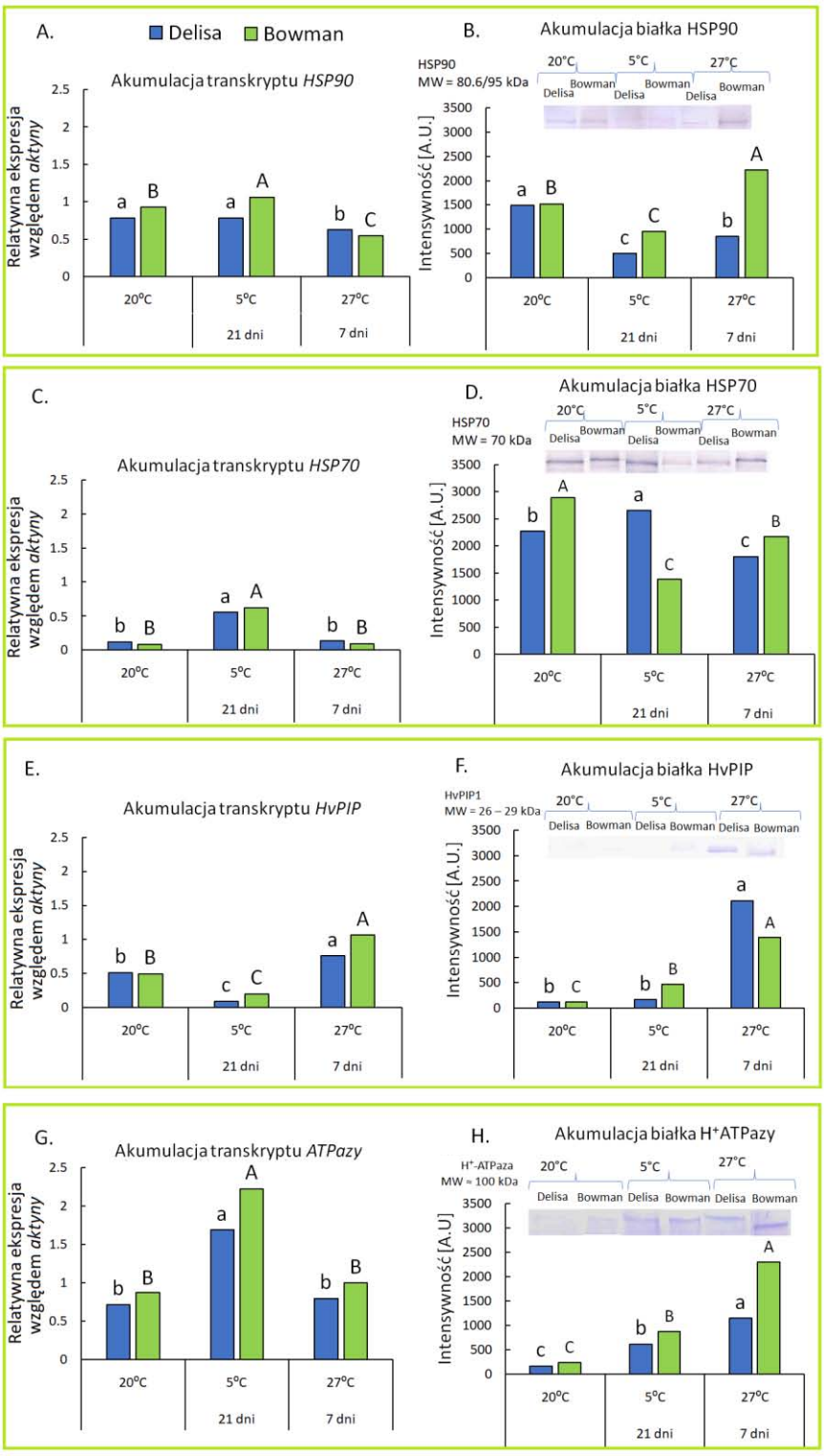
modelowej. Liposomy przygotowywano według zmodyfikowanej procedury opisanej we wcześniejszej pracy autorki (Sadura 2014). *W skrócie,* do *7.69µl EYL (10mM) dodawano 10µl znacznika spinowego (SASL-5 lub SASL-16) o stężeniu 0.1 mM oraz 100 µl brasinosteroidu (BL lub CS) o określonym stężeniu. Całość mieszano za pomocą vortexu przez 30 s, wirowano (quick run przy maksymalnych obrotach), następnie odparowywano metanol pod parami* N_2 *tak by na ściankach probówki powstał film, po czym do próbki dodawano 100 µl buforu CIB (pH 7.5) (50mM Tris-HCl, 5mM EDTA, 0.33mM sorbitol). Całość wytrząsano za pomocą vortexu przez minutę. Tak przygotowaną próbkę umieszczano w kapilarze i rozpoczynano pomiar widma EPR przy narastającej temperaturze od 0°C do 40°C (przyrost temperatury co 5°C). Pomiar prowadzono z wykorzystaniem spektrometru EPR (Miniscope, Niemcy) z kontrolerem temperatury (Magnettech, Niemcy). Otrzymane widma analizowano z wykorzystaniem programu Multiplot, otrzymując parametr uporządkowania membran S oraz korelacyjne czasy rotacji* τ_{2B} *i* τ_{2C} *(parametry mówiące o dynamice molekularnej membran, omówione także w publikacji* ***Sadura i wsp. (2021) – publikacja F****). Przedstawiona zależność pozwala wnioskować o dynamice molekularnej membran. Parametr S oblicza się wg wzorów przedstawionych w pracy* ***Sadura i wsp. (2021) – publikacja F****, odczytując odpowiednie wartości z widm EPR. Na wykresach przedstawiono wartości średnie z 2 pomiarów ± SD.*

6.4.2. Wpływ deficytu i zaburzeń percepcji brasinosteroidów na ekspresję wybranych białek

6.4.2.1. Białka szoku cieplnego – PUBLIKACJA C

(1) W roślinach odmian Delisa i Bowman temperatura wzrostu/aklimacji modyfikowała ekspresję HSP (**Sadura i wsp. 2020a – Publikacja C**). Mimo wyższego poziomu transkryptu *HSP90* po aklimacji w chłodzie, względem kontroli (20°C), szczególnie u odmiany Bowman, poziom białka HSP90 w membranach był zgodnie obniżony u obu odmian (Ryc. 8A, B). Także kilkukrotnie wyższy poziom transkryptu *HSP70* u obu odmian w chłodzie (w porównaniu do kontroli w 20°C) nie miał tak wyraźnego przełożenia na akumulację HSP70 w membranach i cytoplazmie (Ryc. 8C, D).

Aklimacja roślin w 27°C powodowała przejściowy wzrost poziomu transkryptów *HSP70* i *HSP90* u obu odmian w trzecim dniu aklimacji, a następnie (po 7 dniach aklimacji (Ryc. 8A, C)) ponowny ich spadek (*HSP70*) do poziomu kontroli (20°C) lub nawet poniżej poziomu kontroli (*HSP90*) (**Sadura i wsp. 2020a – Publikacja C**). W przypadku akumulacji białek HSP70 i HSP90, wyniki nieco bardziej zależały od odmiany (Ryc. 8B, D). Akumulacja HSP90 była po okresie aklimacji w 27°C podwyższona u odmiany Bowman, a obniżona u odmiany Delisa, w stosunku do kontroli 20°C (Ryc. 8B). Natomiast dla HSP70 u obu odmian zaobserwowano identyczne kierunki zmian akumulacji tego białka po aklimacji w 27°C: obniżenie we frakcji membranowej (Ryc. 8D), ale podwyższenie we frakcji cytoplazmatycznej (**Sadura i wsp. 2020a – Publikacja C**).



Ryc. 8. Zależny od temperatury model zmian w akumulacji transkryptów oraz akumulacji wybranych białek w membranach komórkowych jęczmienia odmiany Delisa i Bowman. (A) transkrypt *HSP90*, (B) białko HSP90, (C) transkrypt *HSP70*, (D) białko HSP70, (E) transkrypt *HvPIP*, (F) białko HvPIP, (G) transkrypt *ATPaza*, (H) białko H^+-ATPaza. *Istotne statystycznie zmiany wywołane wpływem temperatury osobno dla każdej odmiany oznaczono różnymi literami (odmiana Delisa – małe litery; odmiana Bowman – wielkie litery) (test Duncan'a, p≤0.05). MW – masa cząsteczkowa (ang. Molecular Weight).*

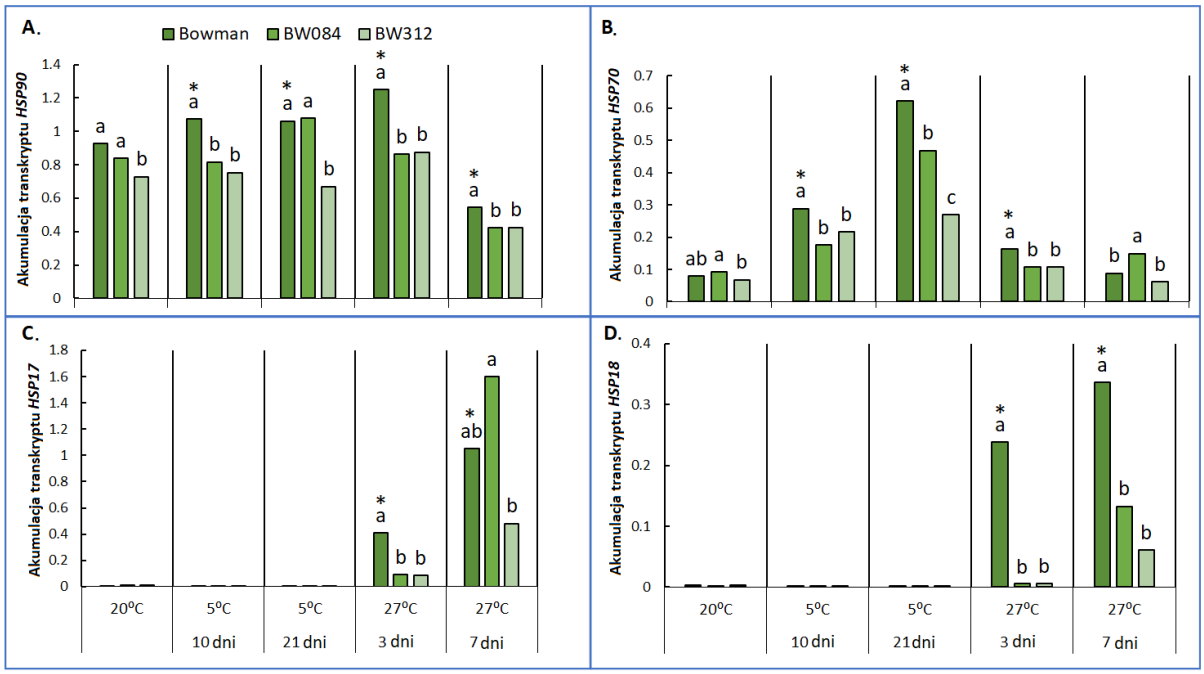
Aklimacja w 27°C przyczyniła się do bardzo silnego zwiększenia poziomu transkryptu niskocząsteczkowych białek szoku cieplnego (sHSP, ang. *small Heat Shock Proteins*): *HSP17* i *HSP18* u obu odmian i efekt ten był szczególnie widoczny po 7 dniach aklimacji. Białek sHSP dostępną metodą nie udało się oznaczyć.

Ze względu na to, iż zmiany w akumulacji transkryptu *HSP* (*HSP90, HSP70, HSP17 i HSP18*) w stosunkowo niedużym stopniu były zależne od odmiany, można przyjąć, że taki jest model zależnej od temperatury transkrypcji *HSP* u jęczmienia (Sadura i wsp. 2020a - Publikacja C).

(2) Analiza wyników otrzymanych dla mutanta 522DK i obu linii bliskoizogenicznych wykazała zmiany w opisanym dla odmian referencyjnych modelu ekspresji HSP w czasie aklimacji do niskiej i wysokiej temperatury. Deficyt BR (z nielicznymi wyjątkami) oraz defekt receptora BRI1 wiązały się najczęściej z obniżonym poziomem akumulacji transkryptów wszystkich *HSP* (Ryc. 9). Efekt ten był w nieznacznym stopniu modyfikowany odmianowo (niewielki wpływ tła genetycznego) i praktycznie nie zaobserwowano wpływu temperatury wzrostu/aklimacji roślin na to zjawisko. Może to wskazywać na **rolę BR jako pozytywnych regulatorów transkrypcji *HSP* u jęczmienia w szerokim zakresie temperatur.**

(3) Defekt receptora BRI1 wiązał się (poza jednym wyjątkiem) ze spadkiem akumulacji białek HSP70 i HSP90. Mogłoby to wskazywać na **udział BR także w regulacji samej biosyntezy białek HSP za pośrednictwem receptora BRI1** (i związanego z nim szlaku transdukcji sygnału). Jednak sam deficyt BR w tkankach nie zawsze i niejednoznacznie wiązał się ze zmianami w biosyntezie HSP – zaznaczał się tu wpływ tła genetycznego, typu mutacji (i związanego z nim poziomu BR w tkankach) oraz temperatury wzrostu/aklimacji roślin.

(4) Wyniki uzyskane w publikacji (**Sadura i wsp. 2020a – Publikacja C**), niewątpliwie wzbogacają wiedzę w aspekcie (1) kierunków zmian ekspresji HSP u jęczmienia w czasie aklimacji do różnych temperatur, (2) regulacji hormonalnej/roli BR w ekspresji HSP. Charakteryzując zmiany ekspresji HSP nie znaleziono jednak prawidłowości pozwalającej jednoznacznie powiązać opisane zmiany dotyczące akumulacji białek HSP z niższą tolerancją na mróz roślin z deficytem BR i defektem receptora BRI1, po aklimacji w 5°C oraz z wyższą tolerancją tych roślin na wysoką temperaturę, po aklimacji w 27°C.



Ryc. 9. Akumulacja transkryptów *HSP90* (A), *HSP70* (B), *HSP17* (C) i *HSP18* (D) u roślin jęczmienia odmiany Bowman i jej linii bliskoizogenicznych BW084 i BW312 rosnących w 20°C i poddanych aklimacji w 5°C i 27°C. *Istotne statystycznie różnice między odmianą a jej liniami w obrębie poszczególnych temperatur, są zaznaczone różnymi literami (test Duncan'a, p≤ 0,05). W przypadku transkryptów HSP17 i HSP18, w temperaturach 20°C i 5°C wykryto jedynie ich śladowe ilości. W celu wykazania różnic wywołanych wpływem temperatury na akumulację transkryptów HSP u odmiany Bowman, dokonano porównania w parach testem t'Studenta, p≤ 0,05 (dla 20°C i 5°C; 20°C i 27°C) i różnice oznaczono gwiazdką „*". Poziom akumulacji transkryptów określono w odniesieniu do genu referencyjnego aktyny.*

6.4.2.2. Akwaporyna (HvPIP) – PUBLIKACJA D

(1) Zarówno u odmiany Bowman jak i Delisa zaznaczył się wpływ temperatury na ekspresję akwaporyny HvPIP (**Sadura i wsp. 2020b – Publikacja D**). W wyniku aklimacji w 5°C zaobserwowano znaczący spadek akumulacji transkryptu *HvPIP*, w porównaniu do 20°C. Odwrotny efekt zaobserwowano w przypadku akumulacji białka HvPIP, którego poziom u roślin jęczmienia poddanych aklimacji w 5°C był wyższy, w porównaniu do 20°C (efekt statystycznie istotny u odmiany Bowman). Ogólnie jednak poziom białka w 20°C i 5°C był bardzo niski, a wzrastał kilkukrotnie dopiero po aklimacji w 27°C, co w tym przypadku korelowało także ze wzrostem akumulacji transkryptu *HvPIP* (Ryc. 8E, F).

(2) Na tle odmiany referencyjnej (Bowman) obie linie bliskoizogeniczne BW084 i BW312 charakteryzowały się zmienionym poziomem ekspresji HvPIP zarówno na poziomie akumulacji transkryptów jak i białek, jednak zjawisko to było zależne od temperatury wzrostu/aklimacji roślin (**Sadura i wsp. 2020b – Publikacja D**). U obu linii rosnących

w 20°C występowała niższa akumulacja transkryptu, ale wyższa akumulacja białka HvPIP, niż u odmiany Bowman. W 5°C obie linie miały zbliżony poziom transkryptu, ale towarzyszył temu nieco obniżony poziom białka HvPIP, w porównaniu do odmiany Bowman. W 27°C obie linie charakteryzowała mniejsza akumulacja transkryptu, względem odmiany Bowman, jednak linia z deficytem BR akumulowała więcej białka HvPIP, a linia z defektem BRI1 znacznie mniej, w porównaniu do odmiany Bowman. Drugi mutant z deficytem BR (522DK), po aklimacji w 27°C, miał podobnie jak BW084 wyższy poziom białka, niż u odmiany referencyjnej (Delisa), jednak było to powiązane także z nieco wyższym poziomem transkryptu *HvPIP*. Podsumowując, otrzymane wyniki wskazują na **powiązanie BR z regulacją ekspresji HvPIP zarówno na poziomie transkrypcji jak i biosyntezy białka, a także na zależność tego procesu m.in. od temperatury wzrostu/aklimacji i w pewnym stopniu (w aspekcie akumulacji transkryptu) od tła genetycznego.**

(3) Rozważając uzyskane wyniki w kontekście nabycia przez rośliny jęczmienia (w wyniku aklimacji w 27°C) tolerancji na wysoką temperaturę, należy przypomnieć, że obie linie bliskoizogeniczne oraz mutant 522DK charakteryzowały się jej wyższym poziomem, niż odmiany referencyjne (**Sadura i wsp. 2019 – Publikacja B**). Biorąc pod uwagę fakt, że w obu odmianach Bowman i Delisa, aklimacja w 27°C wywoływała wzrost poziomu akwaporyn, można przyjąć, że efekt ten mocno spotęgowany u linii BW084 i mutanta 522DK, mógłby się w jakimś stopniu przyczyniać do zwiększenia ich tolerancji na wysoką temperaturę. Jednak zjawisko to nie dotyczyło linii BW312, która w 27°C akumulowała mniej białka HvPIP, niż odmiana Bowman, a mimo to utrzymywała wyższą tolerancję na wysoką temperaturę. W przypadku ekspresji HvPIP w 5°C, różnice między liniami bliskoizogenicznymi i mutantem 522DK a odmianami referencyjnymi są stosunkowo niewielkie (choć niekiedy także istotne statystycznie), a zarazem zasługujące na rozważenie w kontekście tolerancji na mróz. Linie bliskoizogeniczne miały niższą tolerancję na mróz niż odmiana Bowman. Przyjmując, że białko HvPIP ma pewne znaczenie dla przystosowania się gospodarki wodnej jęczmienia do mrozu (jego ilość u odmiany Bowman w chłodzie wzrosła, w stosunku do 20°C), zauważamy, że u obu badanych linii akumulacja HvPIP spadła, zarówno względem odmiany Bowman jak i wartości notowanych u tych linii w 20°C. Można zatem przypuszczać, że efekt ten mógł być jednym z czynników mających wpływ na gospodarkę wodną i ukształtowanie się u obu linii niższej tolerancji na mróz, niż u odmiany referencyjnej. Wspierać tę tezę mógłby także przypadek mutanta 522DK, u którego w 5°C poziom akumulacji HvPIP był zbliżony do poziomu jego akumulacji u odmiany Delisa i jednocześnie tolerancja na mróz obu roślin była na porównywalnym poziomie.

6.4.2.3. Pompa protonowa ATPaza (H^+-ATPaza)

(1) Zarówno u odmiany Bowman jak i Delisa zaznaczył się wpływ temperatury na ekspresję H^+-ATPazy (**Sadura i wsp. 2020b – Publikacja D**). W wyniku aklimacji w 5°C u obu odmian zaobserwowano znaczący (około dwukrotny w 21 dniu) wzrost akumulacji transkryptu *H^+-ATPazy*, w porównaniu do 20°C, czemu towarzyszył kilkukrotny wzrost akumulacji białka H^+-ATPazy. Aklimacja w 27°C, choć u obu odmian nie miała związku ze zmianą akumulacji transkryptu, to wiązała się z wielokrotnym wzrostem akumulacji białek H^+-ATPazy, w porównaniu do 20°C (Ryc. 8G, H). Wzrost poziomu H^+-ATPazy w temperaturze 27°C był znacznie silniejszy niż w 5°C (**Sadura i wsp. 2020b – Publikacja D**). Zjawiska te dość jednoznacznie potwierdzono u obu odmian, co wskazuje, że taki jest **model zmian ekspresji H^+-ATPazy w czasie aklimacji jęczmienia do różnych temperatur** (Ryc. 8G, H).

(2) Obie linie bliskoizogeniczne BW084 i BW312 oraz mutant 522DK charakteryzowały się zmienionym poziomem ekspresji H^+-ATPazy zarówno na poziomie akumulacji transkryptu jak i białka, w porównaniu do odmian referencyjnych (**Sadura i wsp. 2020b – Publikacja D**). Generalnie akumulacja transkryptu *H^+-ATPazy* u linii BW, a także u mutanta 522DK była obniżona niezależnie od temperatury wzrostu/aklimacji, choć efekt ten był znacznie mocniejszy u obu linii BW niż u mutanta 522DK, w porównaniu do odmian referencyjnych. Wskazuje to na **udział BR w regulacji transkrypcji *H^+-ATPazy* jako pozytywnych regulatorów tego procesu.** W 20°C linie BW i mutant charakteryzowały się wyższą akumulacją białka H^+-ATPazy, w porównaniu do odmian referencyjnych. W temperaturze 5°C u linii BW084, BW312 i mutanta 522DK, ilość białka była zbliżona do odmian referencyjnych (niewielki, ale statystycznie istotny wzrost widoczny był u BW312 vs Bowman). Rozbieżność zaobserwowano w przypadku aklimacji w 27°C, tj. mutant 522DK wykazywał wyższy poziom białka H^+-ATPazy, a linie BW084 i BW312 niższy poziom tego białka, w porównaniu do odmian referencyjnych (**Sadura i wsp. 2020b – Publikacja D**).

(3) Nie zaobserwowano wyraźnego modelu zmian, który mógłby przyczyniać się do wyjaśnienia zmienionej tolerancji badanych mutantów na mróz i wysoką temperaturę, w porównaniu do ich odmian referencyjnych.

6.5. Zaburzenia biosyntezy brasinosteroidów oraz dysfunkcja receptora brasinosteroidów a zawartość barwników fotosyntetycznych i wydajność PSII w kontekście aklimacji jęczmienia – Publikacja B

Analizując zmiany w funkcjonowaniu PSII jako istotnego elementu fazy jasnej fotosyntezy, najprostszym podejściem jest oparcie się na ocenie parametru $P.I._{ABS}$ (*ang. performance index*), charakteryzującego ogólną wydajność PSII. Zmiany wartości pozostałych parametrów opisujących funkcjonowanie PSII (ABS/CSm; TRo/CSm; ETo/CSm; DIo/CSm – parametry opisujące przepływ energii od anten fotosyntetycznych do łańcucha przenośników elektronów) zostały omówione w publikacji **Sadura i wsp. (2019) – Publikacja B**.

Wartość $P.I._{ABS}$ u obu badanych odmian spadła w 5°C (w stosunku do kontroli tj. w 20°C), a w 27°C utrzymywała się na poziomie kontroli (**Sadura i wsp. 2019 – Publikacja B**). Na tym tle zarówno bliskoizogeniczne linie BW084 i BW312, jak i mutant 522DK charakteryzowały się, niezależnie od temperatury wzrostu/aklimacji, wyższą wydajnością fotosyntetyczną PSII, niż ich odmiany referencyjne, odpowiednio Bowman i Delisa (**Sadura i wsp. 2019 – Publikacja B**). Nieinwazyjne pomiary zieleni liści przeprowadzone później dla mutanta 522DK i odmiany Delisa (**Sadura i wsp. 2021 – Publikacja F**) wykazały, że liście mutanta charakteryzują się bardziej intensywną zielenią, niż liście odmiany Delisa. Z drugiej strony analiza biochemiczna zawartości chlorofilu w izolowanych chloroplastach udowodniła, że mutanta 522DK i odmianę Delisa charakteryzuje zbliżona zawartość chlorofilu w chloroplastach (**Sadura i wsp. 2021 – Publikacja F**). Zatem wyższa wydajność fotosyntetyczna PSII u 522DK (a także prawdopodobnie u BW084 i BW312) stwierdzona w pomiarach fluorescencji chlorofilu *a*, może wynikać z faktu, iż te półkarłowe rośliny mają jedynie zwiększoną koncentrację barwników na jednostkę powierzchni liścia. Może to stawiać pod pewnym znakiem zapytania rolę BR jako regulatorów w procesie biosyntezy chlorofilu, mimo tego, iż w wielu pracach, w których stosowano BR egzogennie udowodniono pozytywny wpływ tych związków na poziom barwników fotosyntetycznych zwłaszcza w warunkach stresu (**Sadura i Janeczko 2018 – Publikacja A**). Możliwe jednak, że mechanizm tego zjawiska jest inny i wynika np. pośrednio z ogólnej ochronnej roli BR w warunkach stresu (co wiąże się następczo z ograniczeniem degradacji chlorofilu). Zgłębienie roli BR w funkcjonowaniu reakcji świetlnych fotosyntezy wymaga zatem dalszych bardziej szczegółowych badań na poziomie genetycznym i proteomicznym.

7. Podsumowanie

W doświadczeniach przeprowadzonych w ramach przedłożonej pracy doktorskiej starano się przybliżyć znaczenie brasinosteroidów (BR) w kształtowaniu tolerancji jęczmienia na niską i wysoką temperaturę. Obiektem badawczym były rośliny z zaburzeniami biosyntezy i percepcji BR. Biorąc pod uwagę ważną rolę membran komórkowych w procesie przystosowania (aklimacji) roślin do różnych temperatur analizowano udział BR w indukowanych temperaturą zmianach zachodzących w membranach w czasie aklimacji jęczmienia w chłodzie (5°C) i w podwyższonej temperaturze (27°C). Oceniono znaczenie BR jako hormonów mających wpływ na strukturę (skład lipidowy) i własności fizykochemiczne membran oraz jako regulatorów ekspresji wybranych białek membranowych pełniących funkcje ochronne (HSP) oraz transportujących wodę (akwaporyny), a także na ekspresję H^+-ATPazy (pompy protonowej). Badania dotyczące zmian zachodzących w membranach wzbogacono analizami gospodarki hormonalnej i aktywności fotosyntetycznej roślin w czasie aklimacji. Zmiany metaboliczne zachodzące w czasie aklimacji (5°C i 27°C) starano się zinterpretować w świetle tolerancji roślin na mróz (-6°C i -8°C) i wysoką temperaturę (38°C i 45°C).

Ustalono, że głównymi BR jęczmienia obecnymi w największej ilości zarówno w tkance liści jak i w czystych izolowanych chloroplastach są kastasteron i homokastasteron – przedstawiciele grupy BR C_{28} i C_{29}. Jednak w samych chloroplastach stwierdzono występowanie łącznie ośmiu BR w tym rzadziej spotykanych jak: 28-norkastasteron, dolicholid czy homodolichosteron. Zależna od temperatury akumulacja przeważnie dużych ilości BR w chloroplastach jest przesłanką wskazującą na możliwość pełnienia przez te związki funkcji fizjologicznych w tych organellach, ale przybliżenie ich roli u jęczmienia wymagać będzie dalszych badań. Podobnie zresztą jak wyjaśnienie mechanizmów transportu/akumulacji BR w chloroplastach. Stwierdzono bowiem, że mimo obniżonego poziomu BR (kastasteronu) w tkance liści mutanta, w chloroplastach koncentracja tego hormonu utrzymywała się na poziomie zanotowanym u roślin odmiany referencyjnej. Poziom BR u jęczmienia był także regulowany przez temperaturę i korelował z poziomem tolerancji roślin na stres temperaturowy. Największą tolerancją na mróz charakteryzowała się odmiana Bowman, która cechowała się największą zawartością homokastasteronu po procesie aklimacji (hartowania) w 5°C, poprzedzającym ekspozycję na temperatury poniżej zera. Można więc przyjąć, że homokastasteron może odgrywać istotną rolę w aklimacji chłodowej oraz w kształtowaniu tolerancji roślin na mróz. W pewien sposób dowodzi tego także fakt, że rośliny z zaburzeniami w biosyntezie i percepcji BR charakteryzujące się obniżonym

poziomem tego hormonu miały także niższą tolerancję na mróz. Co ciekawe u roślin z zaburzeniami biosyntezy i percepcji BR poddanych aklimacji w 27°C, niższemu poziomowi homokastasteronu (w porównaniu do odmian referencyjnych), towarzyszyła wyższa tolerancja na wysoką temperaturę.

Warto także podkreślić, że o ile defekt receptora BRI1 u roślin BW312 był przyczyną zwiększonej akumulacji kastasteronu (prekursora brasinolidu) oraz samego brasinolidu uważanego za główny ligand tego receptora, zjawisko to nie dotyczyło homokastasteronu. Sugeruje to, że homokastasteron może być ligandem innego białka wiążącego BR niż jedyny scharakteryzowany do tej pory receptor BRI1.

Analizy profilu hormonalnego u jęczmienia rosnącego w różnych temperaturach wskazały, że BR biorą udział w skomplikowanej sieci połączeń hormonalnych i są związane ze szlakami biosyntezy/sygnalingu auksyn, cytokinin, giberelin, kwasu abscysynowego (ABA) czy salicylowego. Ponadto, u roślin z deficytem BR i defektem receptora BR (linii BW084 i BW312) rosnących w 5°C, obniżenie zawartości hormonu stresu ABA wraz ze wzrostem zawartości niektórych giberelin, mogło być jedną z wielu przyczyn ich obniżonej tolerancji na mróz w stosunku do odmiany referencyjnej.

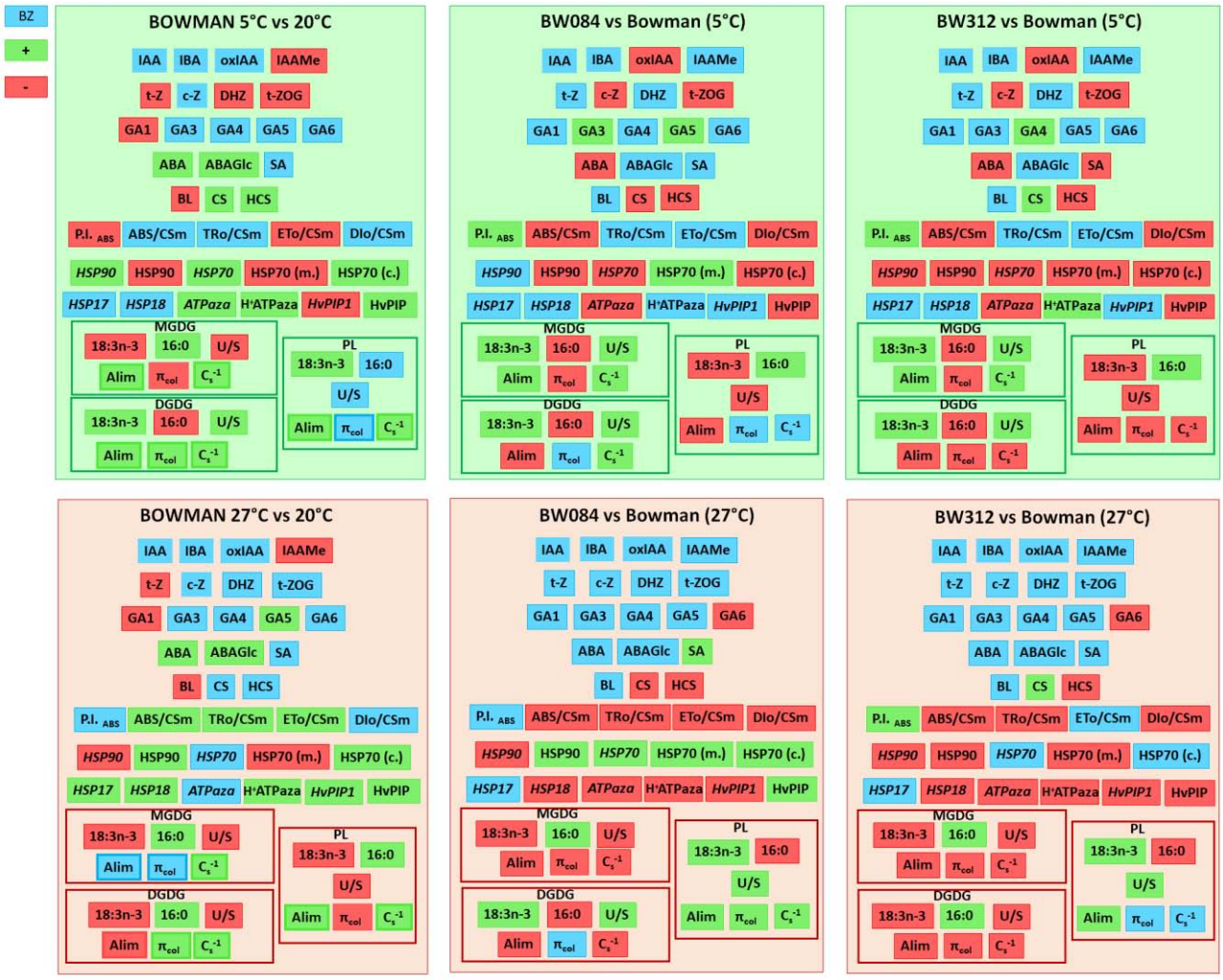
Wyniki analiz składu lipidowego membran komórkowych sugerują, że BR uczestniczą w zależnej od temperatury regulacji biosyntezy kwasów tłuszczowych lub też sterują procesami odpowiedzialnymi za transport/wbudowywanie ich w membrany. Zmiany w składzie lipidowym membran znajdują odzwierciedlenie w zmianach właściwości fizykochemicznych membran, a te z kolei mogą częściowo tłumaczyć obniżoną tolerancję roślin z zaburzeniami biosyntezy/percepcji BR na mróz i zwiększoną tolerancję na wysoką temperaturę. Wykazana metodą wagi Langmuira niższa płynność membran (monowarstw zbudowanych z frakcji digalaktolipidów i fosfolipidów) obu linii BW084 i BW312 hartowanych w 5°C, w porównaniu do odmiany referencyjnej, może być jednym z czynników odpowiedzialnych za obniżoną tolerancję obu linii na mróz (-8°C). Z drugiej strony obniżony stopień płynności membran (monowarstw zbudowanych z frakcji mono- i digalaktolipidów) linii poddanych aklimacji w 27°C na tle odmiany Bowman, może zostać powiązany ze zwiększoną tolerancją roślin na wysoką temperaturę (38°C i 45°C). Dodatkowo można przypuszczać, że poprzez modyfikację struktury i właściwości błon komórkowych BR mogą wywierać także pośrednio wielokierunkowy wpływ na wiele procesów fizjologicznych, zależnych od stanu membran, np. aktywować lub blokować lokowane w membranach białkowe transportery, pompy jonowe i enzymy pośredniczące w ekspresji genów, co jest warunkowane płynnością membran.

Metodą Elektronowego Rezonansu Paramagnetycznego (EPR) badano także wpływ BR na dynamikę molekularną membran chloroplastowych izolowanych z odmiany Delisa i mutanta 522DK (**Sadura i wsp. 2021 - Publikacja F**) oraz membran modelowych (z lecytyny; dane niepublikowane), weryfikując hipotezę, że BR (tak jak sterole) oddziałują bezpośrednio z membranami i stabilizują je. W badaniach wykorzystano mutanta z deficytem BR w liściach oraz jego odmianę referencyjną, jednakże w trakcie prowadzenia eksperymentu ustalono, że w izolowanych chloroplastach (w odróżnieniu od liści) całkowita zawartość BR jest porównywalna, co można uznać za przyczynę stosunkowo niedużych różnic w dynamice molekularnej membran chloroplastów pomiędzy Delisą i 522DK. Również w przypadku membran modelowych nie udało się udowodnić tego zjawiska, zatem kwestia wpływu BR na dynamikę molekularną membran komórkowych pozostaje otwarta do dalszych badań.

W pracy opisano model zmian ekspresji białek szoku cieplnego (HSP), akwaporyny (HvPIP) i H^+-ATPazy u jęczmienia jarego poddanego aklimacji do niskiej i podwyższonej temperatury. Ponadto wykazano, że BR odgrywają rolę pozytywnych regulatorów transkrypcji *HSP* u jęczmienia w szerokim zakresie temperatur. Jak pokazują wyniki uzyskane w przypadku linii BW312, BR uczestniczą także w regulacji biosyntezy białek HSP za pośrednictwem receptora BRI1. BR odgrywają także rolę pozytywnych regulatorów w regulacji transkrypcji *H^+-ATPazy*. BR wydają się być także powiązane z regulacją ekspresji akwaporyny (HvPIP) zarówno na poziomie transkrypcji jak i biosyntezy białka, jednak proces ten dodatkowo zależy w większym stopniu od temperatury wzrostu/aklimacji i w pewnym stopniu (w aspekcie akumulacji transkryptu) od tła genetycznego.

Generalnie przeprowadzenie doświadczeń na dwóch różnych odmianach jęczmienia (o zróżnicowanym tle genetycznym) oraz ich liniach bliskoizogenicznych/mutantach z zaburzeniami w biosyntezie i percepcji BR, pozwala zauważyć, że zarówno tło genetyczne jak i typ mutacji (w tym zaburzenia na różnych etapach biosyntezy BR) w kilku przypadkach miały istotny wpływ na uzyskiwane wyniki. Świadczy to jedynie o złożonej zależności działania BR w sieci metabolicznych powiązań, która dodatkowo modyfikowana jest przez warunki termiczne wzrostu roślin jęczmienia.

Wszystkie ustalone w przeprowadzonych eksperymentach fizjologiczno-biochemiczne zmiany zachodzące na skutek aklimacji w 5°C i 27°C u roślin z deficytem BR i zaburzeniami funkcjonowania receptora BR (BW084 i BW312) na tle wyników uzyskanych dla odmiany referencyjnej (Bowman) zobrazowano na Rycinie 10.



Ryc. 10. Fizjologiczno-biochemiczne zmiany zachodzące w roślinach odmiany Bowman w wyniku aklimacji w 5°C (A) i 27°C (D); punktem odniesienia są rośliny Bowman rosnące w 20°C. Fizjologiczno-biochemiczne zmiany zachodzące w roślinach z zaburzeniami biosyntezy BR (BW084) w temperaturze 5°C (B) i 27°C (E); punktem odniesienia są wyniki uzyskane odpowiednio w 5°C i 27°C dla roślin odmiany referencyjnej (Bowman). Fizjologiczno-biochemiczne zmiany zachodzące w roślinach z defektem receptora BR (BW312) w temperaturze 5°C (C) i 27°C (F); punktem odniesienia są wyniki uzyskane odpowiednio w 5°C i 27°C dla roślin odmiany referencyjnej (Bowman). Kolorem zielonym we wszystkich przypadkach oznaczono wzrost wartości danego parametru, kolorem czerwonym spadek, a brak zmian kolorem niebieskim. *Legenda: IAA – auksyna, kwas indolilo 3-octowy; IBA – auksyna; kwas indolilo-3-masłowy; oxIAA – auksyna, kwas 2-oksindolo-3-octowy; IAAMe – auksyna, ester metylowy kwasu indolilooctowego; t-Z – cytokinina, trans-zeatyna; c-Z – cytokinina, cis-zeatyna; DHZ – cytokinina, dihydrozeatyna; t-ZOG – cytokinina, O-glukozyd trans-zeatyny; GA1 – giberelina A1; GA3 – giberelina, kwas giberelinowy; GA4 – giberelina A4; GA5 – giberelina A5; GA6 – giberelina A6; ABA – kwas abscysynowy; ABAGlc – ester glukozylowy kwasu abscysynowego; SA – kwas salicylowy; BL – brasinosteroid, brasinolid; CS – brasinosteroid, kastasteron; HCS – brasinosteroid, homokastasteron; P.I.$_{ABS}$ – Performance Index (ogólna wydajność PSII); ABS/CSm – energia zaabsorbowana przez anteny fotosyntetyczne; TRo/CSm – energia przekazana na centrum reakcji PSII; ETo/CSm – energia przekazana na łańcuch transportu elektronów; DIo/CSm – energia rozpraszana z PSII w postaci ciepła; HSP70 – transkrypt kodujący białko szoku cieplnego HSP90;* HSP90 *– białko szoku cieplnego 90; HSP70 – transkrypt kodujący białko szoku cieplnego HSP70;* HSP70 (m.) *– białko szoku cieplnego 70 we frakcji membranowej;* HSP70 (c.) *– białko szoku cieplnego 70 we frakcji cytoplazmatycznej; HSP17 – transkrypt kodujący białko szoku cieplnego HSP17; HSP18 – transkrypt kodujący białko szoku cieplnego HSP18; ATPaza – transkrypt kodujący białko błonowe ATPazę;* H$^+$ATPaza *– białko błonowe H$^+$-ATPaza; HvPIP1 – transkrypt kodujący białko błonowe akwaporynę HvPIP;* HvPIP *– białko błonowe akwaporyna; 18:3n-3 – kwas α-linolenowy; 16:0 – kwas palmitynowy; U/S – stosunek nienasyconych do*

nasyconych kwasów tłuszczowych; A_{lim} – parametr określający powierzchnię przypadającą na cząsteczkę lipidu; π_{coll} – ciśnienie powierzchniowe, przy którym następuje kolaps monowarstwy (ciśnienie kolapsu); C_s^{-1} – parametr mówiący o podatności warstw na ściskanie (wytrzymałości mechanicznej warstw oraz stabilności i płynności warstw).

8. Wnioski

Badania z wykorzystaniem roślin jęczmienia z zaburzeniami w biosyntezie/percepcji brasinosteroidów wykazują, że związki te są ważnymi regulatorami metabolizmu o szerokim spektrum działania u tego gatunku. Jakkolwiek nie wszystkie zmiany biochemiczne indukowane w czasie aklimacji (5°C, 27°C) udało się jednoznacznie powiązać ze zmienioną u roślin (z zaburzeniami w biosyntezie/percepcji BR) tolerancją na temperatury ekstremalne (mróz, 38°C i 45°C). Jednak BR należy zakwalifikować jako regulatory istotne z punktu widzenia kształtowania tolerancji na stres temperaturowy i warte dalszych pogłębionych badań zwłaszcza w dobie zmian klimatycznych.

9. Literatura

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10. Streszczenie rozprawy doktorskiej po polsku

Przeprowadzone doświadczenia pozwalają przybliżyć rolę steroidowych hormonów roślinnych brasinosteroidów (BR) u jęczmienia w szczególności w procesach zachodzących w trakcie aklimacji roślin do niskiej i wysokiej temperatury. W badaniach wykorzystano rośliny z zaburzeniami w biosyntezie BR (BW084 (mutacja *HvCPD*) i 522DK (mutacja *HvDWARF*)) oraz rośliny z mutacją *HvBRI1* i defektem receptora BR, (BW312), które analizowano na tle odmian referencyjnych. W tkance liści stwierdzono występowanie trzech BR, a w chloroplastach zidentyfikowano ich osiem. U linii z defektem receptora BR potwierdzono charakterystyczną nadmierną akumulację BR C_{28}, ale nie homokastasteronu, co sugeruje, że związek ten może być ligandem innego receptora niż jedyny poznany BRI1. W czasie aklimacji roślin w 5°C poziom BR w liściach wzrastał, natomiast w 27°C przejściowo obniżał się. Zaburzenia biosyntezy i percepcji BR wiązały się z obniżeniem tolerancji na mróz, ale ze zwiększeniem tolerancji na wysokie temperatury (np. 45°C), u roślin poddanych aklimacji odpowiednio w 5°C i 27°C. Niższą tolerancję BW084 i BW312 na mróz, w stosunku do odmiany referencyjnej, można łączyć z niższą zawartością homokastasteronu (jednego z BR) i kwasu abscysynowego, wyższą zawartością niektórych giberelin oraz z niższą płynnością membran (monowarstw zbudowanych z digalaktolipidów i fosfolipidów). Zwiększona tolerancja roślin z zaburzeniami w biosyntezie/percepcji BR na wysoką temperaturę (na tle odmian referencyjnych) może zostać powiązana z niższą zawartością homokastasteronu i obniżonym stopniem płynności membran (monowarstw zbudowanych z frakcji mono- i digalaktolipidów). W badaniach dynamiki molekularnej metodą Elektronowego Rezonansu Paramagnetycznego weryfikowano hipotezę, że BR oddziałują bezpośrednio z membranami (tak jak sterole) i stabilizują je. Do badań wybrano mutant z deficytem BR (522DK) i jego odmianę referencyjną, jednak w trakcie eksperymentu stwierdzono, że w izolowanych chloroplastach (w odróżnieniu od liści) całkowita zawartość BR była podobna, co można uznać za przyczynę małych różnic w dynamice molekularnej membran chloroplastów pomiędzy odmianą i 522DK. Sugeruje to także istnienie mechanizmów odpowiedzialnych za akumulację BR w chloroplastach niezależnie od zaburzeń ich biosyntezy w komórce. Analizy membran modelowych z wbudowywanymi BR jednak także nie potwierdziły wpływu BR na ich dynamikę. Ustalono, że BR u jęczmienia regulują ekspresję białek szoku cieplnego (HSP), a także zlokowanych w membranach akwaporyn i pompy protonowej, działając zarówno na poziomie transkrypcji jak i biosyntezy białka. W przypadku biosyntezy występuje jednak mocna zależność tej regulacji od temperatury wzrostu i częściowo od tła genetycznego.

11. Streszczenie rozprawy doktorskiej po angielsku (Summary)

The experiments carried out as part of the presented doctoral dissertation allow to bring closer the role of plant steroid hormones brasinosteroids (BR) in barley, in particular in the processes of plant acclimation to low and high temperatures. In the study, plants with disturbances in BR biosynthesis (BW084 (*HvCPD* mutation) and 522DK (*HvDWARF* mutation)) and plants with *HvBRI1* mutation and BR receptor defect (BW312) were used and analyzed against reference cultivars. Three BRs were found in leaf tissue, and eight were identified in chloroplasts. In the BR receptor defective line, a characteristic excessive accumulation of BR C_{28} was confirmed. But it was not observed in case of homocastasterone accumulation what suggest that this compound may be a ligand for other receptor than the only known BRI1. During plant acclimation at 5°C the BR level in leaves increased, while at 27°C it temporarily decreased. Disturbances in BR biosynthesis and perception were associated with decreased frost tolerance and with increased tolerance to high temperatures (e.g. 45°C) in plants acclimated to 5°C and 27°C, respectively. The lower frost tolerance of BW084 and BW312, compared to the reference cultivar, can be associated with a lower content of homocastasterone (one of BR) and abscisic acid, a higher content of some gibberellins and a lower membranes fluidity (monolayers made of digalactolipids and phospholipids). In studies of molecular dynamics of chloroplast membranes with the Electron Paramagnetic Resonance method, the hypothesis that BR interacts directly with membranes (like sterols) and stabilizes them was verified. The BR-deficient mutant (522DK) and its reference cultivar were selected for the study, but during the experiment it was found that in isolated chloroplasts (in contrast to leaves) the total content of BR was similar, which can be considered as the reason for small differences in the molecular dynamics of chloroplast membranes between Delisa cultivar and 522DK mutant. It also suggests the existence of mechanisms responsible for the accumulation of BR in chloroplasts that are not related to disturbances in BR biosynthesis in the cell. Also, analyzes of model membranes with incorporated BR, did not confirm the influence of BR on their molecular dynamics, but this issue remains open for further research. It was found that BR in barley regulates the expression of heat shock proteins (HSPs), as well as membrane-bound aquaporins and proton pumps, acting both at the level of transcription and protein biosynthesis. However, in the case of biosynthesis, there is a strong dependence of this regulation on the growth temperature and partly on the genetic background.

12. 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 nadanie stopnia doktora nauk rolniczych.

Kraków, 16.04.2021r. .....

Oświadczenie Autora pracy

Świadom 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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REVIEW

Physiological and molecular mechanisms of brassinosteroid-induced tolerance to high and low temperature in plants

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Abstract

Brassinosteroids (BRs) are plant hormones that were isolated for the first time in the 1970s. This group currently includes more than 70 compounds that differ in their structure and physiological activity. BRs are present in plants in a free form or in the form of conjugates. BRs are known as plant growth regulators, but they also play a role in the plant response to environmental stresses. In the case of plants that are exposed to low/high temperature, exogenous BRs can counteract growth inhibition and reduce biomass losses as well as increase plant survival. BRs show a multidirectional activity in regulating the metabolism of plants exposed to extreme temperatures. The following BRs actions can be distinguished: changes in membrane physicochemical properties, regulation of the expression of selected genes (including stress-responsive genes), as well as indirect effects on metabolism through other hormones or signalling molecules (such as hydrogen peroxide). This review summarizes the current knowledge about the effects of BRs on the physiological and biochemical processes that occur in plants during exposure to low or high temperatures.

Additional key words: plant acclimation, cell membranes, cold, freezing, gene expression, heat, oxidative damage, photosynthesis, proline.

Introduction: discovery, occurrence, biosynthesis and hormonal nature of brassinosteroids

Brassinosteroids (BRs) were isolated for the first time from *Brassica napus* pollen (Grove *et al.* 1979). This group currently includes more than 70 steroids that differ in structure and physiological activity. BRs are present in a free form and as conjugates in higher plants and algae (Bajguz and Tretyn 2003). In higher plants, BRs occur in all organs in a nano- and pico-gram amount per gram of fresh mass. BRs can be divided into three main groups depending on the number of carbon atoms in the molecule: C_{27}, C_{28}, and C_{29}. Sterols are precursors in the synthesis of all BRs, and campesterol and 24-β-methyl-cholesterol are considered to be precursors of C_{28} BRs (Fujioka and Yokota 1997, Takatsuto *et al.* 1999, Park *et al.* 2000, Schaller 2003, Kim *et al.* 2004, 2006), cholesterol of C_{27} BRs (Fujioka and Yokota 1997, Schaller 2003, Kim *et al.* 2004, 2006), and sitosterol of C_{29} BRs (Fujioka and Yokota 1997, Takatsuto *et al.* 1999, Schaller 2003, Kim *et al.* 2006) (Fig. 1). The main and the first BR biosynthetic pathway was discovered in cultured cells of *Catharanthus roseus* and *Arabidopsis*

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; AsA - ascorbic acid; BAK1 - BRI1 associated receptor kinase1; BES1 - bri1-EMS-supressor1; BIN2 - brassinosteroid insensitive2 kinase; BR - brassinosteroid; BRI1 - cell surface receptor kinase; Brz - brassinazole; BSK1 - brassinosteroid-signalling kinase1; BZR1 - brassinazole resistant1; CAT - catalase; CBFs - C-repeat/dehydration responsive element binding factors; CI - chilling injury; COR - cold-responsive proteins; DHAR - dehydroascorbate reductase; E - transpiration rate; ETR - electron transport rate; F_v/F_m - maximum quantum efficiency of PS II photochemistry; F_v'/F_m' - efficiency of open reaction centres in light; g_s - stomatal conductance; GR - glutathione reductase; GSH - reduced glutathione; HSP - heat shock protein; MDA - malondialdehyde; MDAR - monodehydroascorbate reductase; P_N - net photosynthetic rate; POD - peroxidase; qP - photochemical quenching coefficient; ROS - reactive oxygen species; Rubisco - ribulose--1,5-*bis*-phosphate carboxylase/oxygenase; SOD - superoxide dismutase; Φ_{PSII} - effective quantum yield of PS II photochemistry.

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thaliana for BR type C_{28} brassinolide (Yokota *et al.* 1990, Fuijoka and Yokota 1997, Clouse 2015). The intermediates in this pathway are cathasterone, teasterone, typhasterol, and castasterone in the early C6 oxidation pathway and 6-deoxocathasterone, 6-deoxoteasterone, 6-deoxotyphasterol, and 6-deoxocastasterone in the late C6 oxidation pathway (Fujioka and Yokota 1997).

Brassinosteroids are perceived by the cell surface receptor kinase, BRI1. Binding BR to the extracellular domain of BRI1 induces activation and dimerization of kinase with its co-receptor kinase BAK1 (BRI1-associated receptor kinase1) or its homologue SERK1 (somatic embryogenesis receptor kinase 1). Sequential trans-phosphorylation between the kinase domains of BRI1 and BAK1 activates the kinases and BRI1 in turn phosphorylates the members of two groups of plasma membrane-anchored cytoplasmic kinases, BSK1 (brasinosteroid-signaling kinase1) and CDG1 (constitutive differential growth1). CDG1, and possibly also BSK1, phosphorylates the BRI1-supressor1 (BSU1) phosphatase, which then dephosphorylates and inactivates the GSK3-like kinase brassinosteroid insenitive2 (BIN2). BIN2 directly regulates the phosphorylation status and nuclear accumulation of two homologous transcription factors, BZR1 (brasinazole resistant1) and BES1 (bri1-EMS-suppressor1) (Wang *et al.* 2006, Li and Jin 2007, Wang *et al.* 2014).

Although it was already known in the 1970s that the exogenous BRs stimulates the elongation of stems and cell division in plants (Mitchell *et al.* 1970, Grove *et al.* 1979), these compounds were recognised as a new class of plant hormones only in the 1990s when several genes responsible for the biosynthesis and perception of BRs were discovered. The mutations that are associated with the loss of function of these genes usually lead to growth disturbances (including short hypocotyl, dwarfing of seedlings and mature plants) or to dark green leaves (Yang *et al.* 2011). The ability of these compounds to

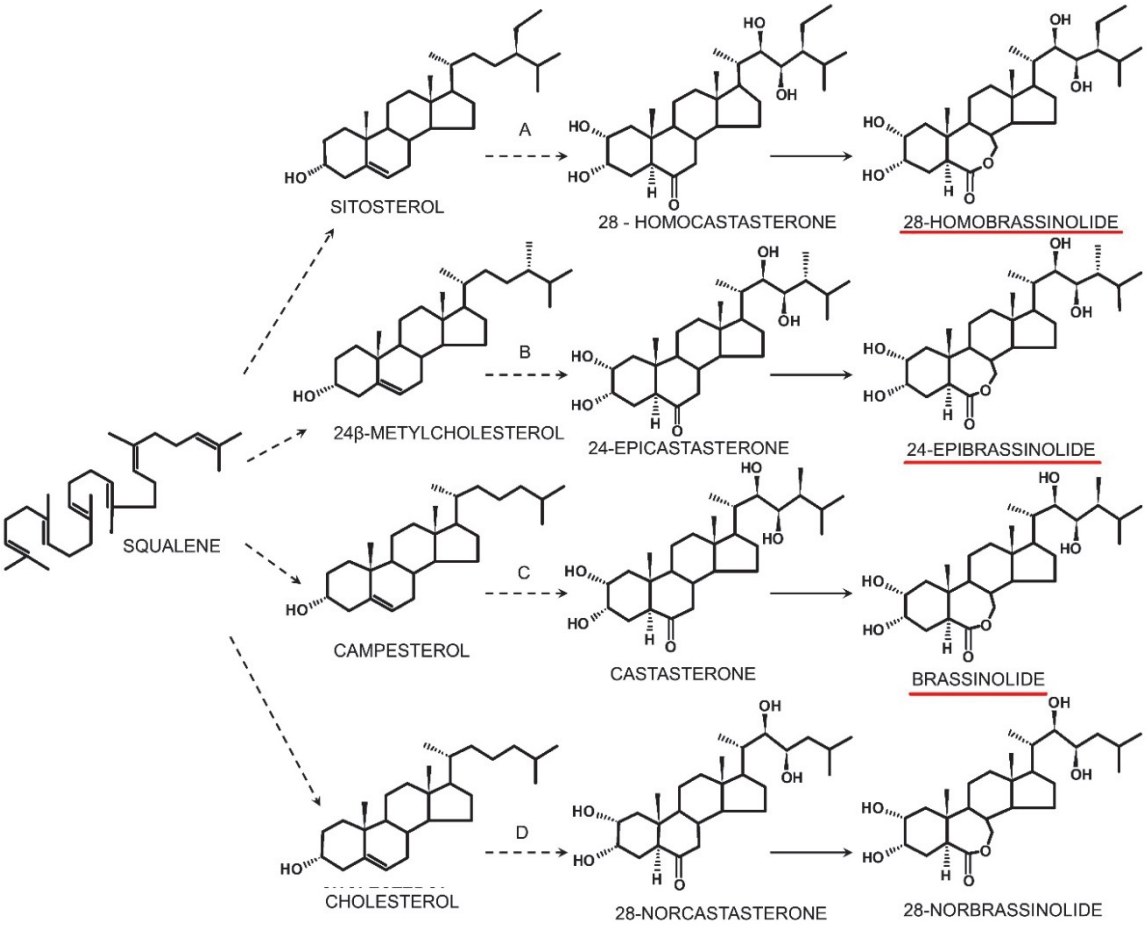

Fig. 1. Simplified model of biosynthesis of selected BRs in plants: *A* - type C_{29} (Takatsuto *et al.* 1999, Schaller 2003, Kim *et al.* 2006), *B* and *C* - type C_{28} (*B* - Winter *et al.* 1999, Park *et al.* 2000, *C* - Takatsuto *et al.* 1999, Park *et al.* 2000, Schaller 2003, Kim *et al.* 2004, 2006), *D* - type C_{27} (Schaller 2003, Kim *et al.* 2004, 2006). BRs tested in experiments with high and low temperature are underlined.

counteract the negative effects of environmental stresses in plants should, however, be considered as a particularly interesting characteristic of BRs due to the possibility of their practical application in agriculture/horticulture (Krishna 2003, Song *et al.* 2016, Shu *et al.* 2017). During their life cycle, plants are usually exposed to different biotic and abiotic stresses, which may result in a decrease in the yield. Among the abiotic stresses, too low or too high temperatures are particularly serious problems in agriculture and horticulture. Species, such as cucumbers, tomatoes, or maize are very sensitive to cold, while frost, especially when occurs under insufficient snow cover on fields, can cause significant yield losses of winter crops, *e.g.*, oilseed rape or cereals. High temperature, in turn, is particularly dangerous to plants when combined with drought. Table 1 Suppl. summarises the data for the temperature ranges, species, and brassinosteroids that were used in order to ameliorate the plant damage.

The structure and properties of cell membranes

Low temperature significantly increases membrane lipid peroxidation due to the increased production of free radicals. Membrane lipid peroxidation is usually estimated based on a spectrophotometric determination of the malondialdehyde (MDA) content (the final oxidation product of polyunsaturated fatty acids) by a reaction with thiobarbituric acid (TBA), which generates a coloured TBA-MDA complex (Hodges *et al.* 1999). An increase in membrane lipid peroxidation was observed in tomato seedlings exposed to day/night (d/n) temperatures of 12/6 °C for 12 d (Cui *et al.* 2016), as well as in tomato fruits that were stored at 1 °C for 3 weeks (Aghdam *et al.* 2012). Spraying of seedlings or fruits with 24-epibrassinolide prior to cold considerably decreased the MDA content compared to seedlings or fruits without BR application. A similar phenomenon was found for the fruits of pepper, eggplant seedlings, and cucumber plants (Hu *et al.* 2010, Wang *et al.* 2012, Jiang *et al.* 2013, Wu *et al.* 2014a). Moreover, cell suspensions of *Chorispora bungeana* that were cultured with 24-epibrassinolide (0.05 mg dm^{-3} or 0.1 μM) showed a marked decrease (by 20 to 40 %) of MDA content compared to the culture that was grown at 0 - 4 °C (for 3 - 5 d) but without 24-epibrassinolide (Liu *et al.* 2009, 2011). The participation of BRs in membrane protection against peroxidation in cold was confirmed by studies on BR mutants. Qu *et al.* (2011) applied 0 °C (24 h) to *A. thaliana* plants and found that the *bri1-116 (-/-)* mutant (BR insensitive null allele of the BR receptor BRI1) reached significantly higher MDA content than the Col-0 wild type. Finally, in tomatoes with a partial deficiency of BRs that were exposed to cold, membrane lipid peroxidation was increased while the overexpression of the BR biosynthetic gene *Dwarf* or the application of 24-epibrassinolide reduced the cold-induced oxidative damage (Xia *et al*. 2017).

In addition to MDA accumulation, ion leakage measurement can be used, which indicates cell membrane permeability. Increased ion leakage is usually interpreted as membrane damage. In the primary leaves of oilseed rape kept at 2 °C, 24-epibrassinolide (0.05 and 1 μM) decreased ion leakage (by about 80 %) compared to the control (Janeczko *et al.* 2007). The authors noted temperature-, tissue-, and concentration-dependent effects of BRs on this parameter. Mango fruits that are stored at 5 °C for 28 d have electrolyte leakage about 40 %, but BR treatment (10 μM) prior to storage reduced this value to 20 % (Li *et al.* 2012). In tomato fruits, stored at 1 °C for three weeks, electrolyte leakage was approximately 85 %, whereas this value was decreased to 70 % in fruits that were treated with 3 μM brassinolide (Aghdam *et al.* 2012). A cell suspension of *C. bungeana* cultured at 0 and 4 °C (3 d) with 24-epibrassinolide (0.1 μM) had approximately a 20 % lower ion leakage compared to the stressed cultures without hormone (Liu *et al.* 2011). Qu *et al.* (2011) applied 0 °C for 24 h to *A. thaliana* plants and found that the *bri1-116 (-/-)* mutant reached approximately 50 % higher ion leakage than the Col-0 wild type. Eremina *et al.* (2016) measured electrolyte leakage in cold-acclimated and frozen leaves of *A. thaliana* wild type and two mutants: BR-hypersignalling line (*BRI1oe*) and BR-signalling defective mutants (*bri1-301*). After freezing at -10 °C (6 h), *BRI1oe* had a lower (by about 20 %) whereas *bri1-301* had a higher (by about 20 %) electrolyte leakage in comparison to the wild type.

Typically, during the process of acclimation to low temperature, membrane structural changes aim at the increasing proportion of unsaturated fatty acids, which improves the fluidity of the membranes. Brassinosteroids may affect the fatty acid composition of cell membranes. An increase in content of unsaturated fatty acids, linoleic acid C18:2 and linolenic acid C18:3, in mango fruits that were treated with brassinolide and stored at 5 °C was higher than in the control. Simultaneously, the content of palmitic acid C16:0, palmitoleic acid C16:1, stearic acid C18:0, and oleate acid C18:1 was slightly reduced by BR (Li *et al.* 2012). In monogalactosyldiacylglycerols (MGDG), a fraction that was isolated from a winter oilseed rape green callus acclimated at 2 °C, 24-epibrassinolide decreased the molar percentage of saturated fatty acids and increased the percentage of unsaturated fatty acids (18:1, 18:2, and 18:3) compared to a callus grown in the cold without BR (Janeczko *et al.* 2009). The MGDG fraction (next to the fraction of digalactosyldiacylglycerols) is considered to be abundant especially in the thylakoid membranes (Johnson and Williams 1989).

Li *et al.* (2012), analysed the plasma membranes of mango fruits that were stored at 5 °C for 28 d by measuring the phase transition temperature using differential scanning calorimetry (DSC) and polar lipid fluidity by electron paramagnetic resonance (EPR). The phase transition temperature in the fruits that are not treated with brassinolide is constant and about 6.5 °C during the storage period, while phase transition temperature in brassinolide-treated plants is markedly decreased. Polar lipid fluidity is determined on the basis of the EPR spectra, which enable the order parameter (S) and rotational correlation time (τ_c; inversely proportional to membrane fluidity) to be calculated. A gradual increase in the value of both parameters is observed with storage time in brassinolide-untreated plants, and there is a decrease in S and τ_c in plants to which this BR was applied. This indicates increased membrane fluidity in the presence of brassinolide. These results correspond to last studies, which were done using a Langmuir bath by Filek *et al.* (2017). When 24-epibrassinolide and 24-epicastasterone are introduced into lipid monolayers, their physicochemical properties changed. Lipids for monolayer formation were isolated from the leaves of wheat growing at 5 and 20 °C and 24-epibrassinolide incorporation into lipid monolayer resulted in the formation of more flexible surface structures (Filek *et al.* 2017). However, 24-epicastasterone induced the opposite effect. These results show importance of the chemical structure of BRs for their interaction with membranes.

High temperature also increases lipid peroxidation. In melon seedlings, a d/n temperature of 42/32 °C for 2 d increased the MDA content compared to the control (Zhang *et al.* 2014). Pretreatment with 24-epibrassinolide (1 mg dm^{-3}) reduced MDA accumulation by 35 %. A similar effect was observed in rice plants grown at high d/n temperatures of 40/30 °C for 7 d (Thussagunpanit *et al.* 2015b). In eggplants at d/n temperatures of 43/38 °C (8 d), 0.1 μM 24-epibrassinolide reduced the MDA content by 16 % compared to plants that were not treated (Wu *et al.* 2014b). Mazorra *et al.* (2011) studied the effect of 45 ºC (4 - 8 h), on tomato plants (wild type and brassinosteroid mutants (mutant with altered BRs sensitivity *curl3*$^{-abs}$, extremely dwarf mutant d^x and dwarf-overexpressing line 35SD). An increase in MDA content was found after 7 d of recovery from heat stress in the Wt, d^x, 35SD and *curl3*$^{-abs}$ plants but 24-epibrassinolide (1 μM) reduced the MDA content.

Concluding remarks: Cell membranes may act as "thermal sensors", which is the primary cause of many other metabolic changes within a cell including the expression of certain genes (Horvath *et al.* 2012). From this point of view, the ability of BRs to induce structural/functional changes in the cell membrane is interesting. Based on presented knowledge, various possible directions of BR action on a membrane can be noted. The first is the influence of BRs on the fatty acid composition towards an increased proportion of unsaturated fatty acids. This suggests the involvement of BRs in the biosynthesis of fatty acids or fatty acid transport and incorporation into cell membranes. Secondly, BRs (similarly as some other sterols) may enter cell membrane directly and modify its properties through, for example, increasing the distance between fatty acid chains, which also may improve the functioning of the membrane under unfavourable temperature conditions. Another mechanism of BR action could be its indirect effect on the inactivation of the excess of reactive oxygen species (ROS) that are generated under stress conditions (see next chapter). ROS-induced damage of constituent membrane elements is manifested, as is mentioned above, by the accumulation of the products of lipid peroxidation. BR-stimulated deactivation of free radicals probably indirectly protects cell membranes and thus lowers lipid peroxidation. A separate issue is the fact that important functional proteins such as water transporting channels and proton pumps are present in the cell membranes. Knowledge of the effect of BR on membrane proteins is rudimentary (Morillon *et al.* 2001, Yang *et al.* 2003) and especially requires studies for low or high temperature conditions.

Mobilization of cellular antioxidant system

Low temperature: A temperature that is too low for optimal growth is accompanied by increased free radical formation (H_2O_2, $O_2^{\bullet-}$, and $^{\bullet}OH$) in cells, which is associated with the activation of the antioxidant system. The antioxidant system consists of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and non-enzymatic antioxidants, including ascorbic acid (AsA) or tocopherols (Liu *et al.* 2009, 2011, Hu *et al.* 2010, Jiang *et al.* 2013, Cui *et al.* 2016). In eggplant seedlings, d/n temperatures of 10/5 for 8 d increased SOD, POD, ascorbate peroxidase (APX), and CAT activities (Wu *et al.* 2014a). The application of 0.1 μM 24-epibrassinolide prior to cold, additionally stimulated the activity of these enzymes, by 31, 120, 33, and 64 %, respectively. Treatment of pepper fruits with 15 μM 24-epibrassinolide caused higher CAT and glutathione reductase (GR) activities during storage at 3 ºC for 18 d (Wang *et al.* 2012). Further, the application of 24-epibrassinolide to two cold-exposed tomato cultivars (tolerant Zhongza9 and sensitive Zhongshu4) increased SOD, POD, and CAT activities by about 50 % (Cui *et al.* 2016). In cold-exposed cucumber plants, 28-homo-brassinolide and 24-epibrassinolide stimulated SOD activity (Hu *et al.*

2010, Farriduddin *et al.* 2011) and Jiang *et al.* (2013) emphasised the significant role of irradiance for post-cold plant regeneration and brassinosteroid action. Cucumber plants were exposed to cold (10/7 °C, 6 d), and then they were regenerated 4 d under high (600 μmol m^{-2} s^{-1}) and low (100 μmol m^{-2} s^{-1}) irradiance. Plants that were treated with 0.1 μM 24-epibrassinolide (spraying on day 5 of cold) and recovered at a high irradiance demonstrated a marked increase in the activities of SOD, APX, monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR), and GR as well as a reduced glutathione/oxidised glutathione (GSH/GSSG) ratio and ascorbate/dehydroascorbate (AsA/DHA) ratio. The plants that recovered under low irradiance had only a slightly higher SOD activity. Finally, the most recent studies of Xia *et al*. (2017) shows that in tomato, BR increases the accumulation of the transcripts of respiratory burst oxidase homolog1 (*RBOH1*) and glutaredoxin (*GRX*) genes, and that BR-induced cold tolerance is accompanied by a higher ratio of reduced/oxidised 2-cysteine peroxiredoxin (2-Cys Prx) and the activation of antioxidant enzymes. The authors suggested that in tomato, BRs enhance cold tolerance through a signalling cascade that involves RBOH1, GRXs, and 2-Cys Prx.

The effect of BRs on antioxidant enzyme activities has also been confirmed in cell suspensions. The application of 24-epibrassinolide (0.1 μM) to *C. bungeana* cell cultures grown at 0 and 4 °C for 3 or 5 d resulted in additional stimulation of the APX, CAT, GR, POD and SOD activities and increase in AsA and GSH content compared to the cell cultures without the added hormone (Liu *et al.* 2009, 2011).

High temperature: The stress caused by a high temperature also activates the antioxidant system. Wu *et al.* (2014b) studied eggplants in the five-leaf stage subjected to high d/n temperatures of 43/38 °C for 8 d and they found a significant increase in the activities of SOD, POD, CAT, and APX by 35, 37, 26, and 14 %, respectively, as well as the AsA and GSH content (by 28 and 25 %, respectively) compared to plants that were not exposed to high temperature. The plants that were previously treated with 0.1 μM 24-epibrassinolide showed increased SOD, POD, CAT, and APX activities by 30, 103, 68, and 22 %, respectively, compared to the plants that were exposed to a high temperature without BR treatment. A marked increase in the AsA and GSH content (48 and 27 %, respectively) was also observed in BR-treated plants compared to stressed, but not BR-treated plants. High temperatures (42/32 °C, 2 d) stimulated the activity of SOD, POD, CAT, and APX in melon (Zhang *et al.* 2014). In this case, 24-epibrassinolide (1 mg dm^{-3}) pre-treatment further enhanced SOD, POD, CAT and APX activities. 24-Epibrassinolide also alleviated the high temperature-induced oxidative stress by increasing the enzymatic and non-enzymatic antioxidant and glyoxalase systems in seedlings of *Ficus concinna* (Jin *et al.* 2015). On the other hand, no effect of 24-epibrassinolide (1 μM) on the activity of antioxidative enzymes was observed in heat-exposed tomatoes (Mazorra *et al.* 2011), which suggests that the effect of BRs may be species or cultivar dependent.

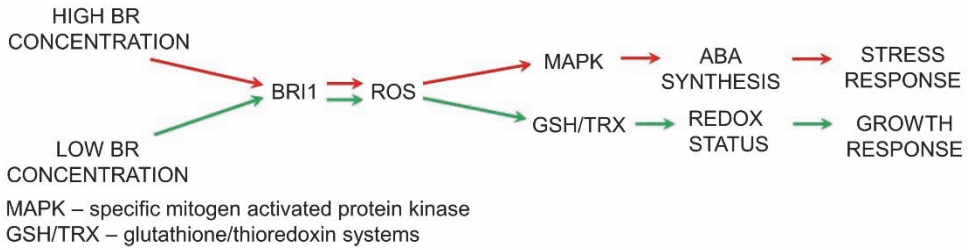

Fig. 2. BR concentration-dependent model of growth and the stress response of plants - role of ROS (modified from Xia *et al.* (2015).

Concluding remarks: According to the best of our knowledge, there is lack of data showing the genetic background of the impact of BR on the antioxidant system under high or low temperature. The results described by Xia *et al.* (2009a) for cucumbers grown under optimal conditions suggest that one of the possible reasons why the activity of antioxidant enzymes increases in the presence of BRs may be higher expressions of the genes coding these enzymes. Xia *et al.* (2009a) also noticed that cucumber plants treated with 24-epibrassinolide (0.1 μM) have higher relative abundance of *CAT*, *POD*, and *MDAR* transcripts than plants that were not treated with BRs. According to many researchers, reactive oxygen species (ROS), in addition to the damaging action on cellular components and structures, also play a signalling role (Kreslavski *et al.* 2012). The concept of BR action on the antioxidant cell system proposed by Xia *et al.* (2009a, 2015) is interesting in this context (Fig. 2). BR receptor activation (as a result of ligand-receptor binding) leads to the production of ROS, *e.g.* H_2O_2, however, spatial and temporal changes in the ROS content depend on the BR content in the tissues. Low BR content can cause a temporary increase in ROS, which stimulates the cell antioxidant system. This leads to a shift in the cell redox balance towards reducing processes and acts as a signal, *e.g.* stimulating photosynthesis and developmental processes. A high BR content in the tissues results in long-term ROS

accumulation, which in turn induces a phosphorylation cascade of mitogen-activated protein kinase. In this case, ROS and kinase stimulate the biosynthesis of abscisic acid, which is the main hormone associated with stress tolerance. In this context, the increased accumulation of BRs as a result of low or high temperature is worth noting (Dockter *et al.* 2014, Pociecha *et al.* 2016). Dockter *et al.* (2014) found that the content of castasterone during the growth of barley is 7.43 pmol g^{-1}(f.m.) at 14 °C, but it increased to 10.31 pmol g^{-1}(f.m.) in plants grown at 26 °C. In turn, Pociecha *et al.* (2016) showed that the castasterone content in rye grown at 18 °C is 4 - 5 pmol g^{-1}(f.m.), however, as a result of six weeks of cold, this content increases to about 14 - 16 pmol g^{-1}(f.m.). This phenomenon can result in the activation of mechanism where ABA is involved (proposed by Xia *et al.* 2015; Fig. 2). On the other hand, BR supply may also slightly increase ROS production even before the onset of stress, which results in a higher antioxidant system activity. The increased activity of antioxidant system in non-stressed plants that were treated with BRs was also reported by Jiang *et al.* (2013).

Photosynthetic pigments

Low temperature: One of the most important symptoms of stress that is caused by a low temperature is a decrease in the content of photosynthetic pigments. Soaking pepper fruits in brassinolide solutions (5, 10, and 15 μM) increases the chlorophyll content by 23, 17, and 24 %, respectively, on the 18th day of storage at 3 °C, compared to plants that were not treated with BR (Wang *et al.* 2012). The senescence of cut leaves of oilseed rape is accelerated at 2 °C, but leaf infiltration with 24-epibrassinolide (0.001 - 1 μM) significantly reduces this phenomenon (Janeczko *et al*. 2007). In 30-d-old cucumber plants, a 40 % reduction in the chlorophyll content after 18 h at 3 - 5 °C was limited to only 16 % after pretreatment with 28-homobrassinolide (10^{-8} M) (Farriduddin *et al*. 2011). In eggplant seedlings, the content of chlorophyll *a*, *b,* and (*a*+*b*) decreased by 32, 57, and 39 %, respectively, during 8 d at 10/5 °C and 24-epibrassinolide (0.05 - 0.2 μM) alleviated this detrimental effects of cold (Wu *et al.* 2014a). He *et al.* (1991) observed that brassinolide improves the greening of etiolated leaves in maize exposed to cold.

High temperature: Stress caused by a high temperature is also accompanied with a decrease in chlorophyll content. In eggplant seedlings exposed to 43/38 °C for 8 d, pretreatment with 0.1 μM 24-epibrassinolide increased the content of chlorophyll *a, b*, and (*a*+*b*) by 17, 27, and 20 %, respectively, compared to heat-exposed plants that were not BR-treated (Wu *et al.* 2014b). The decrease in chlorophyll content was milder in rice supplemented with 24-epibrassinolide prior to exposure to 40/30 °C for 7 d relative to the control (Thussagunpanit *et al.* 2015a). In another study, this rice cultivar treated with 24-epibrassinolide showed an increase in the content of chlorophyll *a*, *b,* and (*a*+*b*) and carotenoids by 86, 74, 83, and 28 %, respectively, on the 7th day of a high temperature exposure, compared to plants that were not treated with BR (Thussagunpanit *et al.* 2015 b). In the plants that are treated with 24-epibrassinolide, the content of individual pigments achieved after 7-d recovery the level of the plants that are not exposed to heat. In maize growing in a net house with maximum temperature of 17.6 - 24.5 ºC and minimum temperature of 2.8 - 7.4 ºC (21 d), decrease in the chlorophyll content is milder when the seedlings are supplemented with 1 μM 24-epibrassinolide (Singh *et al.* 2012). 24-Epibrassinolide also ameliorates photosynthetic pigment losses in melon seedlings exposed to 42/32 ºC for 2 d in the stage of 3 - 4 leaves (Zhang *et al.* 2014).

Concluding remarks: Considering the fact that chlorophyll degradation occurs in plants under stress (Sakuraba *et al.* 2014), it can be assumed that BRs restrict this process. Presumably, BRs affect the synthesis or activity of the enzymes that participate in the processes of chlorophyll breakdown such as Chl *b* reductase (Hörtensteiner and Kräutler 2011). BRs may also participate in regulating chlorophyll biosynthesis. BR-deficient barley mutants at the optimal temperature accumulated a few percent less of chlorophylls compared to wild type (Janeczko *et al.* 2016). Simultaneously, the mutants also had lower content of cytokinins, which are stimulators of chloroplast development and chlorophyll biosynthesis (Parthier 1979). This may suggest an indirect effect of BRs on chlorophyll biosynthesis through cytokinin action. The engagement of BR signalling in chlorophyll accumulation is also suggested by Yoshizawa *et al.* (2014). All of these hypotheses require verification in plants that are exposed to low/high temperatures.

Gas exchange, photosystem activities, and CO_2 assimilation

Low temperature: Gas exchange parameters are very susceptible to environmental changes. In cucumber plants, 28-homobrassinolide (10^{-8} M) and 24-epibrassinolide (0.1 mg dm^{-3}) reduce the cold-induced decreases in

net photosynthetic rate (P_N), stomatal conductance (g_s), internal carbon dioxide concentration (c_i), and transpiration rate (E) (Hu *et al.* 2010, Farriduddin *et al.* 2011). The effect is confirmed by the 24-epibrassinolide treated eggplants exposed to 10/5 °C for 8 d (Wu *et al.* 2014a).

Low temperature usually decreases the efficiency of the photosystems (PS). Fluorescence methods permit a detailed characterization of any disturbances in the energy flow especially within PS II. Cucumber plants in the 3-leaf stage that were sprayed with 24-epibrassinolide (0.1 mg dm^{-3}) prior to exposure to 12/8 °C for 3 d reached higher values of the effective quantum yield of PS II photochemistry (Φ_{PSII}) and photochemical quenching coefficient (q_P) and lower nonphotochemical quenching (NPQ) than plants that were not sprayed (Hu *et al.* 2010). The beneficial effect of this hormone on the efficiency of PS II was also observed during the post-stress regeneration (Hu *et al.* 2010). After the treatment of 28-homobrassinolide (10^{-8} M), 30-d-old cucumber plants reached higher values of PS II maximum quantum yield (variable to maximum fluorescence ratio, F_v/F_m) under cold (3 - 5 °C, 18 h) compared to plants without the BR (Farriduddin *et al.* 2011). Further, the F_v/F_m ratio in cucumber was restored to the level before cold by applying 24-epibrassinolide on the 5th day of cold (at 10/7 °C for 6 d), but only under high irradiance (600 μmol m^{-2} s^{-1}) (Jiang *et al.* 2013). No effect of BR on PS I efficiency was observed in this case. In seedlings of eggplant exposed to 10/5 °C for 8 d, the values of F_v/F_m, potential photochemical efficiency (F_v/F_0), Φ_{PSII}, excitation energy capture efficiency of open centres (F_v'/F_m'), and qP decreased by 9, 32, 17, 9, and 14 %, respectively, and 24-epibrassinolide (0.05 - 0.20 μM) significantly counteracted this phenomenon (Wu *et al.* 2014a). Finally, in cold-treated tomato plants, with a partial deficiency in BRs, F_v/F_m is decreased (Xia *et al.* 2017). Overexpression of the BR biosynthetic gene *Dwarf* or the application of 24-epibrassinolide increased the F_v/F_m under cold (Xia *et al.* 2017).

Under cold, BRs stimulated activity of enzymes responsible for various stages of carbon dioxide binding. The activity of carbonic anhydrase (CA), which participates in the transport of inorganic carbon (Henry 1996), decreased by 24 % in cucumber plants under cold but did not change in plants that were sprayed with 28-homobrassinolide (10^{-8} M) prior to cold (Farriduddin *et al.* 2011). Research conducted by Jiang *et al.* (2013) on cucumber plants exposed to 10/7 °C for 6 d showed that BR play a role in regulating CO_2 binding by ribulose-*bis*-phosphate carboxylase/oxygenase (Rubisco). Plants that were sprayed with 24-epibrassinolide prior to cold treatment and recovered under irradiance of 600 μmol m^{-2} s^{-1} reached a significantly higher Rubisco content, total Rubisco activity, and Rubisco activase (RCA) activity compared to the stressed plants that were not sprayed. This phenomenon did not occur during recovery under irradiance of 100 μmol m^{-2} s^{-1}. An increase in Rubisco activity after 24-epibrassinolide application was also confirmed by Pociecha *et al.* (2016) in a study on winter rye plants exposed to 3 and 6 weeks of cold. On the other hand, in perennial ryegrass, when 24-epibrassinolide (0.25 mg dm^{-3}) was applied before plant exposure to 4 ºC for 6 weeks, the Rubisco activity was reduced by about 30 % but only in sensitive cv. Amarant and not in the resistant cv. Flinston (Pociecha *et al.* 2017). This suggests that the effect of BRs on Rubisco activity in cold may be cultivar dependent.

High temperature: In rice, 24-epibrassinolide limited heat-induced (40/30 °C, 7 d) disturbances of gas exchange. A significant increase was recorded in P_N, g_s, and E by 325, 240, and 235 %, respectively and a decrease in internal CO_2 concentration (c_i) by 97 % in plants treated with 24-epibrassinolide compared to non-treated and heat-exposed plants (Thussagunpanit *et al.* 2015a,b). Positive effect of 24-epibrassinolide (0.005 - 0.20 μM) on the gas exchange in eggplants subjected to 43/38 °C (8 d) was also observed by Wu *et al.* (2014b). In melon plants exposed to 42/32 °C (2 d), spraying with 24-epibrassi-nolide (1 and 1.5 mg dm^{-3}) maintained P_N and g_s on a level close to those at 30/20 °C (Zhang *et al.* 2014). High temperature tolerant and sensitive cultivars of melon grown at 42/32 °C for 2 d also had an improved P_N and higher values of F_v/F_m, F_v'/F_m', Φ_{PSII}, and qP and lower values of F_0 and NPQ after 24-epibrassinolide treatment (Zhang *et al.* 2013, 2014). The decrease in PS II effectivness caused by temperature 43/38 °C (8 d) in eggplant seedlings was compensated by 24-epibrassi-nolide (0.05 - 0.2 μM) (Wu *et al.* 2014b). In rice, 24-epibrassinolide also effectively alleviated the negative effects of 40/30 °C (7 d) on PS II (Thussagunpanit *et al.* 2015 a,b). The minimum fluorescence (F_0) was lower in plants that were sprayed with 24-epibrassinolide before stress, and parameters such as F_m, F_v/F_m, F_v'/F_m' had higher values than those noted in the stressed plants without BR application. The electron transport rate (ETR) in plants treated with 24-epibrassinolide was also significantly improved (Thussagunpanit *et al.* 2015a). An analysis of chlorophyll *a* fast fluorescence kinetics in spring barley exposed to 42 °C (3 h) indicated an abnormal energy flow in PS II that was manifested in a decrease in energy absorption (ABS/CSm), energy flux for trapping (TRo/CSm), and energy flux for electron transport (ETo/CSm) (Janeczko *et al.* 2011). The protective effect of 24-epibrassinolide (0.25 mg dm^{-3}, leaf infiltration before heat) was observed in the first leaf. ABS/CSm, TRo/CSm, and ETo/CSm values were higher by approximately 23, 49, and 69 %, respectively, compared to the values noted in the stressed plants that were not treated with BR. Xia *et al.* (2009a) noticed that after cold (8° C), cucumber plants that were treated with 24-epibrassinolide had higher values of ETR than untreated plants. Application of BR biosynthesis inhibitor brassinazole (Brz) lowered ETR values below the control

values while treatment of plants with combination of Brz + 24-epi-brassinolide restored the values to the control level.

Concluding remarks: BRs regulate gas exchange in plants exposed to low/high temperatures. Improved P_N is often connected with stimulation of the activity of the enzymatic system. Rubisco activity is lower in BR-deficient mutants of barley (under optimal conditions) which may confirm regulatory role of BRs in functioning of the enzyme (Janeczko *et al.* 2016). The BR-stimulated accumulation of the transcripts of genes encoding Rubisco or RCA (Xia *et al.* 2009a,b) was also observed. Xia *et al.* (2009b) showed that BR promotes but Brz inhibits expression of genes encoding the Calvin cycle enzymes required for Rubisco regeneration. All of the described molecular mechanisms of BR action should be confirmed for plants that are cultured at low/high temperature. The simplest reason for the decreased efficiency of PS II during plant exposure to high/low temperatures seems to be a reduced content of photosynthetic pigments (described above). Further, the correct structure and functioning of the thylakoid membranes, where electron carriers are located, is essential to maintain high PS efficiency. The stabilising effect of BRs on the structure and properties of the membranes at low/high temperatures is mentioned in another chapter. Although microscopic analyses show that the application of 24-epibrassinolide maintains the typical shape of the chloroplasts and promotes the formation of grana in the stressed plants, more detailed studies of the effect of BR on photosynthetic light reactions in plants growing at low/high temperature should be done, especially using BR-biosynthesis or signalling mutants. The positive impact of BRs on the thylakoid membranes and chloroplast ultrastructure was confirmed in salt-stressed cucumber plants (Yuan *et al.* 2012). Finally, BRs may also have an indirect influence on CO_2 assimilation by improving plant water management, even if values of E and g_s are usually higher in the BR-treated plants grown under both low or high temperatures.

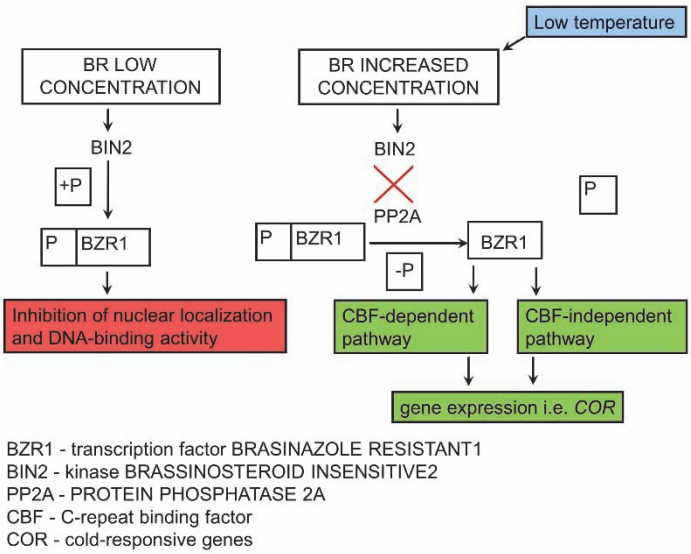

Fig. 3. A model of plant response to low temperature and low [4 - 5 pmol g^{-1} (f.m.)] or increased [14 - 16 pmol g-1 (f.m.)] BR concentration (modified from Wang *et al.* 2014 and Li *et al.* 2017). Brassinosteroid concentrations are exemplary given for rye based on Pociecha *et al*. (2016); P - phosphate. +P - phosphorylation, -P - dephosphorylation, P - phosphate attached to the protein or not attached to protein. The dephosphorylation step of BZR1 is blocked by BIN2 in the case of low BR concentration (Wang *et al*. 2014). A higher content of BRs allows the inactivation of BIN2 and the dephosphorylation of BZR1, which in turn can directly activate BR-responsive genes or act indirectly in a CBF-dependent way (Wang *et al*. 2014, Li *et al*. 2017). For more detail see chapter "Biosynthesis of proteins and accumulation of proline and glycinebetaine".

Sugar accumulation

Low temperature. An increase in sucrose content under cold is especially important for winter plants where it is a part of the hardening before frost. In perennial ryegrass, after pretreatment with 24-epibrassinolide (0.25 mg dm^{-3}) and cold acclimation at 4 °C for 6 weeks, Pociecha *et al.* (2017) observed a significant decrease of glucose and fructose content (in cv. Amarant by about 38 and 41 % and in cv. Flinston by about 28 and 63 %, respectively) compared to untreated plants. It is important that this effect was accompanied by an increase in content of sucrose (54 to 77 %) and also of protective sugars nystose and 1-kestose in both cultivars. The picture was not so

clear in winter rye plants that were grown at 4 °C for six weeks and pretreated with 24-epibrassinolide (Pociecha *et al.* 2016). The content of glucose and fructose decreased (by 33 and 29 %, respectively) and the content of sucrose and nystose increased only in the moderately winter resistant cv. Stach, but in the winter resistant cv. Dańkowskie Złote, a decrease in the glucose (44 %) and fructose (36 %) content was not accompanied by an increase of the sucrose and nystose content (Pociecha *et al.* 2016). Cold induced the accumulation of sugars also in seedlings of cold sensitive maize inbred line LM-17. Application of 24-epibrassinolide (1 μM) further increased the glucose, sucrose, and starch content by about 15, 28 and 45 %, respectively, compared to cold stressed plants without BR treatment (Singh *et al.* 2012).

High temperature. Changes of sugar accumulation under high temperature are not as crucial as in the case of a cold acclimation. A marked decrease in total soluble sugar content was found in rice plants subjected to 40/30 °C (7 d) compared to the control (Thussagunpanit *et al.* 2015b) but an increase in total soluble sugar content (by 23 %) was recorded in plants that were pretreated with 24-epi-brassinolide compared to plants that were subjected to heat without being sprayed with BR. In the same cultivar grown in the field during the hot season, 24-epibrassinolide increased the total soluble sugar content in the straw (by 107 %). The hormone also increased reducing sugar content in the straw (by 72 %) and the starch content in the straw (by 27 %), husk (by 24 %), and seeds (by 35 %) (Thussagunpanit *et al.* 2015a). Another rice cultivar exposed to a temperature of 35/32 °C for 12 h also demonstrated a decrease in the content of soluble sugars, which was further reduced by a mixture of plant growth regulators containing BRs (Fahad *et al.* 2016). Compared to rice, eggplant seedlings subjected to 43/38 °C (8 d) have higher soluble sugar content compared to unstressed plants and the application of 24-epi-brassinolide (0.1 μM) enhanced this effect (Wu *et al.* 2014).

Concluding remarks. In the case of heat treatment, the sugar accumulation in plants may fluctuate possibly due to a dependence on the general performance of photosynthesis. However, the increased sucrose concentration under cold lowers the freezing point of the cell solution, and thus it contributes to a higher resistance to frost. The stimulation of sucrose production along with an accumulation of protective sugars (like nystose) by BRs suggests that these hormones play a role in the process of acquiring frost resistance. A BR-stimulated increase in CO_2 assimilation (described in previous chapter), may be the first step in this process, but BRs may also affect the activity of the enzymes that directly participate in sugar biosynthesis. The involvement of BR in the regulation of sucrose synthesis from glucose and fructose was studied for plants grown under optimal conditions, for example by Yu *et al.* (2004) and Janeczko *et al.* (2016). Yu *et al.* (2004) observed an increase in the activity of the enzymes that are involved in sucrose biosynthesis in cucumber after exogenous BR application. According to Janeczko *et al.* (2016), barley mutants with a reduced BR production accumulated more glucose and fructose and less sucrose than the wild type, which may also confirm dysfunctions in sucrose biosynthesis as a result of a BR deficiency. The mechanisms of the action BR on sugar biosynthesis require further confirmation in plants grown at low/high temperatures.

Biosynthesis of proteins and accumulation of proline and glycinebetaine

Low temperature. The acclimation of plants to low temperature requires an adjustment of a significant part of protein metabolism. In maize seedlings grown in a net house (max. temp. 17.6 - 24.5 ºC, min. temp. 2.8 - 4 ºC; 21 d), the content of total proteins was 28 % lower than in the control in a greenhouse (25/18 °C) but 24-epi-brassinolide (1 μM) ameliorated this decrease (Singh *et al.* 2012).

Among the particular proteins with an accumulation stimulated by BRs under cold is the ATP-synthase β subunit. Cold tolerant and sensitive cultivars of tomato sprayed with 24-epibrassinolide before cold (12/6° C, 12 d) accumulated more of the ATP-synthase β subunit, thereby prevented a cold-induced decrease of its content (Cui *et al.* 2016).

In mango fruits stored at 5 °C (28 d), brassinolide (10 μM) increased the accumulation of the gene transcripts encoding the membrane-associated proteins: temperature induced lipocalin (*TIL*), type II SK2 dehydrin (*TSD*), abscisic stress ripening-like protein (*ASR*), and the remorin family proteins (*REM*) (Li *et al.* 2012). These proteins are associated with the stress response and may also perform important functions in membrane formation and repair. Cold-responsive (COR) proteins belong to hydrophilic polypeptides, which help to stabilise membranes, and thus may protect against damage induced by the cold (Tomashow 1998). According to Eremina *et al.* (2016), BRs enhance plant resistance against freezing stress *via* the regulation of the expression of *COR* genes. BR-controlled basic helix-loop-helix transcription factor (CES), can contribute to the constitutive expression of the *C-repeat/dehydration-responsive element binding factors (CBFs)* transcriptional regulators that control the *COR* gene expression. *CBF1, CBF3*, and that *COR15A* transcript accumulation is increased in the wild type *A. thaliana* as a result of the application of 24-epibrassi-nolide (250 nM). *BRI1oe* (BR-hypersignalling line) mutants have higher

transcriptions of *CBF1* and *CBF3* as well as *COR15A, COR15B*, *COR47*, and *COR78* compared to the wild type. In turn, the BR-signalling defective mutants *bri1-301* and *bri1-1* had a significantly lower accumulation of these transcripts compared to the wild type plants. The effect of BRs on COR proteins was also confirmed by the studies of Kagale *et al.* (2007) and Divi *et al.* (2016), which proved that *COR* genes were up-regulated by BR. According to Li *et al.* (2017), BRs act *via* BZR1 (transcription factor - brassinazole resistant 1) in a CBF-dependent manner in cold but also in a CBF-independent manner (Fig. 3). The dephosphorylation step of BZR1, which is blocked by BIN2 in the case of low BR content, is crucial (Wang *et al*. 2014). A higher content of BRs permits the inactivation of BIN2 and the dephosphorylation of BZR1, which in turn can directly activate BR-responsive genes or act indirectly in a CBF-dependent way (Wang *et al*. 2014, Li *et al*. 2017).

Proline is a specific amino acid that acts as an antioxidant, osmolyte, signalling molecule, and metal chelator (Hayat *et al.* 2012). In eggplants, exposed to 10/5 °C (8 d), a drastic increase in the proline content (by 793 %) was observed compared to plants that were not subjected to cold (Wu *et al.* 2014a). Pretreatment with 24-epibrassinolide (0.1 μM) additionally enhanced this effect by 87 % (Wu *et al.* 2014a). Fruits of tomato that were treated with brassinolide (6 μM) and stored at 1 °C (3 weeks) also had a 65 % higher proline content compared to low-temperature-exposed fruits without BR application (Aghdam *et al.* 2012). Glycine betaine also acts as osmolyte (Ashraf and Foolad 2007). Seedlings of maize treated with 24-epibrassinolide (1 μM) and exposed to cold show an increase of the glycine betaine content by about 85 % compared to chilled plants that were not supplemented with this hormone (Singh *et al.* 2012).

High temperature. In plants at high temperature, protein content may decrease (melon, 42/32 °C, 2 d) or increase (eggplant 43/38 °C, 8 d) (Zhang *et al.* 2014, Wu *et al.* 2014b). The application of 24-epibrassinolide (0.05 - 1.5 mg dm^{-3}) increased protein content by an average of 35 % in both species. A decrease in the protein content at 35/32 °C in rice was ameliorated by a mixture of plant growth regulators with BRs (Fahad *et al.* 2016). Brassinolide (10 nM) increased also the protein content in cell cultures of *Chlorella vulgaris* grown at 30 - 40 °C (Bajguz 2009).

Under high temperature, heat shock proteins (HSP) play a chaperone function and prevent damage to many heat-sensitive cellular proteins. In seedlings of oilseed rape subjected to 45 °C, *hsp101*, *hsp90*, and *hsp70* genes were expressed, and 24-epibrassinolide promoted their transcripts accumulation, especially of *hsp101* and *hsp90* (Dhaubhadel *et al.* 1999, 2002). The 24-epibrassinolide also stimulated the accumulation of small HSPs (sHSPs) after heat treatment. A significant accumulation of *HSP* transcripts (particularly in plants that were treated with 24-epibrassinolide) was found during heat but after heat their accumulation decreased and in BR-treated plants even to a lower level than in control plants (Dhaubhadel *et al.* 2002). It is worth noting that Dockter *et al.* (2014) reported an increased amount of endogenous BRs in plants grown at high temperature. Finally, Samakovli *et al.* (2014) proposed HSP as important players in BRs signalling. Briefly, if a BR is not bound to a receptor, HSP90 in combination with BIN2 in the nucleus prevents the dephosphorylation of BES1 and BZR1 and the expression of BR-responsive genes. If BR is bound to receptor, the HSP-BIN2 complex is transferred to the cytoplasm and the dephosphorylation of BES1 and BZR1 permits the expression of BR-regulated genes.

Eggplant seedlings subjected to 43/38 °C for 8 d as well as melon seedlings subjected to 42/32 °C for 2 d, were characterised by an increase in the proline content compared to the control (Wu *et al.* 2014b, Zhang *et al.* 2014). The application of 24-epibrassinolide (0.1 μM) to the eggplant and 24-epibrassinolide (0.05 - 1.5 mg dm^{-3}) to the melon enhanced this increase. Rice exposed to 35/32 °C and treated with a hormonal mixture containing BRs also had an increased proline content (Fahad *et al.* 2016).

Concluding remarks. Microarray and proteomic analyses of BR-treated plants and BR-deficient mutants proved that many genes are BR-regulated (Yang and Komatsu 2004, Deng *et al.* 2007). A large part of this knowledge has been obtained from studies of plants grown under optimum temperature. More detailed studies should now be devoted to the aspect of low/high temperature treatments, which would be helpful in explaining the mechanisms of BR action under these stresses. Currently, it appears that BRs are engaged in controlling the *COR* expression at low temperature and *HSP* expression at high temperature. They also promote the accumulation of the ATP-synthase β subunit and the transcripts of the *TIL, TSD, ASR,* and *REM*, which are important in the function, formation, and repair of membranes.

Many studies have indicated a positive relationship between plant stress tolerance and the accumulation of proline and glycine betaine (Ashraf and Foolad 2007). The BR-stimulated accumulation of both osmolytes may then support plant acclimation to low or high temperature. The study of Janeczko *et al.* (2016) on barley BR-deficient mutants demonstrated that these plants - under optimum growth conditions and under drought - indeed accumulated less proline than the wild type, which suggests the importance of BR in regulating the proline content. According to Farriduddin *et al.* (2009), BRs activate the proline biosynthesis enzymes.

A search for other proteins that may directly or indirectly contribute to a brassinosteroid-mediated increase in the tolerance to low/high temperature is an open question.

Other effects of BRs application

Low temperature: The acclimation of plants to a low temperature is connected with the significant changes in hormonal homeostasis, especially in the content of the so-called stress hormones. *C. bungeana* cell cultures that were subjected to 0 and 4 °C (3 d) accumulated abscisic acid [ABA);17 and 22 ng g^{-1}(f.m.)], respectively. Cells cultured with 24-epibrassinolide (0.1 μM) accumulated 33 and 27 ng(ABA) g^{-1}(f.m.) at 0 and 4 °C, respectively (Liu *et al.* 2011).

Application of 24-epibrassinolide (0.25 mg dm^{-3}) to perennial ryegrass combined with cold (4 ºC, 6 weeks) resulted in an increased content of the ethylene precursor (ACC) compared to the plants without BR treatment (Pociecha *et al.* 2017). The jasmonic acid content increased only in cv. Flinston and no effect of 24-epibrassinolide on salicylic acid content was noted.

In addition to the well-known effect of BRs on Rubisco or antioxidant enzymes activity, the activity of other important enzymes has also been shown. In cucumber plants under cold (5/3 °C, 18 h), nitrate reductase activity was reduced by 27 % compared to unstressed plants; however, 28-homobrassinolide (10^{-8} M) neutralised this effect (Farriduddin *et al.* 2011). Pectin methylestereases (PMEs) play a significant role in plant cold/frost tolerance as they catalyze the dimethyl-esterification of cell wall polygalacturonans (Micheli 2001, Qu *et al.* 2011). The activity of PMEs was markedly increased in the wild type *A. thaliana* grown at 0 °C (24 h) and less increase was observed in the *bri1-116* homozygous mutant, whereas in the *bzr1-D* mutant, the PME activity was similar as in the wild type (Qu *et al.* 2011). Ammonia-lyase (PAL) is a key enzyme in the metabolism of phenols, which help to protect plants against stress (Lafuente *et al.* 2003). Tomato fruits that were stored at 1 °C (3 weeks) had increased PAL activity; brassinolide further enhanced the effect and simultaneously increased the total phenol content (Aghdam *et al.* 2012). An increase in soluble phenolics was also noted in

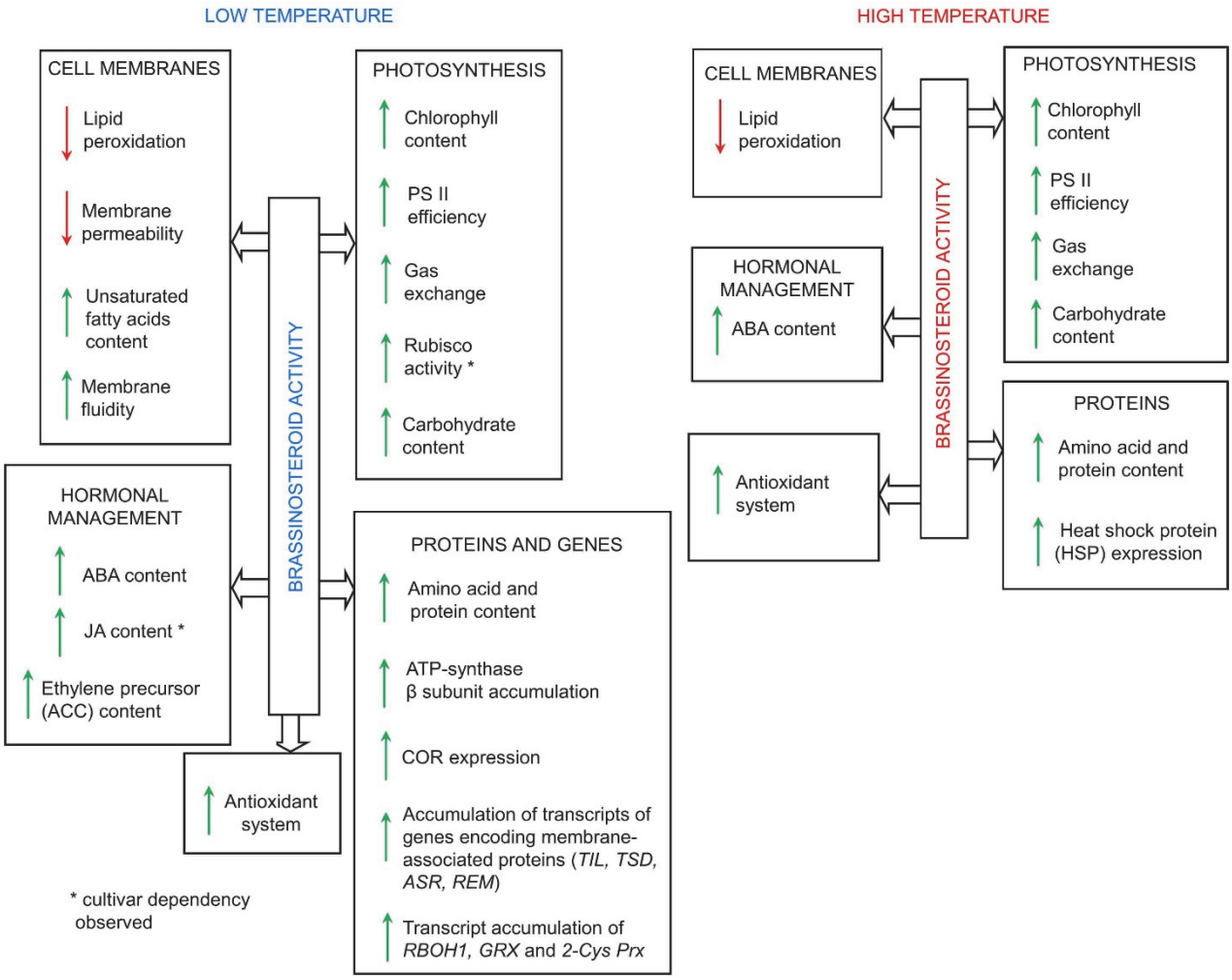

Fig. 4. Multidirectional activity of BRs in the regulation of metabolism of plants exposed to low and high temperatures.

the perennial ryegrass cv. Flinston (but not in cv. Amarant) grown at 4 ºC for 6 weeks and treated with 24-epibrassinolide (0.25 mg dm^{-3}) (Pociecha *et al.* 2017). To the best of our knowledge, there is no data about the effect of BRs on the activation of the above-mentioned enzymes in high temperature.

High temperature: Plant hormonal balance is also changed by a high temperature. *Chlorella vulgaris* cells at temperatures of 30, 35, and 40 °C (3 h) accumulated higher amounts of ABA, which increased along with the increase in temperature to 1.65, 2.2, and 2.6 ng g^{-1}(f.m.), respectively). Addition of 10 nM brassinolide stimulated ABA accumulation at 40 °C to 4.32 ng(ABA) g^{-1}(f.m.) (Bajguz 2009).

Concluding remarks: Plant hormones remain in the connection network and control their own biosynthesis and transport (Weiss and Ori 2007). BRs also contribute to the functioning of this network (Zhang *et al.* 2009), but little is specifically known about their role in terms of temperature stress. As is shown here, BRs increase the accumulation of ABA at low or high temperature. Barley mutants in the *HvDWARF* gene encoding C6-oxidase, which is involved in BR biosynthesis, accumulated a significantly lower amount of ABA compared to the wild type plants (Janeczko *et al.* 2016), which may confirm an interplay between these hormones. During cold hardening of ryegrass, BR also stimulated the accumulation of other stress hormones, jasmonic acid and ethylene precursor ACC. The role of BRs in the hormonal network of plants that are exposed to high temperature and especially to low temperature (significant in hardening to frost) definitely requires further studies.

The detailed mechanisms that are responsible for BR impact on the activity of the aforementioned enzymes remain unknown. We can only theoretically state that BRs act by up-regulating the genes coding those enzymes. It is also possible that there is an indirect effect of BRs *via* the stimulation of the production of chaperones (HSP) that, among others, may help to protect the cell enzymatic system against a loss of activity under temperature stress.

Improvement of plant resistance to low and high temperatures

Low temperature: The viability of cells of *Chorispora bungeana,* measured by 2,3,5-triphenyltetrazolium chloride (TTC) reduction test, decreased during growth at 0 and 4 °C for 3 - 5 d, but supplementation with 24-epibrassinolide (0.05 and 0.1 mg dm^{-3}) counteracted this effect (Liu *et al.* 2009, 2011). Brassinosteroids also reduce the cold damage to plants such as cucumber or maize, which are characterized by a relatively low cold tolerance. About 30 % reduction in the shoot and root length, leaf area, and fresh and dry masses of cucumber exposed to 5/3 °C for 18 h were largely prevented by 10^{-8} M 28-homobrassinolide (Farriduddin *et al.* 2011). In the cucumber cv. Jinchun No. 4, 24-epibrassinolide (0.1 μM) when applied on the 5th day of cold (10/7 °C) counteracted the reductions in the growth and dry mass but the effect was more pronounced in plants treated under high irradiance (Jiang *et al.* 2013). Singh *et al.* (2012) found that seedlings of maize exposed to cold in net house, showed a reduction in plant height (35 %) and fresh mass (24 %) compared to the those in a greenhouse. The pre-treatment with 1 μM 24-epibrassinolide increased plant height and fresh and dry masses by about 15, 36, and 2 %, respectively, compared to the plants without BR pre-treatment. Similar effects were also observed by He *et al.* (1991) for brassinolide pre-treated maize.

The application of BRs provides a chance to minimise fruit damage during cold storage. A marked reduction of chilling injury index (CI) was observed after immersion of tomato cv. Newton fruits in 3 and 6 μM brassinolide solution compared to fruits that were not BR-treated and stored at 1 °C for 3 weeks (Aghdam *et al.* 2012). The similar effect of brassinolide (10 μM) on CI was observed for mango fruits stored at 5 °C for 28 d (Li *et al.* 2012). The BR application limited the sunken lesions of the pericarp and pulp darkening accompanying tissue necrosis. Pepper fruits also responded to storage at 3 °C for 18 d by the occurrence of damage such as surface pitting and calyx discoloration (Wang *et al.* 2012). A gradual increase in the CI index was observed with the duration of fruit storage and the greatest damage was recorded on the last (18th) day. Pre-treatment of the fruits with a 15 μM brassinolide solution alleviated these effects by reducing the CI index by about 69 % compared to the fruits that were stored in the cold without brassinolide treatment.

Relatively new research was dedicated to the possibility of limiting frost damage by BR. Winter rye plants sprayed with 24-epibrassinolide (0.25 mg dm^{-3}) before a 6-week acclimation at 4 °C showed less frost damage and a higher survival rate (Pociecha *et al.* 2016). Winter wheat seedlings sprayed with 24-epibrassinolide (0.25 or 0.05 mg dm^{-3}), cold-acclimated and then exposed to -12°C had a higher survival rate than those that were not BR-treated (Janeczko 2016). Perennial ryegrass acclimated at 4 ºC for 6 weeks and treated with 24-epi-brassinolide (0.25 mg dm^{-3}) had an increased frost tolerance (by 35 %) compared to plants without the BR treatment (Pociecha *et al.* 2017). The importance of BRs in the process of acclimation and frost tolerance was confirmed by a study on *A. thaliana* mutants with impaired BR-signalling (Eremina *et al.* 2016). Compared

to the wild type, BR-hypersignalling mutants *35S:BRI1-GFP (BRI 1oe)* that were exposed to -10 °C (6 h) demonstrated a higher frost tolerance. In turn, *bri1-301* and *bri1-1* mutants with disturbances in BR-signalling exhibited a drastically reduced frost tolerance. The rate of survival of the wild type plants was about 50 %, while for *BRI 1oe*, it was 70 %, and for *bri1-301* only a few percent. Plants of the *bri1-1* mutant did not survive the frost.

High temperature: Bromegrass cell suspension cultures treated with 10 μM 24-epibrassinolide had significantly higher cell viability at high temperatures (40 - 45 ºC, 45 - 90 min) than the untreated cultures (Wilen *et al.* 1995). In one of the first studies that showed the alleviating effects of BRs on whole plants at high temperature, Dhaubhadel *et al.* (1999) assessed the effect of 45 °C (4 h) on oilseed rape and tomato. This temperature was lethal for most of the plants, but 24-epibrassinolide (1 μM) increased plant survival. In the studies of Mazorra *et al.* (2011), all of the tomato cv. Ailsa Craig seedlings and approximately 80 % of cv. Money-maker seedlings survived at 45 °C for 4 h but not for 8 h. 24-Epibrassinolide (1 μM) improved the survival of plants stressed for 5, 6, and 7 h. A temperature of 45 °C (7 h) was then tested on tomato the brassinosteroid mutants: *curl3*$^{-abs}$, *d*^{x}, and 35SD. Approximately 90 % of the *curl3*$^{-abs}$ seedlings survived but only 18 - 30 % of *d*^{x} and 35SD seedlings survived. The survival rate of the *d*^{x} mutants that were treated with 24-epibrassinolide (1 μM) was approximately 2.6-fold higher compared to the *d*^{x} plants that were not treated. 24-Epibrassinolide also improved the survival of the *curl3*$^{-abs}$ seedlings. In eggplant (cv. Huqie 9), a temperature of 43/38 °C (8 d) reduced plant height, stem diameter, and shoot and root fresh masses by 28, 35, 61, and 58 %, respectively, compared to the control (Wu *et al.* 2014b). 24-Epibrassi-nolide (0.1 μM) increased the values by 26, 43, 55 and 43 %, respectively, compared to the control (high temperature, no BR spraying). A high temperature (42/32 °C, 2 d) decreased fresh masses of shoots and roots in melon plants (Zhang *et al.* 2013). 24-Epibrassinolide (1 mg dm^{-3}) treated plants had a higher mass by about 14 - 27 %. 24-Epibrassinolide also decreased the heat injury index as measured by observations of leaf yellowing, curling, and senescence compared to the stressed plants that did not undergo hormone spraying. In another work of these authors on the same species, a higher BR concentration (1.5 mg dm^{-3}) was also effective (Zhang *et al.* 2014). In rice, a temperature of 40/30 ºC (7 d) reduced the shoot and root fresh masses and leaf area, but this effect was limited by 24-epibrassinolide (10^{-8} M) (Thussagunpanit *et al.* 2015b). In the same cultivar grown in the field during the hot season (February to May 2012, Thailand), 24-epi-brassinolide increased the number of filled seeds per panicle by 16 % and increased the seed mass by 35 % (Thussangunpanit *et al.* 2015a).

A high temperature during plant development may negatively affect pollen viability. Tomato pollen that were inoculated on a medium with 1 μM 24-epibrassi-nolide and exposed to 35 ºC for 4 h showed a higher germination, enhanced tube growth, and lower pollen bursting compared to the pollen without BR treatment (Singh and Shono 2003). In rice at a temperature of 35/32 °C, a reduction in pollen fertility, anther dehiscence, number of germinated pollens on the stigma and total number of pollen occurred (Fahad *et al.* 2016). Plants administered with a mixture of growth regulators containing BRs had higher values of the aforementioned parameters in comparison to the stressed plants that had not been sprayed.

Concluding remarks: To conclude, plant resistance to high/low temperatures is an important issue in agriculture and horticulture as well as in fruit and vegetable storage. Many methods are used to improve plant resistance, including applications of protective compounds (Senaratna *et al.* 1988). The results summarised in this chapter show that BRs have the potential to reduce many of the negative effects of low/high temperatures, which encourages the future practical application of BRs.

Conclusions and future perspectives

The negative effects of low or high temperature are reduced in plants to which BRs are applied before or during stress. The following ways of BR action are possible: alterations in the membrane physicochemical properties, regulation of the expression of some genes (*HSP, COR*), and regulation of metabolism through other hormones or signalling molecules (ABA or hydrogen peroxide) (Figs. 4 and 5). Most of the literature on this subject is related to the effect of exogenous BRs on plants that are subjected to low/high temperatures. In the future, more emphasis should be placed on studying the impact of low/high temperatures on BR-biosynthesis/signalling mutants or on plants that are treated with BR inhibitors. This would strengthen the knowledge about the role of these hormones in plant responses to low/high temperatures. Nevertheless, results from studies with the exogenous application of BRs on plants that are exposed to low/high temperatures are a good starting point for agricultural/horticultural practice. Agrochemicals with BRs may help to protect crops against the negative impact of low/high temperatures (*i.e.*, by improving frost resistance). It is worth noting that BRs act in very small concentrations and are natural biodegradable substances. Simultaneously, the use of classical breeding methods or

genetic engineering methods to modulate the BR content in plants or to change the signal transduction pathways that are derived from the BR receptors are promising for the creation of new more stress resistant plants.

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Table 1. Suppl. Plant species, temperature ranges, and brassinosteroids used in the experiments (d/n - day/night, MS - Murashige and Skoog medium).

Authors (year)	Plant species	Temperature and exposure	Brassinosteroid/ concentration/ application method
Janeczko *et al.* (2007)	*Brassica napus* (cv. Lycosmos) - primary leaves and cotyledons	2 ºC - 4 d	24-epibrassinolide/0.05 and 1 µM/ injection into the apoplast of cotyledons or primary leaves, before low temperature treatment
Janeczko *et al.* (2009)	*B. napus* (cv. Górczański) - callus culture	5 ºC - 2 weeks	24-epibrassinolide/ 100 nM/ culture medium
Liu *et al.* (2009)	*Chorispora bungeana* - cultured cells	4 °C - 5 d 0 °C - 5 d	24-epibrassinolide / 0.05 mg dm^{-3}/ transfer on liquid MS medium with hormone 3 d before low temperature treatment
Hu *et al.* (2010)	*Cucumis sativus* (cv. Jinyan No 4) - leaves	12/8 °C (d/n) - 3 d	24-epibrassinolide / 0.1 mg dm^{-3}/ foliar spraying before cold treatment
Farriduddin *et al.* (2011)	*C. sativus* (cv. Summer best) - leaves	8/10 °C - 18 h 3/5 °C - 18 h	28-homobrassinolide / 10^{-8} or 10^{-6} M/ foliar spraying 48 h before low temperature treatment (3 cm^3 $plant^{-1}$)
Liu *et al.* (2011)	*Chorispora bungeana* - cultured cells	4 °C - 3 d 0 °C - 3 d	24-epibrassinolide / 0.1 µM/ transfer on liquid MS medium with hormone, before low temperature treatment
Qu *et al.* (2011)	*Arabidopsis thaliana* (Col-00 *bzr1-D* - BZR1 stabilized mutant	0 °C - 24 h	24-epibrassinolide / 1 µM/ incubation of the seedlings in solution before low temperature treatment
Aghdam *et al.* (2012)	*Soluanum lycopersicum* (cv. Newton) - fruits	stored at 1 °C - 3 weeks	brassinolide / 3 and 6 µM/ immersing of the fruits in solution for 5 min before low temperature treatment
Li *et al.* (2012)	*Magnifera indica* (cv. Zill) - fruits	stored at 5 °C - 28 d	brassinolide / 10 µM/ immersing of the fruits in aqueous solution of brassinolide for 10 min; before low temperature treatment
Singh *et al.* (2012)	*Zea mays* (line LM-17) - leaves	net house: max. 17.6 - 24.5 ºC min. 2.8 - 7.4 ºC - 21 d	24-epibrassinolide/ 0.1 µM, 1 µM, and 10 µM/ foliar spray o 10-d-old plants (10 cm^3 $plant^{-1}$)
Wang *et al.* (2012)	*Capsicum annuum* (cv. Zhongjiao 7) - fruits	stored at 3 °C - 18 d	brassinolide/ 5, 10, and 15 µM/ immersing of the fruits in solution for 20 min before low temperature treatment
Jiang *et al.* (2013)	*Cucumis sativus (*cv. Jinchun No 4) - leaves	10/7 °C (d/n) - 6 d	24-epibrassinolide / 0.1 µM/ foliar spraying after 5 d of the low temperature treatment (5 cm^3 $plant^{-1}$)
Wu *et al.* (2014a)	*Solanum melongena* - leaves	10 /5 °C (d/n) - 8 d	24-epibrassinolide / 0, 0.5, 0.1, 0.2, and 0.4 µM/ foliar spray 1 d before the low temperature treatment
Cui *et al.* (2016)	*Lycopersicum esculentum* (cv. Zhongza9 and Zhongshu4) - leaves	3, 6, 9, 12 d at temperature 12/6 °C (d/n)	24-epibrassinolide / 0.1 µM/ foliar spray before the low temperature treatment (10 cm^3 $plant^{-1}$)
Pociecha *et al.* (2017)	*L. perenne* (cv. Amarant and Flinston) - leaves	4 ºC - 6 weeks	24-epibrassinolide / 0.25 mg dm^{-3}/ foliar spray before low temperature treatment
Pociecha *et al.* (2016)	*Secale cereale* - leaves	-14 °C – 6 h	24-epibrassinolide / 0.25 mg dm^{-3}/ foliar spray before cold acclimation
Wilen *et al.* (1991)	*Bromus inermis* - cultured cells	40 °C - 45 or 90 min 42,5 °C - 45 or 90min 45 °C - 45 min	24-epibrassinolide / 10 µM/ culture medium
Dhaubhadel *et al.* (1999)	*Brassica napus* (cv. Westar) and *Lycopersicon esculentum* (cv. Bonny Best) - leaves	45 °C - 4 h	24-epibrassinolide/ 1 µM/nutrient medium (*in vitro* cultures)
Singh *et al.* (2003)	*Lycopersicon esculentum* (cv. CLN 2026E and Suncherry extra) - pollen	35 °C - 4 h	24-epibrassinolide/ 1 µM/ inoculation with pollen before hig temperature treatment
Bajguz (2009)	*Chlorella vulgaris* - cultured cells	30 ºC - 3 h 35 ºC - 3 h 40 °C - 3 h	brassinolide / 10 nM/ culture medium
Janeczko *et al.* (2011)	*Hordeum vulgare* (cv. Sezam) - leaves	42 °C - 3 h	24-epibrassinolide/ 0.005 and 0.25 mg dm^{-3}/ application directly to apoplast by leaf infiltration; 3 d before high temperature treatment
Mazorra *et al.* (2011)	*Lycopersicon esculentum* (wild type Ailsa Craig and Money-maker; brassino-steroid mutants d^x, 35SD, and *curl3*^{ab}') - leaves	45 °C - 4 to 8 h	24-epibrassinolide / 1 µM/ MS medium, before high temperature treatment
Zhang *et al.* (2013)	*Cucumis melo* (cv. Honglvzaocui and Baiyuxiang) - leaves	42/32 °C (d/n) - 2 d	24-epibrassinolide/ 0.5, 1, and 1.5 mg dm^{-3}/ foliar spray before high temperature treatment (50 cm^3 $plant^{-1}$)
Zhang *et al.* (2014)	*ucumis melo* (cv. Baiyuixiang) - leaves	42/32 °C (d/n) - 2 d	24-epibrassinolide/ 0, 0.05, 0.1, 0.5, 1, and 1.5 mg dm^{-3}/ foliar spray before high temperature treatment (200 cm^3 $plant^{-1}$)
Wu *et al.* (2014b)	*Solanum melongena* (cv. Huquie 9) - leaves	43/38 °C (d/n) - 8 d	24-epibrassinolide/ 0.4, 0.2, 0.1, and 0.005 µM/ foliar spray during the high temperature treatment (50 cm^3 $plant^{-1}$)

Thussagunpanit *et al.* (2015a)	*Oryza sativa* (cv. Pathum Thani 1) - leaves	40/30 °C (d/n) - 7 d	24-epibrassinolide/ 1 nM/ foliar spray 5 d before high temperature treatment (15 cm^3 $plant^{-1}$)
Thussagunpanit *et al.* (2015b)	*Oryza sativa* (cv. Pathum Thani 1) - leaves	40/30 °C (d/n) - 7 d	24-epibrassinolide/ 10^{-8} M/ foliar spray, 1 week before high temperature treatment (10 cm^3 $plant^{-1}$)
Fahad *et al.* (2016)	*Oryza sativa* (cv. IR-64 and Huanghuazhan) - leaves	35/32 °C (d/n) - 12/12 h	Mixture of hormones containing brassinosteroid /4 mg dm^{-3}/ foliar spray before high temperature treatment, combination with other hormones

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: stworzeniu koncepcji publikacji (w tym koncepcji rycin) oraz współpracy przy pisaniu tekstu publikacji.

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Mutations in the *HvDWARF, HvCPD* and *HvBRI1* Genes-Involved in Brassinosteroid Biosynthesis/Signalling: Altered Photosynthetic Efficiency, Hormonal Homeostasis and Tolerance to High/Low Temperatures in Barley

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Abstract

Brassinosteroids (BR) are steroid phytohormones that are involved in the growth and stress response in plants, but the precise mechanisms of their action are still being discovered. In our study we have used BR-deficient barley mutants 522DK and BW084 (which carry missense mutations in the *HvDWARF* and *HvCPD* genes, respectively). We have also used a BR-signalling mutant that harbors missense substitutions in the *HvBRI1* gene. Our aim was (1) to find out if the content of phytohormones in the mutants grown at 20 °C is different than in the wild types and whether/how the content of phytohormones changes after plant acclimation at temperatures of 5 °C and 27 °C?, (2) to characterise the effectiveness of the light reactions of photosynthesis of the barley mutants in comparison to wild types at various temperatures, and (3) to verify the impact of mutations on the tolerance of barley to high and low temperatures. Hormonal characteristics of the BR mutants of barley show the complexity of the interactions between BR and other plant hormones that are additionally modified by temperature and possibly by other factors. The results suggest the participation of BR in auxin catabolism. Further, BR appears to play a role in maintaining the ABA–ABAGlc balance. As for the gibberellin content in plants at a temperature of 20 °C, more in-depth studies will be required to explain the contradictory effects regarding the accumulation of GA3, GA4 and GA5, which appears to be dependent on the type of mutation and connected to the BR level. A fast-kinetic chlorophyll *a* fluorescence analysis has revealed that the mutants had lower values of energy absorption than the wild types, but the values of the energy transferred via the electron-transport chain was maintained at the wild-type level. We presumed that BR are involved in regulating plant acclimation to extreme (low/high) temperatures, thus the BR-deficient and BR-signalling mutants should be less tolerant to low/high temperatures when compared to the wild types. Unexpectedly, all of the mutants showed a higher tolerance to high temperatures than the wild types. The BW084 and BW312 mutants were less tolerant to frost than the wild type, but 522DK had a similar frost tolerance as the reference wild-type cultivar.

Keywords Barley · Brassinosteroid content · Frost · Heat · Phytohormones · PSII efficiency

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Introduction

Temperature stress is, among the abiotic stresses, a particularly serious problem in agriculture and horticulture. Some species are very sensitive to cold, while frost, especially when there is insufficient snow cover on fields, can cause significant yield losses of winter crops. High-temperature stress, in turn, is particularly dangerous to plants when it is combined with drought during the growing season. In the case of barley cultivars, the influence of high temperature on PSII efficiency (Janeczko et al. 2011; Oukarroum et al. 2016) and genes encoding antioxidant enzymes and heat shock proteins (Faralli et al. 2015) was studied. At low temperature conditions the metabolic changes associated with cold adaptation and modifications of expression of the cold-regulated (COR) genes in barley cultivars were also investigated (Stanca et al. 1996). Moreover, characterisation of the CBF (C-repeat binding factor) gene family (which induces the COR expression) was performed in barley cultivars (Skinner et al. 2005). Because all crops are often exposed to extreme temperatures combined with a lack or excess of water, too little or too intense light, nutritional deficits, etc. during their life cycle (Koyro et al. 2012), discovering/studying the mechanisms of the actions of regulators (including plant hormones, for example, brassinosteroids) that stimulate the processes that counteract the effects of stress is very important from the practical point of view. The results of these studies may contribute to obtaining and incorporating new, more resistant cultivars of crops or to prepare agrochemicals that have protective/anti-stress properties for farming applications, especially because abiotic stress is considered to be a major cause of crop losses (Hasanuzzaman et al. 2012; Koyro et al. 2012).

Brassinosteroids (BR) belong to steroid phytohormones that were discovered in the 1970s (Mitchell et al. 1970; Grove et al. 1979). BR are known as regulators of plant growth and development. BR have also been found to be capable of minimising the damage that is caused by various kinds of stresses, including extreme temperatures. In plants that are subjected to high/low temperatures, BR counteract growth inhibition, reduce biomass losses and increase plant survival rates (Sadura and Janeczko 2018). At the cellular/molecular levels, BR have a multidirectional activity that regulates the cellular metabolism of plants that are exposed to temperature stress (Sadura and Janeczko 2018). On the other hand, the growth, development and stress responses in plants are regulated by the coordinated interplay of phytohormones such as auxins, gibberellins (GA), cytokinins, abscisic acid (ABA) or ethylene (Bari and Jones 2009; Small and Degenhardt 2018). Simultaneously, plant hormones control their own biosynthesis and transport. GA interact with ABA in an antagonistic manner—GA stimulate germination, growth and flowering, whereas ABA inhibits these processes (Weiss and Ori 2007; Shu et al. 2018). Auxins promote the influence of GA on the regulation of cell expansion and tissue differentiation by repressing the major negative regulators of the GA signalling—the DELLA proteins—and by regulating the biosynthesis of GA (Weiss and Ori 2007). The ratio of auxin/cytokinin plays an important role in forming shoots and roots (El-Yazal et al. 2015). Cytokinins positively regulate auxin biosynthesis. Although ABA, jasmonates (JA) and salicylic acid (SA) promote stomatal closure, cytokinins and auxins induce stomatal opening during stress (Peleg and Blumwald 2011). Finally, the most important plant physiological process—photosynthesis—is controlled by an integrated system of hormonal interplay (Gururani et al. 2015). In the case of barley cultivars, it is known that phytohormones regulate agronomically important traits (Marzec and Alqudah 2018). However, there are still many unknowns, including barley hormonal regulation under temperature stress. In the case of barley mutants with disturbances in the BR biosynthesis and signalling, according to our best knowledge, hormonal homeostasis has been investigated only in the case of drought stress (Janeczko et al. 2016; Gruszka et al. 2016a). Although BR also contribute to the functioning of the hormonal network, knowledge about this phenomenon is rather limited, and little is known about the influence of various temperatures on the interplay between BR and other phytohormones (Sadura and Janeczko 2018). The impact of BR on the temperature-dependent accumulation of other hormones has been studied in experiments with an exogenous application of BR (Bajguz 2009; Liu et al. 2011; Pociecha et al. 2017). Liu et al. (2011) studied *C. bungeana* cell cultures subjected to 0 °C and 4 °C and observed that on a medium with BR (24-epibrassinolide), cells accumulated more ABA compared to the untreated control. On the other hand, according to Bajguz (2009), algae cells cultured at high temperature on a medium supplemented with BR (brassinolide) contained more ABA compared to cells without BR supplementation. Application of 24-epibrassinolide (0.25 mg dm^{-3}) to perennial ryegrass combined with cold (4 °C, 6 weeks) resulted in an increased content of the ethylene precursor (ACC) compared to the plants without the BR treatment (Pociecha et al. 2017). In view of these data, it would be worth verifying/expanding this knowledge in studies on BR mutants.

Barley (*Hordeum vulgare* L.)—the species used in our experiment, is an important crop plant and the fourth most abundant cereal in both area and tonnage harvested, grown all over the world. Several genes involved in BR metabolism have been identified in barley, and collections of barley mutants with disturbances in BR biosynthesis and signalling

are available (Gruszka et al. 2011a, b, 2016b; Dockter et al. 2014). Among them there are genes that encode the enzymes HvDWARF (represented by the 522DK mutant) and HvCPD (represented by the BW084 mutant) that mediate BR biosynthesis, as well as a gene encoding the BR receptor—HvBRI1 (represented by the BW312 mutant). Various mutations of these genes often result in semi-dwarfism and these semi-dwarf barley mutants, that have disturbances in the BR biosynthesis and signalling, are useful in studies of the mechanisms of BR action in plants and plant interaction with the environment. It is known that semi-dwarf accessions of cereal crops are more tolerant to lodging under unfavourable weather conditions, and, therefore, they have significantly contributed to the success of the so-called 'Green Revolution' in the second half of the twentieth century (Hedden 2003; Chono et al. 2003). However, fine-tuning of the BR-related growth reduction is still required for the efficient breeding of cereal crops, especially when the possibility of global climate change is taken into account. Our recent studies have indicated that in response to drought stress the semi-dwarf barley mutants (BW084 and BW312) exhibited delayed wilting when compared to the wild-type cultivar (Gruszka et al. 2016a). Thus, they may be considered as a potential alternative in future barley breeding programs.

In the present study, we have gained insight into the hormonal homeostasis in the BR-biosynthesis and signalling mutants of barley by answering the following question: is the content of phytohormones in the mutants grown at 20 °C different than in the wild types and whether/how the content of phytohormones changes after plant acclimation at temperatures of 5 °C and 27 °C? Changes in hormonal homeostasis may be accompanied by alterations in the intensity of various physiological processes and may determine the response of plants to stress conditions. That is why we have also characterised the effectiveness of the light reactions of photosynthesis of barley mutants in comparison to wild types at various temperatures. The impact of mutations in the BR biosynthesis and signalling genes on the tolerance of plants to high temperature and frost was also investigated.

Materials and Methods

Plant Material

In our experiments, spring barley (*Hordeum vulgare* L.) cultivars (Delisa and Bowman) and their mutants were selected according to reports by Gruszka et al. (2011b) and Dockter et al. (2014), and the seeds were obtained from the collection of the Department of Genetics, University of Silesia (Poland).

The 522DK mutant is derived from the Delisa cultivar. In this mutant, the G>A substitution was identified at position 1130 of the *HvDWARF* transcript (Gruszka et al. 2011a) and at position 3031 in the gene sequence (Gruszka et al. 2016b), which changes the valine-341 residue into isoleucine. The substituted valine-341 is a highly conserved residue that is present in an analogous position in the homologous DWARF polypeptides in barley, rice, *Arabidopsis* and tomato. Because the *HvDWARF* gene encodes the brassinosteroid C6-oxidase, which is involved in the BR biosynthesis (catalyses production of castasterone), the mutant shows a reduced content of castasterone (Gruszka et al. 2016b).

The material of this study also contained the previously characterised barley Near-Isogenic Lines (NILs), which represent mutants that have defects in BR biosynthesis and signalling, along with the reference cultivar Bowman (Dockter et al. 2014). The BW084 (*brh13.p*) NIL represents a mutant defective in BR biosynthesis, which produces a decreased content of castasterone. The BW084 NIL carries a missense mutation (C2562T substitution) in the *HvCPD* gene, which encodes the C-23α-hydroxylase cytochrome P450 90A1 (CYP90A1) that catalyses the early stages of BR biosynthesis. The C2562T transition causes a substitution of the highly conserved amino acid residue (Pro-445 to Leu). Pro-445 is located within a highly conserved heme binding site in the C-terminal part of the HvCPD enzyme (Dockter et al. 2014).

The BW312 (*ert-ii.79*) NIL is defective in BR perception. The BR perception abnormality is caused by substitutions in the BR receptor kinase-BRI1. Consequently, higher content of castasterone is accumulated in BW312 when compared to the Bowman cultivar. The mutant allele contains a double substitution CC1760/1761AA. The substituted amino acid residue (Thr-573 to Lys) is located in the steroid-binding domain of the BR receptor. The introduction of the charged Lys-573 into the hydrophobic active site surrounding the residues, which destroys charge neutrality, is expected to prevent the binding of the BR molecules (Dockter et al. 2014).

Additionally, cultivars of spring and winter barley (Kucyk and Fridericus, respectively) were used in the preliminary experiments during tests to determine the low and high temperatures to be used for main studies on the mutants.

Experimental Design

Plant Culture

The seeds were germinated for 3 days on plastic vessels (10 cm × 10 cm × 3 cm) with moist filter paper in the dark at 24 °C. There were 40 seeds and 10 mL of water per vessel. After germination, the seedlings were transplanted into pots containing soil. There were 15–20 plants per pot. Each pot (40 cm × 15 cm × 15 cm) contained 3 kg of mixed and sieved soils: 'Eco-ziem universal soil' (Jurków, Poland), soil from the cultivation plots at the University of Agriculture

(Krakow) and sand. The plants were then grown in a growth chamber at 20 °C (d/n) under a 16-h photoperiod for 19 days until they reached the stage with 3–4 leaves. The potted plants were then divided into two groups. The first group was acclimated to 5 °C (d/n) under a 8-h photoperiod for 3 weeks, whereas the second group was acclimated to 27 °C (d/n) under a 16-h photoperiod for 7 days. After the plants had been acclimated, they had four well-developed leaves and sometimes a young fifth leaf. Light intensity in the growth chambers was 170 µmol m^{-2} s^{-1} provided by HPS Philips SON-T AGRO 400 W lamps.

Non-invasive measurements of the fast fluorescence kinetic chlorophyll *a* test were performed on plants before their acclimation (at 20 °C), and then for both groups of acclimated plants (on the last day of acclimation). Similarly, the leaf samples for the analysis of phytohormones were collected from the plants before acclimation, and then on the last day of the acclimation at 27 °C and 5 °C. The exception was the analysis of BR content, where samples were collected: before acclimation, on day 3 of acclimation at 27 °C, on day 7 (last) of acclimation at 27 °C, on day 10 of acclimation at 5 °C, and on day 21 of acclimation at 5 °C.

After the measurements and leaf sampling, the plants acclimated to 5 °C were exposed to frost, whereas the plants acclimated to 27 °C were exposed to heat. The survival rate of the plants was estimated after these exposures.

Frost Test and Estimation of the Plant Survival Rate

After acclimation at 5 °C, the pots with plants of cv. Delisa, cv. Bowman and their mutants were divided into two groups that were exposed to frost in the dark growth chamber separately: − 6 °C (first group), − 8 °C (second group). The starting point was 5 °C, after which the temperature was lowered by 3°/h until the required frost level was reached. The plants were exposed to the frost treatment (exactly − 6 or − 8 °C) for 6 h, and then the temperature was increased by 3°/h until 5 °C was reached. Finally, the temperature was set at 12 °C (d/n) and the plants were left for 2 weeks to recover (12-h photoperiod). After this period, the plant survival rate was estimated based on the Larsen (1978) visual scale (0–9 scale of regrowth after frost treatment), where 0 indicates completely dead plants with no signs of leaf elongation, whereas 9 means no symptoms of injury. For a detailed description of the scale see Pociecha et al. (2016).

The temperatures for testing were selected based on a preliminary experiment where cultures of two barley cultivars (Kucyk—spring barley and Fridericus—winter barley) were prepared. Plants were grown and acclimated at 5 °C identically to the experimental design described above, and then exposed to frost: − 4 °C, − 6 °C, − 8 °C, − 10 °C and − 12 °C. Based on results of the aforementioned regrowth test, the temperatures of − 6 °C and − 8 °C were selected. After exposure to − 6 °C, the regrowth of all of the plants reached a value of 9 points on the Larsen scale, but after exposure to − 8 °C, the plants of the spring cv. Kucyk had a regrowth that was estimated at only four points on the 9-point scale. All of the results of this preliminary test are presented in Supplementary Material: Figure S1A.

Heat Test and Estimation of the Plant Survival Rate

After acclimation at 27 °C, the potted plants of cv. Delisa, cv. Bowman and their mutants were divided into two groups and exposed to temperatures of 38 °C (first group) and 45 °C (second group) for two hours under light conditions in the growth chamber. To examine any heat-induced injury after the heat treatment, the following parameters were analysed: (1) performance index ($P.I._{ABS}$)—photosynthetic parameter that gives information about functioning of the photosystem II; (2) membrane permeability—measured as the amount of electrolyte leakage; (3) leaf blade damage—percentage of the injured (darkened, dried) leaf area in relation to the total leaf area. All leaves of the tested plants were analysed.

The temperatures for the treatment were selected based on the preliminary experiment performed on the barley cultivars Kucyk (spring barley) and Fridericus (winter barley). Plants were grown and acclimated at 27 °C identically to the experimental design described above and were then exposed to heat: 38 °C, 40 °C, 42 °C, 43 °C, 45 °C, 48 °C and 50 °C for 2 h. Based on results, which are presented in Supplementary Material: Figure S1B, C, D, the temperatures of 38 °C and 45 °C were selected for experiments on the mutants and their wild types. These temperatures caused a decrease in the values of the vitality of photosystem II ($P.I._{ABS}$) in both cultivars and resulted in visible, but not lethal, damage to the leaf blade in the spring cv. Kucyk.

Measurements

Fast Kinetics Chlorophyll a Fluorescence

A measurement of chlorophyll *a* fluorescence was performed to estimate the efficiency of photosystem II (PSII) using a Plant Efficiency Analyser (PEA, Hansatech Ltd., King's Lynn, England). Before the measurement, the leaves were adapted to the dark for 30 min. Details of the procedure are given in the paper by Skoczowski et al. (2011). The following phenomenological parameters of the energy flow were calculated using the technical fluorescence parameters that were obtained from the fluorescence curve: the energy absorption by antenna pigments ($ABS/CS = F_m$), the amount of energy trapped in the reaction centre ($TRo/CS = F_v/F_m$ (ABS/CS)), the energy flux for electron transport ($ETo/CS = (F_v/F_m)\ (1 - V_J)\ F_m$), and the dissipation of energy as heat ($DIo/CS = (ABS/CS) - (TRo/CS)$), where CS is the

sample cross section (Strasser et al. 2000). Moreover, the results of the so-called performance index ($P.I._{ABS}$), which is a general indicator of plant vitality, were calculated according to Strasser et al. (2000). The PSII efficiency measurements were performed in 15 replicates for each line/cultivar and treatment. One replicate was one leaf of an individual plant. In the main experiments, in the case of cv. Delisa, cv. Bowman and their mutants, a second leaf (from the base) was measured. In the preliminary experiments, in the fast-growing cvs. Kucyk and Fridericus, these parameters were measured on a third leaf.

Analysis of Brassinosteroids

BRs were isolated and identified using the modified methods of Oklestkova et al. (2017). Briefly, samples of the leaf material (1 g fresh weight) were homogenised using liquid N_2 and 20 mL ice-cold 80% methanol. Samples were then centrifuged. Internal standards with deuterium-labelled BR (25 pmol/sample) were added to the supernatant. Next, the supernatant was passed through Discovery columns (Supelco, Bellefonte, PA USA) and immunoaffinity columns (Laboratory of Growth Regulation, Olomouc, Czech Republic). The samples, which were eluted with cold 100% methanol, were dried and resuspended again in a small amount of methanol to be measured on a UHPLC with a tandem mass spectrometry (UHPLC–MS/MS) using the 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 analyses were performed in four repetitions.

Analysis of Other Phytohormones

Phytohormone analysis was performed as described by Dziurka et al. (2016). All the aerial parts of the plants were cut off, the material was cut into small pieces and 1 g samples were prepared. The extraction and purification procedure was performed as described by Dziurka et al. (2016). The analyses were performed in three independent replicates on a Agilent Infinity 1260 UHPLC apparatus (Agilent, Waldbronn, Germany), which was coupled to a triple quadruple mass spectrometer (6410 Triple Quad LC/MS, Agilent, Santa Clara, CA, USA) equipped with electrospray ionisation (ESI). Separation was performed on an Ascentis Express RP-Amide analytical column (2.7 µm, 2.1 mm × 75 mm; Supelco, Bellefonte, PA, USA). The technical details can be found in the paper by Dziurka et al. (2016). The following hormones were quantified: auxins: indole-3-acetic acid (IAA), oxindole-3-acetic acid (oxIAA), indole-3-butyric acid (IBA), indole-3-acetic acid methyl ester (IAAMe); cytokinins: trans-Zeatin (t-Z), trans-Zeatin-O-glucoside (t-ZOG), dihydrozeatin (DHZ), cis-Zeatin (c-Z); gibberellins: gibberellin A_1 (GA1), gibberellic acid (GA3), gibberellin A_4 (GA4), gibberellin A_5 (GA5), gibberellin A_6 (GA6); abscisic acid (ABA), abscisic acid glucosyl ester (ABAGlc) and salicylic acid (SA). Additionally, the total content of auxin, gibberellin and cytokinin was calculated as the sum of specific components.

Multiple reactions monitoring (MRM) transitions for the analyzed phytohormones and internal standards used are given in Supplementary Material: Table S1.

Membrane Permeability

Pieces from the middle part of a fully expanded leaf were cut into 20 mm sections. A piece of sample from each treatment was placed in separate vials with 10 cm^3 of ultrapure water and shaken (100 rpm, Labmed International, Inc) at 20 °C. After 24 h, the electrical conductivity ($C1$) was measured using a conductometer (CI 317, Elmetron, Poland). The vials with the samples were then frozen for 24 h at − 80 °C, and, after thawing, they were shaken again for 24 h as described above. Then, the conductivity was measured again and the obtained values represented the total ion content ($C2$) in the leaf pieces. Membrane permeability was expressed as the percentage of total electrolyte leakage (EL) ($EL = [C1 \times 100]/C2$). All the measurements were performed in ten biological replicates. One replicate was one leaf of an individual plant. In the main experiments, in the case of cv. Delisa, cv. Bowman and their mutants, the samples were taken from a second leaf. In the preliminary experiments, in the cvs. Kucyk and Fridericus, a third leaf was sampled.

Evaluating Leaf Injuries to Estimate Heat Tolerance

All the leaves from a whole plant were cut off and the percentage of injured (discoloured and withered) leaf area was estimated using a WinDIAS 3 Image Analysis System (Delta-T Devices Ltd, Cambridge, UK).

Statistical Analysis

Statistical analysis (ANOVA, post hoc test) was performed using Statistica 13.1, which is distributed by StatSoft (Tulsa, USA). For the statistical analysis in the study, when more than two groups were compared, Duncan's test was used. The Student's t test, which allows two groups to be compared, was only used to estimate the statistical significances of the effects that were observed in the Delisa cultivar and the 522DK mutant. In Figs. 1, 2, 3, 4, 5, 6 the mean values are plotted with standard error bars.

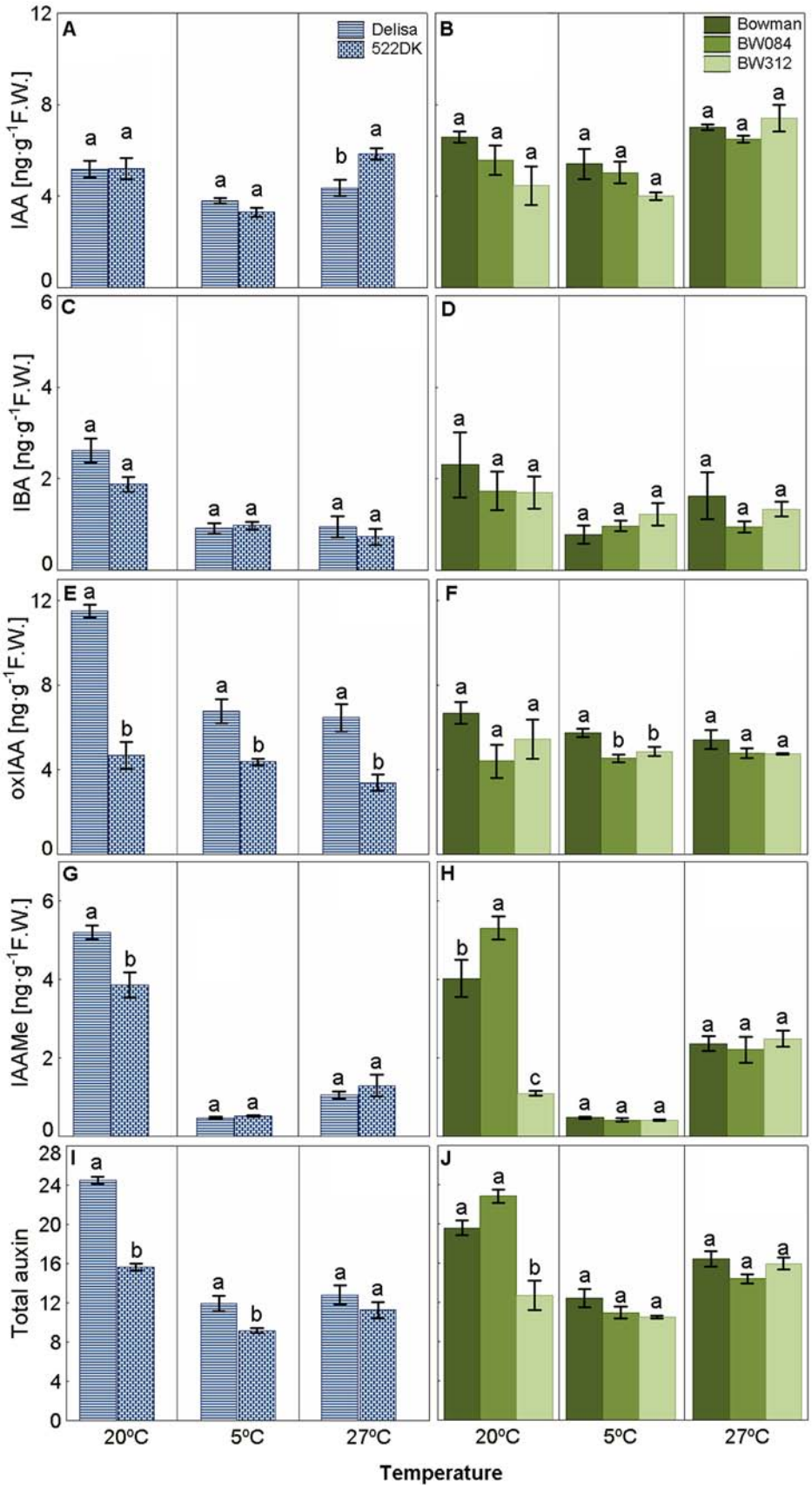

Fig. 1 Content of auxins in the barley cv. Delisa and its 522DK mutant (**a, c, e, g, i**) and in the barley cv. Bowman and its BW084 and BW312 mutants (**b, d, f, h, j**) at 20 °C and after acclimation at 5 °C and 27 °C. The mean values are shown with bars that represent SE. Significant differences between cv. Delisa and 522DK—Student's *t* test; between cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

Results and Discussion

Characterisation of the Hormonal Homeostasis of the Barley BR Mutants Grown at 20 °C, and then Acclimated to 5 °C and 27 °C

Plant hormonal homeostasis is influenced by environmental conditions and, among them, ambient temperature is one of the most important factors. Hormone accumulations, the mechanisms of their biosynthesis or hormone cross talk are the factors that are most often characterised at a temperature of about 20 °C (Janeczko and Swaczynova 2010; Dziurka et al. 2016; Gruszka et al. 2016a, b). Our studies were aimed at characterising the hormonal homeostasis of the BR-biosynthesis and BR-signalling defective mutants incubated at low, optimal or high temperatures. Although brassinosteroids are known as important players in the hormonal network, knowledge about their interplay with other phytohormones is limited, especially in terms of the influence on plant acclimation to altered temperature. Moreover, much of the data originate from experiments that use exogenous BR application and our studies on the BR mutants have enabled some of these findings to be verified.

In our experiment, BR-deficient and BR-signalling defective barley mutants are characterised by the altered hormonal homeostasis compared to the wild types, which provide information about the complicated links between BR biosynthesis/signalling and the regulation of the accumulation of other phytohormones, additionally dependent on the temperature during plant growth.

Brassinosteroids

We were able to determine three brassinosteroids—brassinolide, castasterone and 28-homocastasterone (Tables 1, 2). In comparison to Delisa, the content of castasterone was significantly lower in the 522DK mutant regardless of growth temperature (Table 1). This is a result of disturbances in the BR biosynthetic pathway in 522DK and the data agree with our previous studies of mutants growing in different conditions and different experimental model (Gruszka et al. 2016b). Interestingly, in these earlier studies we did not find brassinolide or 28-homocastasterone in Delisa and 522DK. In the present experiment, brassinolide was detected only in plants growing at a temperature of 20 °C, and the content of this steroid was lower in 522DK than in Delisa. This is an effect of deficiency of the direct brassinolide precursor (castasterone) in the brassinolide biosynthetic pathway (Zhao and Li 2012). The content of 28-homocastasterone was a little lower in 522DK than in Delisa at 20 °C and at 27 °C after 7 days of acclimation and at 5 °C after 21 days of acclimation, but statistical significance was proven only for the mutant at 27 °C. 28-Homocastasterone has a different biosynthetic pathway, independent from castasterone, and is biosynthesised from sitosterol, not from campesterol (Joo et al. 2015). According to these authors, 28-homocastasterone may be converted to castasterone. Perhaps this phenomenon had taken place in the 522DK plants, at 20 °C and after longer periods of acclimation, to alleviate a deficit of castasterone, but this will require further studies.

In comparison to Bowman, the content of castasterone was significantly higher in the signalling mutant BW312 regardless of growth temperature (Table 2). This agrees with our previous studies on this mutant cultured in a different experimental model (Gruszka et al. 2016a). According to data published by Gruszka et al. (2016a), BW084 was characterised by a dramatically decreased (but detectable) content of castasterone. In the present studies, the content of castasterone was below the detection limit. Similarly, as in the cases of Delisa and 522DK, brassinolide was only detected in Bowman and BW312 growing at 20 °C. In

Table 1 Content of brassinosteroids in the barley cv. Delisa and its mutant 522DK growing at 20 °C and after acclimation at 5 °C (10 and 21 days) and 27 °C (3 and 7 days)

Plant growth temperature	Barley	Brassinolide (pg g^{-1} F.W.)	Castasterone (pg g^{-1} F.W.)	28-Homocastasterone (pg g^{-1} F.W.)
20 °C	Delisa	145 ± 30^{a}	214 ± 13^{a}	2214 ± 474^{a}
	522DK	21 ± 3^{b}	76 ± 7^{b}	1649 ± 384^{a}
5 °C (10 days)	Delisa	< *LOD*	714 ± 169^{a}	1940 ± 218^{a}
	522DK	< *LOD*	358 ± 75^{b}	1982 ± 391^{a}
5 °C (21 days)	Delisa	< *LOD*	736 ± 169^{a}	8537 ± 1736^{a}
	522DK	< *LOD*	446 ± 88^{b}	6596 ± 1538^{a}
27 °C (3 days)	Delisa	< *LOD*	197 ± 21^{a}	870 ± 189^{a}
	522DK	< *LOD*	74 ± 6^{b}	927 ± 276^{a}
27 °C (7 days)	Delisa	< *LOD*	164 ± 14^{a}	2437 ± 282^{a}
	522DK	< *LOD*	91 ± 17^{b}	1712 ± 143^{b}

Significant differences between averages (±SE) for cv. Delisa and 522DK (Student's *t* test, $P \leq 0.05$) for each temperature are indicated by different letters. < LOD below limit of detection

Table 2 Content of brassinosteroids in the barley cv. Bowman and its mutants BW084 and BW312 growing at 20 °C and after acclimation at 5 °C (10 and 21 days) and 27 °C (3 and 7 days)

Plant growth temperature	Barley	Brassinolide (pg g^{-1} F.W.)	Castasterone (pg g^{-1} F.W.)	28-Homocastasterone (pg g^{-1} F.W.)
20 °C	Bowman	102 ± 21^{b}	112 ± 20^{b}	3149 ± 497^{a}
	BW084	$< LOD$	$< LOD$	2583 ± 683^{ab}
	BW312	269 ± 43^{a}	465 ± 99^{a}	1630 ± 116^{b}
5 °C (10 days)	Bowman	$< LOD$	268 ± 56^{b}	3816 ± 906^{a}
	BW084	$< LOD$	$< LOD$	2523 ± 255^{b}
	BW312	$< LOD$	869 ± 170^{a}	2799 ± 469^{ab}
5 °C (21 days)	Bowman	$< LOD$	368 ± 97^{b}	$11{,}175 \pm 2014^{a}$
	BW084	$< LOD$	$< LOD$	7234 ± 1544^{b}
	BW312	$< LOD$	1255 ± 278^{a}	7690 ± 1056^{b}
27 °C (3 days)	Bowman	$< LOD$	119 ± 8^{b}	1090 ± 230^{a}
	BW084	$< LOD$	$< LOD$	807 ± 180^{a}
	BW312	$< LOD$	343 ± 83^{a}	1101 ± 313^{a}
27 °C (7 days)	Bowman	$< LOD$	145 ± 7^{b}	2935 ± 605^{a}
	BW084	$< LOD$	$< LOD$	1504 ± 277^{b}
	BW312	$< LOD$	590 ± 99^{a}	1974 ± 230^{b}

Significant differences between averages (±SE) for cv. Bowman and its mutants (Duncan's test, $P \leq 0.05$) for each temperature are indicated by different letters. <LOD below limit of detection

BW084, brassinolide was below the detection limit. The content of brassinolide in BW312 was twice as high as in Bowman, which was accompanied by (and probably resulted from) an elevated level of castasterone in this mutant. Generally, it is noticeable that longer cold acclimation increased the level of castasterone and 28-homocastasterone in all tested mutants and wild types, sometimes even by a few times in comparison to that at 20 °C. This is the same tendency as earlier noted in cold acclimated winter rye (Pociecha et al. 2016) and winter wheat (Janeczko and Oklestkova, unpublished data), so probably the phenomenon is characteristic, at least, for cereals. On the other hand, in plants acclimated to 27 °C (7 days), the content of BR was maintained at a similar level to that at 20 °C or was even lower.

Auxins

Auxin and BR interplay is reviewed by Zhang et al. (2009), but little is known about endogenous auxin homeostasis in relation to BR deficiency or disturbances in BR signalling. The following auxins were detected in our experiment: IAA, IBA, oxIAA and IAAMe (Fig. 1a–j). At 20 °C and at 5 °C, the content of IAA in the wild-type Delisa and 522DK was at a similar level, whereas at 27 °C the level of this auxin was increased in 522DK. In the wild-type Bowman and its mutants, the content of IAA remained at a similar level regardless of the temperature conditions. Our earlier studies (Janeczko et al. 2016; Gruszka et al. 2016a) have shown no evidence that barley BR-mutants have increased IAA content, which is generally in agreement with current findings. Simultaneously, the exogenous application of brassinolide on *Arabidopsis* (Col-0) and mutant *det2* plants induced *IAA5* and *IAA19* gene expression gradually and continuously, but it did not increase the endogenous level of IAA (Nakamura et al. 2003). IBA content in all our tested mutants was similar as in the wild types regardless of the growth temperature. Mutant 522DK had a lower level of oxIAA (50–70%) compared to Delisa at all temperatures. In the BW084 and BW312 mutants (compared to Bowman), a similar slight tendency was observed, but statistical significance was only achieved at 5 °C. To the best of our knowledge, our analysis has revealed the auxin metabolite oxIAA in barley for the first time. OxIAA is considered to be inactive, or only a slightly active product of IAA oxidation in the catabolic pathway of this hormone (Reinecke and Bandurski 1983; Pěnčík et al. 2013). It has been established that the oxIAA content increases in response to an increased auxin level (Pěnčík et al. 2013). The lower content of oxIAA in our barley mutants may theoretically mean that the level of IAA was maintained at a normal (defined for wild types) level due to the mechanism of the limited deactivation of IAA. This suggests a role for BR in auxin catabolism at which a lower level of BR limits the deactivation of auxins.

IAA can be also conjugated, for example, to amino acids or sugars or converted to methyl IAA ester (IAAMe) (Qin et al. 2005). The role of such conjugates is primarily to store or transport the auxins as well as to detoxify any excess IAA and to protect free acids from peroxidative degradation (LeClere et al. 2002). Our studies are the first to report the presence of IAAMe in barley plants with disturbances in the BR biosynthesis and signalling. In our work, at 20 °C, the content of IAAMe was lower in the 522DK mutant than

in Delisa (by about 30%), but it was increased in BW084 (20%) compared to Bowman. In BW312, the content of IAAMe was lower by 70% compared to Bowman. At 5 °C and 27 °C, no changes were observed for this compound between the mutants and their wild types. According to Qin et al. (2005), exogenously applied IAAMe had stronger effects than free IAA, which indicates that IAA methylation may have a role in regulating the IAA activities. The complex regulation of the IAAMe accumulation is shown by the opposite effect that was obtained at 20 °C for the two BR-biosynthesis mutants with a different mutation type (and different BR level). Simultaneously, the dramatically lower content of IAAMe in the BW312 mutant has provided information about the role of BR signalling in regulating the accumulation of this compound. It is known that BR regulate auxin signalling. BZR1 (Brassinazole Resistant1—a major transcription factor regulating the BR-dependent gene expression) interacts with the ARF proteins (Auxin Response Factors—the class of transcriptional regulators) to target multiple auxin signalling components and the genes that are involved in the auxin metabolism (Tian et al. 2018).

The total auxin content decreased in the 522DK mutant at 20 °C and 5 °C (but not at 27 °C) compared to the wild-type Delisa. In the Bowman-derived mutants, the total auxin content was significantly decreased only in the mutant BW312 at a temperature of 20 °C but remained unchanged in all other samples.

To summarise, a BR-deficit or disrupted signalling of BR have no direct impact on the accumulation of particular active auxins but does affect the accumulation of auxins in methylated or oxidised forms, thus suggesting a role for BR in auxin metabolism in barley.

Cytokinins

We were able to detect t-Z, c-Z, DHZ and t-ZOG in the barley (Fig. 2a–j). According to the literature, exogenous application of BR on plants may influence the accumulation of cytokinins (Kudryakova et al. 2013). Brassinolide-treated transgenic *A. thaliana* plants (carrying the GUS reporter linked to the promoter for the *ARR5* gene of primary response to cytokinins) had an increased cytokinin level, by about 48%, in their leaves compared to untreated plants (Kudryakova et al. 2013). In the leaves of barley treated with 24-epibrassinolide under the white light of a fluorescent lamp, the content of tZ, cZ and DHZ increased by about 60, 66 and 150%, respectively, whereas the content of t-ZOG decreased (43%) (Efimova et al. 2017). Taking this into consideration, we have assumed that in our experiment the amount of cytokinins could be lower, especially in the BR-deficient mutants. Generally, the assumptions were verified in plants with mutation in the *HvCPD* gene growing at temperatures of 5 °C and 20 °C. At 20 °C, BW084 had a lower content of individual cytokinins and total cytokinins than Bowman, although in the cases of t-Z and t-ZOG only a statistically insignificant tendency was observed. In BW084 at 5 °C, c-Z and t-ZOG content was only lowered a few percent. The decrease in cytokinins in the BR-deficient mutants was not observed in plants with the mutation in the *HvDWARF* gene. The only exception was 14%-higher content of DHZ in the 522DK mutant compared to Delisa, observed at 5 °C. Interestingly, in our earlier studies, this mutation was correlated with a lowered cytokinin level (Janeczko et al. 2016). That experiment was, however, conducted under different conditions, at natural spring light in a hall without sidewalls and with a transparent (foil) roof. We have suspected that the different conditions of plant growth in the previous and present experiments, particularly the effect of light, are the cause of these differences. The role of light in regulating cytokinin homeostasis and physiological effects is well known (Kudryakova et al. 2013; Roman et al. 2016; Dobisova et al. 2017).

Finally, a marked decrease in the cytokinin content in the BR-signalling defective mutant was observed suggesting a link between BR signalling and cytokinin biosynthesis pathways in barley. At 20 °C and 5 °C, BW312 had a lower content of all cytokinins (by average about 20%) in comparison to Bowman, although, in the case of t-Z statistical significance was not proven. According to Kudryakova et al. (2013), BR may be involved in regulating the genes in the cytokinin-signalling pathway via an increase in the cytokinin levels.

Gibberellins

Interplay between BR and gibberellins is rather complicated, multilevel and depends on species or growth conditions (Jager et al. 2005; Tong et al. 2014; Unterholzner et al. 2015). *Arabidopsis* mutants deficient in BR signalling had impaired biosynthesis of bioactive GA, which is connected with defective expression of the GA-biosynthetic genes (Unterholzner et al. 2015). According to Jager et al. (2005), BR negatively regulated GA20 levels in pea. GA20 levels were affected by the BR content, but this did not result in clear alterations in the level of the bioactive GA1. On the other hand, the studies of Tong et al. (2014) have shown that BR greatly induces the expression of D18/GA3ox-2, one of the GA biosynthetic genes, which caused an increase in the bioactive GA1 level in rice seedlings.

In our studies, the following gibberellins were detected: GA1, GA3, GA4, GA5 and GA6 (Fig. 3a–l). These studies have shown that the level of BR may be important in barley tissue and, more generally, the abnormalities at different stages of the BR biosynthetic pathways, which are catalyzed by the mutated enzymes (HvCPD in BW084 and HvDWARF in 522DK) seem to affect the accumulation of

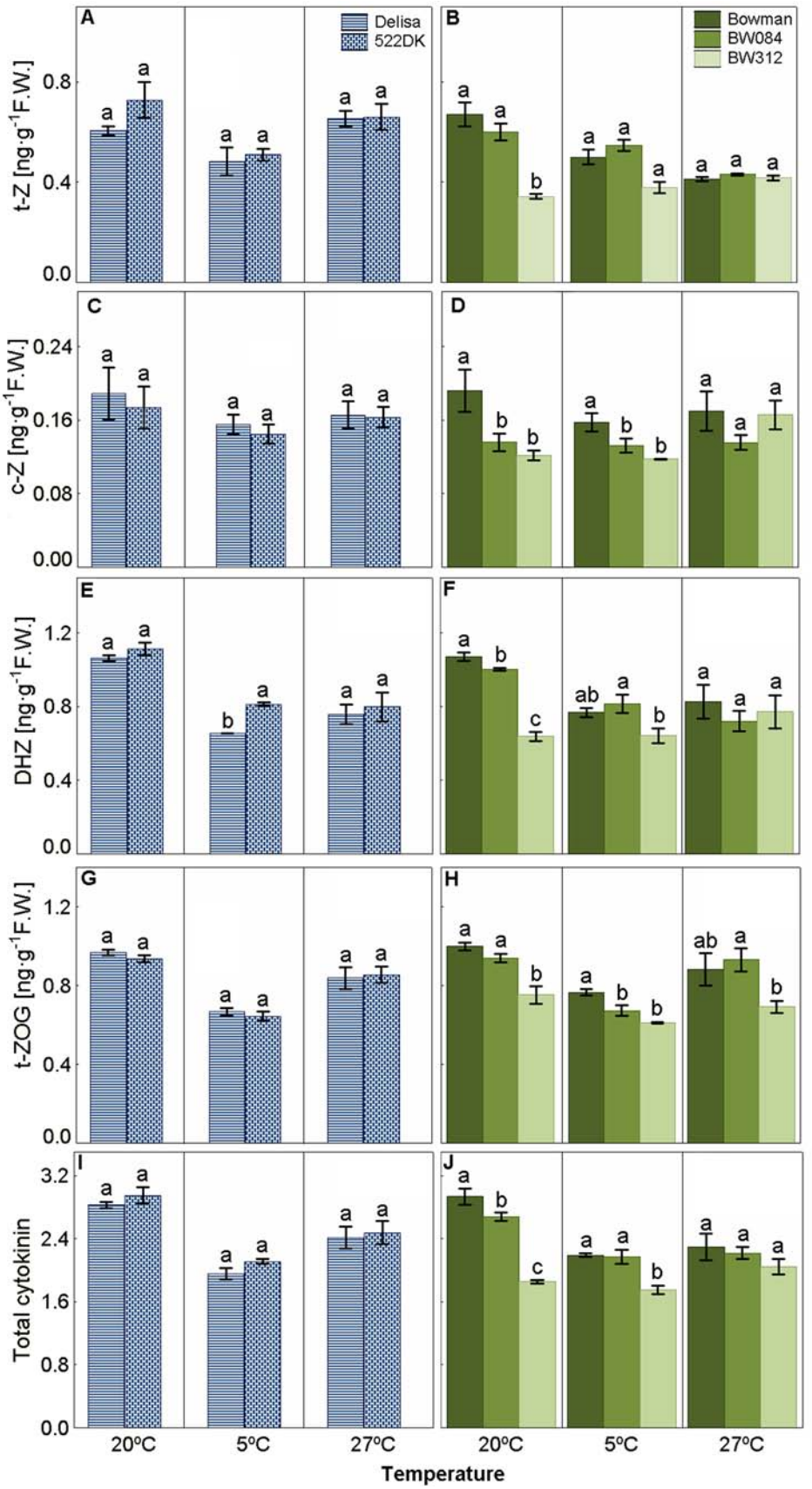

Fig. 2 Content of cytokinins in the barley cv. Delisa and its 522DK mutant (**a, c, e, g, i**) and in the barley cv. Bowman and its BW084 and BW312 mutants (**b, d, f, h, j**) at 20 °C and after acclimation at 5 °C and 27 °C. The mean values are shown with *bars* that represent SE. Significant differences between cv. Delisa and 522DK—Student's *t* test; between cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

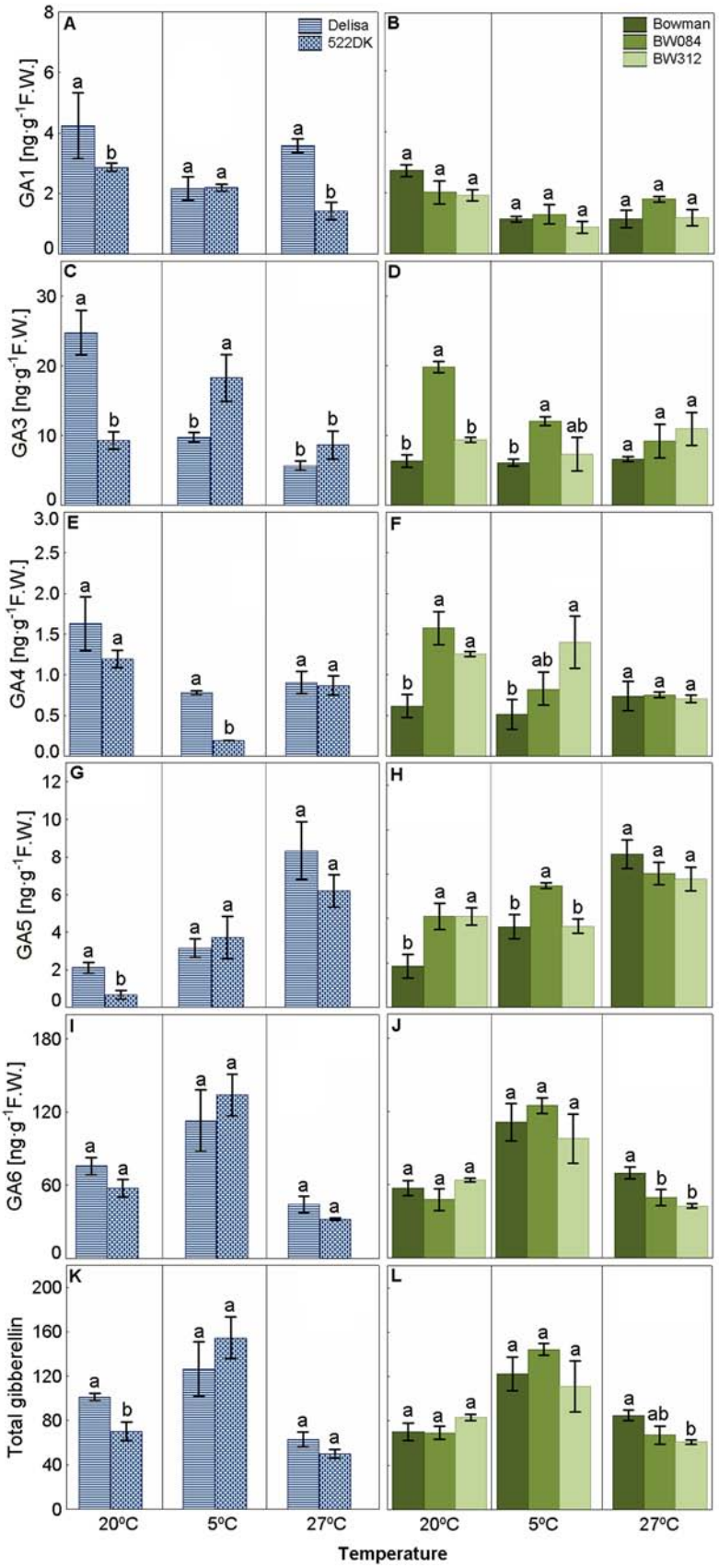

Fig. 3 Content of gibberellins in the barley cv. Delisa and its 522DK mutant (**a, c, e, g, i, k**) and in the barley cv. Bowman and its BW084 and BW312 mutants (**b, d, f, h, j, l**) at 20 °C and after acclimation at 5 °C and 27 °C. The mean values are shown with *bars* that represent SE. Significant differences between the cv. Delisa and 522DK—Student's *t* test; between cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

various representatives of gibberellins. The opposite effects were observed for the mutants carrying substitutions in the *HvCPD*, and *HvDWARF* genes, especially in terms of GA3, GA4 and GA5. Especially at 20 °C and partly at 5 °C (GA4, GA5), the content of the aforementioned gibberellins decreased in 522DK whereas they increased in BW084. Interestingly, at 5 °C, GA3 content increased in both mutants. As for GA3, this compound was discovered in the fungus *Gibberella fujikuroi* (Hedden and Sponsel 2015), but later its occurrence was proven in plants, for example, *Fagus sylvatica, Pinus pinaster, Malus hupehensis*, and *Triticum aestivum* (Fernández et al. 1997; Delatorre et al. 2017; Mao et al. 2017; Janeczko et al. 2018). Mao et al. (2017) even found that treatment of *Malus hupehensis* apple seedlings with brassinolide decreases GA3 content in comparison to untreated plants. No changes were noted in the GA3 level in BW312 in comparison to Bowman.

No differences were observed in the GA6 content regardless of the temperature conditions between 522DK and Delisa (Fig. 3i). The same was observed for Bowman and its mutants at temperatures of 20 °C and 5 °C, but at 27 °C the content of GA6 was significantly lower in both mutants than in Bowman (Fig. 3j).

The total gibberellin content was about 30% lower in the 522DK mutant compared to the wild type at 20 °C only. As for Bowman and its mutants, the total gibberellin content was only lower in the mutant BW312 compared to Bowman (at 27 °C).

Different levels of particular BR compounds, which are caused by mutations in different genes (*HvCPD* and *HvDWARF*) that encode enzymes catalyzing different steps of the BR biosynthetic pathway, are probably significant for modifying gibberellin accumulation in different ways. Changes in the content of some of the gibberellins studied in the BR-signalling defective mutant also suggest a connection between the BR signalling and the gibberellin biosynthetic pathways in barley.

Abscisic Acid

Abscisic acid (ABA) and its conjugate form ABAGlc were detected in our study (Fig. 4a–d). According to the literature, exogenous BR increase the accumulation of this stress hormone at both low and high temperatures, which was found in *Chorispora bungeana* cell cultures (Liu et al. 2011), algal cells (Bajguz 2009) and *Brassica napus* seedlings (Kurepin et al. 2008). So, we have presumed that the BR-deficiency characteristic for the 522DK and BW084 mutants will be correlated with a lowered content of ABA. In fact, our earlier studies on barley, grown outside and under natural light conditions, have shown that free ABA content was decreased in the 522DK mutant compared to the wild type Delisa in optimally watered plants and, especially, in drought-stressed plants (Janeczko et al. 2016). Here, in an experiment conducted in the artificial light of sodium lamps, the effect was not observed in 522DK (Fig. 4a). The content of ABAGlu was also unchanged in this mutant when compared to Delisa and regardless of the growth temperature (Fig. 4c). In BW084, ABAGlu was also unchanged, whereas free ABA content was lowered only in plants at 5 °C (Fig. 4b, d). In our opinion, similar to cytokinins, the light spectrum (and perhaps intensity) plays an important role in the biosynthesis of ABA. It has been described that ABA concentration is regulated/elevated strongly, for instance, by the UV-B radiation that is present in the natural light spectrum (Tossi et al. 2009). It seems that the role of light conditions on the hormonal homeostasis of the barley BR mutants should be more closely studied in the future.

On the other hand, Hu and Yu (2014) reported an interplay between ABA and BR signalling (BR repressed the ABA signalling) in *Arabidopsis thaliana* L., whereas, according to Wang et al. (2018), ABA inhibits BR signalling. Our work has also suggested a connection between ABA content and BR signalling, although it depends on the temperature during plant growth. Especially interesting results were observed at 20 °C, where the BR-signalling mutant (BW312) simultaneously accumulated a lower ABA content but an increased content of the abscisic acid glucosyl ester (ABAGlc) (Fig. 4b, d). ABAGlc is a hydrolysable ABA conjugate of which deconjugation enables the formation of free ABA in response to stress conditions, and this, in turn, enables ABAGlc to participate in the maintenance of ABA homeostasis (Burla et al. 2013). In our work, the phenomenon observed at a temperature of 20 °C was not observed at 5 °C or 27 °C, possibly because, under these more stressful temperatures, ABAGlc was more intensively mobilised to form free ABA to obtain an amount as high as is only possible for the BW312 mutant.

Salicylic Acid

The literature describing interplay between SA and BR is scarce. An artificial increase of the BR level via exogenous application of 24-epibrassinolide to perennial ryegrass, combined with low temperature conditions (4 °C, 6 weeks) did not cause statistically significant changes in the salicylic acid content (Pociecha et al. 2017). On the other hand, we did not observe any changes in the SA level in either BR-deficient mutant at 20 °C and 5 °C (Fig. 4e, f). The BR deficiency was accompanied by an increase in the SA content only in the BW084 mutant at 27 °C (Fig. 4f). This, once again, suggests the importance of the stage in the BR biosynthesis pathway at which the deficiency occurs (the mutations in the *HvCPD* and *HvDWARF* genes), which are connected with the difference in

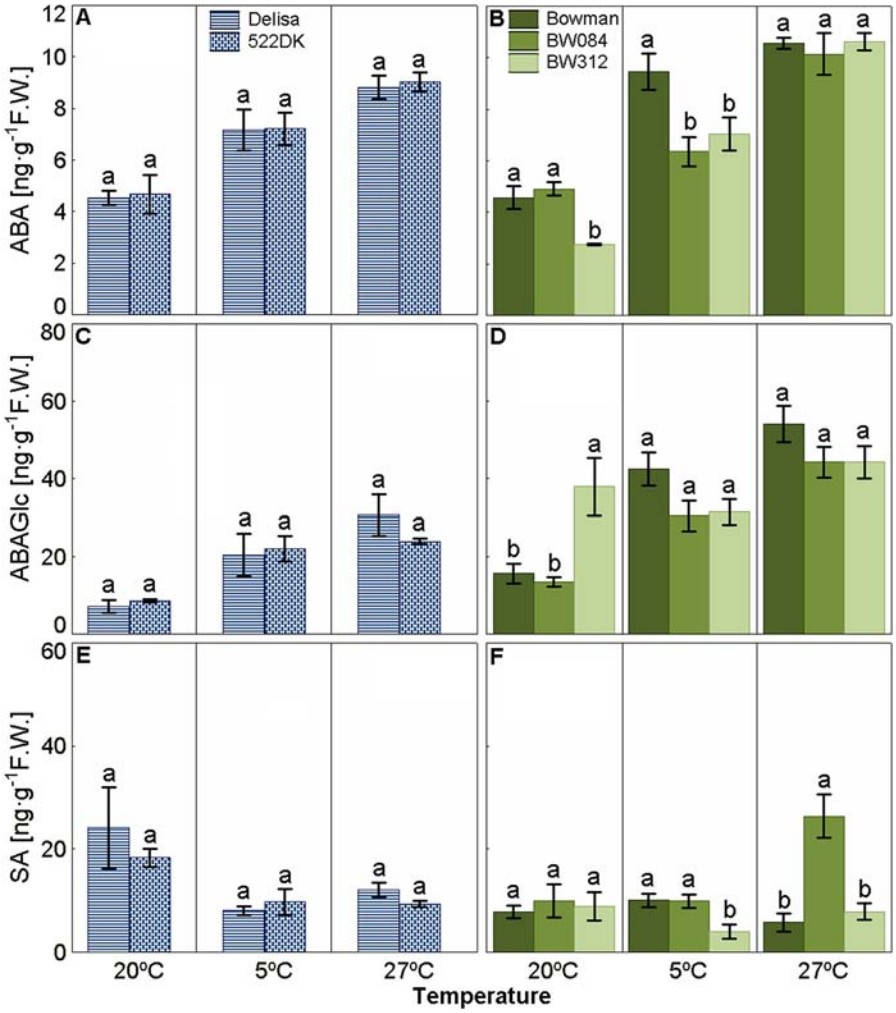

Fig. 4 Content of ABA, ABAGlc and SA in the barley cv. Delisa and its 522DK mutant (**a, c, e**) and in the barley cv. Bowman and its BW084 and BW312 mutants (**b, d, f**) at 20 °C and after acclimation at 5 °C and 27 °C. The mean values are shown with *bars* that represent SE. Significant differences between the cv. Delisa and 522DK—Student's *t* test; between the cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

the BR content, and the effect of the temperature during plant growth on the possibility of the SA-BR crosstalk in barley.

Divi et al. (2010) showed some links between BR and SA in experiments on temperature-stressed plants of *Arabidopsis*. Also, in our study the content of SA was lower in the BR-signalling mutant at 5 °C, thereby suggesting some connection between the BR- and SA-signalling pathways also in barley.

Supplementary Materials: Table S2 provides an insight into the complicated hormonal homeostasis in the BR barley mutants. The table summarises the effect of mutations in the genes participating in the BR biosynthetic or signalling pathways on phytohormone accumulation, and dependence on the ambient temperature.

Characterisation of the PSII Efficiency of the Barley BR Mutants Grown at 20 °C, and then Acclimated to 5 °C and 27 °C

Many studies have shown that exogenous BR stimulate plant photosynthesis—the efficiency of both light and dark reactions (for a review see Holá 2011). Analysis of the fast kinetics of chlorophyll *a* fluorescence in high temperature-stressed barley revealed that exogenous BR can increase PSII performance (Janeczko et al. 2011), which is why we have presumed that a BR deficit may have a negative influence on PSII functioning. However, analysis of the fast kinetic of fluorescence of chlorophyll *a* revealed that BR-deficient barley mutants (522DK, BW084) had a better performance of the light reactions of photosynthesis that are related to PSII (Fig. 5a–j). This was generally expressed by elevated values of the performance index ($P.I._{ABS}$) when compared to the respective wild type cultivars (Fig. 5i, j). Detailed analysis of the phenomenological fluxes (ABS/CSm, TRo/CSm, ETo/CSm, DIo/CSm) has revealed that, in comparison to the respective wild type cultivars, all the BR-deficient mutants had significantly lower losses of energy (DIo/CSm). Simultaneously, the energy flux for electron transport (ETo/CSm) was increased at 20 °C in the 522DK and BW084 mutants and at 5 °C—especially in the mutant 522DK. At 27 °C, ETo/CSm was unchanged in 522DK or slightly lower in BW084. The phenomenon took place in

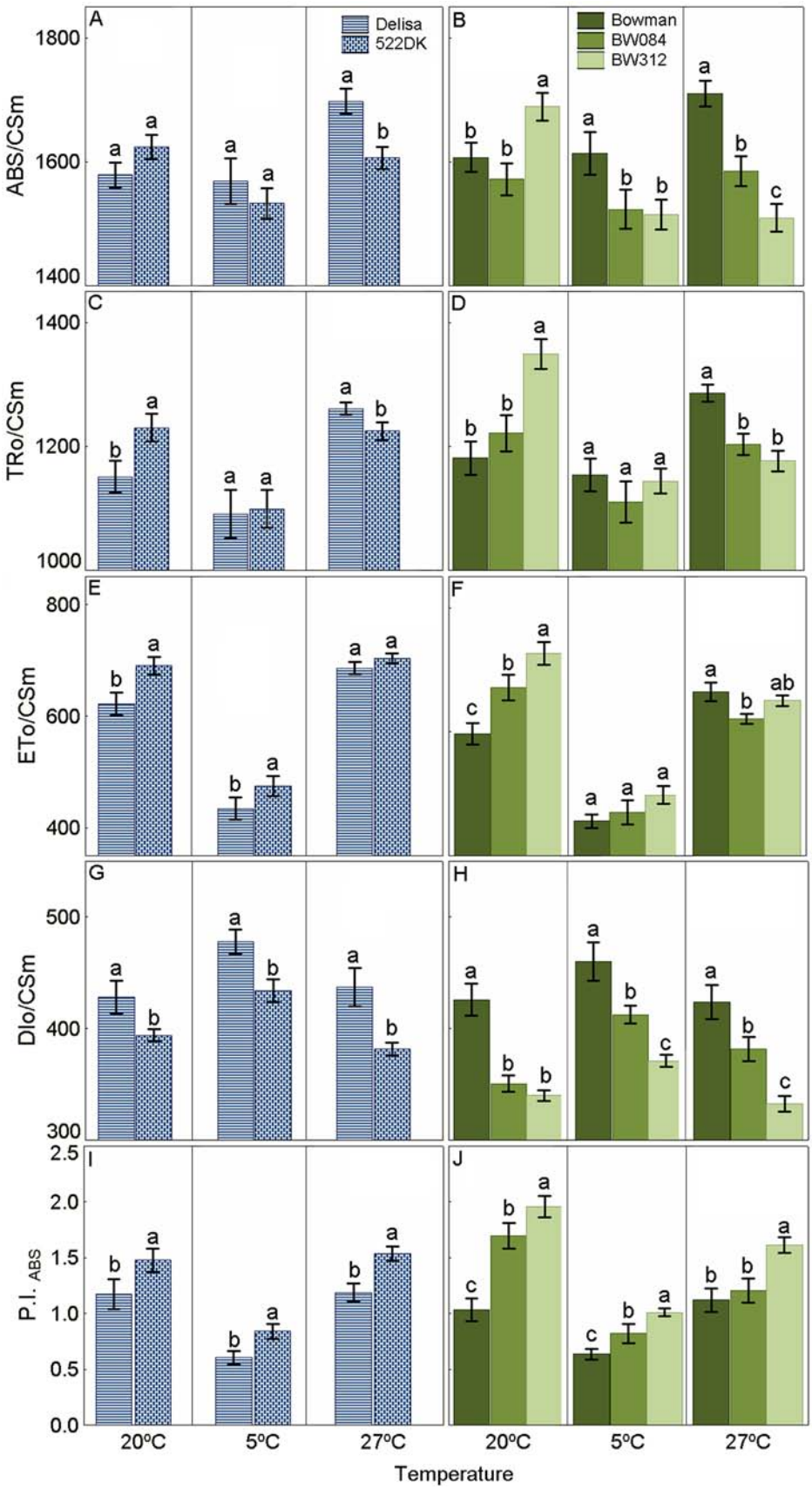

Fig. 5 Efficiency of PSII of the barley cv. Delisa, 522DK mutant, cv. Bowman, BW084 and BW312 mutants at 20 °C and after acclimation at 5 °C and 27 °C as expressed by phenomenological fluxes: **a, b** the absorption of energy ABS/CSm; **c, d** energy flux for trapping TRo/CSm; **e, f** energy flux for electron transport (ETo/CSm); **g, h** energy dissipation DIo/CSm; **i, j** performance index ($P.I._{ABS}$). The mean values are shown with *bars* that represent SE. Significant differences between cv. Delisa and 522DK – Student's *t* test; between cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

the situation when energy absorption by antennas (ABS/CSm) was similar or even decreased in the mutants in comparison to their wild type cultivars, which has provided information about more efficient energy conservation in the BR-deficient mutants.

Results obtained in the present experiment, which was carried out under artificial light are in accordance with our earlier studies, in which optimally watered BR-deficient mutants 522DK and 527DK grown under natural light conditions also had better performance of the PSII reactions (Janeczko et al. 2016). Although the chlorophyll content was lower in the mutants, the carotenoid level was higher, which in this case might have a positive effect on energy capture and PSII efficiency (Janeczko et al. 2016). As for other data from the literature, there are only a few studies characterising the PSII efficiency in plants with changed levels of endogenous BR. Data obtained by Wu et al. (2008) seem to contradict our observations because in transgenic rice with an increased BR accumulation, an increased maximum quantum yield of the primary photochemistry of PSII (F_v/F_m) was observed when compared to the wild type (Wu et al. 2008). On the other hand, these results seemed to be dependent on the light intensity and the best effect was achieved at 150 µmol m^{-2} s^{-1}. Anyway, according to Wu et al. (2008), the mechanism of BR action on the light reactions of photosynthesis remains unknown. The authors suspect that the repression of expression of genes encoding the U-box ubiquitin ligase and the chloroplast protease (observed in the microarray data) might have prolonged the half-life of some of the photosynthetic proteins.

In our experiment, a slightly different situation was observed in the case of the BR signalling mutant BW312. BW312, when grown at 20 °C, had values of energy absorption by antennas (ABS/CSm) higher than the wild type Bowman, which allowed plants to reach the highest of all experiment values observed of TRo/CSm, ETo/CSm and $P.I._{ABS}$. Interestingly, at the end of acclimation at 5 °C and 27 °C, this mutant had an ABS/CSm much lower than the wild type but maintained ETo/CSm on a level similar to Bowman, probably due to much lower energy loses (DIo/CSm). All this allowed BW312 to reach higher $P.I._{ABS}$ values than Bowman at 5 °C and 27 °C.

To conclude, our work has allowed characterisation of the PSII efficiency in the BR-deficient and signalling-defective mutants, but the role of BR content and BR signalling for functioning of the light reactions of photosynthesis will require further and more detailed proteomic and genetic studies.

Tolerance of the Barley BR Mutants to Frost and High Temperatures

Frost Tolerance

After exposure of all tested mutants and their wild types to − 6 °C, plant regrowth was very effective and reached from eight to nine points on the Larsen scale (Fig. 6a, b). After exposure to a temperature of − 8 °C, plants of Delisa cultivar and the 522DK mutant had a similar level of regrowth of about three points on the Larsen scale. After the same temperature treatment of Bowman, the plants had regrowth at a level of about eight points, whereas the BW084 and BW312 mutants were characterised with regrowth at about four and six points, respectively. The results of the regrowth test have shown that both these mutants suffered higher damage at − 8 °C than the wild-type Bowman. We have presumed that the BR in plants are involved in regulating their acclimation to low temperatures, thus BR-deficient and BR-signalling mutants should be less tolerant to low temperatures, compared to the wild types. Our presumption was made based on our earlier studies (Pociecha et al. 2016, 2017) that showed increased frost tolerance after an application of exogenous BR in winter rye and perennial ryegrass, together with an increased accumulation of castasterone after cold acclimation (cold hardening) and before frost exposition (Pociecha et al. 2016). Our hypothesis has been verified positively only partly. The castasterone accumulation in the BW084 mutant was below the detection limit and was accompanied by a decrease in its tolerance to frost. On the other hand, the BR deficiency in 522DK did not cause the effect of lowered frost tolerance, but it should be noted that in this mutant castasterone was still present at the level of 446 pg g^{-1} F.W. Further, we suspect that 28-homocastasterone may be more important for plant frost tolerance than castasterone. Our parallel experiments on winter wheat (Janeczko and Oklestkova, unpublished data) have shown that after cold acclimation a higher content of 28-homocastasterone was characteristic for more frost-tolerant cultivars of winter wheat. The correlation was not so clear for castasterone. In the present experiment on barley the Delisa cultivar was less frost-tolerant than Bowman because its regrowth after − 8 °C was at three points, whereas for Bowman it reached seven points (Fig. 6a, b). Simultaneously, after acclimation at 5 °C, 28-homocastasterone in Delisa was at the level of 8537 pg g^{-1} F.W., whereas in Bowman it reached 11,175 pg g^{-1} F.W. The content of 28-homocastasterone was not different in the 522DK mutant in comparison with Delisa and, simultaneously, plants had similar regrowth after − 8 °C. The situation is different in Bowman and BW084, where the mutant had a significantly lower content of 28-homocastasterone than Bowman and simultaneously weaker regrowth after exposure to − 8 °C.

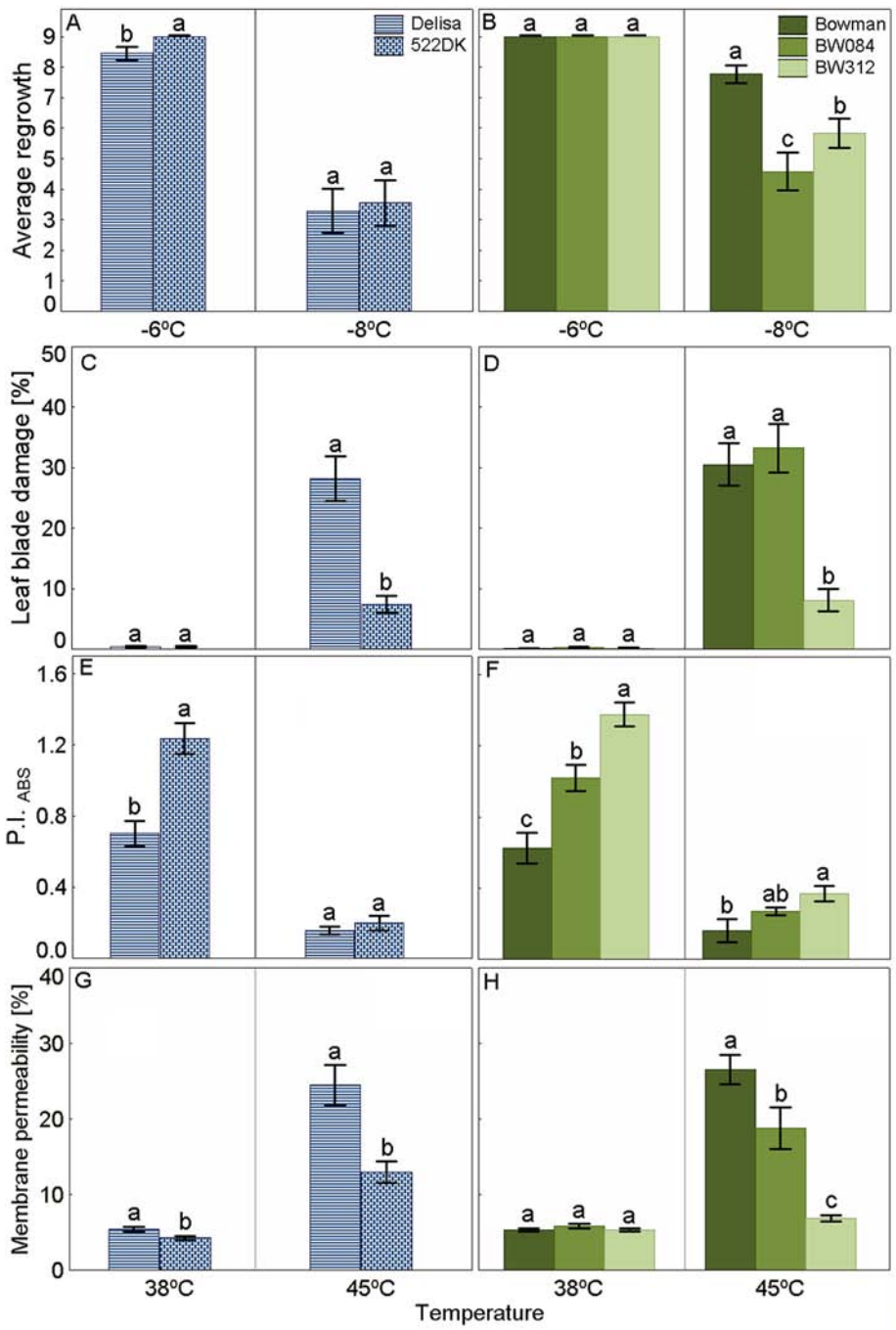

Fig. 6 Tolerance of the barley cv. Delisa, 522DK mutant, the cv. Bowman, BW084 and BW312 mutant to frost (**a, b**) and high temperatures (**c**–**h**). **a, b** average regrowth; **c, d** leaf blade damage; **e, f** photosynthesis efficiency of PSII measured as performance index ($P.I._{ABS}$); **g, h** membrane permeability. The mean values are shown with *bars* that represent SE. Significant differences between the cv. Delisa and 522DK – Student's *t* test; between cv. Bowman and its mutants—Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters

Interestingly, regrowth of the BR-signalling mutant BW312 was little better than BW084, although it was still weaker than in Bowman. BW312 accumulated a very high level of castasterone and it is likely that, in this case, this steroid positively influenced frost tolerance although via an unknown mechanism. Steroids of this kind may interact with cell membranes and stabilise them, especially at low temperatures (Li et al. 2012; Filek et al. 2017), however this requires further studies. As mentioned, BW312 had better survival after frost than BW084 but still lower than Bowman. This phenomenon is probably connected to the BR-signalling disturbances and this finding is in accordance with results published by Eremina et al. (2016). Eremina et al. (2016) studied the role of BR in the process of plant hardening to frost on *Arabidopsis thaliana* L. mutants with impaired BR signalling. The BR-signalling mutants *bri1-301* and *bri1-1* had a decreased tolerance to frost. According to these authors, BR improved the frost tolerance of plants by regulating the expression of *COR* (cold-responsive) genes. The BR-hypersignalling line *35S:BRI1-GFP (BRI1oe)* mutant

showed a higher frost tolerance and higher accumulation of the *COR15A, COR15B, COR47, COR78* transcripts.

Finally, our experiment has provided knowledge about the hormonal homeostasis in barley that has been cold acclimated (hardened) before frost treatment. Among the results from the hormone analyses, the results for the ABA accumulation seem to be the most important, as they may explain the differences in frost tolerance between our tested genotypes. ABA is a well-known factor whose increase is correlated with low temperature-stress conditions (Welbaum et al. 1997; Gusta et al. 2005; Janeczko et al. 2018) and is considered to be a hormone, which protects plants against freezing (Veisz et al. 1996). An accumulation of higher amounts of ABA correlates with low temperature resistance in maize (Janowiak et al. 2003). In our experiment, the Delisa cultivar, which is more sensitive to frost, accumulated less ABA in the acclimation process at 5 °C than the more tolerant cultivar (Bowman). In addition, the ABA content in the 522DK mutant was no different than that of the wild-type Delisa. This corresponds with the same cold tolerance in Delisa and 522DK. In the case of Bowman, both of its mutants were less tolerant to frost and, at the same time, accumulated less ABA than Bowman. These results have painted a picture of the relationship between frost tolerance and the level of BR and ABA accumulation in barley.

High Temperature Tolerance

We have presumed that BR in plants are involved in regulating their acclimation to high temperature, thus BR-deficient and BR-signalling mutants should be less tolerant to high temperatures when compared to the wild types. Unexpectedly, all the mutants show a higher tolerance to high temperatures than their respective wild types.

After exposure to a temperature of 38 °C, leaf blade damage (Fig. 6c, d) and membrane permeability (Fig. 6g, h) were at a similar level in all the plants tested. Only the measurement of the fast-kinetic chlorophyll *a* fluorescence indicated differences in the efficiency of the photosystem II, which was expressed by the $P.I._{ABS}$. parameter (Fig. 6e, f). After treatment at 38 °C all the mutants gained significantly better/higher values of this parameter than their respected wild types. For example, the mutants that had disturbances in the BR biosynthesis, 522DK and BW084, had 76% and 63% higher values of $P.I._{ABS}$, respectively, compared to Delisa and Bowman. Temperature of 45 °C caused markedly higher damage for all the plants tested. Interestingly, all the mutants, except BW084, were characterised by weaker injuries than the wild types. The leaf blade damage for the 522DK mutant was 74% lower than for the Delisa cultivar. In the case of the BW084 mutant, no differences were observed in the leaf blade damage compared to Bowman. Less damage was observed for BW312 when compared to Bowman and BW084. A much lower membrane permeability—from about 30% to more than 70%—was observed for all the mutants tested compared to their wild types. Changes in the values of the $P.I._{ABS}$ parameter were not observed at 45 °C for the 522DK and BW084 mutants compared to the wild-type Delisa and Bowman, respectively. However, the $P.I._{ABS}$ value was higher in BW312 than in Bowman.

In an earlier experiment, Mazorra et al. (2011) tested the effect of an elevated temperature (45 °C) on the tomato altered BR-sensitive mutant BR (*curl3*$^{-abs}$) and an extreme dwarf mutant (d^x). The *curl3* mutant is characterised as being BR insensitive (Koka et al. 2000; Bishop 2003). The d^x mutant lacks castasterone, but has an elevated level of its precursor 6-deoxocastasterone (Bishop 2003).

In the experiment of Mazorra et al. (2011), approximately 90% of the *curl3*$^{-abs}$ seedlings survived high temperatures (< 30% of the wild type also survived), which is in line with the results obtained for our BR-signalling mutant BW312.

At the same time, Mazorra et al. (2011) found no differences between the survival rate of the wild-type tomato and the survival of the BR-deficient mutant d^x. According to the authors, the basic thermotolerance of the tomato mutants was independent of the endogenous level of BR. In our work, regardless of the mutated gene that had caused the BR-deficiency, both the 522DK and BW084 mutants showed even better tolerance to high temperatures than their wild types, but after acclimation at 27 °C. Generally, the results from the publication by Mazorra et al. (2011) and our results give a different picture than obtained in the experiments using exogenous BR treatments (Dhaubhadel et al. 1999, 2002; Mazorra et al. 2011; Zhang et al. 2013, 2014; Wu et al. 2014; Thussagunpanit et al. 2015a, b). In these types of experiments, the artificially increased BR content via their exogenous application stimulated the tolerance to heat. In our work we have presumed that if the BR content decreased in the BR biosynthetic mutants, these plants would have worse thermotolerance—but they did not.

Determining the mechanism of the phenomenon of the better tolerance of the BR mutants to high temperatures will require further studies, but with the present knowledge, in our opinion, part of the explanation of the phenomenon may be simple and seems to be connected to plant height and leaf area. High-temperature treatment is accompanied by increased transpiration, which may additionally cause a water deficit in the leaves along with all the physiological and biochemical consequences that this induces. The mutants tested were semi-dwarf (Gruszka et al. 2011a; Dockter et al. 2014), therefore the areas of the aerial parts of the mutant plants were smaller than in the wild types. This is connected with lower transpiration, which in this case, may have limited the negative physiological consequences of water loss.

Finally, taking into account the fact that mutants can be part of breeding programs to develop new barley cultivars, information about their tolerance to environmental stresses may be useful.

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Author Contributions IS ad EP made frost and high temperature tests. IS measured PS II efficiency. IS made extraction of all phytohormones. AJ made purification of brassinosteroids on immunoaffinity columns, MD measured phytohormone content (except of brassinosteroids) on UHPLC–MS. JO and ON measured brassinosteroid content on UHPLC–MS/MS. DG provided seeds of mutants for experiment and gave suggestion about interpretation of data. AJ conceived the experimental idea, AJ is coordinator of the project 2015/17/B/NZ9/01695 and she designed experiments and choose all other scientists to cooperation. IS and AJ analyzed and interpreted data. AJ coordinated writing of manuscript. All authors read article and suggested corrections.

Compliance with Ethical Standards

Conflict of interest The authors have no conflict of interest to declare.

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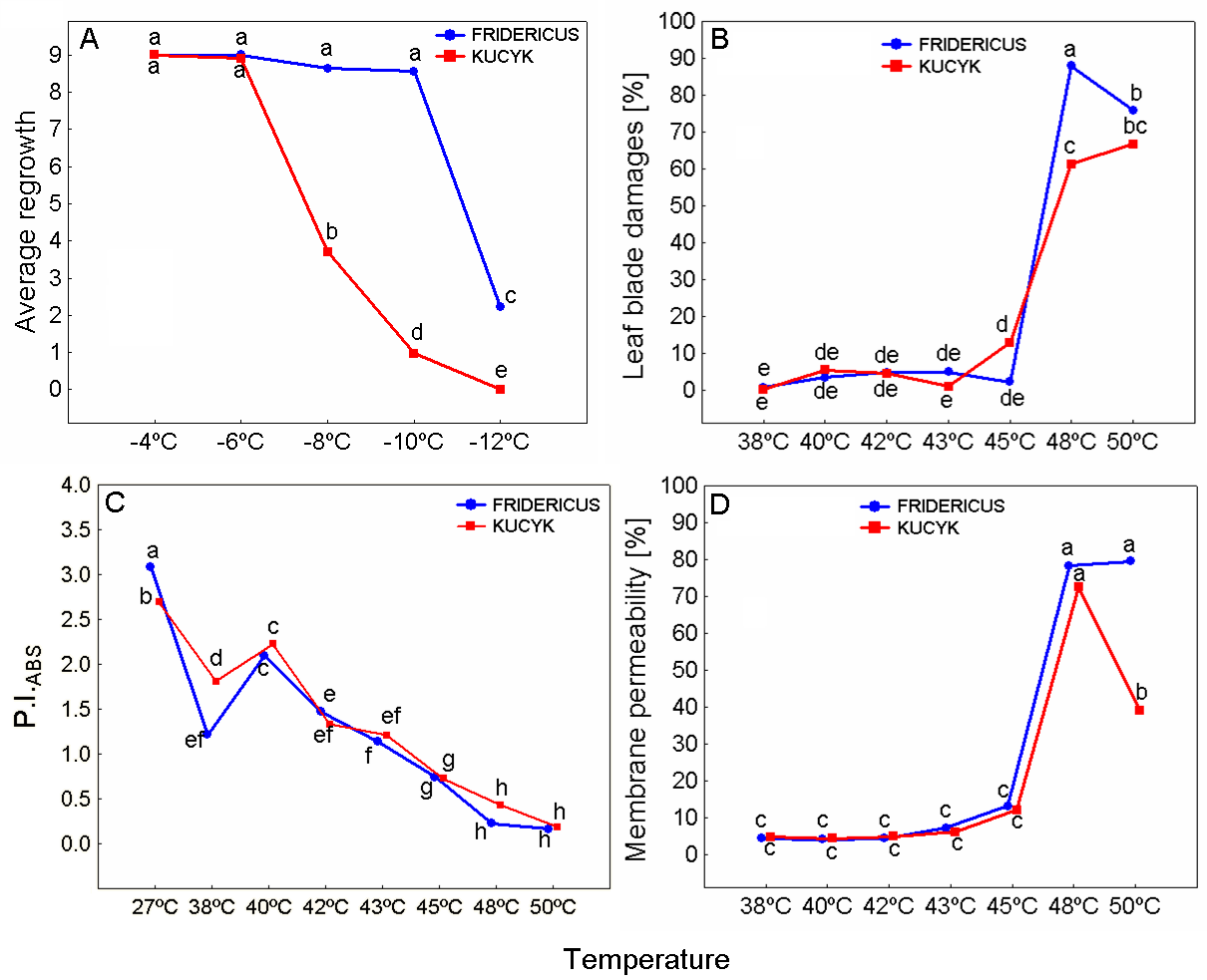

Fig. 1. **Supplementary material**. Test of the tolerance of barley (winter cultivar cv. Fridericus and spring cultivar cv. Kucyk) to frost temperatures ranging from -4°C to -12°C (A) and high temperatures ranging from 38°C to 50°C (B-D). A – average regrowth; B – leaf blade damage; C – photosynthesis efficiency of PSII measured as the Performance Index ($P.I._{ABS}$); D – membrane permeability. Values marked with the same letters did not differ significantly according to the Duncan test ($P \leq 0.05$) for both cultivars and all of the temperatures. In the case of the $P.I._{ABS}$, data that was measured at 27°C are additionally shown.

Table S1. Multiple reactions monitoring (MRM) transitions for the analysed phytohormones and internal standards used: positive ion mode (+ESI), capillary voltage 4 kV, gas temperature 350 °C, gas flow 12 l/min and nebuliser pressure 35 psi.

Compound	Type of ion	Quantifier transition (precursor/product ions)	Fragmentor voltage (V)	Collision energy (V)	MRM Start Time (min)
t-ZOG	$[M+H]^+$	382.1/202.1	142	17	1.6
t-Z	$[M+H]^+$	220.2/136.3	85	9	
c-Z	$[M+H]^+$	220.2/136.3	85	9	
DHZ-N15	$[M+H]^+$	226.2/140	124	18	
DHZ	$[M+H]^+$	222.2/136	124	18	
oxIAA	$[M+H]^+$	192.2/146.1	54	9	4.0
SA-D4	$[M+H]^+$	143.2/125.2	80	14	8.5
SA	$[M+H]^+$	139.2/121.2	80	14	
IAA-D5	$[M+H]^+$	181.1/135.1	38	14	
IAA	$[M+H]^+$	176.1/130.3	51	9	
GA3	$[M-H_2O+H]^+$	329.3/311.3	100	14	
GA1-D2	$[M-H_2O+H]^+$	333.3/287.2	58	9	10.3
GA1	$[M-H_2O+H]^+$	331.3/285.3	100	14	
ABAGlc	$[M-H_2O+H]^+$	409.2/247.1	104	14	
GA6	$[M-H_2O+H]^+$	329.3/283.3	104	14	12.4
ABA-D6	$[M-H_2O+H]^+$	253.4/191.3	80	14	16.7
ABA	$[M-H_2O+H]^+$	247.4/187.2	80	14	
GA5	$[M-H_2O+H]^+$	285.1/267.1	96	5	
IAAMe-D5	$[M+H]^+$	195.2/134.2	80	5	18.3
IAAMe	$[M+H]^+$	190.2/130.2	80	5	
IBA	$[M+H]^+$	204.1/186.4	69	9	
GA4-D2	$[M-H_2O+H]^+$	317.3/271.2	88	9	20.9
GA4	$[M-H_2O+H]^+$	315.3/269.3	100	14	

The monitored hormones ranked in elution order were: *trans*-zeatin-O-glucoside (t-ZOG), *trans*-zeatin (t-Z) and *cis*-zeatin (c-Z), [$^{15}N_4$]dihydrozeatin (DHZ-N15, used as ISTD), dihydrozeatin (DHZ), 2-oxo-indole-3-aceticacid (oxIAA), [$^2H_4$]salicylic acid (SA-D4, ISTD), salicylic acid (SA), [$^2H_5$]indole-3-acetic acid (IAA-D5, ISTD) and indole-3-acetic acid (IAA), gibberellic acid (GA3), [$^2H_2$]gibberellin A_1 (GA1-D2, ISTD), gibberellin A_1 (GA1), abscisic acid glucosyl ester (ABAGlc), gibberellin A_6 (GA6), [$^2H_6$]*cis*,*trans*-abscisic acid (ABA-D6, ISTD), *cis*,*trans*-abscisic acid (ABA), gibberellin A_5 (GA5), [$^2H_5$]indole-3-aceti acid methyl ester (IAAMe-D5, ISTD), indole-3-aceti acid methyl ester (IAAMe), indolebutyric acid (IBA), [$^2H_2$]gibberellin A_4 (GA4-D2, ISTD), gibberellin A_4 (GA4).

Table S2. Changes in content of the particular phytohormones in barley in relation to mutated genes and plant growth temperature [20ºC, 5ºC (21 days), 27ºC (7 days)].
+ increase in comparison to the reference wild type cultivar;
- decrease in comparison to the reference wild type cultivar;
NC - not changed in comparison to wild type;
NM - not measurable
The plant material in our studies included the barley (*Hordeum vulgare* L.) BR-deficient mutant 522DK (mutation in the *HvDWARF* gene) from the Delisa cultivar, the barley BR-deficient mutant BW084 (mutation in the *HvCPD* gene**)**, the BR-signalling mutant BW312 (mutation in the BR receptor gene *HvBRI1*) and their reference cultivar Bowman.

Phytohormone	**Mutated genes**								
	HvDWARF			***HvCPD***			***HvBRI1***		
	20ºC	**5 ºC**	**27 ºC**	**20ºC**	**5 ºC**	**27 ºC**	**20ºC**	**5 ºC**	**27 ºC**
Brassinosteroids									
Brassinolide	-	NM	NM	NM	NM	NM	+	NM	NM
Castasterone	-	-	-	NM	NM	NM	+	+	+
Homocastasterone	NC	NC	-	NC	-	-	-	-	-
Auxins									
IAA	NC	NC	+	NC	NC	NC	NC	NC	NC
oxIAA	-	-	-	NC	-	NC	NC	-	NC
IAAMe	-	NC	NC	+	NC	NC	-	NC	NC
IBA	NC	NC	NC	NC	NC	NC	NC	NC	NC
Cytokinins									
t-Z	NC	NC	NC	NC	NC	NC	-	NC	NC
c-Z	NC	NC	NC	-	-	NC	-	-	NC
DHZ	NC	+	NC	-	NC	NC	-	NC	NC
t-ZOG	NC	NC	NC	NC	-	NC	-	-	NC
Gibberellins									
GA1	-	NC	-	NC	NC	NC	NC	NC	NC
GA3	-	+	NC	+	+	NC	NC	NC	NC
GA4	NC	-	NC	+	NC	NC	+	+	NC
GA5	-	NC	NC	+	+	NC	+	NC	NC
GA6	NC	NC	NC	NC	NC	-	NC	NC	-
ABA	NC	NC	NC	NC	-	NC	-	-	NC
ABAGlc	NC	NC	NC	NC	NC	NC	+	NC	NC
SA	NC	NC	NC	NC	NC	+	NC	-	NC

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OŚWIADCZENIE WSPÓŁAUTORA

Wyrażam zgodę na wykorzystanie przez Iwonę Sadura publikacji: Sadura Iwona, Pociecha Ewa, Dziurka Michał, Oklestkova Jana, Novak Ondrej, Gruszka Damian, Janeczko Anna (2019) Mutations in the *HvDWARF*, *HvCPD* and *HvBRI1* genes – involved in brassinosteroid biosynthesis/signalling: altered photosynthetic efficiency, hormonal homeostasis and tolerance to high/low temperatures in barley. [Journal of Plant Growth Regulation 38: 1062-1081] w przewodzie doktorskim opartym na zbiorze artykułów opatrzonych tytułem: „Rola brasinosteroidów w procesach aklimatyzacyjnych roślin jęczmienia do niskiej i wysokiej temperatury."

Oświadczam, że mój udział w wyżej wymienionej pracy polegał na wykonywaniu wraz z doktorantką (I.Sadura) testów tolerancji badanych roślin jęczmienia na mróz i wysoką temperaturę oraz na dyskusji z doktorantką na temat otrzymanych wyników.

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Olomouc, 2.03.2021

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I declare that my participation in the above-mentioned work consisted in: UHPLC-MS/MS measurement of brassinosteroid content in barley.

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: przygotowaniu materiału nasiennego (w tym nasion linii bliskoizogenicznych i mutantów) do badań oraz współpracy przy pisaniu manuskryptu (analiza/dyskusja aspektów genetycznych).

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Oświadczam, że mój udział w wyżej wymienionej publikacji polegał na: stworzeniu koncepcji badań do projektu 2015/17/B/NZ9/01695, wyborze zespołu naukowców do współpracy, zapewnieniu finansowania z racji pełnienia roli kierownika projektu, koordynowaniu pracy doktorantki (I. Sadura) oraz kierowaniu analizą i interpretacją otrzymanych wyników w czasie procesu tworzenia publikacji.

Anna Janeczko

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HSP Transcript and Protein Accumulation in Brassinosteroid Barley Mutants Acclimated to Low and High Temperatures

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Abstract: In temperature stress, the main role of heat-shock proteins (HSP) is to act as molecular chaperones for other cellular proteins. However, knowledge about the hormonal regulation of the production of the HSP is quite limited. Specifically, little is known about the role of the plant steroid hormones—brassinosteroids (BR)—in regulating the HSP expression. The aim of our study was to answer the question of how a BR deficit or disturbances in its signaling affect the accumulation of the HSP90, HSP70, HSP18, and HSP17 transcripts and protein in barley growing at 20 °C (control) and during the acclimation of plants at 5 °C and 27 °C. In barley, the temperature of plant growth modified the expression of HSPs. Furthermore, the BR-deficient mutants (mutations in the *HvDWARF* or *HvCPD* genes) and BR-signaling mutants (mutation in the *HvBRI1* gene) were characterized by altered levels of the transcripts and proteins of the HSP group compared to the wild type. The BR-signaling mutant was characterized by a decreased level of the *HSP* transcripts and heat-shock proteins. In the BR-deficient mutants, there were temperature-dependent cases when the decreased accumulation of the *HSP70* and *HSP90* transcripts was connected to an increased accumulation of these HSP. The significance of changes in the accumulation of HSPs during acclimation at 27 °C and 5 °C is discussed in the context of the altered tolerance to more extreme temperatures of the studied mutants (i.e., heat stress and frost, respectively).

Keywords: brassinosteroids; acclimation process; small HSPs (sHSPs); HSP70; HSP90; temperature stress

1. Introduction

While abiotic stresses, including extreme temperatures, are a natural part of changing environmental conditions, they are also a serious problem for agriculture. Low- and high-temperature stresses are considered to be some of the major sources of abiotic stress for crop plants [1], and they cause yield losses, among others, in cereals [2]. Plants can acclimate to extreme temperatures (such as frost or heat) via temporary exposure to stress factors of a lower intensity such as cold or warm conditions [3,4]. Cold acclimation is particularly important for winter plants. Temperature stress initiates metabolic changes in plants and activates defense mechanisms such as an enhanced expression of the stress-responsive genes including the genes coding proteins from the protective group, which are called heat-shock proteins (HSPs) [5–7]. These proteins were first discovered in heat-treated

plants and five classes of HSP are now known. They are characterized by their different molecular weights, namely, HSP100, HSP90, HSP70, HSP60, and small heat-shock proteins (sHSP) [5–7]. The main role of HSPs is to act as molecular chaperones. They are responsible for regulating protein folding, as well as its accumulation, location, translocation, and degradation in all plant and animal species [6–8]. In addition to being molecular chaperones, HSPs also have other functions. HSP90 plays an important role in the functioning and translocation of signaling proteins (e.g., kinases, calmodulin, actin) (for a review, see Al-Whaibi [6]; Park and Seo [7]), while HSP70 is thought to be the most abundant heat-shock protein in eukaryotic cells. It is responsible for protecting plant cells from the damaging effects of heat stress, and it is also thought that it assists denatured protein refolding and prevents its aggregation [9]. The main functions of sHSPs are to degrade any misfolded proteins and to prevent irreversible unfolding or incorrect protein aggregation. They are also thought to play a role in acclimating plants to high-temperature stress. Unlike other HSPs, the activity of sHSPs is independent of ATP (for a review, see Al-Whaibi [6]; Park and Seo [7]). Horváth et al. [10] showed that sHSPs bind to specific domains that are located in cell membranes and modulate their properties and physical states. The cell membrane is an important element in counteracting the negative effects of abiotic stresses (including extreme temperatures) and, according to the work of Horváth et al. [10], cell membranes are considered to be "thermal sensors" (membrane sensor hypothesis); thus, they are thought to be the main cause of many of the metabolic changes within a cell, including the expression of certain genes. Like physiological/biochemical processes, the expression of HSPs may be regulated/modulated by phytohormones. However, knowledge about the hormonal regulation of the production of the HSP production is quite limited. Little is known about the role of the plant steroid hormones, brassinosteroids (BRs), in the expression of HSPs. BRs were discovered in *Brassica napus* pollen in the 1970s [11,12]. BRs occur in almost all parts of a plant and, to date, about 70 BRs were isolated. BRs can prevent or reduce injuries caused by many environmental stresses including extreme temperatures [13,14]. In plants that are exposed to high/low temperatures, the application of BR changes the physicochemical properties of the plant plasma membrane, regulates photosynthesis and sugar production, and generally controls the various directions of the metabolism of plants through its interactions with other phytohormones (for a review, see Sadura and Janeczko [14]). Studies on BR mutants revealed that the endogenous content of BRs or disturbances in BR signaling have an impact on the temperature stress tolerance of *Arabidopsis thaliana*, tomato, and barley plants [15–17]. BR mutants with a disturbed BR signaling or with a BR deficit resulting from mutations of the gene encoding enzymes that control the specific steps of BR biosynthetic pathways are generally good models for studying the role of these phytohormones in plants. Therefore, the plant material that was used in the present study included the barley BR mutants 522DK, BW084, and BW312 [18,19]. The 522DK mutant, which was derived from a Delisa cultivar, was induced via chemical mutagenesis in the Delisa cultivar; it has a mutation in the *HvDWARF* gene encoding brassinosteroid C6-oxidase, which is involved in BR biosynthesis [18,20]. This mutation is connected to a lower content of castasterone, brassinolide, and 28-homocastasterone by about 60%, 85%, and 25%, respectively, in the 522DK mutant compared to the wild-type Delisa [17]. The BW084 and BW312 mutants are semi-dwarf near-isogenic lines (NILs) that were derived from a Bowman cultivar [19]. The BW084 (*brh13.p*) carries a mutation in the *HvCPD* gene, which encodes the barley C-23α-hydroxylase cytochrome P45090A1 (CYP90A1) that catalyzes the early stages of BR biosynthesis. This mutation causes a significantly lower level of castasterone and brassinolide (below the detection limit) and an 18% lower content of 28-homocastasterone than the wild-type Bowman cultivar [17]. The BW312 (ert-ii.79) mutant has a double substitution (CC1760/1761AA) in the *HvBRI1* gene encoding the BR receptor kinase, BRI1, which results in a disturbance in the binding of the BR molecules, as well as a significantly higher content of BR [17,19]. After acclimation at 27 °C, all these mutants were characterized by a higher tolerance to high temperatures (38–45 °C) [17]. However, after acclimation at 5 °C, the frost tolerance of mutants was unchanged (522DK, −8 °C), slightly higher (522DK, −6 °C) or lower (BW084 and BW312, −6 °C and −8 °C) [17]. Studies of the expression of heat-shock proteins could, thus, provide knowledge

about the hormonal regulation of the production of HSPs (the role of BRs) and could potentially help explain the molecular background of the changed tolerances to temperature stress of BR mutants. The aim of the study was to answer the following main question: How do a BR deficit and disturbances in its signaling affect the accumulation of the HSP90, HSP70, HSP18, and HSP17 transcripts and protein in barley that is grown at 20 °C (control) and during the acclimation of plants at 5 °C and 27 °C?

2. Results and Discussion

2.1. Presence of Heat-Shock Proteins in Barley Membrane and Cytosolic Fractions

In cells, heat-shock proteins can be found in the cytoplasm, in specific organelles such as the mitochondria or the endoplasmic reticulum, and, generally, in the cell membranes. The localization can be different for specific HSPs. Our studies on barley enabled us to prove the presence of HSP90 in a membrane fraction that was isolated from barley plants (Figure 1C–F); however, it was below the detection limit in the cytosolic fraction for our method. According to Xu et al. [21], HSP90 mainly occurs in the cytoplasm and only sometimes in the endoplasmic reticulum, mitochondria and chloroplasts, but recent studies showed that HSP90 can also interact with the cell membranes and affect their structure [22].

On the other hand, immunodetecting the HSP70 protein using the primary antibody against cytoplasmic HSP70 revealed the presence of this protein in both the membrane and the cytosolic fractions that were isolated from barley (Figure 2C–J). This finding is in agreement with the literature. HSP70 was originally identified as being one of the most abundant proteins produced in response to an elevated temperature that induces thermotolerance [23]. Currently, these 70-kDa heat-shock proteins are known to be upregulated during many cellular stresses, and they are essential during normal growth. HSP70 is a multigenic family, and the various members that constitute this family are present in different cellular compartments [9,24]. Recently, it was found that the HSP70 protein incorporates into the lipid bilayer and interacts with the cell membranes [25]. It was explained that the insertion of HSP70 into the plasma membrane is the gateway for its export to the extracellular matrix in the form of vesicles, where they play a signaling role to alert and activate the necessary defense machinery against harmful conditions.

As for the sHSPs, in our study, HSP17 and HSP18 were not found in the cell membrane or cytosolic fraction of the barley, although the *sHSP* transcripts were present and even drastically increased after acclimation at 27 °C. It is likely that these proteins typically accumulate under extreme high-temperature stress, usually higher than 40 °C [26–28]. The high accumulation of the *sHSP* transcripts in plants might be interpreted as a mechanism that prepares plants for more extreme temperature. Simultaneously, as discussed by Dhaubhadel et al. [29], the presence of a specific messenger RNA (mRNA) of HSPs in cells is not necessarily accompanied by an active translation or a translation that is in accordance with their levels. According to the literature, sHSPs are located rather in the thylakoid membranes where they can act as "membrane-stabilizing factors" by influencing the integrity of the cell membrane during stress [6,10,12].

2.2. Changes in the Accumulation of the HSP90 Transcript and Protein in the Barley BR Mutants and Wild-Type Plants Growing at 20 °C and Acclimated at 5 °C and 27 °C

In our experiment, the acclimation of the cultivar Delisa at 5 °C did not cause any significant differences in the accumulation of the *HSP90* transcript compared to the plants in the control conditions (20 °C) (Figure 1A). The barley cultivar Bowman at 5 °C was characterized by an increased accumulation of the *HSP90* transcript compared to the plants at 20 °C (Figure 1B), which is in agreement with the findings of Krishna et al. [30] and Kagale et al. [31]. These authors observed an increase in the accumulation of the *HSP90* transcript in *Brassica napus* seedlings at 5 °C and in *Arabidopsis thaliana* at 2 °C.

In our barley, the accumulation of the HSP90 protein in the cell membranes was significantly lower after 21 days at 5 °C than in the respective plants at 20 °C in both the Delisa and the Bowman cultivars (Figure 1C–F). Earlier, Krishna et al. [30] observed a gradual increase in the accumulation of the HSP90 protein in the leaf tissue of *B. napus* at 5 °C, while Vítámvás et al. [32] found that, in winter wheat, the accumulation of the HSP90 protein decreased at 6 °C if compared to the control plants.

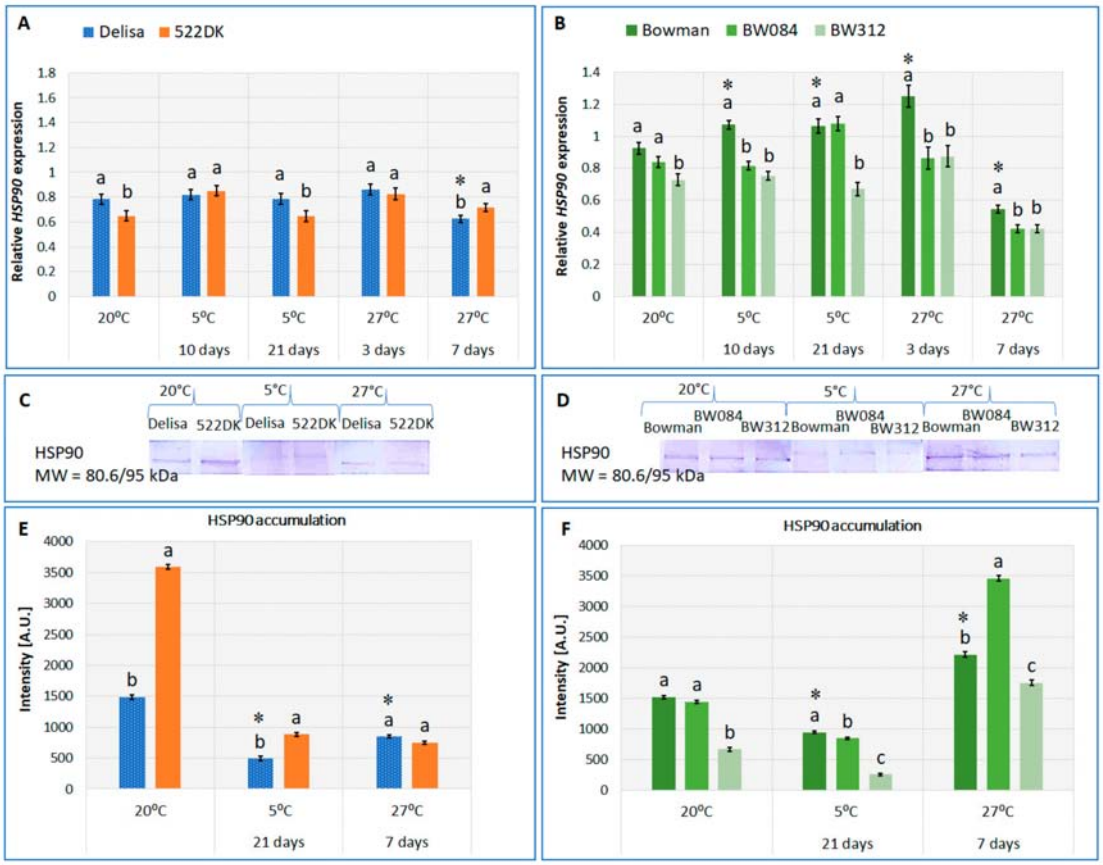

Figure 1. Changes in relative transcript level of heat-shock protein 90 (*HSP90*) (**A**,**B**) and the accumulation of HSP90 (**C**–**F**) in the barley cell membrane fraction isolated from the cultivar (cv.) Delisa, 522DK mutant, cv. Bowman, and the BW084 and BW312 mutants growing in different temperature conditions. The transcript levels are presented as the fold change in the expression of a specific gene in the specific samples compared to the reference gene *actin*. The visualized bands corresponding to the HSP90 protein were identified as described in Section 3. In total, 15 µg of proteins were loaded onto the gel. MW—molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder). A.U.—arbitrary units. The statistical differences between the cv. Delisa and its mutant 522DK (Student's *t*-test, $p \leq 0.05$) and between the cv. Bowman and its mutants (Duncan's test, $p \leq 0.05$) for each temperature are indicated by different letters. Additionally, the accumulation of the transcript and protein in the Delisa and Bowman cultivars at different temperatures was also compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $p \leq 0.05$), and the statistical differences are indicated by an asterisk.

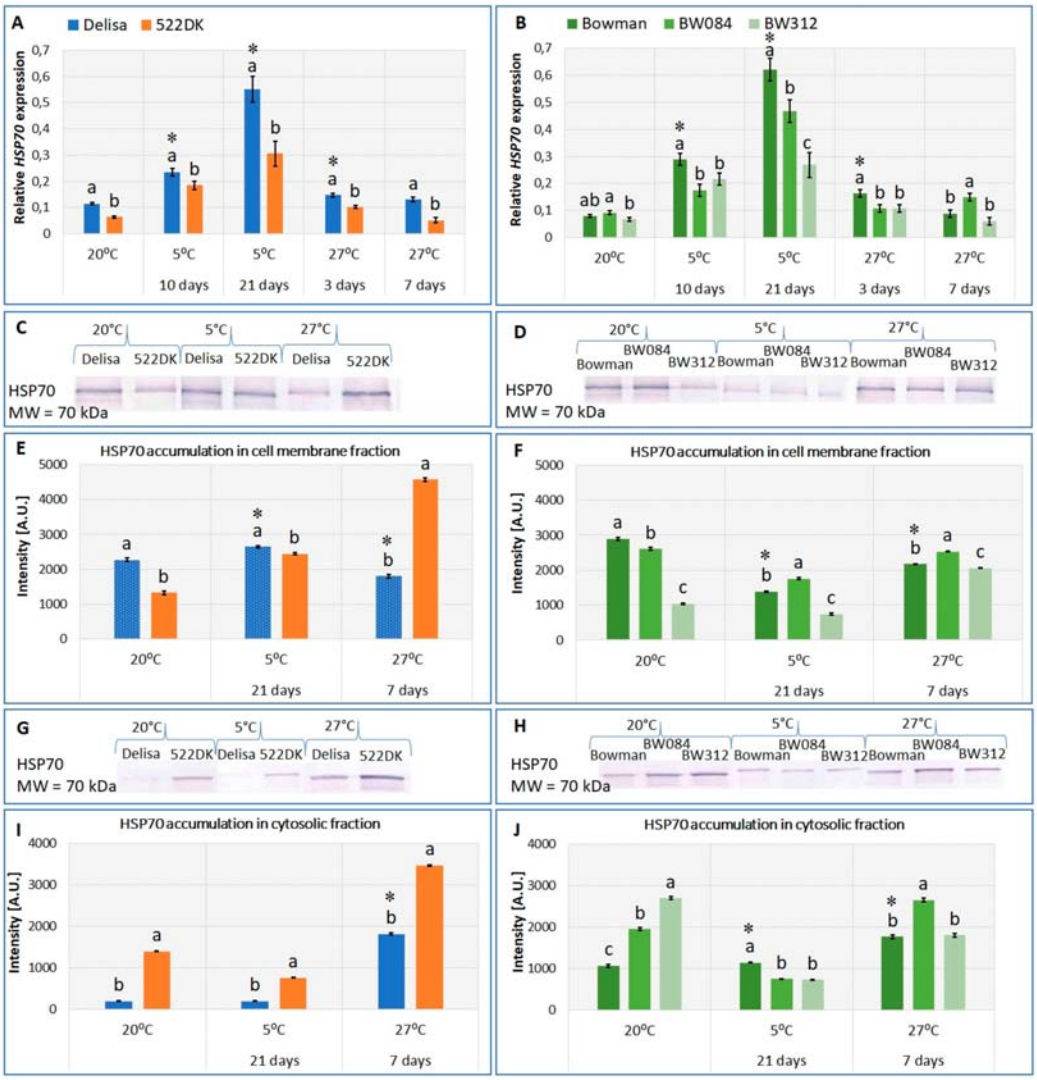

Figure 2. Changes in the relative transcript level of *HSP70* (**A**,**B**) and the accumulation of the HSP70 protein in the barley cell membrane fraction (**C**–**F**) and in the cytosolic fraction (**G**–**J**) of the cv. Delisa, 522DK mutant, cv. Bowman, and the BW084 and BW312 mutants growing in different temperature conditions. The transcript levels are presented as the fold change in the expression of a specific gene in the specific samples compared to the reference gene *actin*. The visualized bands correspond to the HSP70 protein identified as described in Section 3. In total, 10 µg of the proteins from the cell membrane fraction and the cytosolic fractions were loaded onto the gel. MW—molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder). A.U.—arbitrary units. Statistical differences between the cv. Delisa and its mutant 522DK (Student's *t*-test, $p \leq 0.05$) and between the cv. Bowman and its mutants (Duncan's test, $p \leq 0.05$) for each temperature are indicated by different letters. Additionally, the accumulations of the transcript and protein in the Delisa and Bowman cultivars at different temperatures were also compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $p \leq 0.05$) and the statistical differences are indicated by an asterisk.

As for studies of BR mutants, a decrease in the accumulation of the *HSP* transcript in the BR mutants (compared to the wild types) was sometimes accompanied by an increase in the accumulation of the protein but sometimes not (Figure 1 A–F). For example, the level of the *HSP90* transcript was lower in the 522DK than in the Delisa at 20 °C and 5 °C (after 21 days), while the protein accumulation in the membrane fraction was doubled in this mutant in comparison to wild type. Simultaneously, at 5 °C and 20 °C, a lower accumulation of the *HSP90* transcript in BW312 (compared to the Bowman)

was also accompanied by a lower accumulation of the protein. Hence, an interesting phenomenon can be observed; the disturbances in BR biosynthesis (in the BW084 and 522DK mutants) were reflected by a decreased accumulation of the *HSP90* transcript after a shorter or longer exposure to the low temperature, respectively (effect at the transcriptional level) (Figure 1A,B). Interestingly, the disturbance in BR biosynthesis in the 522DK mutant resulted in an increase in the HSP90 protein accumulation under both control (20 °C) and stress conditions (5 °C) for the Delisa cultivar (effect at the translational level) (Figure 1C,E). However, the mutation in the BW084 mutant did not have any significant effect at 20 °C (or had only a weak effect at 5 °C) at the translational level (Figure 1D,F). On the other hand, a disturbance in the BR signaling in the BW312 mutant led to a significant decrease in the transcript and protein accumulations compared to the Bowman cultivar under at control and low temperature (Figure 1B,D,F). Thus, the disturbance in BR signaling had an effect at both transcriptional and translational levels. It can also be inferred that the BR insensitivity affects the function of the major transcription factors that are required for the BR-dependent gene [33–35]. To the best of our knowledge, there is only one article that discussed the effect of BR on the expression of HSP90 in plants at low temperature. *Arabidopsis thaliana* that was acclimated at 2 °C (three days) and treated with 1 µM 24-epibrassinolide had a similar accumulation of the *HSP90* transcript level to the BR-untreated plants [31].

Furthermore, in our experiment, the *HSP90* transcript and the HSP90 protein levels were analyzed in both cultivars that were acclimated at a temperature of 27 °C. In the Bowman and Delisa, after a transient increase in the accumulation of the *HSP90* transcript on the third day of the acclimation at 27 °C (statistically significant effect only in the Bowman), there was a lower level of this transcript on the seventh day (Figure 1A,B). On the seventh day of the acclimation, the HSP90 protein accumulation in the cell membranes was about 30% lower in the Delisa but was higher in the Bowman compared to the control (20 °C) (Figure 1C–F). From the literature, it is well known that high temperatures stimulate the synthesis of HSPs in plants [5]. The known effects are primarily related to the effects of extremely high temperatures (heat shock). The expression of *HSP90* genes was higher in carrot cell lines at a temperature of 38 °C [36]. Pavli et al. [37] investigated the effect of heat stress (47 °C; 180 min) on the expression of *HSP90* in four sorghum genotypes and found that an increase in the relative accumulation of the *HSP90* transcript was followed by a decrease. In the leaves of *Brassica napus*, heat (45 °C; 2 h) increased the accumulation of the *HSP90* transcript, as well as the accumulation of the HSP90 protein [30]. In this context our studies provide some new knowledge about the expression of HSP90 during the process of acclimation at slightly lower temperatures, which may lead to an acquired thermotolerance as mentioned by Altschuler and Mascarenhas [3].

Furthermore, we observed how disturbances in BR signaling or BR deficiency modified the patterns of the changes that were described for the wild-type cultivars at an acclimating temperature of 27 °C. In the 522DK mutant, there was no impact of the BR deficiency on the accumulation of *HSP90*, especially after three days of acclimation at 27 °C. After seven days of acclimation, 522DK accumulated about 15% more of the *HSP90* transcript than its wild-type cultivar (Figure 1A). No difference in the protein content was observed between the wild type and the mutant (Figure 1C,E). After both three and seven days of acclimation at 27 °C, BW084 accumulated about 35% and 28% less of the *HSP90* transcript, respectively, than the wild-type Bowman, while the accumulation of the HSP90 protein was about 60% higher than in the Bowman. For BW312, the accumulation of the HSP90 protein (similar to the transcript; Figure 1B) was about 20% lower than in the Bowman after seven days at 27 °C (Figure 1D,F).

Although BRs are known to play a role in enhancing the high-temperature stress tolerance in plants [38], the available data regarding their influence on the expression of HSP90 are different. Dhaubhadel et al. [29,39] reported that, after heat stress (*Brassica napus* and tomato), the accumulations of HSPs (including HSP90) at the transcript and protein levels were higher in the BR-treated plants than in the untreated control. Hovewer, Kagale et al. [31] later reported that the exogenous application of BRs in wild-type *Arabidopsis* exposed to a high temperature did not change the accumulation of the

HSP90 transcript compared to the BR-untreated plants. Since there is no information on the effect of BR on the expression of HSP90 during the process of acclimation at slightly lower temperatures, our studies provide new knowledge on this subject.

2.3. Changes in the Accumulation of the HSP70 Transcript and Protein in the Barley BR Mutants and Wild-Type Plants Growing at 20 °C and Acclimated at 5 °C and 27 °C

Both the Delisa and the Bowman cultivars accumulated significantly more of the *HSP70* transcript after 10/21 days of acclimation at 5 °C than at 20 °C (Figure 2A,B). After acclimation at 5 °C, the Delisa accumulated about 13% more of the HSP70 protein in the cell membrane fraction than the control plants (20 °C) (Figure 2C,E). In contrast to the Delisa, the cold-acclimated Bowman accumulated about 50% less of the HSP70 protein in the cell membrane fraction than the plants that were not acclimated (Figure 2D,F). In the cytosolic fraction, the HSP70 accumulation did not differ for the Delisa growing at 5 °C and 20 °C (Figure 2G,I). The HSP70 accumulation was statistically significantly higher for the cold-acclimated Bowman compared to the plants growing at 20 °C, although only by about 9% (Figure 2H,J). The results of our study correspond to the effects that were described in the literature, especially in the case of the accumulation of the *HSP70* transcript. The effect of temperature on the transcript and protein levels of HSP70 in three tomato genotypes was investigated by Kubienová et al. [23]. A temperature of 4 °C increased the accumulation of the *HSP70* transcript in the two genotypes and also increased the accumulation of the HSP70 protein in one of the three genotypes. The cold acclimation (6 °C) of winter wheat (*Triticum aestivum* L.) increased the accumulation of the HSP70 protein in a crude extract of crowns [32]. Similar results were also obtained on rice roots that were cold acclimated at 10 °C [40], pea stems (10/2 °C; day/night) [41], and chicory roots [42].

To the best of our knowledge, there were no studies concerning the effect of BR on the accumulation of HSP70 in cold-acclimated plants. The results of our study show that both BR-deficient mutants, 522DK and BW084, accumulated significantly less of the *HSP70* transcript (by about 44% and 24%, respectively) after 21 days of cold acclimation than their respective wild types (Figure 2A,B). The BR-signaling mutant BW312 was also characterized by a markedly lower level of the accumulation of the *HSP70* transcript after both 10 and 21 days of cold compared to the Bowman cultivar (Figure 2B). The accumulation of the HSP70 protein in the cell membrane fraction was lower in the 522DK mutant than in the Delisa both at 20 °C and after acclimation at 5 °C (Figure 2C,E). However, the 522DK mutant accumulated significantly more of the HSP70 protein in the cytosolic fraction than its wild type at 20 °C and 5 °C (Figure 2G,I). The accumulation of the HSP70 protein in the cell membrane fraction of BW084 was lower than in the Bowman at 20 °C but higher than in the Bowman after growing at 5 °C (Figure 2D,F). The opposite tendency was observed in the cytosolic fraction in which HSP70 was accumulated in a higher amount in BW084 than in the Bowman at 20 °C but in a lower amount in BW084 than in the Bowman at 5 °C (Figure 2H,J). Compared to the Bowman (at 20 °C), the BW312 mutant was characterized by about a 70% lower accumulation of HSP70 in the cell membrane fraction, while there was an increase of about 70% in the accumulation of this protein in the cytosolic fraction (Figure 2D,F,H,J). After acclimation at 5 °C, the accumulation of HSP70 in this mutant was lower compared to the wild type in both the membrane and the cytosolic fraction (Figure 2D,F,H,J).

In both the Delisa and the Bowman cultivars, three days of acclimation at 27 °C increased the accumulation of the *HSP70* transcript compared to the control (20 °C); however, after seven days of acclimation, its level returned to that of the control plants (Figure 2A,B). In the case of the accumulation of the HSP70 protein, a different tendency was observed for the cell membrane and cytosolic fractions of the Delisa and Bowman. In the cell membrane fraction, the HSP70 levels were significantly lower than in the control plants (20 °C) (Figure 2C–F). In the cytosolic fraction, there was a noticeable increase in both the Delisa and the Bowman cultivars (Figure 2G–J). As mentioned earlier, high temperatures usually increase the accumulation of the *HSP70* transcript and protein in plants, but this is mainly associated with more extreme temperatures than those tested in our studies. Heat stress (40.5 °C) increased the accumulation of the HSP70 transcript and protein in tomato compared to the control (20 °C) [23]. After

one hour at 37 °C, there was a significant increase for all 10 studied members in the stress 70 family in spinach and tomato seedlings, which was followed by a decrease during the next 11 h of heat shock treatment [43].

In our work, we also investigated the effect of a temperature of 27 °C on the expression of HSP70 in the BR mutants. The BR-deficient mutant 522DK that was acclimated at 27 °C for three and seven days accumulated significantly less of the *HSP70* transcript than its wild-type Delisa (Figure 2A). A similar tendency was observed for the BW084 mutant that was acclimated to a high temperature for three days; however, after seven days of growing at 27 °C, the *HSP70* transcript level was higher in this mutant than in the wild-type Bowman (Figure 2B). Despite some differences in the accumulation of the transcript in both of the BR-deficient mutants at 27 °C, the accumulation of the HSP70 protein was significantly higher in the cell membrane fraction and cytosolic fraction in both mutants compared to their respective wild types (Figure 2C–J).

After acclimation at 27 °C, the BR-signaling mutant (BW312) had generally a decreased accumulation of the *HSP70* transcript compared to the wild type (Figure 2B). The mutant also accumulated less of the HSP70 protein in the cell membrane fraction than the Bowman, while the HSP70 level in the cytosolic fraction was similar to that in the wild type (Figure 2D,F,H,J).

The significance of BR in regulating the expression of HSP70 under heat stress was also studied by Dhaubhadel et al. [29] and Kagale et al. [31], but for temperatures much higher than 27 °C. The BR-deficient mutants of *Arabidopsis thaliana* at 43 °C accumulated more of the *HSP70* transcript than the unstressed plants, while, in the mutants that were treated with 24-epibrassinolide, the accumulation of the *HSP70* transcript was lower compared to the BR-untreated plants. As for the accumulation of the HSP70 protein, Dhaubhadel et al. [29] showed that treating *Brassica napus* plants with BR under heat stress (45 °C) increased the accumulation of HSP70 compared to the untreated plants.

Regarding HSP70, we also observed a changed relationship between the presence of HSP70 in the cytosolic fraction and membrane fraction for both the mutants and the wild types. Earlier, Armijo et al. [25] also found that HSP70 can be incorporated into the lipid bilayer in artificial cell membranes. In the mutants that were cultured especially at 20 °C, the relationship between HSP70 that was present in the cytosolic fraction and HSP70 in the membranes was disturbed compared to the wild types (Supplementary Figure S1,). All of the mutants accumulated more HSP70 in the cytosolic fraction than in the membrane fraction at 20 °C compared to the wild types. This relationship was also slightly changed at 5 °C, although it was unchanged at 27 °C (data not shown). This may suggest that BRs somehow participate in regulating the balance between the HSP70 present in the cytoplasm and that incorporated into the membranes; however, once again, because the phenomenon is dependent on temperature, it requires more detailed studies.

2.4. Changes in the Accumulation of the HSP18 and HSP17 Transcripts in the Barley BR Mutants and Wild-Type Plants Growing at 20 °C and Acclimated at 5 °C and 27 °C

In our studies, the acclimation of the barley cv. Delisa and Bowman at a low temperature did not change the accumulation of the *HSP17* transcript compared to the control growing at 20 °C (Figure 3A,B), and only trace amounts of the transcript were detected at both temperatures. Transcript *HSP18* was below the detection limit in plants at 5 °C, although some traces were observed at 20 °C (control) (Figure 3C,D). No accumulation of the HSP17 and HSP18 proteins was detected in either the cell membranes or in the cytosolic fraction of the plants that were acclimated to a low temperature. According to the literature, however, the connection between cold treatment and the expression of sHSPs in some plant species may exist. For example, sHSPs take part in the mechanisms of tomato chilling tolerance [44]. Moreover, the overexpression of the chloroplast-localized sHSPs increased the cold tolerance in tomato [45]. On the other hand, the role of BRs in the expression of the small heat shock protein during a plant's acclimation to a low temperature is unknown. In light of this, we assume that our studies are the first to present the slight differences in the level of accumulated transcripts that were only found between the Bowman and its mutants (not between the Delisa and 522DK) (Figure 3A–D). In the control conditions of 20 °C, the accumulation of the *HSP17* and *HSP18* transcripts

was slightly lower in the Bowman than in its mutants. At a low temperature, only the *HSP17* transcript was observed, and only during acclimation (10th day) was a slightly increased amount observed in the case of BW084 (compared to the Bowman). This effect disappeared after 21 days in cold.

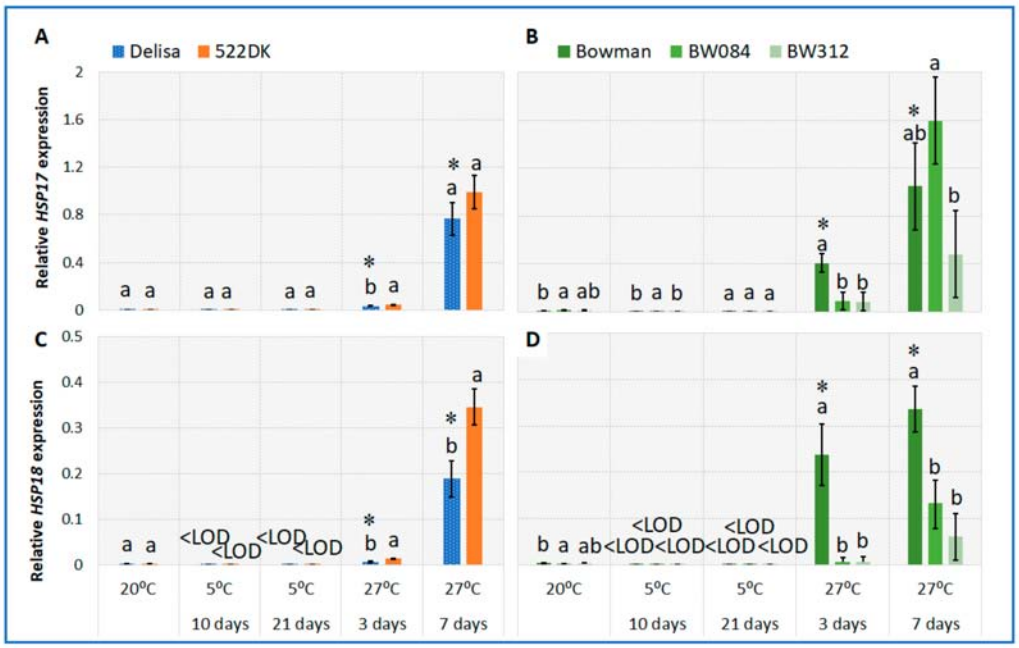

Figure 3. Relative transcript level of *HSP17* and *HSP18* in the barley leaves of the cv. Delisa, 522DK mutant (**A**,**C**), cv. Bowman, and the BW084 and BW312 (**B**,**D**) mutants at 20 °C and during and after acclimation at 5 °C and 27 °C. The transcript levels are presented as the fold change in the expression of a specific gene in the specific samples compared to the reference gene *actin*. The statistical differences between the cv. Delisa and its mutant 522DK (Student's *t*-test, $p \leq 0.05$) and between cv. Bowman and its mutants (Duncan's test, $p \leq 0.05$) for each temperature are indicated by different letters. Additionally, the accumulations of the transcript in the Delisa and Bowman cultivars at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $p \leq 0.05$) and the statistical differences are indicated by an asterisk. LOD: below detection limit.

Heat stress intensively induces the accumulation of the sHSP transcript and protein in plants [5,27,28,46–48]. The temperatures that were tested were within a range of 37–45 °C, but less is known about the expression of the sHSP during the process of plant acclimation in which a gradual increase in the temperature improves the thermotolerance of plants to extremely high temperatures [4]. In our studies, during acclimation at 27 °C, the Delisa and Bowman accumulated significantly more of the *HSP18* and *HSP17* transcripts compared to the plants in the control conditions (20 °C) (Figure 3A–D). Interestingly, the effect was really strong after seven days of acclimation and much weaker after three days, especially in the Delisa. The plant reaction was then much slower than in the case of heat shock (described in literature), where there was an increase in the transcript accumulation after several hours. Moreover, it is likely that the plants at 27 °C had an increased transcript accumulation of the sHSP as part of the preliminary acclimation mechanisms that prepare plants for more extreme temperatures. As was explained at the beginning of this section, we were not able to detect any of the small heat-shock proteins in the isolated cytosolic or membrane fractions.

In our studies, we also focused on changes in *sHSP* during the acclimation of the BR mutants at 27 °C. After three days, the transcript level (*HSP17* and *HSP18*) in the BR-deficient mutant 522DK was higher than in the wild-type Delisa (Figure 3A,C). After seven days, this tendency was maintained, although only in the case of *HSP18* was the difference between 522DK and the Delisa statistically significant. In contrast to 522DK, after three days of acclimation, the second BR-deficient mutant had a significantly lower accumulation of the *HSP17* and *HSP18* transcripts compared to its wild type

(Figure 3B,D). After seven days, the effect was only maintained in the case of *HSP18*. The BR-signaling mutant BW312 had a drastically lower accumulation of both transcripts compared to the wild type after three and seven days of acclimation (Figure 3B,D). As already mentioned for the mutants, we also did not detect any HSP17 and HSP18 proteins in the cytosolic or membrane fractions. Although we realize that it can be difficult to visualize these proteins due to their low abundance and a significant increase in their content only at high temperatures, we decided to perform such an experiment in order to determine whether a mutation in the BR synthesis or BR signaling in barley plants can affect the expression of the studied sHSPs.

According to the literature, BRs are thought to be a factor that induces sHSP synthesis in heat-stressed plants [31,39,49], but these studies were performed only for extremely high temperatures. The 24-epibrassinolide-treated *Brassica napus* seedlings accumulated more *sHSP* transcripts at 45 °C than the untreated ones [39]. BR-treated tomato accumulated a higher amount of mitochondrial small heat-shock proteins than the BR-untreated control at 38 °C [49]. Kagale et al. [31] studied the effect of 43 °C on the accumulation of the class II *sHSP* transcript in two BR-deficient mutants of *Arabidopsis thaliana*. One of the mutants accumulated *sHSP* even before heat stress (in the control conditions of 22 °C), while the second one did not. A high temperature increased the accumulation of the *sHSP* transcript in both mutants compared to the plants in the control conditions (22 °C). Interestingly, treating the mutants with 24-epibrassinolide even decreased the accumulation of the *sHSP* transcript in both mutants.

2.5. General Comments

The role of brassinosteroids as positive regulators of the expression of HSP in barley seems to be proven mainly by the results that were obtained for the BR-signaling mutant. In most cases, the mutant with a defective BR receptor had a lower accumulation of the *HSP* transcripts and HSP proteins compared to the wild type regardless of the plant growth/acclimation temperature. The results that were obtained for both BR-deficient mutants (BW084 and 522DK) confirm that BRs are among the players that regulate HSP expression. Interestingly, however, the accumulation of the *HSP70* or *HSP90* transcripts was most often lower in the mutants, although this was also often accompanied by an increased level of the heat-shock proteins. According to the literature [29], the levels of heat-shock protein may be higher in heat-treated plants (during the recovery period), while the transcripts that correspond to these proteins can be present at higher levels in an untreated control. In addition, our results shows that, contrary to the disturbances in BR signaling, a decrease in the level of BRs (BRs were drastically lowered in BW084, while relatively slightly lowered in 522DK) did not always act negatively on the accumulation of the HSP protein. What is more surprising, however, is that there were some cases in which the direction of the changes was different in 522DK and BW084. For example, the level of HSP90 at 5 °C was higher in the 522DK mutant than in the wild type (Delisa), while, in BW084, it was slightly lower than in its wild type (Figure 1C–F). The differences in the transcript and protein accumulation patterns that were observed between both BR biosynthetic mutants seem to reflect the fact that the mutants have defects at different stages of the BR biosynthesis pathway [19]. The 522DK mutant carried the missense mutations in the *HvDWARF* gene, while, in BW084, it was in the *HvCPD* gene. *HvDWARF* encodes the C6-oxidase enzyme that is involved in the later stages of BR biosynthesis, while the enzyme that is encoded by *HvCPD* acts during the earlier BR biosynthetic stages. The phenomenon of the different expressions of HSP depending on the mutation that was connected to BR biosynthesis was also observed by Kagale et al. [31]. At 22 °C, the *Arabidopsis* BR-deficient mutant *det2-1* accumulated *HSP* transcripts, while a second BR-deficient mutant *dwf4* did not. Further, the influence of the genetic background cannot be underestimated, since both mutants come from different cultivars, which are characterized by different levels of other hormones [17]. Because BRs, when regulating the physiological/biochemical processes, act in a complicated network with other hormones (and also interact with them), the regulatory mechanism of biosynthesis of some proteins including HSP may be more complex.

The directions of changes of the accumulation of the transcripts and proteins of the HSP group in mutants compared to the wild types at various temperatures are summarized and visualized in Supplementary Tables S1 and S2.

The plant growth temperature (20 °C, control) as well as the acclimation of plants at 5 °C or 27 °C, affected the expression of the HSP in all of the mutants. Changes in the expression of the selected heat-shock proteins during the acclimation of mutants may, however, only partly explain the reasons for the altered tolerance of the mutants to a high temperature or frost that was described in our earlier studies [17]. In relation to the tolerance to frost, attention should be paid, in particular, to changes in the expression of HSP90 observed during acclimation. After acclimation at 5 °C, the BW084 and BW312 mutants were characterized by a lower survival rate in frost (−6 °C and −8 °C) than the Bowman reference cultivar [17]. At the same time, these mutants accumulated less of the protective HSP90 protein during acclimation at 5 °C. HSP90 is indicated as being important for low-temperature tolerance [30]. The 522DK mutant accumulated more HSP90 after acclimation than the Delisa and had an unchanged (at −8 °C) or even a slightly higher frost tolerance (−6 °C) than the wild type [17]. It is worth mentioning that, compared to BW312 or BW084, 522DK also accumulated more HSP90 before acclimation at 5 °C (Figure 1C–F). This could also have some protective significance for the plants at the beginning of cold acclimation.

In turn, in the case of the higher tolerance of the mutants to a high temperature (38–45 °C) than the wild type [17], more attention should be paid to changes in the HSP70 protein level, particularly in the mutants of BR biosynthesis. Both of these mutants accumulated more HSP70 than the wild type after acclimation at 27 °C. This tendency, however, did not apply to the BR-signaling mutant, in which the HSP70 level was similar to the wild-type Bowman, although the tolerance to a high temperature was higher, thus other factors had to be additionally responsible for its higher tolerance.

The main findings of the whole work are summarized/concluded in Section 4.

3. Materials and Methods

3.1. Plant Material

The spring barley (*Hordeum vulgare* L.) cultivars (Delisa and Bowman) and their mutants (522DK, BW084, and BW312) were used in our study. The mutants were derived from the collection of the Department of Genetics at the University of Silesia (Katowice, Poland). Both the cultivars and the mutants were selected according to the works by Gruszka et al. [18] and Dockter et al. [19].

3.2. Plant Culture and Experimental Design

The plants were cultivated as described by Sadura et al. [17]. Briefly, after germination, the plants were cultivated in a growth chamber at 20 °C (16 h photoperiod) for about three weeks and then the plants were divided into two groups. The first group was acclimated at 5 °C (day/night) under an 8-h photoperiod for 21 days, while the second group was acclimated at 27 °C (day/night) under a 16-h photoperiod for seven days. After acclimation, the plants had four well-developed leaves and sometimes a young fifth leaf. Light intensity in the growth chambers was 170 $\mu mol \cdot m^{-1} \cdot s^{-1}$ (HPS Philips SON-T AGRO 400-W lamps).

Samples for the analyses of the accumulation of a transcript (*HSP90*, *HSP70*, *HSP18*, and *HSP17*) were taken from the plants (the middle part of the second leaf) at 20 °C (control, before acclimation) and during/after acclimation (on the 10th and 21st days at 5 °C; on the third and seventh days at 27 °C).

To determine the accumulation of protein (HSP90, HSP70, HSP18, and HSP17), two types of samples were prepared: cytosolic fraction samples and membrane fraction samples. The aerial parts of the seedlings were cut off and the cell membrane fraction and cytosolic fraction were immediately isolated and then frozen at −80 °C for further analysis of the proteins. The samples were taken at 20 °C (control, before acclimation) and after acclimation (on the 21st day at 5 °C and on the seventh day at 27 °C).

3.3. Isolation of the Membrane and Cytosolic Fractions

The cell membranes were isolated according to a modified protocol of Sommarin et al. [50] and Janeczko et al. [51]. In total, 100 g of leaves were homogenized in 400 mL of a cell fraction isolation buffer (pH 7.8) containing 10 mM Tris/HCl, 0.25 M sucrose, 1 mM ethylene diaminetetraacetic acid (EDTA), and 2.5 mM dithiotreitol (DTT) (Sigma-Aldrich, Poznań, Poland) using a Camry CR 4050 blender. The crude extract was filtered through two layers of fiber and centrifuged for 10 min at 10,000× *g* (Beckman L3-50, Beckman Coulter, Palo Alto, CA, USA) in order to eliminate any residues after plant extraction. The supernatant was centrifuged for 30 min at 80,000× *g* (Beckman L8-M, Beckman Coulter, Palo Alto, CA, USA). The obtained pellet was the cell membrane fraction, and the supernatant was considered to be the cytosolic fraction. Then, 15 mL of the supernatant was densified with 2.5 g of Sephadex for 30 min. The samples that were obtained were used to analyze the accumulation of protein.

3.4. Accumulation of the Transcripts of HSP90, HSP70, HSP18, and HSP17: RNA Isolation, Complementary DNA (cDNA) Synthesis, and Real-Time PCR Reaction

The accumulation of the transcripts of *HSP90*, *HSP70*, *HSP18*, and *HSP17* was determined using quantitative PCR amplification and analyzed using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The collected samples (approximately 0.05 g of the central part of the second leaf) were frozen in liquid nitrogen and then the mRNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The concentration and quality of the mRNA was assessed using a Q5000 UV–Vis Spectrophotometer (Quawell, San Jose, CA, USA). Next, the genomic DNA was eliminated by adding 2 µL of mRNA (approximately 600 ng) to a mixture of 2 µL of a genomic DNA (gDNA) Wipeout Buffer and 10 µL of RNase-free water (included in the QuantiTectReverse Transcription (RT) Kit, Qiagen, Hilden, Germany). After a 2-min incubation at 42 °C, the template RNA that was obtained was added to a reverse-transcription master mix (containing 1 µL of Quantiscript Reverse Transcriptase, 4 µL of a Quantiscript RT Buffer, and 1 µL of RT Primer Mix (QuantiTectReverse Transcription Kit, Qiagen, Hilden, Germany), and the reverse-transcription reaction was performed. The concentration and quality of the RNA and cDNA were assessed using a Q5000 UV–Vis spectrophotometer (Quawell, San Jose, CA, United States). The PCR amplifications of the *HSP90*, *HSP70*, *HSP18*, and *HSP17* transcripts were run in triplicate as described by Jurczyk et al. [52]. The primers and probes were designed using Primer Express Software v 3.0.1 (Applied Biosystems by Life Technologies, Foster City, CA, USA). The primer and probe sequences are listed in Table 1. The levels of the *HSP90*, *HSP70*, *HSP18*, and *HSP17* transcripts were determined relative to *actin* as the reference gene [53]. Five biological replicates (analyses for different cDNAs) were made.

Table 1. Sequence origins and primer and probe sequences used in the study. ID—identifier.

Gene Name	GenBank ID	Forward Primer	Reverse Primer	TaqMan MGB Probe
HSP17	Y07844.1	CGACACCTTCCGCTCCAT	CGGCCGTCTCGCTGTT	FAM–TCCCGGCGTTCTCT–MGB
HSP18	X64561.1	CGTATTCGAGTCGGAGCCATT	TCACAACTGTATTTAGGCTGCAGAA	FAM–CTCGCACACACATCAA–MGB
HSP70	L32165.1	CCTCAATGTGGCTAGGATCATCAAT	CCACCCCTCTTGTCCAAACC	FAM–CTGCTGCTGCTATTGC–MGB
HSP90	AY325266.1	GTTCAAGGCTGTCCTGTTTGTTC	GTTGTTGGCCTTCTTCTTGTTGTC	FAM–CCCCTTCGACCTCTTC–MGB
Actin	AY145451.1	GCAACTGGGATGACATGGAGAAAAT	GCCACACGGAGCTCATTGTA	FAM–CTGGCATCACACTTTC–MGB

3.5. Analysis of the Protein Content in the Cell Membrane and the Cytosolic Fractions

The protein content was determined based on the Sedmak and Grossberg [54] procedure. Firstly, 2 µL of the microsomal fraction was mixed with 2 µL of a 10% water solution of Triton X-100 (Sigma-Aldrich, Poznań, Poland) and 196 µL of the cell fraction isolation buffer before being kept on ice for 15 min. Then, 3 mL of water and 1 mL of Bradford reagent (BioRad, Munich, Germany) were added. After 10 min, the absorbance was measured (596 nm) using a UV–Vis spectrometer Lambda Bio 20 (Perkin Elmer, Waltham, MA, USA). The measurements were performed in triplicate. Bovine serum albumin (BSA) (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The BSA was diluted in the cell fraction isolation buffer and 2 µL of a 10% water solution of Triton X-100 was added. For the cytosolic fraction, 185 µL of the cell fraction isolation buffer, 15 µL of the cytosolic fraction, 3 mL of water, and 1 mL of Bradford reagent were mixed. The absorbance was then measured (596 nm).

3.6. Analysis of the Accumulation of HSP90, HSP70, HSP18.5, and HSP17.7 Using Immunoblotting

The same amount of proteins (selected after optimization and testing sample dilutions in range of 2.5 - 30 µg), which were isolated from the analyzed samples, was loaded and separated on 12% SDS-PAGE (1-mm polyacrylamide gel) based on the Laemmli [55] procedure and blotted to the nitrocellulose membrane (1 h at 45.5 mA (7–9 V)) using a BioRad semi-dry transfer) (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked with low-fat milk powder that was diluted in a Tris-buffered saline/Tween (TBS-T) buffer (containing 0.9% NaCl and 10 mM Tris) overnight. Subsequently, the membranes were washed four times for 5 min with TBS-T buffer and probed in the appropriate antibodies for 1.5 h (Agrisera, Vännäs, Sweden) (anti-HSP90, 1:3000; anti-HSP70, 1:3000; anti-HSP18.5, 1:1000; anti-HSP17.7, 1:1000). Next, the membranes were washed with a TBS-T buffer (four times for 5 min) and probed in alkaline phosphatase-conjugated secondary anti-rabbit antibody for 1.5 h (Sigma-Aldrich, Poznań, Poland) (dilution: HSP90 1:2000, HSP70 1:3000, HSP18.5 1:4000, HSP17.7 1:2000). Dilutions of all of the antibodies were applied according to the protocol provided by Agrisera after optimization. Three independent trials were performed. The protein content was quantified by a densitometric analysis of the visualized band intensity staining using ImageJ software (NIH, Bethesda, MD, USA). The averages are expressed as arbitrary units (A.U.) correlated with the area under densitometric curves. The densitometry was performed using ImageJ software (NIH, Bethesda, MD, USA).

3.7. Statistical Analysis

The statistical analysis (ANOVA, post hoc test) was done using Statistica 13.1 (StatSoft, Tulsa, OK, USA). Duncan's test was used to compare more than two groups (Bowman cultivar and its mutants BW084 and BW312). Student's *t*-test was used to compare two groups (Delisa cultivar and its mutant 522DK). The values marked with the same letters in specific figures did not differ significantly. Additionally, the accumulations of the transcripts and proteins in the Delisa and Bowman cultivars at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $p \leq 0.05$). The statistical significances are indicated by an asterisk (*). The averages on the figures are presented together with the standard error bars.

4. Conclusions

This work provides information on changes in HSPs in barley wild types and brassinosteroid mutants during plant growth at 20 °C and acclimation at 5 °C or at 27 °C, which usually enables plants to develop a better tolerance to more extreme temperatures.

The main findings of the work are as follows:

(1) In the tested Delisa and Bowman cultivars, the temperature of the growth/acclimation affected the expression of the HSPs. Acclimation at 5 °C increased the *HSP90* transcript only in the Bowman (compared to 20 °C). Acclimation at 27 °C decreased the *HSP90* transcript and drastically

increased the *sHSP* transcripts in both cultivars. Acclimation at 5 °C and 27 °C increased the *HSP70* transcript in both cultivars. As for the respective protein accumulation, the results were more cultivar-dependent, but for both cultivars, identical directions of changes were observed for the accumulation of HSP90 (lower in the membrane fraction at 5 °C) and HSP70 accumulation (lower at 27 °C in the membrane fraction but increased in the cytosolic fraction).

(2) The role of brassinosteroids as positive regulators of the expression of HSPs seems to be proven by the results that were obtained for the BR-signaling mutant. In most cases, the mutant with a defective BR receptor had a lower accumulation of the *HSP* transcripts and HSP proteins compared to the wild type, regardless of the plant growth/acclimation temperature. The results that were obtained for the BR-deficient mutants (BW084 and 522DK) may additionally confirm that BRs are among the players that regulate the expression of HSPs. The results, however, also show that lowering the level of BRs (BRs were drastically lower in BW084, but only relatively slightly lower in 522DK) does not always act negatively on the expression of HSPs. Moreover, the genetic background of cultivars from which the biosynthetic mutants were derived also seems to be important for HSP expression, because BRs may act in complicated network with other phytohormones.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/5/1889/s1.

Author Contributions: I.S. and A.J. isolated the cell membrane fraction and cytosolic fraction and analyzed the protein content in the isolated fractions. I.S. and M.L.-K. analyzed the accumulation of the heat-shock proteins after the method was optimized. I.S. quantified the results by densitometry using ImageJ software. I.S. and B.J. measured the accumulation of the transcripts of the heat-shock proteins. D.G. provided the seeds of the mutants for the experiment and made suggestions regarding the interpretation of the data. All of the authors analyzed and interpreted the data. A.J. coordinated the writing of manuscript. All of the authors read the article. A.J. was the coordinator of the project 2015/17/B/NZ9/01695, designed the experiments, and invited all of the other scientists to cooperate and to suggest corrections. All authors have read and agreed to the published version of the manuscript.

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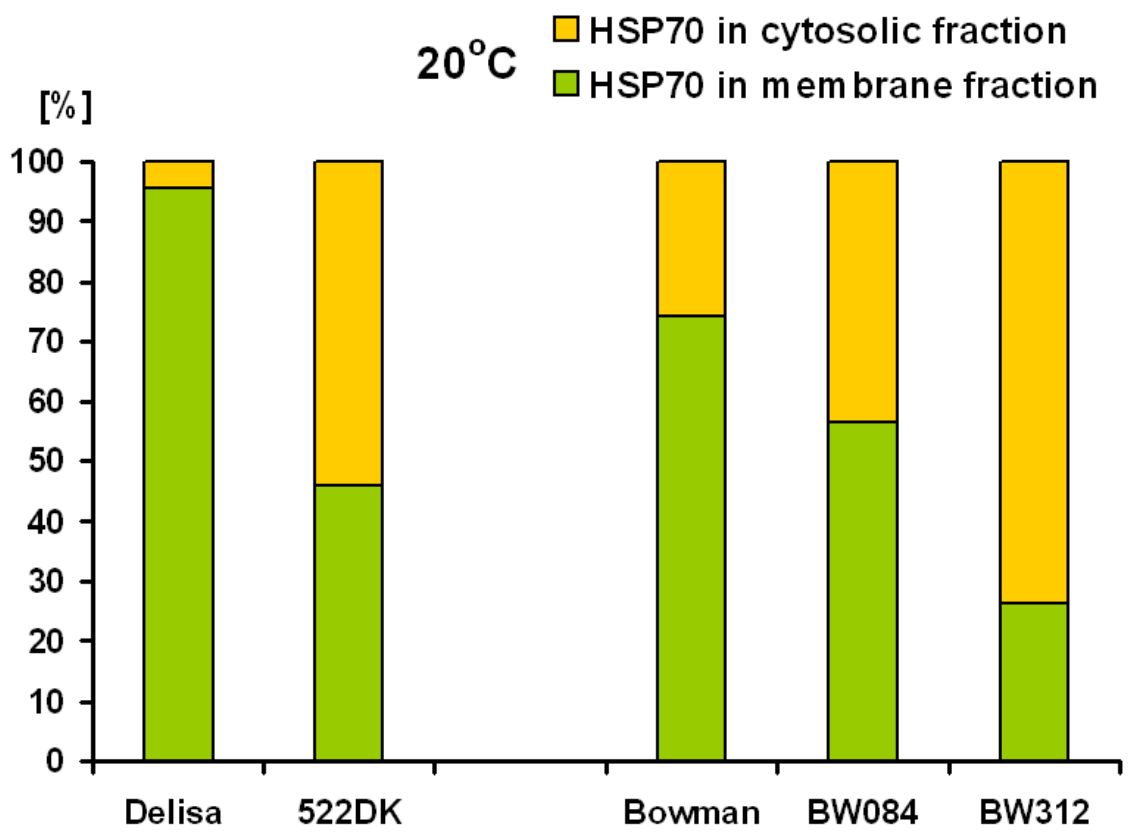

Fig. S1 Percentage relationship of the HSP70 protein in the cytosolic fraction to the HSP70 in the membrane fraction in barley wild types (Delisa and Bowman) and the mutants 522DK, BW084 and BW312 that had been cultured at 20°C. Data are expressed as the % of the total accumulation of HSP70, which was calculated as the sum of HSP70 in the cytosolic fraction and in the membrane fraction from Figure 2 E, F, I, J.

Table S1. Changes in the accumulation of the *HSP90, HSP70, HSP18* and *HSP17* transcripts in barley in relation to a mutation and the temperature of growth (+ increase compared to the wild type; – decrease compared to the wild type; NC not changed compared to the wild type). The plant material in our studies included the barley (*Hordeum vulgare* L.) BR-deficient mutant 522DK (mutation *HvDWARF*) from the Delisa cultivar, the barley BR-deficient mutant BW084 (mutation in the *HvCPD* gene**)**, the BR-signalling defective mutant (BW312, mutation in the *HvBRI1* gene) and their reference cultivar Bowman. < LOD below limit of detection

Transcript	**Genetic mutation**														
	HvDWARF					***HvCPD***					***HvBRI1***				
	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)
HSP90	**-**	**NC**	**-**	**NC**	**+**	**NC**	**-**	**NC**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
HSP70	**-**	**-**	**-**	**-**	**-**	**NC**	**-**	**-**	**-**	**+**	**NC**	**-**	**-**	**-**	**NC**
HSP18	**NC**	**<LOD**	**<LOD**	**+**	**+**	**+**	**<LOD**	**<LOD**	**-**	**-**	**NC**	**<LOD**	**<LOD**	**-**	**-**
HSP17	**NC**	**NC**	**NC**	**+**	**NC**	**+**	**+**	**NC**	**-**	**NC**	**NC**	**NC**	**NC**	**-**	**NC**

Table S2. Changes in the accumulation of the HSP90 and HSP70 proteins in barley in relation to a mutation and the temperature of growth (+ increase compared to the wild type; – decrease compared to the wild type; NC not changed compared to the wild type). The plant material in our studies included the barley (*Hordeum vulgare* L.) BR-deficient mutant 522DK (mutation in the *HvDWARF* gene) from the Delisa cultivar, the barley BR-deficient mutant BW084 (mutation in the *HvCPD* gene**)**, the BR-signalling defective mutant (BW312, mutation in the *HvBRI1* gene) and their reference cultivar Bowman.

Protein	**Genetic mutation**								
	HvDWARF			***HvCPD***			***HvBRI1***		
	20ºC	5 ºC	27 ºC	20ºC	5 ºC	27 ºC	20ºC	5 ºC	27 ºC
HSP90 (cell membrane)	+	+	**NC**	**NC**	-	+	-	-	-
HSP70 (cell membrane)	-	-	+	-	+	+	-	-	-
HSP70 (cytosolic fraction)	+	+	+	+	-	+	+	-	**NC**

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: nauczeniu doktorantki (I.Sadura) procedury homogenizacji materiału roślinnego oraz oznaczania białka na potrzeby metody Western Blott i Immunoblot, nauczeniu metody Western Blott i Immunoblot, nadzorowaniu pracy doktorantki przy wykonywaniu w/w analiz w materiale roślinnym (jęczmień).

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: nauczeniu doktorantki (I.Sadura) procedury izolacji RNA i procedury wykonania odwrotnej transkrypcji, nadzorowaniu pracy doktorantki przy wykonywaniu w/w analiz w liściach pozyskanych z badanych roślin jęczmienia. Ponadto praca moja polegała na wykonaniu analizy RT-PCR i pomocy przy interpretacji wyników.

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: przygotowaniu materiału nasiennego (w tym nasion linii bliskoizogenicznych i mutantów) do badań oraz współpracy przy pisaniu manuskryptu (analiza/dyskusja aspektów genetycznych).

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: stworzeniu koncepcji badań do projektu 2015/17/B/NZ9/01695, wyborze zespołu naukowców do współpracy, zapewnieniu finansowania z racji pełnienia roli kierownika projektu, koordynowaniu pracy doktorantki (I. Sadura) - w tym przekazaniu doktorantce wiedzy do przeprowadzenia procedury izolacji frakcji membranowej i cytoplazmatycznej z badanych roślin jęczmienia i pomiaru w nich zawartości białka, pomocy przy interpretacji otrzymanych wyników, koordynowaniu tworzenia publikacji.

Anna Janeczko

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Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature

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ABSTRACT

The integral parts of the cell membranes are the functional proteins, which are crucial for cell life. Among them, proton-pumping ATPase and aquaporins appear to be of particular importance. There is some knowledge about the effect of the temperature during plant growth, including stress-inducing temperatures, on the accumulation of the membrane proteins: plasma membrane H^+-ATPase and aquaporins, but not much is known about the effect of the phytohormones (i.e. brassinosteroids (BR)) on control of accumulation of these proteins. The aim of our study was to answer the question of how a BR deficit and disturbances in the BR perception/signalling affect the accumulation of plasma membrane H^+-ATPase (PM H^+-ATPase), the aquaporin HvPIP1 transcript and protein in barley growing at 20 °C and during its acclimation at 5 °C and 27 °C. For the studies, the BR-deficient mutant 522DK (derived from the wild-type Delisa), the BR-deficient mutant BW084 and the BR-signalling mutant BW312 and their wild-type Bowman were used. Generally, temperature of growth was significant factor influencing on the level of the accumulation of the *H^+-ATPase* and *HvPIP1* transcript and the PM H^+-ATPase and HvPIP1 protein in barley leaves. The level of the accumulation of the *HvPIP1* transcript decreased at 5 °C (compared to 20 °C), but was higher at 27 °C than at 20 °C in the analyzed cultivars. In both cultivars the protein HvPIP1 was accumulated in the highest amounts at 27 °C. On the other hand, the barley mutants with a BR deficiency or with BR signalling disturbances were characterised by an altered accumulation level of PM H^+-ATPase, the aquaporin HvPIP1 transcript and protein (compared to the wild types), which may suggest the involvement of brassinosteroids in regulating PM H^+-ATPase and aquaporin HvPIP1 at the transcriptional and translational levels.

1. Introduction

The cell membrane plays an important role in the interaction of a cell with the environment and also regulates the transport of various substances inside and outside of a cell. Among others, both the lipid and protein components of the membrane alter dynamically as a result of changes in the temperature during plant growth (Los and Murata, 2004; Zheng et al., 2011). A membrane-sensing hypothesis, which assumes that membranes are the thermal sensors of cells, has even been proposed (Horváth et al., 2012). Many biochemical reactions, which are crucial for cell life, occur with the participation of membrane-bound proteins (Brown, 2011). Among the many membrane-bound proteins, proton-pumping ATPase and aquaporin appear to be very important. Plasma membrane H^+-ATPase (PM H^+-ATPase) is part of a large family of membrane proteins that are responsible for the active transport of cations or other compounds across the membranes and this process is coupled with ATP hydrolysis. The PM H^+-ATPase releases protons from a cell in order to create a proton motive force that has a membrane potential of 120–160 mV (negative inside) and a pH gradient of 1.5–2 units (acid outside) (Sze et al., 1999). It is known that PM H^+-ATPase is also involved in other functions that are crucial for appropriate plant growth including salt tolerance, intracellular pH regulation and cellular expansion (Morsomme and Boutry, 2000). Aquaporins, on the other hand, are transmembrane proteins, which were discovered as water channels that transport water through the cell membranes. In addition to water, aquaporins can transport, among others, CO_2, urea, ammonia, silicon and O_2 (Maurel et al., 2008; Kapilan et al., 2018). There is a family of major intrinsic proteins (MIP) and they are classified into five subfamilies, which include the plasma membrane intrinsic proteins (PIPs) that are located in the organs that are distinguished by huge

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fluxes of water e.g. the vascular tissues. Their main function in plants is to regulate the transmembrane water transport when the water flow needs to be modified or when it is very low (Kapilan et al., 2018). They are also known to participate in regulating the root water transport in response to different environmental factors and in facilitating water transport through the inner leaf tissues during transpiration (Maurel et al., 2008). Its transport activity may be controlled by multiple mechanisms that are involved in regulating the accumulation of transcripts or proteins or in subcellular trafficking (Maurel et al., 2008).

From the physiological and biochemical points of view, maintaining the integrity and fluidity of the cell membranes is very important for plants to survive under high/low temperature stress (Zheng et al., 2011). For example, according to Los and Murata (2004), low temperatures change the lipid composition of the cell membranes by increasing the content of unsaturated fatty acids, which is connected with membrane rigidification, while high temperatures cause the fluidisation of the cell membranes. However, knowledge about effect of temperature stress on the membrane proteins, including the accumulation or activity of PM H^+-ATPase and the aquaporin proteins, is rather scarce. The response of the PM H^+-ATPase to low temperatures in cucumber roots has been relatively well described (Ahn et al., 2000; Janicka-Russak et al., 2012; Muzi et al., 2016). On the other hand, cold lowers the expression of the *aquaporin* (*PIP*) transcripts (Maurel et al., 2008). Simultaneously, however, not much is known about the effect of phytohormones on the activity of these proteins (Suga et al., 2002; Falhof et al., 2016). There are only a few articles that discuss the effects of exogenously applied brassinosteroids (BR) on the levels of the PM H^+-ATPase or aquaporin (Morillon et al., 2001; Suga et al., 2002) and vacuolar V-ATPase and aquaporin (Yang et al., 2003). For example, Suga et al. (2002) investigated the effect of exogenously applied brassinosteroid (0.1 μM brassinolide, BL) as well as 1 mM gibberellic acid (GA_3) or 0.1 mM abscisic acid (ABA) on the accumulation of the plasma membrane aquaporin isoforms (RsPIPs) in radish seedlings. GA_3 and ABA lowered the amount of the RsPIP2-1 protein, while the BL did not cause any significant changes. A good model for studies of the role of hormonal regulation (combined with the impact of temperature) on the functioning of ATPase or aquaporins could be mutants with disturbances in hormonal biosynthesis. To the best of our knowledge, the number of articles that specifically discuss brassinosteroid mutants in terms of the expression of PM H^+-ATPase or aquaporins is low and among them, there are no works that are specifically dedicated to this aspect in cereal plants. Brassinosteroids are the plant steroid hormones that are responsible for the processes of plant growth and modulating the plant responses to environmental stresses (Bajguz and Hayat, 2009; Sadura and Janeczko, 2018). In our studies, barley brassinosteroid mutants (522DK, BW084 and BW312) were used (Gruszka et al., 2011; Dockter et al., 2014). The 522DK and BW084 are mutants with disturbed BR biosynthesis which results in lower level of BR in comparison to wild type. In mutant BW312 as a result of mutation, BR receptor partly losses its function (BR perception is weaken), BR signaling is disturbed and endogenous BR level increased.

Our earlier studies have shown that all of these mutants, after acclimation at 27 °C had higher tolerance to high temperature (38 − 45 °C) in comparison to their wild types (Sadura et al., 2019). Simultaneously after acclimation at 5 °C, mutants BW084 and BW312 had lower tolerance to frost in comparison to respective wild type cultivar Bowman (Sadura et al., 2019). On the other hand, frost tolerance of 522DK was similar to tolerance of its wild type cultivar Delisa. The phenomenon was partly explained by altered mutant's hormonal homeostasis (mainly ABA accumulation) but mechanism of changed tolerance remained generally unknown. Since both for frost and partly for heat tolerance important is cell water management and also cellular transport of water and other substances, in present work we would like to focus on answering the following question - how brassinosteroid deficit and disturbances in its signaling influence on PM H^+-ATPase and HvPIP1 (aquaporin) transcript and protein accumulation in barley growing at 20 °C and after plant's acclimation at 5 °C and 27 °C?

2. Materials and methods

2.1. Plant material

The seeds of the spring barley (*H. vulgare* L.) cultivars (Delisa and Bowman) and their 522DK, BW084 and BW312 mutants were derived from the collection of the Department of Genetics, University of Silesia (Katowice, Poland). The cultivars and mutant lines were selected according to the works of Gruszka et al. (2011) and Dockter et al. (2014).

The 522DK mutant was obtained from the Delisa cultivar and is characterised by a G > A substitution at position 1130 of the *HvDWARF* transcript (Gruszka et al., 2011) and at position 3031 in the gene sequence (Gruszka et al., 2016), which causes substitution of the valine-341 residue by isoleucine. The *HvDWARF* gene encodes the brassinosteroid C6-oxidase, which takes part in BR biosynthetic pathways and this is the cause of the lower content of BR (mainly castasterone) in the mutant (Gruszka et al., 2016; Sadura et al., 2019). The BW084 and BW312 mutants are Near-Isogenic Lines of Bowman cultivar (Dockter et al., 2014). The BW084 (*brh13.p*) carries the C2562 T missense mutation in the *HvCPD* gene encoding barley C-23α-hydroxylase cytochrome P450 90A1 (CYP90A1), which catalyses the early steps of the BR biosynthesis. The C2562 T mutation results in a substitution of the highly conserved amino acid residue (Pro-445 to Leu). Pro-445 is situated within the highly conserved heme binding site in the C-terminal part of the HvCPD protein (Dockter et al., 2014). Mutant is characterized with lower content of 28-homocastasterone while content of brassinolide and castasterone is even below detection limits (Sadura et al., 2019). The BW312 (*ert-ii.79*) mutant that is defective in the BR perception has a double substitution (CC1760/1761AA) in the BR receptor kinase BRI1. The substituted amino acid residue (Thr-573 to Lys) is present in the steroid-binding island domain of the BR receptor. The charged Lys-573 in the hydrophobic active site vicinity, destroys the charge neutrality, and may handicap the binding of BR molecules (Dockter et al., 2014). This mutation causes significant increase in BR (castasterone, brassinolide and 28-homocastasterone) content (Sadura et al., 2019).

2.2. Plant growth and experimental design

The seeds were put on moist filter paper on plastic vessels (10 cm × 10 cm × 3 cm) for germination in the dark at 24 °C for three days. To each vessel 40 seeds and 10 ml of water were added. After germination, the seedlings were planted into pots with soil (15–20 plants/pot 40 cm × 15 cm × 15 cm). Each pot was filled with 3 kg of soil prepared from: "Eco-ziem Universal soil" (Eko-Ziem s.c., Jurków, Poland), soil from the cultivation plots at the University of Agriculture (Kraków) and sand (1:1:0.5). The plants were kept for three weeks in a growth chamber (20 °C d/n; 16 h photoperiod). Light intensity: 170 μmol $m^{-1} \cdot s^{-1}$ emitted by HPS Philips SON-T AGRO 400 W lamps. When plants developed three to four leaves, the pots with plants were separated into two groups. The first group was acclimated to 5 °C (8 h photoperiod, 21 days). The second group was acclimated to 27 °C (16 h photoperiod for seven days). After acclimation, plants usually developed four to five leaves.

Samples for the analyses of the transcripts (*H^+-ATPase* and aquaporin *HvPIP1*) were collected from the plants (the central part of the second leaf) before the acclimation (20 °C), and then on the last day of the acclimation at 5 °C (21st day) and 27 °C (7th day). Additional samples were also collected at the halfway point of the acclimation (10th day at 5 °C and 3rd day at 27 °C).

To determine the accumulation of protein, two types of samples were collected. To obtain the first group of samples, the aerial parts of the seedlings were cut off and the cell membrane fraction was immediately isolated and then frozen at − 80 °C for the further analysis of

Table 1
Sequence origins and primers and probes sequences used in the study.

Gene name	GenBank ID	Forward primer	Reverse primer	TaqMan MGB Probe
HvPIP1;1	AB286964.1	CTGGCGGGCACATCAAC	CGTCAGCGACAGCTTCCT	FAM-ACCTTCGGGCTGTTCC-MGB
ATPase1	AF308816.1	AGACAGGAAGTACCTGAGAAATCCA	TGGTCTCGGCACTGTCATG	FAM-CACCAGCAGAATCC-MGB
Actin	AY145451.1	GCAACTGGGATGACATGGAGAAAAT	GCCACACGGAGCTCATTGTA	FAM-CTGGCATCACACTTTC-MGB

the proteins. To get the second group of samples, the aerial parts of the seedlings were cut off, frozen (−80 °C) and kept in order to prepare a fresh crude leaf extract immediately before the protein analysis. The aim of using this approach was to compare the results of the protein analyses that were obtained from both types of samples. Similar to the preparations of the transcripts, samples were collected from plants growing at 20 °C, 5 °C (21st day) and 27 °C (7th day). Additional samples were also collected at the halfway point of acclimation (10th day at 5 °C and 3rd day at 27 °C) in order to have the leaves cut off and frozen to get the crude extract for further protein analysis.

2.3. Biochemical analysis

2.3.1. Isolation of the cell membranes

The cell membranes were isolated based on a modified protocol of Sommarin et al. (1985) and Janeczko et al. (2008). About 100 g of plant material were homogenised using a Camry CR 4050 blender in 400 ml of a 10 mM Tris/HCl buffer (pH 7.8) containing 0.25 M sucrose, 1 mM EDTA and 2.5 mM dithiotreitol (DTT). All of the reagents were purchased from Sigma-Aldrich (Poznań, Poland). The crude extract was filtered and centrifuged for ten minutes at 10 000 × *g* (Beckmann L8-M, rotor SW 27 and 28, Palo Alto, CA, USA) to remove any residues after the plants had been homogenised. The supernatant was centrifuged for 30 min at 80 000 × *g* (Beckmann L8-M, rotor SW 27 and 28, Palo Alto, CA, USA). The obtained pellet constituted the cell membrane fraction. The samples that were obtained were used to analyse the accumulation of proteins.

2.3.2. Measurement of the protein concentration in the cell membrane fractions

The protein concentration was estimated according to Sedmak and Grossberg (1977). Two µl of a 10 % water solution of Triton X-100 (Sigma-Aldrich, Poznań, Poland) was mixed with 2 µl of the microsomal fraction and 196 µl of a buffer to isolate the cell fractions and kept for 15 min in ice. Next, 3 mL of water and 1 mL of a Bradford reagent (BioRad, Munich, Germany) were added. After 10 min, the absorbance was recorded (596 nm) using a UV/VIS spectrometer Lambda Bio 20 (Perkin Elmer, Norwalk, USA). Measurements were carried out in three replicates. Bovine serum albumin – BSA (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The BSA for spectrophotometric measurements was diluted in the same buffer as buffer used to isolate the cell membrane fractions and 2 µl of a 10 % water solution of Triton X-100 was added.

2.3.3. Measurement of the protein concentration in the crude leaf extract

The aerial parts of the seedlings (five to six plants) were collected and then they were cut into about 0.5 cm fragments and 1 g of the sample was prepared. The samples were homogenised in 2.5 mL of a Tricine buffer containing 100 mM Tricine, 3 mM $MgSO_4$, 1 mM DTT, 3 mM EGTA, adjusted to pH 8.0 with 1 M Tris. After the homogenisation, which was carried out at 4 °C, the samples were centrifuged for five minutes at 35 060 × *g* (Hettich, Tuttingen, Germany). The supernatant was collected and the concentration of the protein in the obtained crude extract was measured according to Bradford (1976) using a Bio-Rad Protein Assay Kit with BSA as the standard. Four measurements were taken per sample.

2.3.4. Accumulation of the transcripts of H^+-ATPase and HvPIP1: RNA isolation, cDNA synthesis and real-time PCR reaction

The accumulation of the *ATPase* and *HvPIP1* transcripts was measured using real-time quantitative PCR amplification and the analyses were carried out using a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Samples (approximately 0.05 g of the central part of the second leaf) were collected and frozen in liquid nitrogen. Next, the mRNA was isolated using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Subsequently, the genomic DNA was eliminated by adding 2 µl of mRNA (corresponding to approximately 600 ng of RNA) to 2 µl of the gDNA Wipeout Buffer and 10 µl of RNase-free water (included in a QuantiTectReverse Transcription Kit, Qiagen, Hilden, Germany). After 2 min of incubation at 42 °C, the obtained mixture was added to a reverse-transcription master mix (containing 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript RT Buffer and 1 µl of RT Primer Mix) (QuantiTectReverse Transcription Kit, Qiagen, Hilden, Germany) and a reverse-transcription reaction was performed at 42 °C (15 min). The concentration and quality of the RNA and cDNA were estimated using a Q5000 UV/VIS spectrophotometer (Quawell, San Jose, CA, USA). The PCR amplifications of the *ATPase1* and *HvPIP1;1* transcripts were performed in triplicate, as described by Jurczyk et al. (2012). The primers and probes for the target genes and an endogenous control gene were designed using Primer Express Software v 3.0.1 (Applied Biosystems, Foster City, CA, USA) and their sequences are listed in Table 1. The levels of the *ATPase1* and *HvPIP1;1* transcripts accumulations were determined relative to *Actin* as the reference gene for five biological replicates (five different cDNAs from five different plants).

2.3.5. Analysis of the accumulation of the cell membrane proteins PM H^+-ATPase and HvPIP1 using immunoblotting

Equal amount of proteins present in the extracts isolated from analysed material was loaded on 12 % polyacrylamide gels and electrophoretically separated as described by Laemmli (1970). The separated proteins were blotted to a nitrocellulose membrane for 2 h at 45,5 mA (7 − 9 V) using a BioRad semi-dry transfer. The membranes were blocked overnight with low-fat milk powder that had been diluted in a TBS-T buffer (containing 0.9 % NaCl and 10 mM Tris). Next, the membranes were washed with a TBS-T buffer four times for 5 min and then incubated in the appropriate primary antibody (Agrisera) (anti-PIP1, 1:2000, for 1 h 30 min; anti-H^+ATPase, 1:2000, for 1 h 30 min). Then, the membranes were washed again with the TBS-T buffer (four times for 5 min) and incubated in Alkaline Phosphatase – conjugated secondary anti-rabbit antibody (Sigma-Aldrich) (1:2000, for 1 h 30 min). Three independent replicates were performed. Quantification of analysed protein level was performed on the basis of staining intensity of bands corresponding with PIP1 or PM H^+-ATPase, performed by ImageJ software (NIH, USA).

2.4. Statistical analysis

Statistical analysis (ANOVA, post hoc test) was performed using Statistica 13.1 (StatSoft, Tulsa, OK, USA). When more than two groups were compared, the Duncan's test was used (for Bowman, BW084, BW312). When two groups were compared (Delisa, 522DK), the Student's *t*-test was used. Values marked with the same letters in the figures did not differ significantly. Additionally, the accumulations of

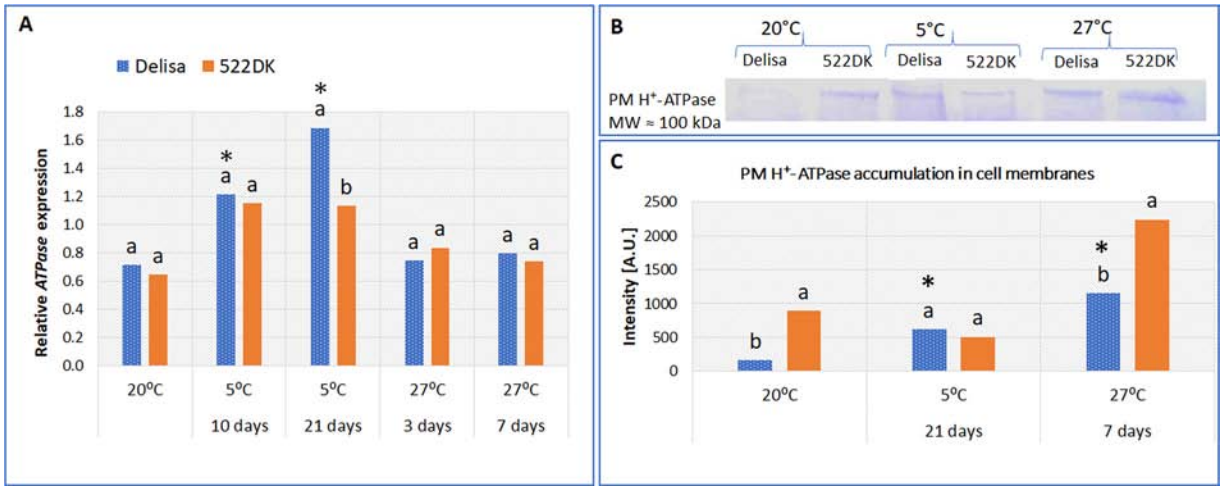

Fig. 1. Changes in the relative *ATPase* transcript level (A) and the PM H^+-ATPase protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Delisa and its mutant (522DK) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein identified using the anti- PM H^+-ATPase antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 20 µg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated PM H^+-ATPase was estimated based on densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Delisa and its mutant 522DK – (Student's *t*-test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulation of the transcript and protein concentration in the Delisa cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

the transcripts and proteins in the Delisa and Bowman cultivars at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an asterisk (*).

3. Results and discussion

3.1. Accumulation of the H^+-ATPase transcript and protein in the barley BR mutants and wild type plants growing at 20 °C and acclimated at 5 °C and 27°C

In our studies, low temperature (5 °C) gradually increased the relative transcript level of *H^+-ATPase* in both the Delisa and Bowman barley cultivars compared to the temperature 20 °C (Fig. 1A and 2 A). Finally, after 21 days at 5 °C, relative transcript level in both cultivars was doubled compared to the level that had been observed at 20 °C. A similar, although much weaker, tendency was observed for all of the tested mutants with a brassinosteroid deficit or signalling disturbances. According to literature, the exposure of cucumber roots to low temperatures resulted in a gradual decrease of the *H^+-ATPase* gene expression from day 1–6 of chilling, compared to the control plants (Ahn et al., 2000). Similarly, a decreased relative *H^+-ATPase* expression was obtained by Janicka-Russak et al. (2012) for cucumber roots that had been exposed to a low temperature for three days. However, prolonged exposure to a low temperature (for 6 days) increased the expression levels of four of the six genes encoding PM H^+-ATPase that were tested compared to the control plants.

The BR mutants derived from the Bowman cultivar, BW084 and

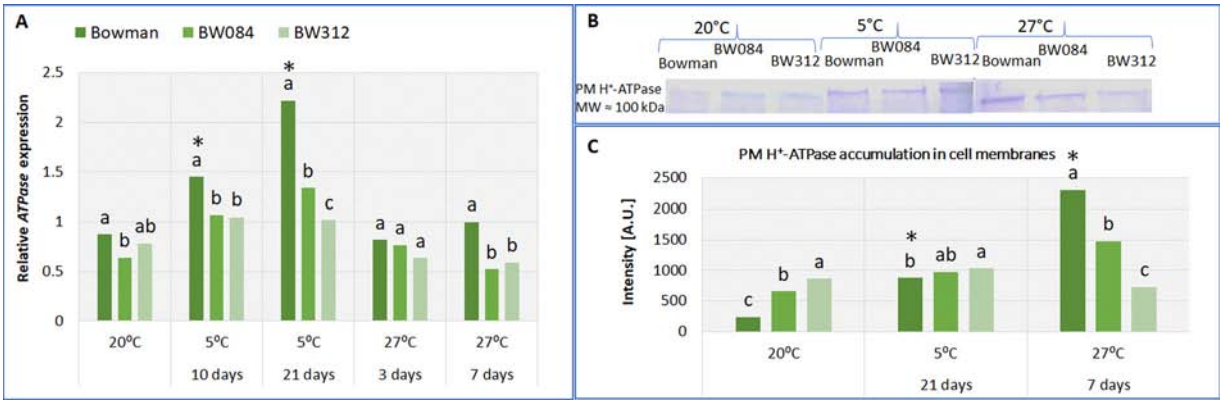

Fig. 2. Changes in the relative *ATPase* transcript level (A) and the PM H^+-ATPase protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Bowman and its mutants (BW084 and BW312) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein that was identified using the anti- PM H^+-ATPase antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 20 µg of proteins from the cell membrane fractions was applied on each lane. The amount of the accumulated PM H^+-ATPase was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Bowman and its mutants (BW084 and BW312) – (Duncan's test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Bowman cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

BW312, showed a lower (few percent to about 50 %) level of the *H*$^+$-*ATPase* transcript accumulation than their respective cultivar at all tested temperatures but especially at 5 °C (Fig. 2 A). Mutant 522DK had a statistically significant lower level of the transcript than Delisa only at 5 °C (Fig. 1A). Then in the case of the mutants with BR biosynthesis disturbances, the effect was more spectacular for mutant BW084 than for mutant 522DK. It should be kept in mind that the mutants represent defects at various stages of the BR biosynthesis, as they carry mutations in two different loci (Dockter et al., 2014; Gruszka et al., 2016). BW084 has a mutation in early stages of the biosynthesis of BR and produces two main BR (brassinolide or castasterone) at a level that is sometimes impossible to detect (Sadura et al., 2019). Mutant 522DK has a defect in the late stage of BR biosynthesis and only produces a lower content of BR (on average 30 %) at 5 °C (Sadura et al., 2019). Generally the effect (lowered transcript level) was especially prominent under the stress conditions (5 °C) and in this case the effect was independent on the genetic background of the analyzed mutants. The lower level of the transcript accumulation in BR-deficient mutants may suggest that BR are one of regulators that are necessary for controlling the accumulation of the *H*$^+$*-ATPase* transcript. The effect, which was significant also in the case of mutant BW312, means that BR signalling is also important for regulation of accumulation of this transcript.

When the levels of the accumulation of the PM H$^+$-ATPase protein in the Delisa and Bowman cultivars was compared at 20 °C and 5 °C, a few times higher accumulation of this protein was observed at 5 °C (Figs. 1B and C and 2 B and C) and these data are in agreement with the literature. Kim et al. (2013) found that the leaves and roots of camelina (*Camelina sativa* L.) and rapeseed (*Brassica napus* L.) that had been exposed to low temperature (2 °C/1 day) accumulated a higher amount of the PM H$^+$-ATPase protein than the plants that had not been exposed to temperature stress. Prolonged low temperature treatment changed the accumulation of the PM H$^+$-ATPase protein in both species, however it was dependent on whether the roots or leaves were examined. Low temperature also increased the accumulation of PM H$^+$-ATPase in the plasma membrane fraction of *A. thaliana* plants and this effect corresponded with the results obtained for the relative expression of the *H*$^+$-*ATPase* genes – exposure to low temperature increased their levels (Muzi et al., 2016). This is especially in agreement with our results obtained for cv. Delisa and Bowman because, as was mentioned, the accumulation of *H*$^+$*-ATPase* transcript (simultaneously with the PM H$^+$-ATPase protein accumulation) was clearly higher after exposure to 5 °C (if compare to 20 °C).

All of the BR mutants grown at 20 °C accumulated a few times more of the PM H$^+$-ATPase protein in their membrane fraction than their respective cultivars (Figs. 1B and C and 2 B and C), but at 5 °C the effect was not noted (522DK) or hardly visible in the case of BW084 and BW312. It could suggest that, in some temperature conditions (especially at 20 °C), BR are negative regulators of the production of this protein in barley. We suggest that this BR-dependent regulation of the PM H$^+$-ATPase protein accumulation may occur in this case rather at the translational level.

There are not many studies related to effect of the plant exposure to higher temperatures on the accumulation of the PM H$^+$-ATPase transcript or protein. That is why our work provides new information in this field. The temperature of 27 °C that was tested in our experiment was not as extreme and was maintained for a longer period. The accumulation of the *H*$^+$*-ATPase* transcript remained unchanged in the Delisa and Bowman cultivars at 20 °C and at 27 °C (Figs. 1A and 2 A). However, the accumulation of the PM H$^+$-ATPase protein was few times higher at 27 °C than at 20 °C in both cultivars (Figs. 1B and C and 2 B and C). The effect of much higher temperature was earlier studied by Liu et al. (2009). Authors found that in pea leaves, heat (38 °C) caused an increase in the accumulation of the PM H$^+$-ATPase transcript and protein after 1 h of high-temperature treatment and that after a longer period (3 h) its amount decreased.

The BR deficiency that was connected with disturbances at the late stage of biosynthesis (in 522DK mutant) had no effect on the accumulation of the *H*$^+$*-ATPase* transcript at 27 °C (after 3 or 7 days) (Fig. 1A). The mutant was however characterized by increased accumulation of the PM H$^+$-ATPase protein (Fig. 1 B and C). The accumulation of the transcript in the BW084 mutant (strong BR deficit) and the BW312 mutant (BR perception disturbances) growing at 27 °C was unchanged after 3 days but lowered after 7 days in comparison to Bowman (Fig. 2A). It was accompanied also by lowered level of PM H$^+$-ATPase protein in comparison to Bowman wild type (Fig. 2B and C). Comparing results obtained for two BR deficient mutants 522DK and BW084 we can say that the observed opposite effect (especially for protein accumulation) was probably dependent on the genetic background of the analyzed mutants.

Not much is known about the role of BR in regulating the functioning of PM H$^+$-ATPase. Jakubowska and Janicka (2017) found that the relative expression of the *H*$^+$*-ATPase* genes in cucumber roots that had been treated with 10 nM 24-epibrassinolide (one of the brassinosteroids) markedly increased expression of three out of seven genes compared to the control (not treated with 24-epibrassinolide). In general, this may be in agreement with our findings where the BR-deficient mutants (especially BW084 in which the defect occurs at the early step of the BR biosynthesis) showed lower accumulation of the *H*$^+$*-ATPase* transcript.

Jakubowska and Janicka (2017) also found that both the control cucumber roots and the roots that had been treated with 24-epibrassinolide accumulated a similar amount of the PM H$^+$-ATPase protein. In this context, our data are slightly different because depending on the temperature, genotype and the mutation, the plants had different accumulations of the PM H$^+$-ATPase protein (most often increased or decreased, rarely unchanged). This difference may be a result of different approaches – exogenous BR treatment in the study by Jakubowska and Janicka (2017) and use of the BR-deficient and insensitive mutants in our experiments. The difference may also stem from different plant species and organs tested.

3.2. Accumulation of the aquaporin (HvPIP1) transcript and protein in barley BR mutants and wild type plants growing at 20 °C and acclimated at 5 °C and 27 °C

In both the Delisa and Bowman cultivars, there was a marked decrease in the relative *HvPIP1* expression during the cold acclimation (5 °C) compared to temperature 20 °C (Figs. 3A and 4 A). Many earlier studies have shown that cold decreases the expression of the *aquaporin* (*PIP*) transcripts in plant roots or shoots (for review see Maurel et al. (2008)), thus our results correspond with reported observations. On the other hand, not much is known about the effect of higher temperatures on the accumulation of the *PIP* transcripts in plants and known studies usually concerned the impact of temperature about 40 °C. Our studies of barley cv. Delisa and cv. Bowman that had been exposed 3 or 7 days to a less extreme temperature 27 °C, showed that the accumulation of the *HvPIP1* transcript (compared to 20 °C) was finally increased by a third (cv. Delisa) or doubled (cv. Bowman). Earlier, Christou et al. (2014) described the effect of heat stress (40 °C) on the accumulation of the *PIP* transcripts in strawberry roots. They found a rapid accumulation of the *PIP* transcript after 1 h of the stress treatment. However, the accumulation of the *PIP* transcript after 8 h of the heat treatment was similar to that of the control. In our work, all of the tested mutants showed similar tendencies in the *HvPIP1* transcript accumulation as their respective cultivars after both cold acclimation and after exposure to a higher temperature (27 °C) in comparison to 20 °C.

In our studies, lower accumulation of the *PIP* transcript in both cultivars (at 5 °C compared to 20 °C) was generally accompanied by a similar accumulation of the HvPIP1 protein in Delisa at 20 °C and 5 °C (Fig. 3B and C) and a slightly higher accumulation of the HvPIP1 protein at 5 °C than at 20 °C in Bowman (Fig. 4B and C). An accumulation of the protein HvPIP1 is not always correlated with the same

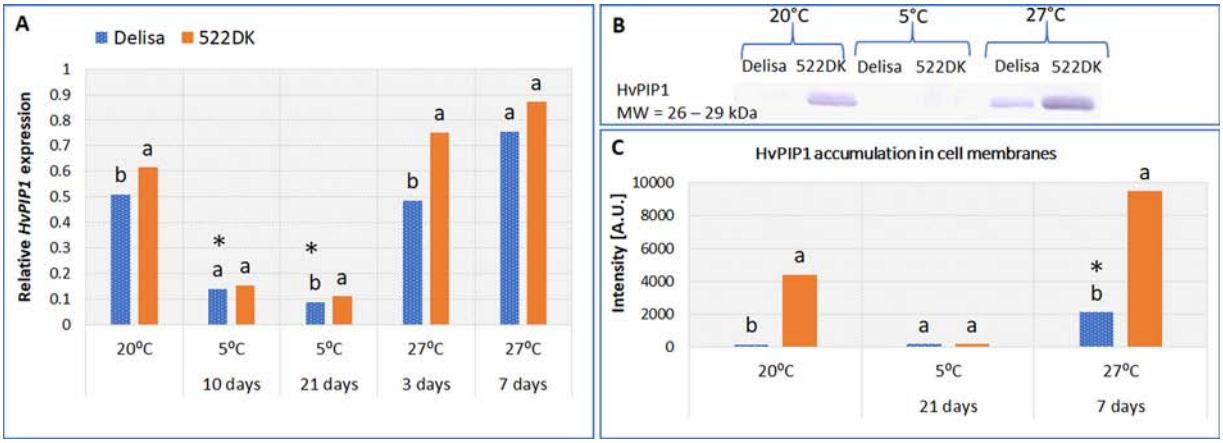

Fig. 3. Changes in the relative *HvPIP1* transcript level (A) and the HvPIP1 protein accumulation (B, C) in barley cell membrane fractions of wild type Delisa and its mutant (522DK) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein that was identified using the anti-HvPIP1 antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 7.5 μg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated HvPIP1 was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which are correlated to the area under the densitometric curves. Significant differences between cv. Delisa and its mutant 522DK – (Student's *t*-test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Delisa cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

accumulation of the transcript. Aroca et al. (2005) showed that maize roots accumulated significantly more of the PIP protein after chilling than the control plants, but the opposite effect was observed for the expression levels of the *PIP* genes. However, at 27 °C (compared to 20 °C) both cultivars accumulated markedly higher amount of HvPIP1 protein (Fig. 3B and C and 4 B and C), which also correspond with higher *HvPIP1* transcript accumulation.

In our experiment, the BR-deficient mutant 522DK generally had a higher accumulation of the aquaporin transcript than Delisa, while mutant BW084 (and signalling mutant BW312) showed the opposite tendency (Figs. 3A and 4 A). However, both BR-deficient mutants had a significantly increased accumulation of the HvPIP1 protein in the membrane fraction (at 20 °C and 27 °C, Figs. 3B and C and 4 B and C). Mutant BW084 had a slightly lower accumulation of HvPIP1 at 5 °C than the Bowman cultivar, while in 522DK there was no difference compared to Delisa. BW312 accumulated a higher level of the HvPIP1 protein at 20 °C but a lower level at 5 °C and 27 °C compared to Bowman (Fig. 4B and C). To the best of our knowledge, very little is known about the effect of BRs on the accumulation of aquaporins in plants. Morillon et al. (2001) showed that brassinolide may control the activity of aquaporins in *A. thaliana*. On the other hand, brassinolide had no significant effect on the accumulation of the aquaporin proteins in the cell membrane fraction of radish seedlings (Suga et al., 2002).

For a comparison, the accumulation of PM H^+-ATPase and HvPIP1 was also analysed for the leaf crude extracts. Such analysis would be faster and simpler than analysis using procedure with cell membrane isolation. The results however differed from those obtained for the cell membrane fraction (Figs. 1 and 2, Supplementary Material). Accumulation of PM H^+-ATPase and HvPIP1 (or other proteins) is usually calculated per total protein concentration in sample. Protein concentration in pure membrane fraction is however differ than in crude leaf extract (Figs. 3 and 4, Supplementary Material). Moreover in

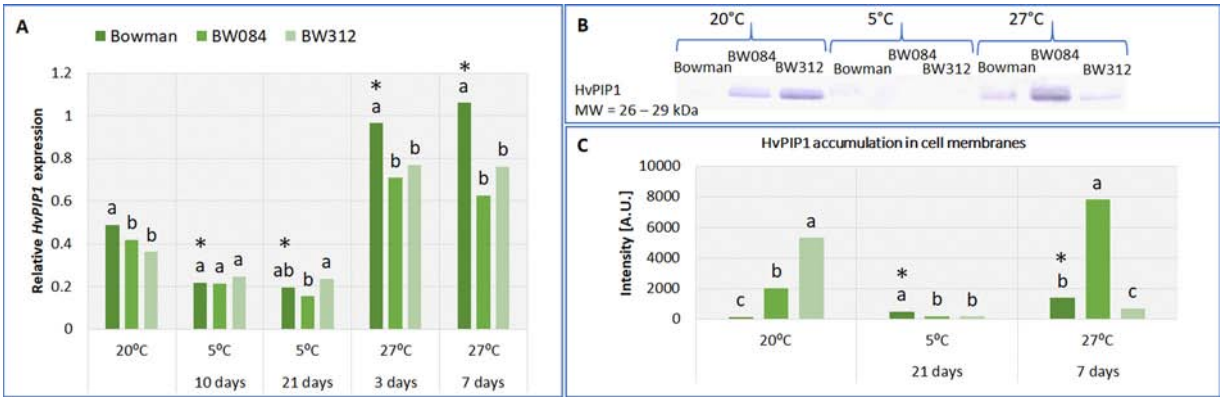

Fig. 4. Changes in the relative *HvPIP1* transcript level (A) and the HvPIP1 protein accumulation (B, C) in the barley cell membrane fractions of the wild-type Bowman and its mutants (BW084 and BW312) at 20 °C and after acclimation at 5 °C and 27 °C. The results of the accumulation of the transcript are presented as the fold change in the expression of a specific gene in the given samples compared to the endogenous reference gene – *actin*. The visualised band corresponded with the protein identified using the anti-HvPIP1 antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 7.5 μg of proteins from the cell membrane fractions was applied on each lane. The amount of accumulated HvPIP1 was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units (A.U.), which correlated with the area under the densitometric curves. Significant differences between cv. Bowman and its mutants (BW084 and BW312) – (Duncan's test, $P \leq 0.05$) for each temperature are indicated by different letters. Moreover, the accumulations of the transcript and protein in the Bowman cultivar at different temperatures were compared. The comparisons were performed in pairs (for 20 °C and 5 °C; 20 °C and 27 °C) (Student's *t*-test, $P \leq 0.05$) and significant differences are indicated by an "*".

the case of the leaf extract, we additionally dealt with the cytoplasmic proteins. The difference in the accumulation of PM H^+-ATPase and HvPIP1 in dependency on analyze type is surely result of the different concentration of protein in the cell membrane fraction and the crude leaf extract. Analyses of the accumulation of the membrane proteins such as ATPase or PIP should be then rather conducted on the isolated membrane fraction and should be calculated more precisely per membrane protein concentration (additionally measured after the addition of a surfactant). ATPase or PIP analyze in the crude leaf extract seems to be less reliable, because the results per soluble proteins are mainly calculations of those proteins that are present in the cytoplasm.

To conclude, in barley wild types, temperature of growth/acclimation is significant factor influencing on the level of the accumulation of the *ATPase* and *HvPIP1* transcript and the H^+-ATPase and HvPIP1 protein. The barley mutants with the BR deficiency and with BR signalling disturbances were characterised by the altered levels of *H^+-ATPase* transcript, the aquaporin *HvPIP1* transcript and the accumulation of the proteins (compared to wild types) (Table 1 A and B, Supplementary Material), which may suggest the involvement of BRs in regulating the accumulation of PM H^+-ATPase and aquaporin HvPIP1 on the transcriptional and translational levels. The phenomenon was dependent on temperature of plant growth/acclimation but there was not observed pattern of changes which could help to explain described earlier (Sadura et al., 2019) altered tolerance of mutants to heat shock or frost.

Author contributions

IS and AJ made isolation of cell membrane fraction and analyzed protein concentration in isolated fraction. IS and MLK prepared samples of crude leaf extracts and analyzed protein concentration in this material. IS and MLK made analysis of PM H^+-ATPase and HvPIP1 proteins accumulation after method optimization. IS made quantification of the results by densitometry using ImageJ software. IS and BJ measured accumulation of the transcripts *H^+-ATPase* and *HvPIP1*. DG provided seeds of mutants for experiment and gave suggestion about interpretation of data. AJ was coordinator of the project 2015/17/B/NZ9/01695, designed experiments and choose all other scientists to cooperation. All authors analyzed and interpreted data. IS wrote the manuscript under AJ supervision. All authors read article and suggested corrections.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.153090.

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Supplementary material

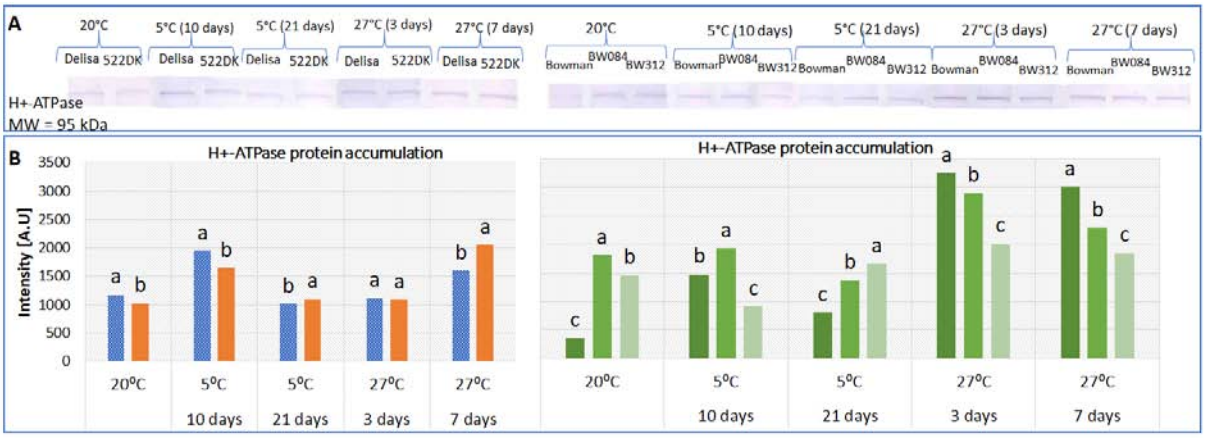

Figure 1S. PM H^+-ATPase protein accumulation in the barley leaves of cv. Delisa, the 522DK mutant, cv. Bowman, the BW084 and BW312 mutants at 20°C and after acclimation at 5°C and 27°C. The visualised band corresponded with the protein that was identified using the anti- PM H^+-ATPase antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 20 µg of proteins from leaf extracts was applied on each lane **(A)**. The amount of accumulated PM H^+-ATPase was estimated based on a densitometric analysis of the band intensity staining. The values are presented as arbitrary units, which correlated with the area under the densitometric curves **(B)**. Significant differences between cv. Delisa and its mutant 522DK – (Student's t-test, $P \leq 0.05$); between cv. Bowman and its mutants – Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters.

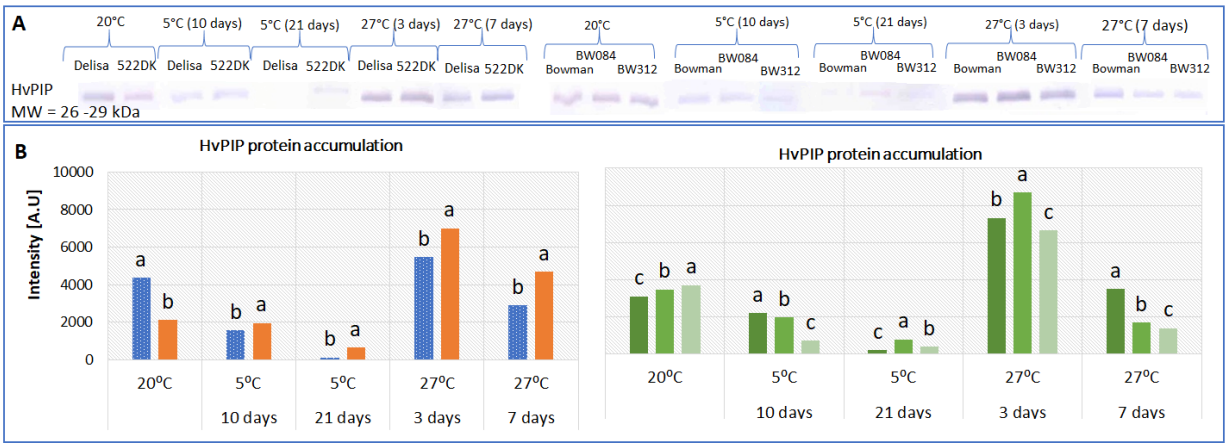

Figure 2S. HvPIP1 protein accumulation in the barley leaves of cv. Delisa, 522DK mutant, cv. Bowman, BW084 and BW312 mutants at 20°C and after acclimation at 5°C and 27°C. The visualised band corresponded with the protein that was identified using the anti-HvPIP1 antibody. MW – molecular weight standard (Thermo Scientific PageRuler Prestained Protein Ladder), 10 µg of proteins from leaf extracts was applied on each lane **(A)**. The amount of accumulated HvPIP1 was estimated after a densitometric analysis of the band intensity staining. The values are presented as arbitrary units, which correlated with the area under the densitometric curves **(B)**. Significant differences between cv. Delisa and its mutant 522DK – (Student's t-test, $P \leq 0.05$); between cv. Bowman and its mutants – Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters.

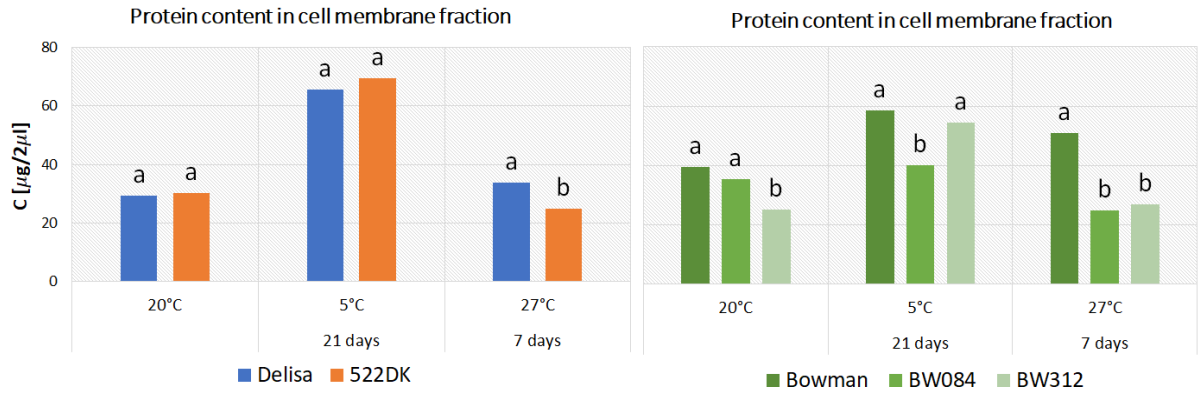

Figure 3S. Protein concentration in the barley cell membrane fractions of cv. Delisa, 522DK mutant, cv. Bowman, BW084 and BW312 mutants at 20°C and during acclimation at 5°C and 27°C. Significant differences between cv. Delisa and its mutant 522DK – (Student's t-test, $P \leq 0.05$); between cv. Bowman and its mutants – Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters.

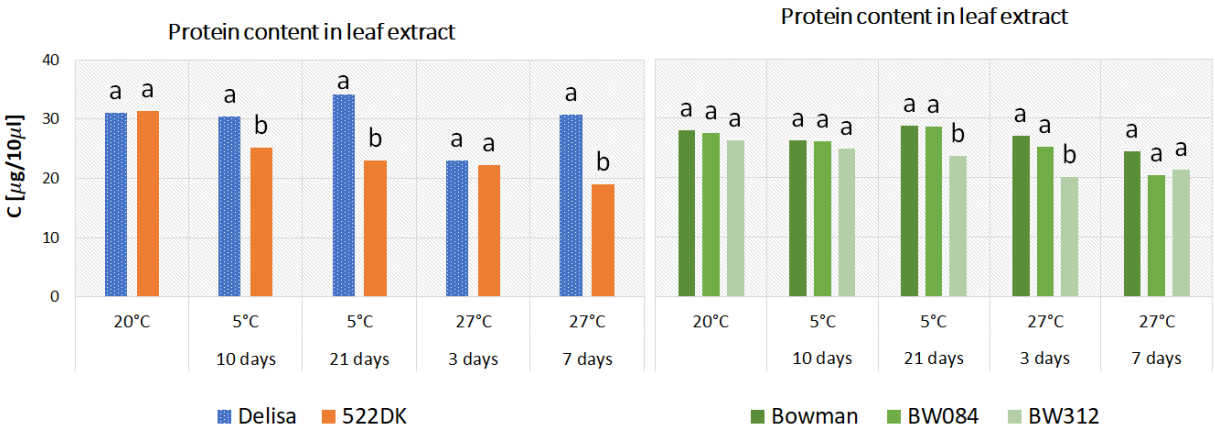

Figure 4S. Protein concentration in the barley leaves of cv. Delisa, 522DK mutant, cv. Bowman, BW084 and BW312 mutants at 20°C and during acclimation at 5°C and 27°C. Significant differences between cv. Delisa and its mutant 522DK – (Student's t-test, $P \leq 0.05$); between cv. Bowman and its mutants – Duncan's test, ($P \leq 0.05$) for each temperature are indicated by different letters.

Table S1A. Changes of accumulation of *HvPIP1* and *H^+-ATPase* transcripts in barley in relation to mutation type and temperature of growth (+ increase in comparison to wild type; - decrease in comparison to wild type; NC not changed in comparison to wild type). The plant material in our studies included the barley (*Hordeum vulgare* L.) BR-deficient mutant 522DK (mutation *HvDWARF*) from the Delisa cultivar, the barley BR-deficient mutant BW084 (mutation *HvCPD***)**, the BR-signalling defective mutant (BW312, mutation *HvBRI1*) and their reference cultivar Bowman.

Transcript	**Genetic mutation**														
	HvDWARF					***HvCPD***					***HvBRI1***				
	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)	20°C	5°C (10 days)	5°C (21 days)	27°C (3 days)	27°C (7 days)
H^+-ATPase	**NC**	**NC**	**-**	**NC**	**NC**	**-**	**-**	**-**	**NC**	**-**	**NC**	**-**	**-**	**NC**	**-**
HvPIP1	**+**	**NC**	**+**	**+**	**NC**	**-**	**NC**	**NC**	**-**	**-**	**-**	**NC**	**NC**	**-**	**-**

Table S1B. Changes of accumulation of HvPIP1 and PM H^+-ATPase proteins in barley in relation to mutation type and temperature of growth (+ increase in comparison to wild type; - decrease in comparison to wild type; NC not changed in comparison to wild type). The plant material in our studies included the barley (*Hordeum vulgare* L.) BR-deficient mutant 522DK (mutation *HvDWARF*) from the Delisa cultivar, the barley BR-deficient mutant BW084 (mutation *HvCPD***)**, the BR-signalling defective mutant (BW312, mutation *HvBRI1*) and their reference cultivar Bowman.

Protein	**Genetic mutation**								
	HvDWARF			***HvCPD***			***HvBRI1***		
	20ºC	5 ºC	27 ºC	20ºC	5 ºC	27 ºC	20ºC	5 ºC	27 ºC
PM H^+-ATPase	**+**	**NC**	**+**	**+**	**NC**	**-**	**+**	**+**	**-**
HvPIP1	**+**	**NC**	**+**	**+**	**-**	**+**	**+**	**-**	**-**

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Kraków, dn. 2.03.2021r.

OŚWIADCZENIE WSPÓŁAUTORA

Wyrażam zgodę na wykorzystanie przez Iwonę Sadura publikacji: Sadura Iwona, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020) Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature. [Journal of Plant Physiology 244:153090] w przewodzie doktorskim opartym na zbiorze artykułów opatrzonych tytułem: „Rola brasinosteroidów w procesach aklimatyzacyjnych roślin jęczmienia do niskiej i wysokiej temperatury."

Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: nauczeniu doktorantki (I.Sadura) procedury homogenizacji materiału roślinnego oraz oznaczania białka na potrzeby metody Western Blott i Immunoblot, nauczeniu metody Western Blott i Immunoblot, nadzorowaniu pracy doktorantki przy wykonywaniu w/w analiz w materiale roślinnym (jęczmień).

Marta Libik-Konieczny

czytelny podpis

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OŚWIADCZENIE WSPÓŁAUTORA

Wyrażam zgodę na wykorzystanie przez Iwonę Sadura publikacji: Sadura Iwona, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020) Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature. [Journal of Plant Physiology 244:153090] w przewodzie doktorskim opartym na zbiorze artykułów opatrzonych tytułem: „Rola brasinosteroidów w procesach aklimatyzacyjnych roślin jęczmienia do niskiej i wysokiej temperatury."

Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: nauczeniu doktorantki (I.Sadura) procedury izolacji RNA i procedury wykonania odwrotnej transkrypcji, nadzorowaniu pracy doktorantki przy wykonywaniu w/w analiz w liściach pozyskanych z badanych roślin jęczmienia. Ponadto praca moja polegała na wykonaniu analizy RT-PCR i pomocy przy interpretacji wyników.

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: przygotowaniu materiału nasiennego (w tym nasion linii bliskoizogenicznych i mutantów) do badań oraz współpracy przy pisaniu manuskryptu (analiza/dyskusja aspektów genetycznych).

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Anna Janeczko

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czytelny podpis

cells

MDPI

Article

The Impact of Mutations in the *HvCPD* and *HvBRI1* Genes on the Physicochemical Properties of the Membranes from Barley Acclimated to Low/High Temperatures

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Abstract: (1) Background: The study characterized barley mutants with brassinosteroid (BR) biosynthesis and signaling disturbances in terms of the physicochemical/structural properties of membranes to enrich the knowledge about the role of brassinosteroids for lipid metabolism and membrane functioning. (2) Methods: The Langmuir method was used to investigate the properties of the physicochemical membranes. Langmuir monolayers were formed from the lipid fractions isolated from the plants growing at 20 °C and then acclimated at 5 °C or 27 °C. The fatty acid composition of the lipids was estimated using gas chromatography. (3) Results: The BR-biosynthesis and BR-signaling mutants of barley were characterized by a temperature-dependent altered molar percentage of fatty acids (from 14:0 to 20:1) in their galactolipid and phospholipid fractions in comparison to wild-type (WT). For example, the mutants had a lower molar percentage of 18:3 in the phospholipid (PL) fraction. The same regularity was observed at 5 °C. It resulted in altered physicochemical parameters of the membranes (A_{lim}, π_{coll}, Cs^{-1}). (4) Conclusions: BR may be involved in regulating fatty acid biosynthesis or their transport/incorporation into the cell membranes. Mutants had altered physicochemical parameters of their membranes, compared to the WT, which suggests that BR may have a multidirectional impact on the membrane-dependent physiological processes.

Keywords: brassinosteroids; cell membranes; Langmuir monolayers; galactolipids; plant acclimation to high/low temperature; phospholipids

1. Introduction

During their life cycle, plants are exposed to changing environmental conditions—biotic (pathogenic microorganisms) or abiotic (extreme temperature, drought, flooding, nutritional depletion, too low or too intense light, UV radiation, etc.). Among the abiotic stresses, temperature stress is a particularly serious problem for crop production. Some species are sensitive to cold (cucumber, maize). Frost, on the other hand, especially with an insufficient snow cover on the fields, can cause significant yield losses of winter crops including winter cereals. High-temperature stress is particularly dangerous for plants when combined with a water deficit. Gradually increasing temperature allows plants to acclimate and tolerate further increases in temperatures, which normally might be lethal.

On the other hand, cold can acclimate plants to freezing temperatures and in the winter plants, this is known as the cold-hardening (cold acclimation) process. During acclimation, metabolic adjustments occur in plant cells that include, among others, changes in the hormonal homeostasis, an elevated biosynthesis of the proteins with chaperone properties (i.e., heat shock proteins) or enhancement of the antioxidant system. Crucial changes that stimulate the acquisition of frost tolerance influence the cell membranes, especially in cold acclimation. Since membranes are considered to be "thermal sensors" (according to the membrane sensor hypothesis [1], they are thought to elicit other metabolic changes within a cell, including gene expression. In the lipid part of the cell membrane, acclimating to elevated temperatures or cold causes essential physicochemical adjustments, one of which is the modification of the fatty acid composition. Higher temperatures usually initiate a decrease in the level of unsaturated acids in the lipid part of a membrane [2]. In cold, the composition of the fatty acids of the membrane lipids is expected to become more unsaturated [3]. This unsaturation of the fatty acids causes a decrease in the phase transition temperature and increases the "fluidity" of the hydrophobic phase. The main fatty acids from groups 16C and 18C (especially 18:3 or 18:2), present in cells in the highest amounts, have a significant physiological role in plant response to abiotic but also biotic stresses [2,3]. In winter plants, low temperature stimulates biosynthesis of 18:3 acid, resulting in an increase of unsaturation of membrane lipids thus better acclimation to unfavorable conditions of growth in winter [3,4]. On the other hand, a lowered accumulation of 18:3 (and increased lipid saturation) is beneficial for instance for better thermostability of the photosynthetic apparatus at higher temperatures [2]. Polyunsaturated fatty acids can also be released from membranes in response to an attack by biotic agents [5–7]. Fatty acid 18:3 may directly activate NADPH-oxidase and generate reactive oxygen intermediates after inoculation with bacteria. On the other hand, lipid-derived metabolites produced by oxidation of fatty acids (18:3 or 18:2)—oxylipins like jasmonic acid—are an integral part of plant defense against pathogens.

Plant growth hormones/regulators such as brassinosteroids (BR) can improve the tolerance of plants to low- or high-temperature stress [8]. BR are plant steroid hormones that have been extensively studied during the past three decades. These studies have revealed many of the mechanisms of their action in the process of growth and development stimulation as well as plant stress tolerance [9]. However, little is known about the impact of BR on the plant membrane structure (lipid part) and the membrane physicochemical properties, which are, as was mentioned, important in the process of plants acclimating to various temperatures.

The effects of the exogenous administration of BR on the lipid FA composition in plants have been found in several previous studies [10–13]. Further, Li et al. [12] in studies using electron paramagnetic resonance observed an increase in the membrane fluidity in the presence of brassinolide in mango, which was an important step for developing an improved plant tolerance to low temperature. Our studies [14,15] showed that the structural properties of the cell membranes were differentiated by the presence of brassinosteroids, and therefore, the role of BR in improving the tolerance of winter wheat to low temperatures was suggested. In Langmuir monolayer studies, 24-epibrassinolide and 24-epicastasterone were introduced into lipids that had been obtained from the aerial part of winter wheat seedlings that had been cultured at 5 °C or 20 °C. It was suggested that the tested BR (similar to sterols) entered the cell membrane directly and modified its properties by, for example, increasing the distance between the fatty acid chains, which might improve the functioning (flexibility) of the membrane in low temperatures [14,15]. As 24-epicastasterone induced a slightly different effect than 24-epibrassinolide, these results also showed the importance of the chemical structure of BR for their interactions with membranes [14].

The novelty of the presented studies, contrary to studies with the exogenous BR treatments, was the use of barley BR-deficient and BR-insensitive mutants to verify how disturbances in the BR biosynthesis (mutation in the *HvCPD* gene) and the BR signaling (mutation in the *HvBRI1* gene) in barley change the FA lipid composition and the physicochemical properties of the cell membranes. We characterized the natural lipid monolayers that had been obtained directly from these barley

mutants (with a decreased or increased content of endogenous BR), which had been acclimated at 5 °C and 27 °C. It is worth mentioning that our earlier studies [16] revealed that the mutants (after acclimation at 27 °C) had acquired a heat tolerance that was higher than the wild-type. After acclimation at 5 °C, both mutants had a lower frost tolerance. In our earlier articles, we also described changes in the protein component of the cell membranes (aquaporins, heat shock proteins) that might modify the mutants' tolerance to high temperature or frost [17,18]. In the current work, we focused on the lipid part of the cell membranes. The aim was to investigate the dependence between the FA composition of the individual lipid fractions and changes in the physicochemical properties of the membrane structure, which determines their permeability, stiffness/fluidity, and ability to be penetrated by various compounds including hormones. The characteristics of the physicochemical properties of membranes are increasingly used to explain the subtle changes in the structure of lipids that occur during the physiological processes (review Rudolphi-Skórska and Sieprawska [19]). In the current work, this approach enabled (I) the dependence between an increased/decreased level of the endogenous BR in leaf tissue and structural-functional properties of membranes to be discussed and (II) the role of BR in the low/high-temperature tolerance mechanism that involves the modifications of the membranes to be deliberated.

2. Plant Material and Experimental Design

The BR-deficient mutant BW084 and the BR-insensitive mutant BW312, which are near the isogenic lines (NILs) of the wild-type (WT) Bowman cultivar, were selected for the studies. The homogenous genetic background simplifies comparative physiological analysis [20,21]. BW084 has a mutation in the *HvCPD* gene coding enzyme that mediates the early BR biosynthetic steps and the brassinolide or castasterone content in this mutant are significantly reduced when compared with the WT [16,22]. The BR-signaling mutant BW312 has a mutation in the gene that encodes the BR receptor *HvBRI1* and due to the BR-insensitivity accumulates more BR than the WT cultivar [16,22]. The barley seeds were obtained from the collection of the University of Silesia (Katowice, Poland). The plant cultures, growth conditions, and experimental design are described in detail in the work by Sadura et al. [16]. Briefly, after seed germination, the plants were transferred to pots (15–20 plants/pot) and then cultured in a growth chamber (16 h photoperiod, 20 °C) for about three weeks. When the plants had developed three to four leaves, the pots were divided into two groups. One group was acclimated at 5 °C (21 days, 8 h photoperiod), while the second was acclimated at 27 °C (16 h photoperiod) for seven days. The leaf samples for the analyses were collected from the plants before acclimation (20 °C) and after acclimation at 5 °C or 27 °C.

2.1. Lipid Extraction and Fatty Acid Composition Measurement

The lipids of the membranes were extracted from the leaves using a mixture of methanol/chloroform (2:1) and were then re-extracted with chloroform according to a modified method of Bligh and Dyer [23], which was described in detail by Gzyl-Malcher et al. [24] and Janeczko et al. [10]. The lipid fractions, which included phospholipids (PL) and the glycolipids (monogalactosyldiacylglycerols [MGDG] and digalactosyldiacylglycerols [DGDG]), were separated on silica acid using column chromatography as was described in the work Janeczko et al. [10]. The purity of obtained fractions was checked by thin-layer chromatography (TLC). The fatty acid (FA) esterification was made based on a modified AOAC Official Method 991,39 [25]. The equipment for the analysis on the gas chromatograph were a chromatograph TRACE GC ULTRA (Thermo Electron Corporation, Milano, Italy), a flame ionization detector (FID), and a STABILWAX column (30 m—0.25 mm—0.25 μm). The individual fatty acid methyl esters were identified by comparing them to the standard mixture of Supelco 37 component FAME Mix (Sigma-Aldrich, Poznań, Poland). Fatty acids from 14:0 to 21:1 were detected (14:0—myristic acid; 16:0—palmitic acid; 16:1n-9—*cis*-7-hexadecenoic acid; 16:1n-7—cis-9-hexadecenoic (palmitoleic acid); 18:0—stearic acid; 18:1 n-9—oleic acid; 18:1 n-7—vaccenic acid; 18:2 n-6 (18:2)—linoleic acid; 18:3 n-3 (18:3)—linolenic acid; 20:0—arachidic acid and 20:1—eicosenoic acid). The data are expressed as

the molar percentage of a specific fatty acid in relation to all of the fatty acids that were measured. The degree of the unsaturation of the individual lipid fractions was determined based on the calculated ratios of the unsaturated to saturated FA (U/S) and 18:3/18:2.

2.2. The Langmuir Monolayers and Their Physicochemical Parameters

Fractions of extracted polar lipids, i.e., PL, DGDG, and MGDG, were used for the experiments using the Langmuir technique (Minitrough, KSV; Finland). The monolayers were produced by spreading chloroform solutions of the lipids on the surface of the water (as the subphase). The Langmuir monolayers were compressed at a rate of 3.5–4.6 $Å^2$/molecule × min. A Platinum Wilhelmy plate connected to an electrobalance was used to detect the surface pressure (accuracy of ±0.1 mN/m). All of the experiments were performed at 25 °C. The measurements were repeated three/four times to confirm a high recurrence of the obtained isotherms (±0.1–0.3 $Å^2$).

Based on the dependence of the surface pressure (π) versus the area per lipid molecule (A), the parameters that characterized the structure of the monolayers were 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. Moreover, the static compression modulus was calculated as $C_s^{-1} = -(d\pi/d\ln A)$. This parameter indicates the mechanical resistance of the layers and provides information on the stability and fluidity of a layer.

2.3. Statistical Analysis

Statistical analysis (ANOVA, post hoc test) was performed in Statistica 13.1 (StatSoft, Tulsa, USA). Duncan's test was used to compare the averages of data obtained for the WT Bowman, BW084, and BW312 (Figures 1 and 3, Table 1). Values marked with the same letters did not differ significantly ($p \leq 0.05$). Values on Figures 1, 3 and Figure S1—supplementary materials are given ±SD.

Table 1. Composition of the fatty acids from the three classes of lipids (MGDG, DGDG, PL) that were isolated from the barley WT Bowman and its BW084 and BW312 mutants cultured at 20 °C and then acclimated at 5 °C or 27 °C. Any significant differences between WT Bowman and its mutants (Duncan test, $p \leq 0.05$) for each temperature, lipid fraction, and fatty acid (separately) are indicated by different letters.

Temperature	Plants	Fatty Acids [mol %]										
		14:0	16:0	16:1n-9	16:1n-7	18:0	18:1n-9	18:1n-7	18:2n-6	18:3n-3	20:0	20:1
MGDG												
20 °C	Bowman	0.321 ^{b}	5.270 ^{c}	0.049 ^{b}	0.069 ^{a}	2.695 ^{c}	1.208 ^{b}	0.190 ^{b}	4.458 ^{b}	85.335 ^{a}	0.075 ^{c}	0.046 ^{a}
20 °C	BW084	0.584 ^{a}	8.271 ^{a}	0.094 ^{a}	0.071 ^{a}	4.201 ^{a}	1.340 ^{a}	0.252 ^{a}	5.216 ^{a}	79.399 ^{c}	0.121 ^{a}	0.049 ^{a}
20 °C	BW312	0.298 ^{b}	6.011 ^{b}	0.035 ^{c}	0.061 ^{a}	3.040 ^{b}	1.005 ^{c}	0.207 ^{b}	5.180 ^{a}	83.800 ^{b}	0.088 ^{b}	0.031 ^{b}
5 °C	Bowman	0.257 ^{b}	7.380 ^{a}	0.041 ^{b}	0.046 ^{a}	3.900 ^{a}	1.157 ^{a}	0.191 ^{a}	3.483 ^{a}	82.928 ^{c}	0.152 ^{a}	0.078 ^{a}
5 °C	BW084	0.233 ^{b}	5.128 ^{c}	0.032 ^{b}	0.052 ^{a}	3.767 ^{b}	0.770 ^{c}	0.173 ^{a}	2.648 ^{c}	86.709 ^{a}	0.141 ^{a}	0.034 ^{c}
5 °C	BW312	0.293 ^{a}	5.702 ^{b}	0.057 ^{a}	0.056 ^{a}	3.288 ^{c}	1.066 ^{b}	0.195 ^{a}	3.043 ^{b}	85.840 ^{b}	0.093 ^{b}	0.056 ^{b}
27 °C	Bowman	0.387 ^{c}	7.858 ^{b}	0.031 ^{c}	0.067 ^{a}	4.874 ^{c}	1.017 ^{b}	0.211 ^{a}	6.640 ^{b}	78.427 ^{a}	0.147 ^{b}	0.030 ^{a}
27 °C	BW084	0.484 ^{b}	9.282 ^{a}	0.074 ^{b}	0.069 ^{a}	6.646 ^{a}	1.046 ^{b}	0.222 ^{a}	6.600 ^{b}	74.951 ^{b}	0.196 ^{a}	0.026 ^{a}
27 °C	BW312	0.582 ^{a}	9.369 ^{a}	0.222 ^{a}	0.054 ^{b}	5.767 ^{b}	1.172 ^{a}	0.205 ^{a}	7.425 ^{a}	74.527 ^{c}	0.159 ^{b}	0.030 ^{a}
DGDG												
20 °C	Bowman	0.228 ^{c}	13.474 ^{a}	0.103 ^{b}	0.085 ^{ab}	1.998 ^{a}	1.606 ^{b}	0.354 ^{ab}	3.821 ^{c}	77.904 ^{a}	0.028 ^{b}	0.061 ^{a}
20 °C	BW084	0.326 ^{a}	13.532 ^{a}	0.106 ^{b}	0.091 ^{a}	1.899 ^{b}	1.733 ^{a}	0.370 ^{a}	4.228 ^{b}	77.151 ^{b}	0.040 ^{a}	0.061 ^{a}
20 °C	BW312	0.246 ^{b}	12.920 ^{b}	0.189 ^{a}	0.072 ^{b}	1.689 ^{c}	1.524 ^{c}	0.336 ^{b}	4.783 ^{a}	77.803 ^{a}	0.030 ^{b}	0.057 ^{a}
5 °C	Bowman	0.178 ^{a}	12.986 ^{a}	0.124 ^{a}	0.084 ^{a}	1.582 ^{b}	1.364 ^{a}	0.341 ^{a}	2.678 ^{c}	80.131 ^{c}	0.051 ^{b}	0.075 ^{a}
5 °C	BW084	0.140 ^{b}	11.694 ^{b}	0.037 ^{c}	0.075 ^{b}	1.167 ^{c}	1.022 ^{c}	0.314 ^{a}	3.070 ^{a}	82.039 ^{a}	0.046 ^{b}	0.070 ^{a}
5 °C	BW312	0.132 ^{b}	11.728 ^{b}	0.054 ^{b}	0.064 ^{c}	1.809 ^{a}	1.171 ^{b}	0.320 ^{a}	2.852 ^{b}	81.413 ^{b}	0.083 ^{a}	0.047 ^{b}
27 °C	Bowman	0.320 ^{b}	17.561 ^{b}	0.058 ^{c}	0.082 ^{a}	2.630 ^{a}	1.446 ^{a}	0.344 ^{a}	6.093 ^{b}	70.943 ^{b}	0.038 ^{a}	0.056 ^{a}
27 °C	BW084	0.298 ^{b}	15.433 ^{c}	0.065 ^{b}	0.085 ^{a}	2.217 ^{b}	1.077 ^{b}	0.328 ^{a}	5.988 ^{b}	73.967 ^{a}	0.041 ^{a}	0.042 ^{b}
27 °C	BW312	0.562 ^{a}	18.933 ^{a}	0.220 ^{a}	0.071 ^{b}	2.595 ^{a}	1.457 ^{a}	0.331 ^{a}	6.987 ^{a}	68.074 ^{c}	0.057 ^{a}	0.026 ^{c}
PL												
20 °C	Bowman	0.165 ^{b}	22.483 ^{b}	0.045 ^{b}	0.070 ^{a}	1.200 ^{b}	3.839 ^{a}	0.427 ^{b}	20.456 ^{b}	50.346 ^{a}	0.117 ^{b}	0.220 ^{a}
20 °C	BW084	0.169 ^{b}	22.393 ^{b}	0.054 ^{a}	0.070 ^{a}	1.264 ^{ab}	3.654 ^{b}	0.506 ^{a}	22.228 ^{a}	48.504 ^{c}	0.174 ^{a}	0.221 ^{a}
20 °C	BW312	0.198 ^{a}	23.345 ^{a}	0.040 ^{c}	0.069 ^{a}	1.305 ^{a}	2.661 ^{c}	0.404 ^{b}	22.005 ^{a}	49.017 ^{b}	0.124 ^{b}	0.168 ^{b}
5 °C	Bowman	0.136 ^{a}	22.258 ^{b}	0.028 ^{a}	0.052 ^{b}	1.064 ^{a}	2.409 ^{a}	0.329 ^{a}	16.411 ^{c}	56.357 ^{a}	0.087 ^{c}	0.231 ^{a}
5 °C	BW084	0.119 ^{b}	23.162 ^{a}	0.034 ^{a}	0.061 ^{a}	1.071 ^{a}	1.992 ^{b}	0.329 ^{a}	21.462 ^{a}	50.779 ^{c}	0.151 ^{a}	0.160 ^{c}
5 °C	BW312	0.108 ^{b}	22.730 ^{a}	0.019 ^{b}	0.048 ^{b}	1.113 ^{a}	2.393 ^{a}	0.348 ^{a}	18.826 ^{b}	53.552 ^{b}	0.112 ^{b}	0.196 ^{b}
27 °C	Bowman	0.246 ^{a}	25.844 ^{a}	0.070 ^{a}	0.072 ^{b}	1.640 ^{a}	3.585 ^{a}	0.481 ^{a}	20.618 ^{b}	46.404 ^{c}	0.106 ^{c}	0.139 ^{a}
27 °C	BW084	0.246 ^{a}	24.314 ^{b}	0.073 ^{a}	0.083 ^{a}	1.409 ^{c}	2.493 ^{c}	0.435 ^{b}	19.856 ^{c}	49.991 ^{a}	0.143 ^{b}	0.096 ^{b}
27 °C	BW312	0.227 ^{b}	24.122 ^{b}	0.072 ^{a}	0.069 ^{b}	1.523 ^{b}	2.691 ^{b}	0.397 ^{c}	22.808 ^{a}	46.957 ^{b}	0.156 ^{a}	0.101 ^{b}

3. Results

3.1. Fatty Acid Composition of the Membrane Lipids from Barley Growing at 20 °C and the Physicochemical Properties of the Lipid Monolayers

Focusing on fatty acid 18:3 (PL fraction), which was present in the highest percentage (approx. 50% of the FA pool), it can be observed that the amount of this acid was significantly lower at 20 °C in both tested mutants, BW312 and BW084, compared to the WT Bowman (Table 1). This phenomenon was accompanied by an increased content of 18:2. The content of the third acid (saturated 16:0) in the mutants at 20 °C was higher (BW312) or remained unchanged (BW084). The level of 18:0, present, however, at a low percentage, was increased in the signaling mutant, but not in the BR-biosynthesis mutant. In the MGDG and DGDG fractions, the content of 18:3 acid was much higher than in the PL fraction and reached approximately 80%. The mutants in most cases also exhibited a lower content of 18:3 in these fractions compared to the WT Bowman. In all of the tested fractions, the 18:3/18:2 ratio was lower in the mutants compared to the WT (Figure 1A,C,E). The U/S ratio was lower in both of the mutants for the MGDG fraction (Figure 1B). The results were less evident for the DGDG and PL (Figure 1D,F).

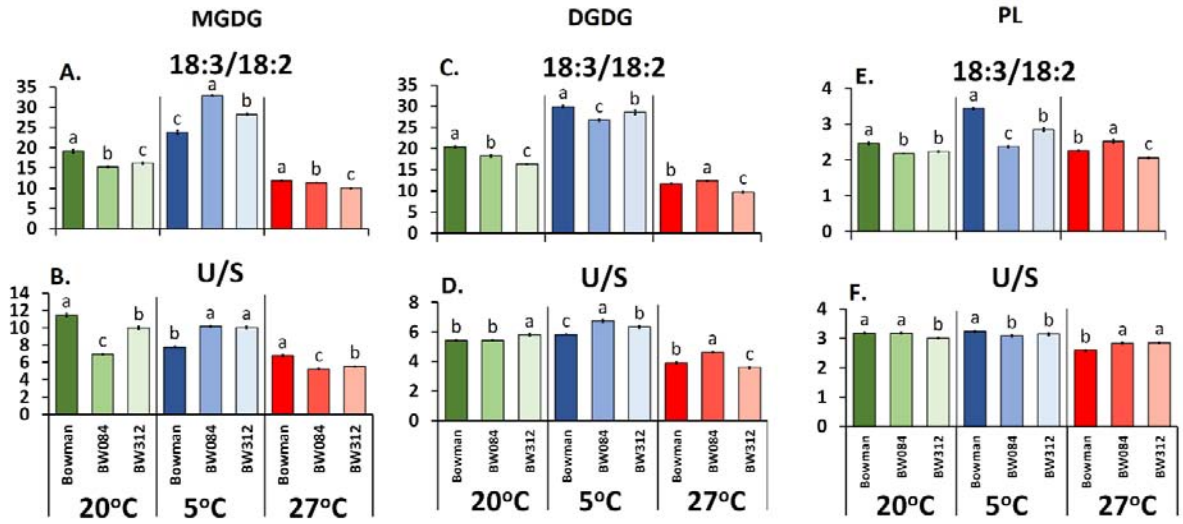

Figure 1. The ratio of fatty acid 18:3 to 18:2 (18:3/18:2) and unsaturated fatty acids to saturated fatty acids (U/S) for the specific lipid fractions (galactolipids and phospholipids) that were isolated from the leaves of the barley WT Bowman, mutant BW084 and mutant BW312 cultured at 20 °C and then acclimated at 5 °C and 27 °C. Any significant differences between WT Bowman and its mutants (Duncan test, $p \leq 0.05$) for each temperature are indicated by different letters. (A,B) Fraction of galactolipids (MGDG); (C,D) Fraction of galactolipids (DGDG); (E,F) Fraction of phospholipids (PL).

Other FA were also detected in the PL, MGDG, and DGDG fractions: 14:0, 16:1, 18:1, 20:0, and 20:1 (Table 1). These FA were, however, present at a very low percentage (below 3% and much lower). At 20 °C (as also after acclimation at 5 °C and 27 °C), slight changes were observed within the molar percentage of these FA in the mutants compared to the WT.

An analysis of the physicochemical properties of the membranes based on the dependence of the surface pressure (π) versus the lipid area (A) in individual fractions (Figure 2A–I) and the physicochemical parameters that were calculated based on these data (Figure 3A–I), it was found that for the plants growing at 20 °C, the values of A_{lim} in the PL fraction were generally similar both for the WT Bowman and its mutants, whereas for the galactolipids, the highest A_{lim} were obtained for the WT Bowman. The π_{coll} parameter significantly differentiated the lipids of the studied fractions that had been derived from the genotypes growing at 20 °C. The WT exhibited higher values of this parameter in the PL fraction and the lowest in galactolipids compared to mutants. The last parameter (C_s^{-1}) had

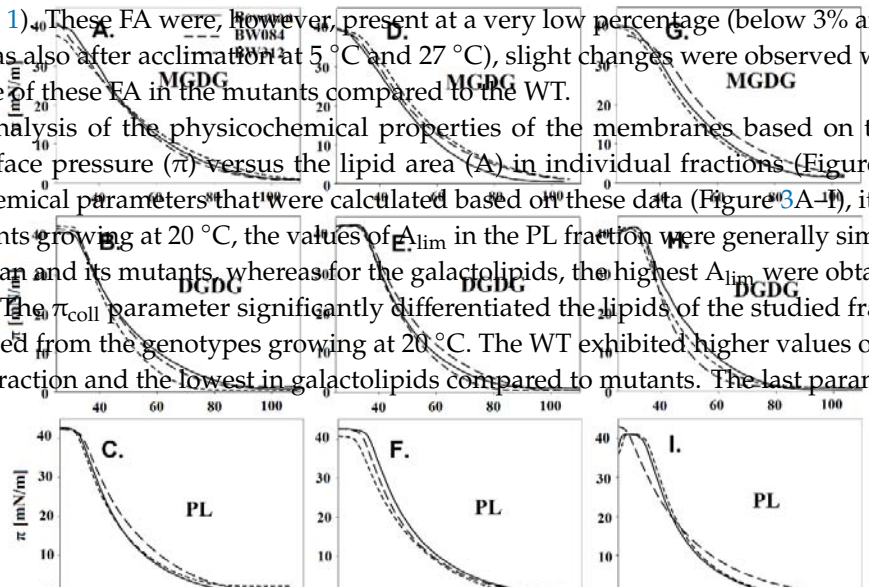

Figure 1. The ratio of fatty acid 18:3 to 18:2 (18:3/18:2) and unsaturated fatty acids to saturated fatty acids (U/S) for the specific lipid fractions (galactolipids and phospholipids) that were isolated from the leaves of the barley WT Bowman, mutant BW084 and mutant BW312 cultured at 20 °C and then acclimated at 5 °C and 27 °C. Any significant differences between WT Bowman and its mutants (Gaussian test, $p < 0.05$) for each temperature are indicated by different letters. (**A**, **B**) Fraction of galactolipids (MGDG); (**C**, **D**) Fraction of galactolipids (DGDG); (**E**, **F**) Fraction of phospholipids (PL)

the highest values for the BW312 in the PL fraction (although similar to the WT) and DGDG, while generally, the lowest values were for the MGDG lipids.

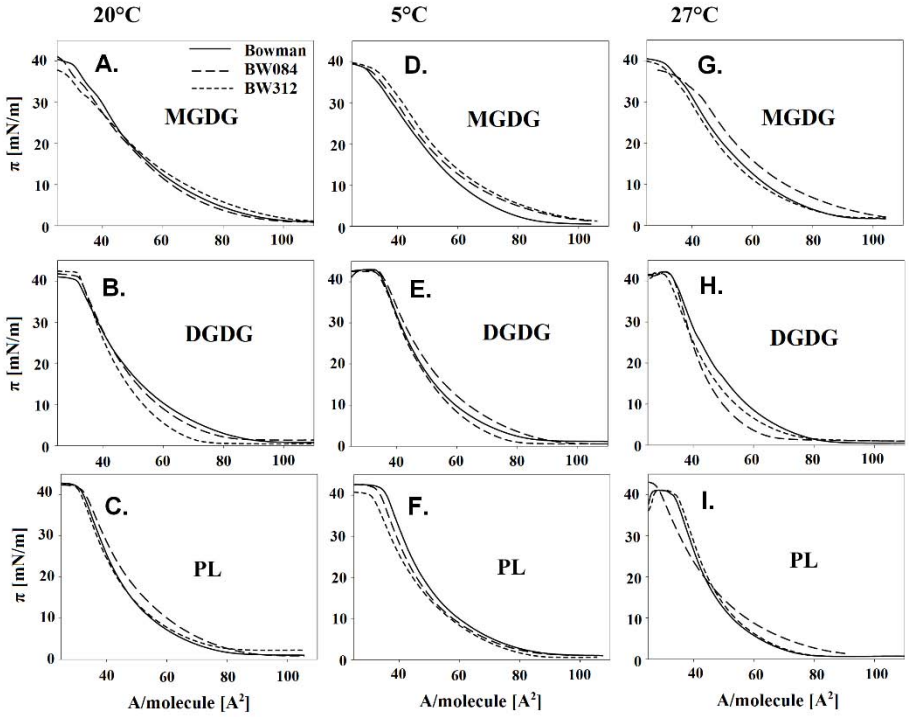

Figure 2. Exemplary Langmuir isotherms (surface pressure, π, versus area per molecule, A) for the monolayers of the galactolipids, MGDG (**A**–**C**) and DGDG (**D**–**F**), and phospholipids, PL (**G**–**I**) that were obtained from the leaves of the barley WT Bowman, mutant BW084, and mutant BW312 cultured at 20 °C (**A**–**C**) and then acclimated at 5 °C (**D**–**F**) and 27 °C (**G**–**I**).

3.2. Fatty Acid Composition of the Membrane Lipids from the Barley That Had Been Acclimated at 5 °C and the Physicochemical Properties of the Lipid Monolayers

Similar to 20 °C, the decrease in the 18:3 content in the FA pool of PL at 5 °C was accompanied by an increase in the 18:2 content in both mutants when compared with the WT Bowman (Table 1). At 5 °C, the content of 16:0 acid was higher in both mutants than in the WT. In the MGDG and DGDG fractions, the highest percentage was 18:3 (more than 80% of the FA pool).

At the low temperature, the 18:3/18:2 ratio was higher in the mutants for the MGDG but lower for the DGDG and PL fractions compared to the WT (Figure 1A,C,E). The opposite effect was observed for the U/S (for DGDG). Value A_{lim} at 5 °C was higher in the mutants (MGDG fraction) and was accompanied by a higher C_s^{-1} and lower π_{coll} values (Figure 3A–C). In the DGDG and PL fractions, the pattern of the changes of those three parameters was, with some exceptions, a little similar to that recorded at 20 °C. Especially for BW312, all of the values of A_{lim}, π_{coll}, and C_s^{-1} were at 5 °C always lower than in the WT. Figure S1A–I additionally illustrates the direction of the changes of the A_{lim}, π_{coll}, and C_s^{-1} parameters in the mutants and the WT Bowman at 5 °C in relation to 20 °C (expressed as 100%).

3.3. Fatty Acid Composition of the Membrane Lipids from the Barley Acclimated at 27 °C and the Physicochemical Properties of the Lipid Monolayers

Generally, the percentage of 18:3 in the PL fraction that was isolated from the plants acclimated at 27 °C was generally the lowest in all of the plants that were tested compared to 20 °C and the low temperature (Table 1). However, the level of this fatty acid was higher in the mutants compared to the WT, which was the opposite effect to that observed at 20 °C and 5 °C. The molar percentage of

saturated 16:0 acid decreased in all of the mutants at 27 °C while the molar percentage of 18:2 decreased in the BW084 but increased in the BW312 (if compare to WT Bowman). In the MGDG and DGDG fractions, the content of 18:3 at 27 °C decreased in the BW312. As for BW084, the molar percentage of 18:3 was increased in DGDG but decreased in MGDG, in comparison to WT Bowman. The 18:3/18:2 ratios and U/S were generally the lowest for all of the galactolipid fractions compared to the values that were noted at 20 °C and 5 °C (Figure 1A–D). The 18:3/18:2 ratios and U/S were lower in the MGDG fraction in the mutants than in the WT. In DGDG, these ratios were higher in BW084 but lower in BW312 than in the WT. As can be seen in Figure 3A–F, the values of A_{lim}, π_{coll}, and C_s^{-1} for the fraction of galactolipids were lower in the mutants than in the WT. The exception was π_{coll} in the BW084, which was similar to the WT in DGDG fraction. For the PL fraction, the A_{lim} value was higher in both mutants and it was accompanied by higher π_{coll} and C_s^{-1} values compared to WT Bowman but only in the BW084. The changes of these physicochemical parameters (A_{lim}, π_{coll}, and C_s^{-1}) for the tested mutants and the WT Bowman, which were calculated additionally in relation to the temperature of 20 °C (100%), are presented in Figure S1A–I.

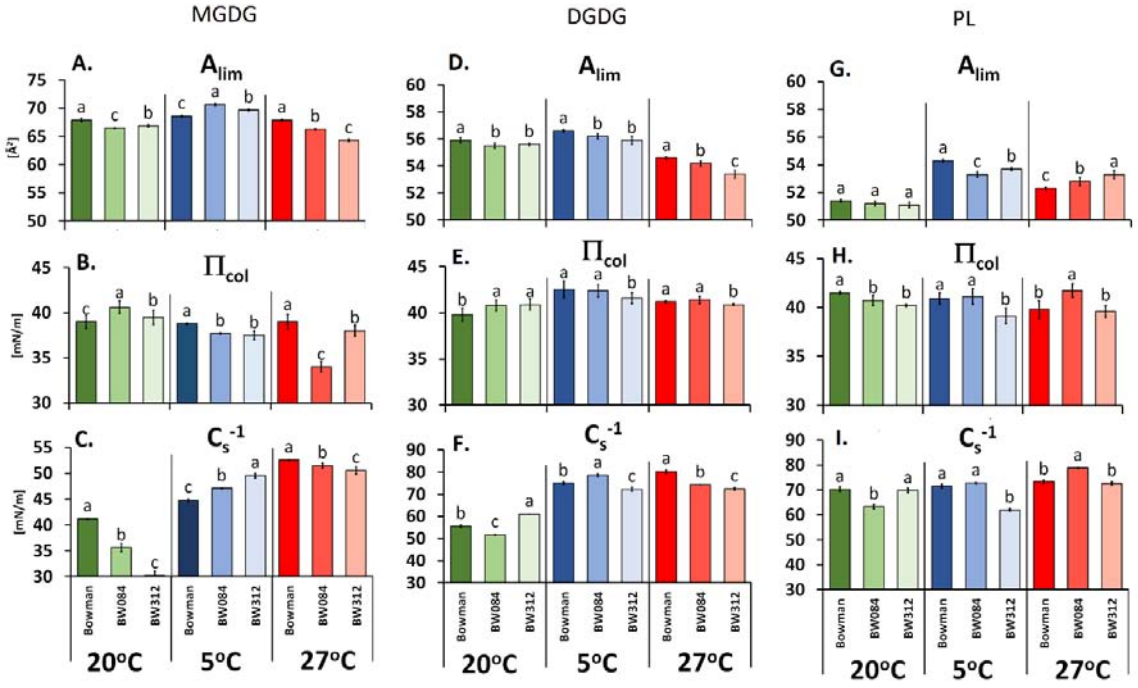

Figure 3. Physicochemical parameters: the limiting area per molecule (A_{lim} [Å^2]), collapse pressure (π_{coll} [mN/m]) and compression modulus (C_s^{-1} [mN/m]) of the monolayers that were prepared from the galactolipids—MGDG and DGDG (A–F) and phospholipids—PL (G–I) obtained from the leaves of the barley WT Bowman, mutant BW084, and mutant BW312 cultured at 20 °C and then acclimated at 5 °C and 27 °C. Any significant differences between WT Bowman and the mutants ($p \leq 0.05$) for each temperature are indicated by different letters.

3.2. Fatty Acid Composition of the Membrane Lipids from the Barley That Had Been Acclimated at 5 °C and the Physicochemical Properties of the Lipid Monolayers

Similar to 20 °C, the decrease in the 18:3 content in the FA pool of PL at 5 °C was accompanied by an increase in the 18:2 content in both mutants when compared with the WT Bowman (Table 1). At 5 °C, the content of 16:0 acid was higher in both mutants than in the WT. In the MGDG and DGDG fractions, the highest percentage was 18:3 (more than 80% of the FA pool).

At the low temperature, the 18:3/18:2 ratio was higher in the mutants for the MGDG but lower for the DGDG and PL fractions compared to the WT (Figure 1A,C,E). The opposite effect was observed for the U/S (for DGDG). The value A_{lim} at 5 °C was higher in the mutants (MGDG fraction) and was accompanied by a higher C_s and lower π_{coll} values (Figure S1A–C). In the DGDG and PL fractions, the pattern of the changes of those three parameters was, with some exceptions, a little similar to that recorded at 20 °C. Especially for BW312, all of the values of A_{lim}, π_{coll}, and C_s^{-1} were at 5 °C always lower than in the WT. Figure S1A-I additionally illustrates the direction of the changes of the A_{lim}, π_{coll}, and C_s^{-1} parameters in the mutants and the WT Bowman at 5 °C in relation to 20 °C (expressed as 100%).

4. Discussion

Our work, which was devoted to mutants with the impaired biosynthesis and signaling of BR, permitted the results that have been obtained for fatty acids (especially 18:3) in the experiments in which BRs were applied exogenously to be partly confirmed [10,12,13]. Both the BR-deficiency and BR-signaling disorders in the mutants were reflected in the modification of the fatty acid composition/proportion in the individual lipid fractions. Earlier, Janeczko et al. [10] reported that the exogenous application of BR into a culture of oilseed rape calli increased the molar percentage of 18:3 in their PL fraction at 20 °C. In the current work, the mutants with the defects of BR biosynthesis and signaling, growing at 20 °C had, as expected, a lower molar percentage of 18:3 in PL fraction.

The same regularity was observed at 5 °C. The mutant BW084 had a lower molar percentage of 18:3 (in PL fraction), while BR-sprayed mango fruits, which subsequently were grown in the cold for 14–21 days, were characterized by an increased molar percentage of 18:3 in the polar lipid fraction [12]. Thus, the effects of an increasing BR level in tissues through its exogenous administration were, as expected, exactly the opposite of the effects that were caused by the BR deficit in the mutant. However, the proper functioning of the BR receptors was also important because, despite the increased BR level that is characteristic for the mutant BW312 [16,22], abnormal BR signaling often had a similar or the same effect as a BR deficiency. Although it appears that BR regulates the biosynthesis of the FA or their transport/incorporation into the cell membranes, this issue will require a more detailed investigation and explanation of the mechanism. The relationship between BR and FA was, of course, modified by the plant growth/acclimation temperature. Highly unambiguous results were obtained for the PL fraction (more characteristic for the plasma membrane) and 18:3 present within about 50% in the FA pool. Consistently, in both tested mutants, BR-biosynthesis or BR-signaling disorders were associated with a decrease in the content of this fatty acid in the PL fraction at 5 °C and 20 °C, but an increase in its content at 27 °C. It is assumed that for the better "adaptation" of membranes to temperature, the percentage of unsaturated FA should increase at 5 °C and decrease at 27 °C [26]. This is considered to be one of the steps in plant acclimation. Therefore, in the case of the PL fraction, the analyzed mutants had little less favorable parameters than the WT. The opposite situation we had observed in the galactolipid fractions. At 5 °C, the mutants had a higher percentage of 18:3 than the WT, while at 27 °C, the mutants were characterized by a lower content of 18:3, especially in the case of MGDG. Since galactolipids are more typical for the chloroplast membranes and it is known that the MGDG fraction constitutes about 55% of the thylakoid membrane lipids [27], this may partly explain why the mutants maintained better efficiency of photosystem II (PSII) at 5 °C and 27 °C when compared to the WT [16]. Moreover, studies on *Arabidopsis thaliana* mutants that carry mutations in the genes encoding the MGD-synthase also confirm the contribution of MGDG fraction to photosynthesis efficiency. These mutations led to a significant reduction in the ability of the mutant plants to conduct photosynthesis [28]. In our experiment more 18:3 (more fluidic thylakoid membranes) at low temperature and less 18:3 (less fluidic thylakoid membranes) at a higher temperature may provide a better environment to membrane-located processes at these temperatures, and PSII is located in the thylakoid membranes. In fact, as mentioned in the introduction, the accumulation of 18:3 is beneficial for the thermostability of the photosynthetic apparatus at higher temperatures [2]. Our earlier work [16] showed that mutants at 5 °C and 27 °C were characterized by higher (than WT) values of $P.I._{ABS}$ informing about general PSII efficiency. In detail, mutants maintained comparable to WT plants energy transfer to electron transport chain (ETo/CSm) but it was accompanied by lower requirements of absorbed energy (ABS/CSm) and connected to lower energy loss as a heat (DIo/CSm).

According to a detailed analysis of the physicochemical properties of the lipid fractions, it seems that a greater amount of modifications between the tested plants were associated with the galactolipids rather than the PL fraction, and the MGDG fraction in particular. These results confirm the importance of the chloroplast structure in the plant response to the temperature changes. Thus, the physicochemical galactolipid modifications could be an important step, through FA composition changes, in the thylakoid membrane "adaptations" (stiffness/fluidity) that enable the proper functioning of photosystems at 5 °C and 27 °C. The value of the limiting area per molecule (A_{lim}) usually increases at 5 °C compared to 20 °C (which was also visible in the lipid fractions of genotypes analyzed in our study (Figure 3A,D,G) and provides information about any increase in membrane fluidity [29]. It is worth noting that, in the main lipid fraction of thylakoids (MGDG), the increase in A_{lim}—indicating the increase in membrane fluidity at 5 °C—was greater in the mutants than in the WT Bowman (Figure 3A). The opposite effects, a decrease in A_{lim} and the fluidity of monolayers were caused by an elevated temperature up to 27 °C (Figure 3A), and once again, the effect was stronger in the mutants than in the reference WT cultivar and concerned not only MGDG but the DGDG fraction as well. In both cases, 5 °C and 27 °C, the A_{lim} values corresponded well with a higher molar percentage of 18:3 in the MGDG fraction as well as a

higher ratio of 18:3/18:2 and U/S. This correlation is consistent with expectations because linolenic acid, which contains three double bonds in the *cis* configuration, has the greatest impact on increasing the distance between the lipid hydrocarbon chains [30]. It is worth noting that in the MGDG fraction, present mainly in thylakoids, the higher values of membrane fluidity in the mutants at 5 °C and lower at 27 °C, compared to the WT Bowman, could be one of the reasons for the higher efficiency of photosystem II that was observed in the mutants [16]. According to Escribá et al. [31], even small changes in the lipid compositions can affect the physicochemical properties of the membrane, such as its fluidity and, as a result, affect the biochemical function of the signaling and transport proteins that are located in the membrane.

As mentioned, BR seems to be one of the hormones that regulate the biosynthesis of the main fatty acid—18:3 and/or its incorporation into the membranes. Such regulation may influence various physiological processes that are related to the membranes (such as the light reactions of photosynthesis), however, the question arises as to what significance this has for the frost tolerance or high-temperature tolerance of the whole plant that is acquired as a result of acclimation. Our work [16], showed that despite the metabolic disorders that differentiated the mutants from the WT Bowman, the mutants (after acclimation at 27 °C) had a higher tolerance to temperatures around 40 °C than the WT. In contrast, their frost tolerance (measured after acclimation at 5 °C) was lower than in WT Bowman. In our other studies [17,18], we were trying to explain this phenomenon by analyzing the changes in important membrane proteins. In the current study, we attempted to explain it by analyzing the physicochemical properties of the lipid membranes from plants that had been acclimated at 5 °C (thus hardened to frost) or acclimated at 27 °C (thus more tolerant to a much higher temperature).

The observed changes in membrane saturation, which were characterized by the ratio of the most common fatty acids that were present in the plant membranes, i.e., 18:3/18:2, were usually accompanied by changes in the physicochemical parameters in the model membrane system that had been obtained from the lipid fractions. The clearest correlation was observed in the monolayers of the lipids from plants acclimated at 5 °C. In the analyzed mutants, the parameters that were calculated for the monolayers showed that a higher unsaturation (18:3/18:2 and U/S, MGDG fraction, Figure 1A,B) was associated with a higher value of A_{lim}—surface area per single lipid molecule (Figure 3A)—thereby illustrating a higher degree of membrane fluidity. Lower unsaturation (18:3/18:2 and U/S, PL fraction, Figure 1E,F) was associated with a decrease in the surface area per single lipid molecule (A_{lim}, Figure 3G). While the lack of this regularity was observed in some cases, it can be explained by the fact that A_{lim} is also affected by other factors, such as the charges that are localized on the polar part of lipids. Moreover, as was mentioned above, an increase in the surface area per single lipid molecule (A_{lim}) usually means a higher degree of the fluidity of the monolayer while a decrease of A_{lim} is connected to a lower degree of fluidity. It is believed that higher membrane fluidity is more beneficial for better frost tolerance [32,33]. As the analyzed mutants had a lower frost tolerance than the WT Bowman after acclimation at 5 °C [16], the obtained results may at least partly explain the reason for this. Only the monolayers of MGDG had higher A_{lim} values, and consequently a higher degree of fluidity, whereas the DGDG or PL monolayers which mainly build the plasma membrane did not. Based on this model study, it can be suspected that the natural cell membranes of the mutants also have a lower degree of fluidity than the WT Bowman membranes at 5 °C and that this could be one of the factors that influence the higher frost susceptibility of the mutants in comparison with the WT Bowman [16]. Interestingly, more membrane injuries (measured as electrolyte leakage) were reported by Qu et al. [34] and Eremina et al. [35] in the *Arabidopsis* BR-signaling mutants that had been exposed to temperatures of 0 °C and below, which also confirms the connection of BR to the membrane "adaptation" to this stress.

Moreover, the mutants acclimated at 27 °C were less susceptible to heat stress (about 40 °C) than the WT [16]. In both mutants, A_{lim} for the MGDG and DGDG monolayers reached a lower value, than in the WT Bowman, which indicates a lower degree of fluidity. This feature is more desirable as an "adaptation" to high-temperature stress. It is worth mentioning that the mutants had lower membrane

injuries after high-temperature exposure (estimated based on electrolyte leakage) than the WT [16]. To conclude, the changes leading to a lower degree of fluidity that were observed in the membranes of the analyzed mutants could be part of mechanisms that are associated with the improved tolerance of these mutants to the heat stress.

An analysis of the relationships between the other physicochemical parameters revealed that the pressure at which the monolayer collapses (π_{coll}) and the compression modulus (Cs^{-1}) in mutants were also changed when compared to the WT Bowman and were dependent on the temperature of plant growth/acclimation (Figure 3B,C,E,F,H,I and Figure S1A–I). The values of these parameters provide additional information on the stability and flexibility of the monolayers as a result of the strength of the interactions that occur between the saturated and unsaturated FAs [29,30]. The increase in the π_{coll} value (the value of the surface pressure at which a layer collapses) may result from better geometric alignment of the lipid particles (usually for saturated acids), but it may also be modified by the electrostatic interactions between polar lipid parts. The value of this parameter was most often lower after acclimation at 5 °C and 27 °C for the mutant with BR-signaling disorders (for all of the fractions) and in the galactolipid fraction MGDG for the BR-biosynthesis mutant.

All of the changes that occur in the physicochemical and structural state of membranes (as a result of modification in the lipid composition for membrane acclimation to lower/higher temperatures) can also influence the possibility of the interactions and locations of various compounds (sterols, steroid hormones, etc.) in the membranes. The fact that the mutants, compared to the WT, had altered physicochemical and structural parameters characterizing the membranes shows how wide and multidirectional the impact of brassinosteroids can be on the membrane-dependent physiological processes.

Sometimes the directions of changes in the parameters studied were different in the mutant with BR deficit in comparison to a mutant with the BR-signaling disorder. A possible explanation can be that the BR-deficient mutant, however, produces low amounts of BR which still can interact with the BR receptor to induce a physiological responses to BR (i.e. connected to lipid biosynthesis). In the case of the mutant with BR-signaling disorder, despite BR overproduction (resulting from the feedback mechanism), signal perception is disturbed and the physiological response is also disturbed.

5. Conclusions

Brassinosteroids seem to be one of the players that regulate fatty acid biosynthesis or their transport/incorporation into the cell membranes. The BR-biosynthesis and BR-signaling mutants of barley were characterized by a temperature-dependent altered molar percentage of fatty acids (from 14:0 to 20:1) in their galactolipid and phospholipid fractions, which suggests that BR play a role in lipid management, although the mechanism of this regulation requires further studies. The fact that the BR-biosynthesis and BR-signaling mutants had altered physicochemical parameters of their membranes, compared to the WT, shows that BR may have a multidirectional impact on the membrane-dependent physiological processes.

Supplementary Materials: The following can be found at http://www.mdpi.com/2073-4409/9/5/1125/s1. **Figure S1**. Changes in the values of the A_{lim}, π_{coll} and C_s^{-1} parameters for the barley brassinosteroid mutants (BW084 and BW312) and the WT Bowman growing at 5 °C and 27 °C in relation to 20 °C (when 20 °C is expressed as 100%). (**A**, **B**, **C**) Data calculated for monolayers of monogalactosyldiacylglycerols (MGDG); (**D**, **E**, **F**) Data calculated for monolayers of digalactosyldiacylglycerols (DGDG); (**G**, **H**, **I**) Data calculated for monolayers of phospholipids (PL).

Author Contributions: A.J. is the coordinator of the project 2015/17/B/NZ9/01695 and designed the entire experimental model. I.S. isolated the lipids; M.F. supervised the lipid isolation; E.R.-S. with help of I.S. performed the Langmuir trough measurements; M.F. and E.R.-S. interpreted the results of the Langmuir trough measurements; D.G. provided the seeds of the mutants and WT Bowman and characterized plants genetically; I.S. and E.R.-S. performed the statistical analysis and prepared the table/figures under the supervision of A.J.; GC analysis of FA composition was made by technical staff. A.J., M.F., and E.R.-S. wrote the manuscript; all of the authors read and approved the manuscript. All authors have read and agreed to the published version of the manuscript.

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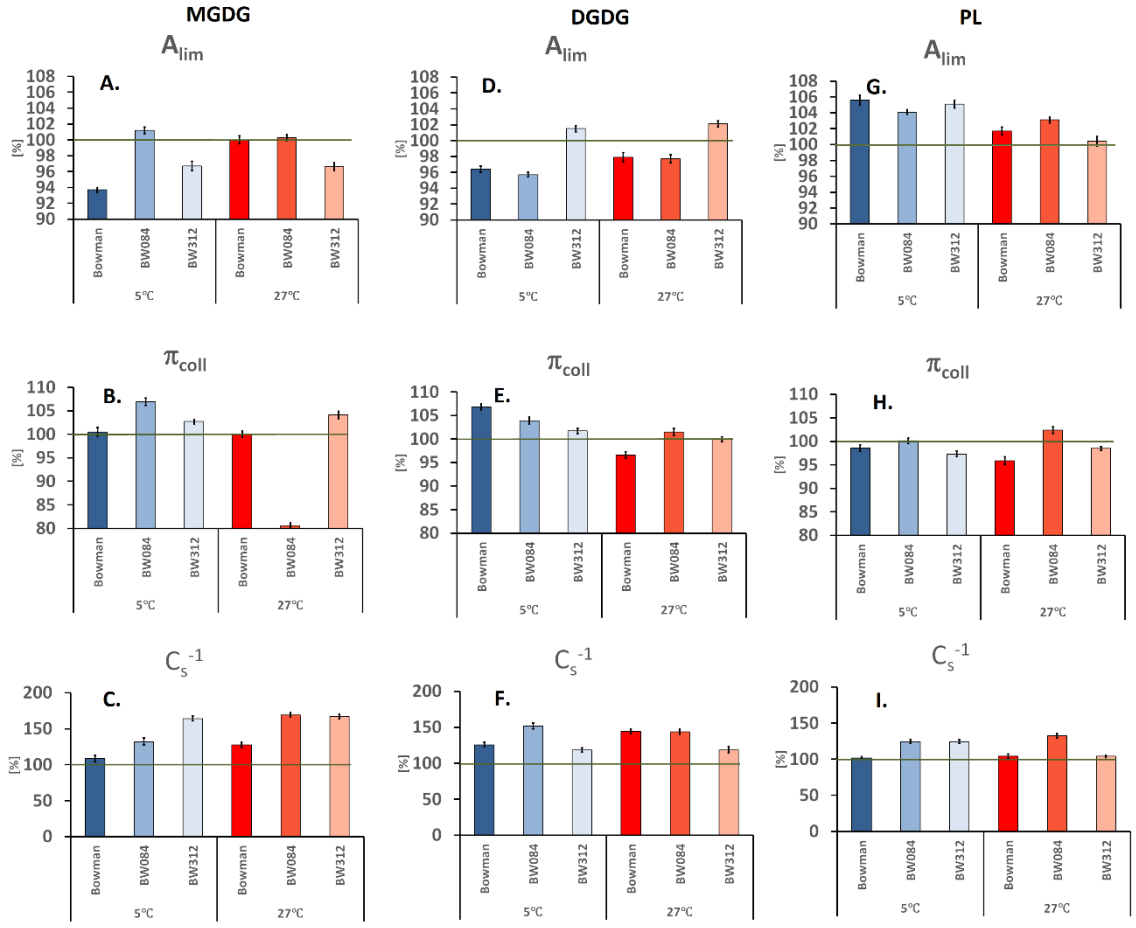
Figure S1. Changes in the values of the A_{lim}, π_{coll} and C_s^{-1} parameters for the barley brassinosteroid mutants (BW084 and BW312) and the WT Bowman growing at 5°C and 27°C in relation to 20 °C (when 20°C is expressed as 100%). (**A**, **B**, **C**) Data calculated for monolayers of monogalactosyldiacylglycerols (MGDG); (**D**, **E**, **F**) Data calculated for monolayers of digalactosyldiacylglycerols (DGDG); (**G**, **H**, **I**) Data calculated for monolayers of phospholipids (PL).



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Article

Molecular Dynamics of Chloroplast Membranes Isolated from Wild-Type Barley and a Brassinosteroid-Deficient Mutant Acclimated to Low and High Temperatures

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Abstract: Plants have developed various acclimation strategies in order to counteract the negative effects of abiotic stresses (including temperature stress), and biological membranes are important elements in these strategies. Brassinosteroids (BR) are plant steroid hormones that regulate plant growth and development and modulate their reaction against many environmental stresses including temperature stress, but their role in modifying the properties of the biological membrane is poorly known. In this paper, we characterise the molecular dynamics of chloroplast membranes that had been isolated from wild-type and a BR-deficient barley mutant that had been acclimated to low and high temperatures in order to enrich the knowledge about the role of BR as regulators of the dynamics of the photosynthetic membranes. The molecular dynamics of the membranes was investigated using electron paramagnetic resonance (EPR) spectroscopy in both a hydrophilic and hydrophobic area of the membranes. The content of BR was determined, and other important membrane components that affect their molecular dynamics such as chlorophylls, carotenoids and fatty acids in these membranes were also determined. The chloroplast membranes of the BR-mutant had a higher degree of rigidification than the membranes of the wild type. In the hydrophilic area, the most visible differences were observed in plants that had been grown at 20 °C, whereas in the hydrophobic core, they were visible at both 20 and 5 °C. There were no differences in the molecular dynamics of the studied membranes in the chloroplast membranes that had been isolated from plants that had been grown at 27 °C. The role of BR in regulating the molecular dynamics of the photosynthetic membranes will be discussed against the background of an analysis of the photosynthetic pigments and fatty acid composition in the chloroplasts.

Keywords: brassinosteroids; chloroplast membranes; temperature stress; barley; molecular dynamics; EPR

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1. Introduction

Among the abiotic stresses, temperature stress is a global problem that mainly causes a decrease in the yield of most crop plants in agriculture and horticulture [1,2]. Some cereal species are sensitive to cold (maize), while other species (winter wheat) are sensitive to low temperatures, especially when there is poor snow cover on fields, which causes frost injuries that lower the yield. On the other hand, high temperatures may be particularly

dangerous to plants when there is a water deficit in the summer vegetation season, which has increasingly happened in recent years due to climate changes. Periods of cold (e.g., 5 °C) harden plants (e.g., winter cereals) and enable them to better survive frost during winter. In turn, exposure to warmth may increase the ability of plants to survive high-temperature stress (e.g., 40 °C). Among the plant strategies for counteracting the negative effects of abiotic stresses (including extreme temperatures) [3], biological membranes are an important element. According to Horvath et al. [4], membranes can be considered to be "thermal sensors", and are the primary cause of many other metabolic changes within cells and their organelles. During acclimation to changing temperature conditions, the alterations in the fluidity of the biological membranes are connected with changes in the proportion of unsaturated fatty acids, which results in a rearrangement of the membrane structure and its properties. The membrane properties can also be altered by incorporating various components into their structure, such as tocopherols, sterols, steroids [5], or carotenoids [6].

Many of the physiological phenomena that occur in cells could be due to the multidirectional effect of hormones on the plant metabolism, which is also the basis for the adaptability of organisms in order to function in changing environmental conditions. One plant hormone group is the brassinosteroids (BR), which are responsible for regulating both plant growth and development as well as their response to stress. BR belong to the steroid phytohormones that were first isolated from oilseed rape pollen in the 1970s [7,8]. To date, more than 70 BR are known, including brassinolide, 24-epibrassinolide, 28-homocastasterone, etc. Bajguz and Hayat [9] showed that in plants that had been exposed to temperature extremes, BR counteract the inhibition of growth, reduce the biomass losses and increase the plant survival rate. This is the result of the multidirectional activity of BR at the cellular and molecular level [10]. The function of BR in plants is still being explained and relatively little is known about their impact on the plant membrane properties. Recently, we found that BR may be involved in regulating the accumulation of the proteins that are incorporated in the cell membranes of barley plants (heat shock proteins, proton pumps and aquaporins) [11,12]. Our most recent studies revealed that barley mutants with a BR deficiency or a BR insensitivity were characterised by a different fatty acid composition in the cell membranes, which resulted in altered membrane physicochemical properties that could have an impact on the membrane-dependent physiological processes [13].

Studies of the compounds that are incorporated into the membranes have made a huge contribution to understanding the processes that occur in the biological membrane. In the case of thylakoids that had been obtained from the diatom *Phaeodactylum tricornutum*, studies of the diadinoxanthin cycle have provided information about the molecular dynamics of the thylakoid membrane and about the influence of the diadinoxanthin cycle pigment on this effect [14]. Strzałka et al. [15] investigated changes in the physical properties (general membrane lipid fluidity, dynamic orientational order parameter) of the vacuolar membranes compared to the protein body membrane in germinating pumpkin (*Cucurbita* sp.) seeds. Similar studies were performed on thylakoid membranes that had been isolated from barley during leaf senescence [16]. These measurements are taken using electron paramagnetic resonance (EPR) spectroscopy, which permits the direct study of the systems (including biological systems) that contain unpaired electrons. In biological systems, compounds that contain unpaired electrons are present in the products of biochemical transformations, e.g., photosynthesis. EPR can also be used to study systems that do not contain unpaired electrons naturally. In this case, artificial paramagnetic centres (spin labels) that contain a nitroxyl group, e.g., 5-doxyl stearic acid (5-SASL) or 16-doxyl stearic acid (16-SASL) are incorporated into the studied object. In the case of the biological membranes, the EPR spectra can provide information, among others, about the degree of order (S) and the rotational correlation times (τ_B and τ_C) of the molecules that contain a spin label, which is helpful in determining the molecular dynamics of the membranes [14,15].

In the presented study, we focused on the chloroplast membranes, which are crucial elements of photosynthetic machinery, and affect the level and quality of plant yields [17].

Bearing in mind the role of membranes as the first cellular line to react to changing temperatures (and also the importance of chloroplasts for the crucial plant process of photosynthesis), the aim of the study was to broaden the knowledge about the changes in the molecular dynamics of the chloroplast membranes during the acclimation of a plant to extreme temperatures and the influence of BR on these properties. By studying wild-type barley plants and a BR-deficient mutant, we wanted to answer the following detailed questions: (1) is the content of BR in the chloroplast membranes different in the wild-type plants than in a BR-biosynthesis mutant grown at an optimal growth temperature, i.e., 20 °C? (2) How does the temperature of acclimation (5 °C and 27 °C) modify the accumulation of BR in the chloroplast membranes of the wild type and mutant? (3) Are there differences in the molecular dynamics of the chloroplast membranes from wild-type barley and a mutant that is cultured at 20 °C? (4) How does a lower (5 °C) or higher (27 °C) temperature during plant growth modify the molecular dynamics of the chloroplast membranes? Finally, the role of BR as regulators of the molecular dynamics of the photosynthetic membranes will be discussed in light of the chloroplast content of the photosynthetic pigments and fatty acid composition in the BR-deficient barley mutant and in the respective wild type.

2. Material and Methods

2.1. Plant Material, Experimental Design and Sampling

The objects of study were the barley cultivar Delisa (wild type) and its semi-dwarf mutant (522DK), which has disturbances in the BR biosynthesis. The mutant was described in the work by Gruszka et al. [18]. The mutant was obtained via the chemical mutagenesis of the cultivar Delisa. The 522DK mutant has a G > A substitution at position 1130 of the *HvDWARF* transcript [18] and at position 3031 in the gene sequence [19], which causes the change of the valine-341 residue into isoleucine. The substituted valine-341 is a highly conserved residue that occurs in similar position in the homologous DWARF polypeptides from barley, *Arabidopsis*, rice and tomato. Since the *HvDWARF* gene encodes the brassinosteroid C6-oxidase, which is an important factor in the BR-biosynthesis (it catalyses the production of castasterone), the mutant 522DK has a lowered content of castasterone [19] and it also has a decreased level of other BR [20].

The seeds were germinated for three days at 24 °C in the dark. After germination, the seedlings were transplanted into pots (40 cm × 15 cm × 15 cm) (about 40 seedlings per pot) containing soil ('Eco-ziem universal soil' (Jurków, Poland), soil from the cultivation plots at the University of Agriculture (Cracow, Poland), sand and 'Substral Osmocote—a universal substrate' (Scotts Poland sp. z o.o., Warsaw, Poland) at a ratio of 8:4:2:4, respectively), after which they were grown for 18 days at 20 °C (16 h photoperiod). Then, the plants were divided into two groups. Each group consisted of three pots with plants of the wild-type cultivar and three pots with plants of the mutant. In the first group, the temperature was lowered to 5 °C (21 days, 8 h photoperiod) and in the second group, it was increased to 27 °C (7 days, 16 h photoperiod). Light intensity in the growth chambers was 170 μmol m^{-2} s^{-1} and was provided by HPS Philips SON-T AGRO 400 W lamps. Because the current studies are a continuation of our earlier works, the durations of the acclimation and the photoperiods were identical to those that were described in our earlier articles [11,12,20–22]. According to literature data, both cold acclimation and acclimation to higher temperatures enable plants to acquire a greater tolerance to frost or heat, respectively [23–25]. Our previous studies [20] showed that after acclimation at 5 °C, both the wild type and mutant were characterised by a comparable tolerance to frost (−8 °C) while the mutant 522DK had an even better frost tolerance at −6 °C compared to the wild-type Delisa. After acclimation at 27 °C, the mutant had a much better heat tolerance (tested at 38 and 45 °C) compared to the wild type.

Before cutting off the plants, leaf greenness was measured using a chlorophyll meter for the plants that had been grown at 20 °C and then in the plants that had been acclimated at 5 °C and 27 °C. The plants grown at 20 °C had four leaves, while the acclimated plants had four well-developed leaves and sometimes a young fifth leaf. The samples to isolate

the chloroplasts were collected from the wild-type and mutant plants that had been grown at 20 °C and from the plants that had been acclimated at 5 °C and 27 °C. It was possible to isolate about 1.5 g (0.3 g per sample) of chloroplasts from 20 to 25 plants that weighed around 40 g. Three samples containing 0.3 g of chloroplasts (each sample from plants from different pot) were collected to analyse the BR. For the other analyses, each sample containing 0.3 g chloroplasts were first suspended in a 1 mL chloroplast isolation buffer (CIB) buffer (see *Isolation of Chloroplasts* section) and these suspensions were used to further analyse the content of proteins, photosynthetic pigments, and the fatty acid composition. The proteins were also analysed in order to standardise the samples to be used for the EPR studies.

2.2. Leaf Greenness Measurement

Leaf greenness, which corresponds to the leaf chlorophyll concentration, was measured using a chlorophyll meter (SPAD 502; Konica Minolta, Tokyo, Japan, (SPAD—Soil Plant Analysis Development)) and the values of greenness are expressed in arbitrary SPAD units. The measurement was taken in the middle part of the fourth fully developed leaf in three technical replications per each leaf and the average value was calculated for each leaf. The leaves from nine plants were measured—three plants from each pot.

2.3. Isolation of Chloroplasts from Barley Leaves

The chloroplasts were isolated based on a modified protocol of Block et al. [26] and Filek et al. [27]. About 100 g of the aerial part of the barley (mainly leaves) were homogenised using a Camry CE 4050 blender in 400 mL of a chloroplast isolation buffer (CIB) (pH 7.5) that contained 50 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.33 mM sorbitol. The crude extract was filtered and centrifuged for three minutes at 300× *g* (Hettich zentrifugen Universal 320R, rotor 1494, Tuttlingen, Germany) in order to remove any residues after plant homogenisation. Next, the supernatant was centrifuged for ten minutes at 1200× *g* (Hettich zentrifugen Universal 320R, rotor 1494, Tuttlingen, Germany). The obtained pellet contained isolated chloroplasts. The purity of the isolated chloroplasts was checked under a microscope (Nikon Eclipse E600, Tokyo, Japan) (Figure S1). The entire isolation process was performed in a cold room (4–6 °C). Next, in order to break down the chloroplasts, samples were frozen in liquid nitrogen and thawed in two cycles; this was especially important in EPR studies. Breakdown of chloroplasts was checked under microscope (Nikon Eclipse E600, Tokyo, Japan). Frozen samples were kept at −80 °C until the day of analysis.

2.4. Analysis of the Protein Content in Chloroplasts

The protein concentration was estimated according to Sedmak and Grossberg [28]. Two microlitres of a 10% water solution of Triton X-100 (Sigma-Aldrich, Poznań, Poland) were mixed with 2 µL of the chloroplasts that had been suspended in CIB and 196 µL of a CIB buffer (see *Isolation of chloroplasts* section) and kept for 15 min on ice. Ten microlitres of each sample were placed into separate wells of a plate (96-well polystyrene titration plate with a flat bottom, FL Medical, Torreglia, Italy) and then, 200 µL of a Bradford reagent (BioRad, Munich, Germany) (diluted with water 1:4) was added. After 10 min, the absorbance was recorded (595 nm) using a Synergy^{TM}2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). The measurements were carried out in three replicates. Bovine serum albumin (BSA) (Sigma-Aldrich, Poznań, Poland) was used as the calibration standard. The BSA for spectrophotometric measurements was diluted in the same buffer as the buffer that had been used to isolate the chloroplasts (CIB buffer).

2.5. Analysis of the Fatty Acid Composition in Chloroplasts

The fatty acids (FA) were extracted by homogenising the isolated chloroplasts. Samples of the chloroplast suspension in the CIB buffer (0.15 mL each sample) were suspended in 0.20 mL of toluene and transferred into screw-capped glass test tubes. Subsequently,

1.50 mL of methanol and 0.30 mL of an 8.0% HCl solution were added. The test tubes were vortexed and then incubated at 45 °C overnight in order to turn the extracted fatty acids into fatty acid methyl esters (FAME). After cooling to room temperature, the FAME were extracted by adding 1 mL of hexane and 1 mL of water [29]. The test tubes were vortexed, and then the hexane layers were analysed using gas-liquid chromatography (GC). The n-hexane extracts were analysed chromatographically on an Agilent 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) that was equipped with a flame ionisation detector (FID) and a capillary column. The certified reference material 37 FAME MIX (Supelco, CRM 47885) and an internal standard (biphenyl) were used to identify and quantify the fatty acid profiles. The analyses were conducted using a gas chromatograph equipped with a split/splitless injector. An ionic liquid fused silica capillary column (SLB-IL100, Supelco, 28884-U) (30 m × 0.25 mm ID × 0.2 mm film thickness) with a matrix 1,9-di(3-vinylimidazolium)nonane bis(trifluoromethanesulfonyl)imide phase was operated under the following programmed conditions: 50–240 °C at 3 °C min^{-1} for 30 min (detector and injector temperatures of 240 °C), autosampler injection mode and volume of 0.5 µL and split (10:1) with helium 6.0 as the carrier gas (velocity 40 cm min^{-1}). FAME were identified by comparing them with the standard mixture (Sigma-Aldrich, Poznań, Poland) and their retention time.

2.6. Determining the Carotenoid and Chlorophyll Content in Chloroplasts

Eighty percent aqueous acetone (2.09 mL) was added to the samples of the suspension of chloroplasts in the CIB buffer (0.01 mL each sample). The samples were vortexed and centrifuged (8 min, 12.100× *g*) (Eppendorf Mini-Spin, Hamburg, Germany). The UV-ViS (Jasco870, Easton, MD, USA) measurements were taken at wavelengths of 663.2, 664.8 and 470 nm. The measurements were taken in three replicates. The amounts of chlorophylls and carotenoids were calculated according to following Equations [30]:

$$C_a = 12.25 \cdot A663.2 - 2.79 \cdot A664.8 \quad (1)$$

$$C_b = 21.50 \cdot A664.8 - 5.10 \cdot A663.2 \quad (2)$$

$$C_{a+b} = 7.15 \cdot A663.2 + 18.71 \cdot A664.8 \quad (3)$$

$$C_{x+c} = (1000 \cdot A470 - 1.82 \cdot C_a - 85.02 \cdot C_b)/198 \quad (4)$$

where C_a is the concentration of chlorophyll *a*; C_b is the concentration of chlorophyll *b*; C_{a+b} is the sum of the chlorophyll *a* and *b* concentration; and C_{x+c} is the concentration of carotenoids.

2.7. Analysis of the Brassinosteroids in the Chloroplasts

Samples of the isolated chloroplasts (0.3 g each) were homogenised in 80% methanol and enriched to the internal standard with deuterium-labelled BR (25 pmol/sample, Olchemim s.r.o., Olomouc, Czech Republic). Next, the samples were centrifuged and the supernatant was passed through Discovery DPA-6S columns (Supelco, Bellefonte, PA, USA) and immunoaffinity columns (Laboratory of Growth Regulation, Olomouc, Czech Republic) [31]. The brassinosteroids that had been eluted with cold 100% methanol from the IA columns were dried and resuspended in 40 µL of methanol in order to measure them on a UHPLC using a tandem mass spectrometry (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) [31,32]. The analyses were performed in three repetitions and each repetition included about 0.3 g of the isolated chloroplasts.

2.8. Electron Paramagnetic Resonance (EPR) Measurements

The molecular dynamics of the cell membranes were determined according to the modified protocol described by Strzałka et al. [15] and was monitored using EPR spectroscopy

with spin labels 5-doxyl stearic acid (5-SASL, Santa Cruz Biotechnology, Heidelberg, Germany) and 16-doxylstearic acid (16-SASL, Sigma-Aldrich, Poznań, Poland), which provides information about the molecular dynamics of the region near the polar heads and the membrane interior, respectively. The spin labels were incorporated into the chloroplast membranes at room temperature by vortexing. About 100–300 µL of the chloroplast membranes (corresponding to 30 µg of protein) were vortexed for 20 min with 5 µL of 10 mM spin labels in methanol [15]. To remove any traces of the spin labels in the supernatant, the samples were centrifuged three times at 4500× *g* for two minutes at room temperature. The pellet that was obtained was resuspended in 50 µL of a 10mM CIB buffer (its composition is described in the *Isolation of chloroplasts* section) in order to obtain a final cell membrane concentration yield that corresponded to 30 µg of the protein (the protein content was measured using the Bradford method and no significant differences were observed between wild-type Delisa and the 522DK mutant). The EPR spectra of the spin label as a function of the temperature was recorded using an X band EPR spectrometer (Miniscope, Berlin, Germany) that was equipped with a Temperature Controller (Magnettech, Berlin, Germany) with a temperature range of 0 to 40 °C at intervals of 5 °C. For the plants that had been acclimated at 5 °C, the range of the measurement was between 0 °C and 25 °C and the interval was 2.5 °C. The EPR measurements were performed at microwave powers between 3.2 mW and 10.0 mW with a sweep width of 12–15 mT, a modulation amplitude of 0.1 mT and a microwave frequency of about 9.4 GHz. Two biological replicates with two to three technical replicates were done. The obtained EPR spectra were analysed with MultiPlot 2.0 Software (Magnettech GmBH, Berlin, Germany) (Figure 1).

The parameters that were required to calculate the dynamic orientational order parameter (S) and the rotational correlation times (τ_{2B} and τ_{2C}) are presented in Figure 1. The values of the S parameter were calculated using the following Equation [33]:

$$\text{where } S = 0.5407\,(A'_{||} - A'_{\perp})/a_o \tag{5}$$

$$a_o = (A'_{||} + 2A'_{\perp})/3 \tag{6}$$

The rotational correlation times (τ_{2B} and τ_{2C}) were calculated according to the following Equations [34]:

$$\tau_{2B} = 6.51 \cdot 10^{-10} \cdot \Delta H_0 \cdot ((h_0/h_-)^{\frac{1}{2}} - (h_0/h_+)^{\frac{1}{2}})\ [s] \tag{7}$$

and

$$\tau_{2C} = 6.51 \cdot 10^{-10} \cdot \Delta H_0 \cdot ((h_0/h_-)^{\frac{1}{2}} + (h_0/h_+)^{\frac{1}{2}} - 2)\ [s] \tag{8}$$

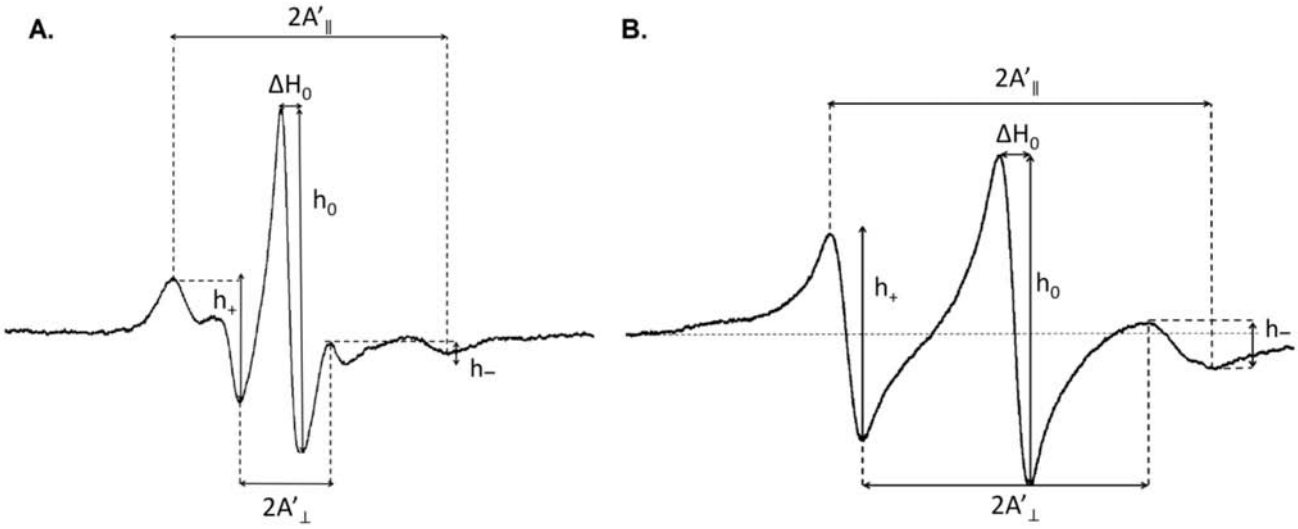

Figure 1. Schematic electron paramagnetic resonance (EPR) spectra of the SASL-5 (**A**) and SASL-16 (**B**) spin labels that had been incorporated into the chloroplast membranes. The spectral parameters that were analysed are indicated ($2A'_{\perp}$ and $2A'_{||}$, ΔH_0, and h_+, h_0 and h_-).

2.9. Statistical Analysis

The statistical analysis (ANOVA, post hoc test) was performed using Statistica 13.1 (StatSoft, Tulsa, OK, USA). For the statistical analysis in the study, when the Delisa and 522DK were compared, Student's t-test was used ($p \leq 0.05$). Values marked with the same letters in Figures 2 and 3 and Table 1 did not differ significantly (the comparisons were performed separately for each temperature of plant growth). Moreover, the accumulations of BR (Figure 3) in the Delisa cultivar and mutant at different temperatures were also compared (Student's t-test, $p \leq 0.05$). The comparisons were performed in pairs (Delisa for 20 °C and 5 °C; mutant for 20 °C and 5 °C; Delisa for 20 °C and 27 °C; mutant for 20 °C and 27 °C) and any significant differences are indicated by an "*". The significant differences in the results for the molecular dynamics (Figure 4) between the wild-type Delisa and the mutant are indicated by an asterisk (*); the comparisons were performed separately for each EPR measurement temperature.

Table 1. Composition of the fatty acids of the chloroplast membranes isolated from the barley wild-type Delisa and its 522DK mutant cultured at 20 °C, 5 °C, and 27 °C. Any significant differences between Delisa and the mutant (Student's t-test, $p \leq 0.05$) for each temperature are indicated by different letters. <LOD—below limit of detection.

Fatty Acids [%mol]	Growth Temperature					
	20 °C		5 °C		27 °C	
	Delisa	522DK	Delisa	522DK	Delisa	522DK
10:0	<LOD	0.79	0.47 a	0.50 a	<LOD	0.35
12:0	4.00 a	3.93 a	4.49 a	4.37 a	4.12 b	4.60 a
14:0	0.28 a	0.25 a	0.23 a	0.19 a	0.29 a	0.21 b
16:0	16.54 b	18.76 a	16.16 a	17.41 a	17.20 a	16.11 a
16:1	6.29 a	4.95 a	4.25 a	4.23 a	6.45 a	3.36 a
18:0	3.53 a	3.24 a	3.37 a	3.47 a	2.96 a	2.59 a
18:1 $^{\Delta 9\ cis}$	1.05 b	1.84 a	0.93 b	1.15 a	1.36 a	1.54 a
18:2 $^{\Delta 6\ cis}$	4.24 a	4.32 a	3.05 a	3.01 a	6.85 b	7.64 a
18:3 (3)	63.87 a	61.85 a	66.45 a	65.33 a	60.43 a	63.28 a
20:1	0.19 a	0.06 a	0.34 a	0.34 a	0.34 a	0.31 a
18:3/18:2	15.09 a	14.38 a	22.29 a	21.70 a	8.84 a	8.29 a
U/S	3.11 a	2.72 b	3.01 a	2.86 a	3.08 a	3.20 a

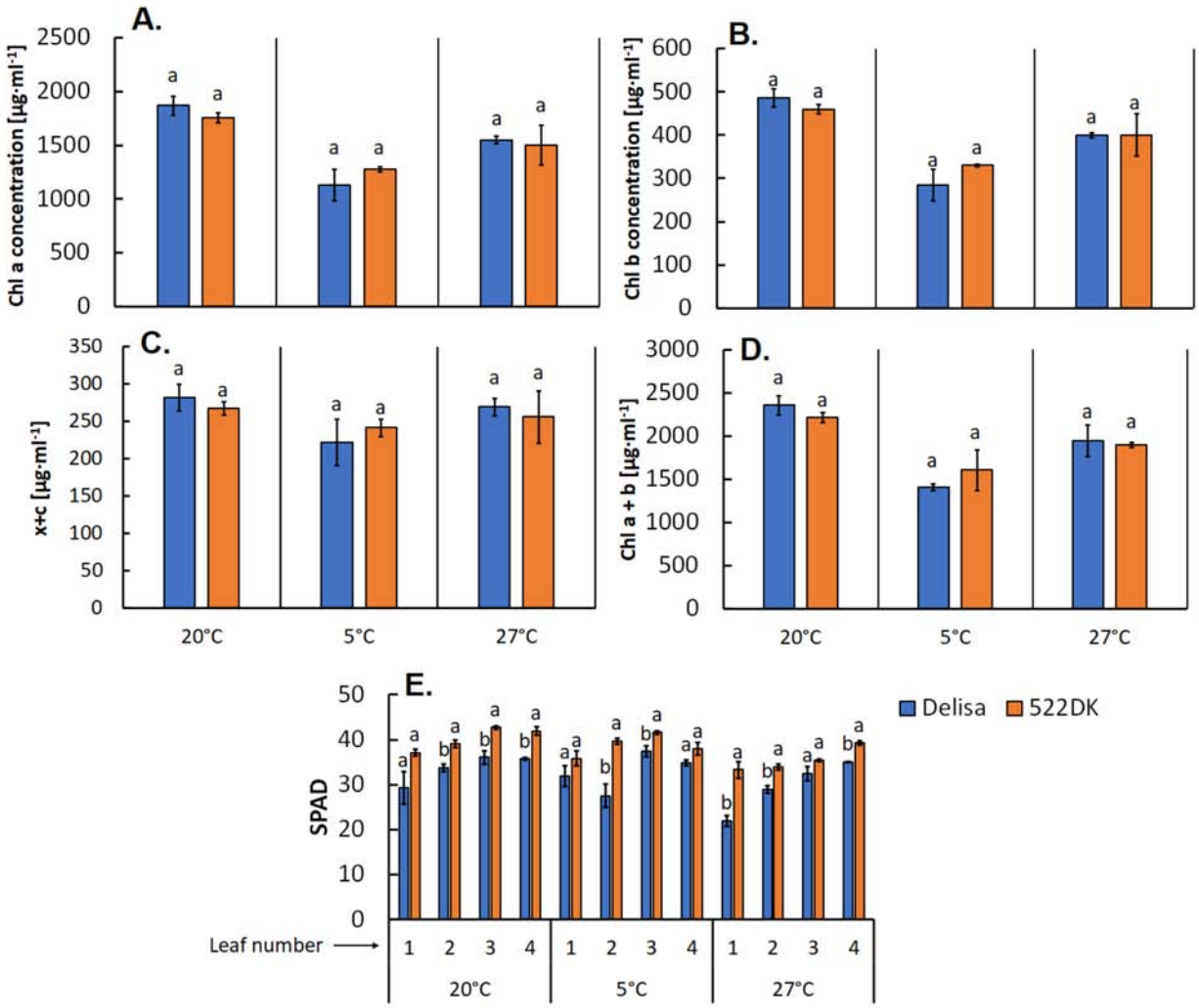

Figure 2. Concentration of chlorophyll (Chl) *a* (**A**), chlorophyll *b* (**B**), xantophylls and carotenoids (x + c) (**C**), and total chlorophyll (*a* + *b*) (**D**) in a suspension of chloroplasts from the barley wild-type Delisa and its 522DK mutant. (**E**) The greenness intensity [SPAD units] of 1st–4th leaf of Delisa and its mutant 522DK. Plants had been cultivated at 20 °C and acclimated at 5 °C and 27 °C. Mean values (±SE) marked with the same letters (separately for plant growth temperature) did not significantly differ according to Student's *t*-test ($p \leq 0.05$).

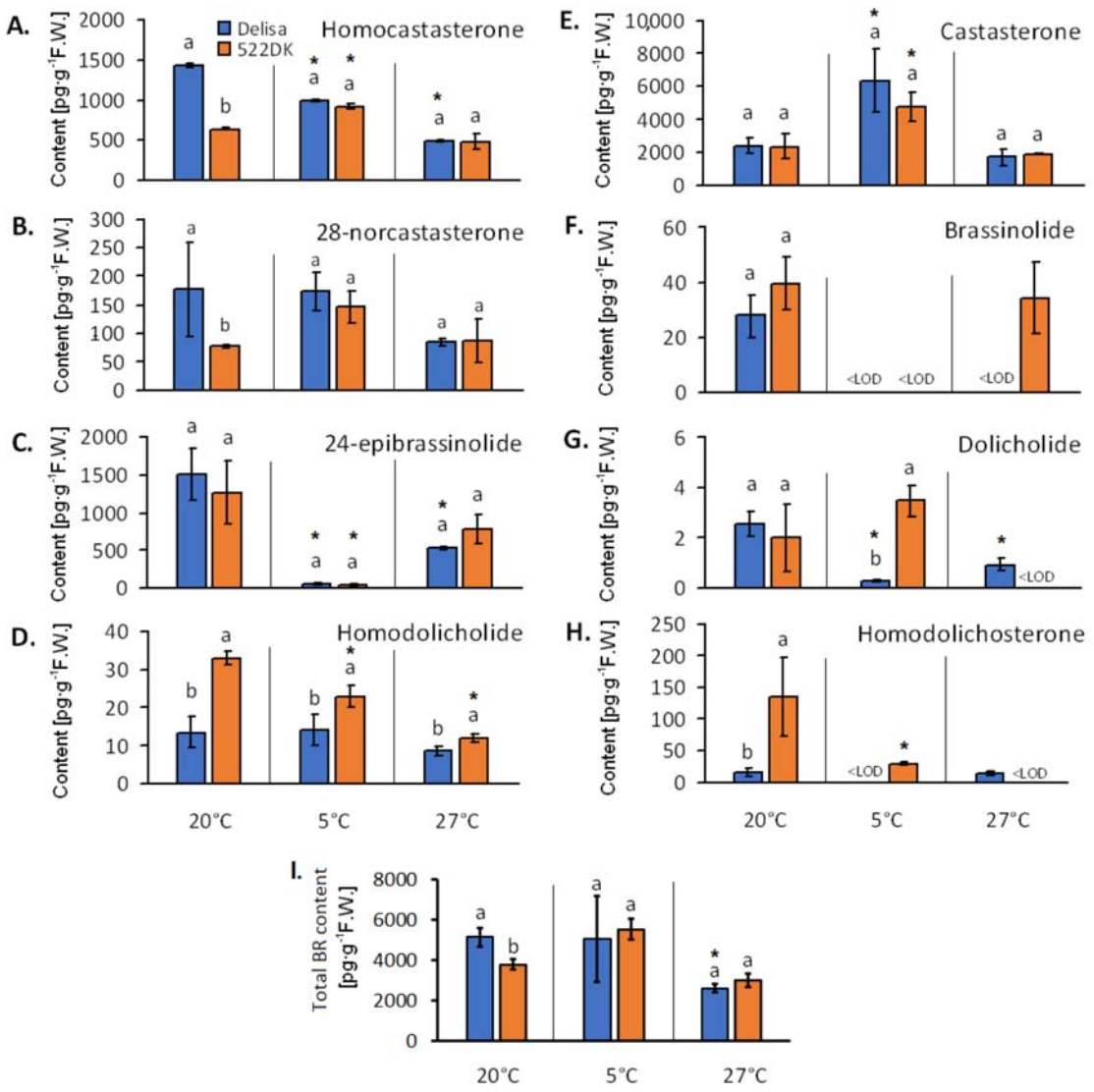

Figure 3. Content of brassinosteroids in the chloroplasts of the barley wild-type Delisa and the BR-deficient mutant 522DK that had been grown at 20 °C and acclimated at 5 °C or 27 °C. (**A**) Homocastasterone, (**B**) 28-norcastasterone, (**C**) 24–epibrassinolide, (**D**) Homodolicholide, (**E**) Castasterone, (**F**) Brassinolide, (**G**) Dolicholide, (**H**) Homodolichosterone, (**I**) Total BR content. <LOD–below detection limit. Mean values (±SD) marked with the same letters are not significantly different according to Student's t-test ($p \leq 0.05$) (separately for each growth temperature). Additionally, in order to show the impact of temperature on the BR level in Delisa and mutant, the comparisons (Student's *t*-test, $p \leq 0.05$) were performed in pairs (Delisa at 20 °C and 5 °C; mutant at 20 °C and 5 °C; Delisa at 20 °C and 27 °C; mutant at 20 °C and 27 °C) and any significant differences are indicated by an "*".

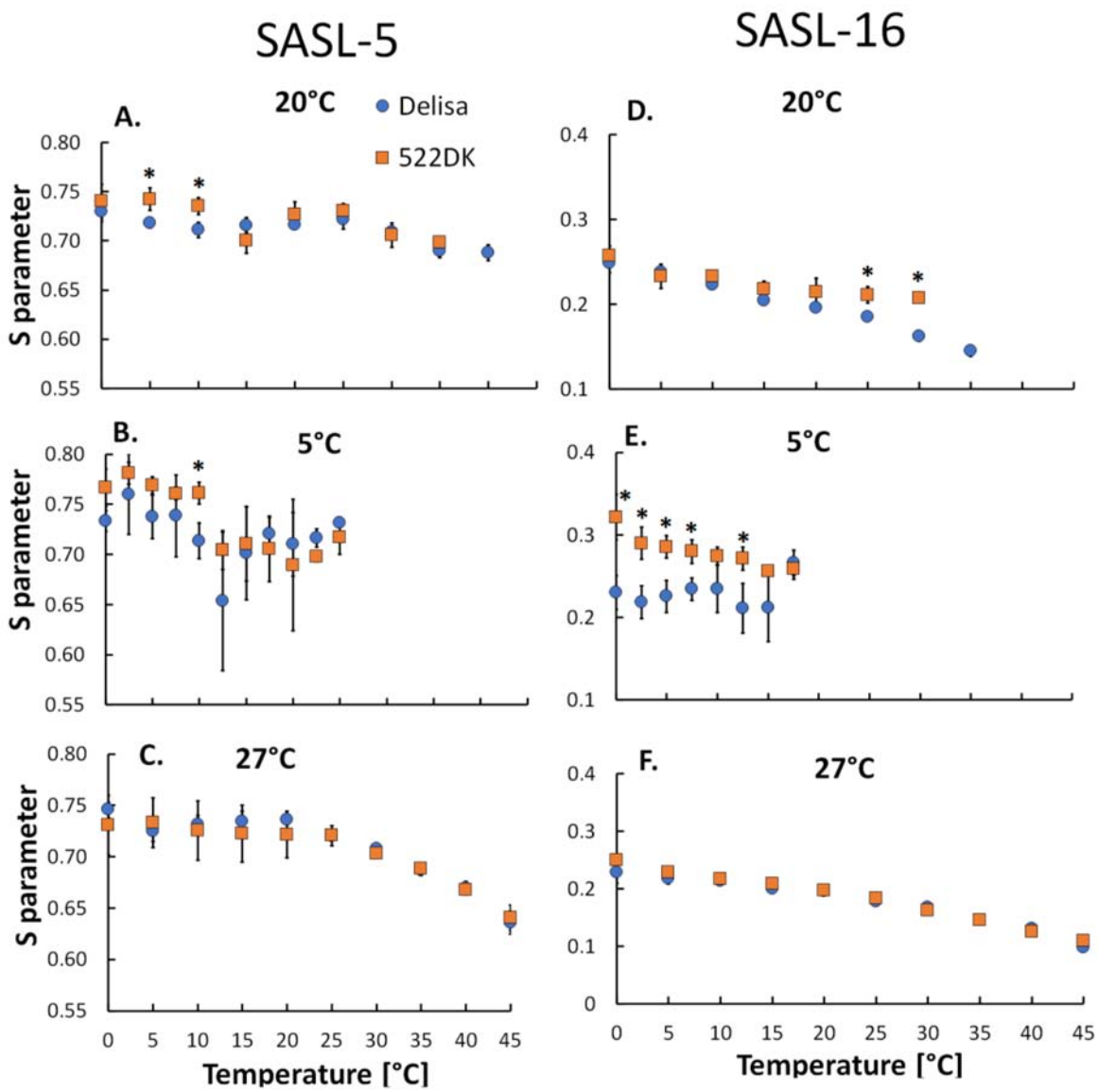

Figure 4. Dependence of the dynamic orientational order parameter S that was calculated for the chloroplast membranes that had been isolated from the barley wild-type Delisa and its mutant 522DK with incorporated SASL-5 (**A**–**C**) and SASL-16 (**D**–**F**) on temperature. Plants had been cultivated at 20 °C and acclimated at 5 °C and 27 °C. Average data are given ± SE and significant differences between the wild type and mutant are indicated with an asterisk (*); comparisons were made separately for each EPR measurement temperature.

3. Results and Discussion

3.1. Characteristic of Selected Chemical Components of the Chloroplast Membranes Isolated from Barley Grown at 20 °C, 5 °C, and 27 °C

3.1.1. Fatty Acid Composition of the Chloroplast Membranes

Only a few FA had differences in content between the wild-type Delisa and its 522DK mutant and even when they were statistically significant, they were not huge. Those that were most abundant in the chloroplast membranes were 18:3 (about 60%) and 16:0 (about 16–18%). The content of the other FA, including $18{:}1^{\Delta 9cis}$ or $18{:}2^{\Delta 6cis}$, was less than a few percent. What is important is that at all of the tested temperatures, there were no differences between the mutant and Delisa for the main FA (18:3). As for the other FA, for example, at 20 °C the 522DK mutant was characterised by a higher content of 16:0 and $18{:}1^{\Delta 9cis}$ than the Delisa cultivar, while at 5 °C, only $18{:}1^{\Delta 9cis}$ content was higher in the 522DK mutant.

For the plants that had been acclimated to a high temperature, the 522DK mutant was characterised by a higher level of 18:2$^{\Delta 6cis}$ than the Delisa cultivar (Table 1).

There were no significant differences between Delisa and its mutant at any growth temperature for the 18:3/18:2 ratio. Only at 20 °C was there a significantly higher value of the unsaturated FA to saturated FA ratio (U/S) for Delisa than for the 522DK mutant (Table 1).

Fatty acids are the main component of the biological membranes and their composition plays a huge role in the membrane properties including membrane fluidity [35,36]. According to Hölzl and Dörmann [37], chloroplasts are the major sites for FA synthesis in plant cells and 16:0, 18:1 $^{\Delta 9cis}$, 18:2 $^{\Delta 9cis,12cis}$ and 18:3 $^{\Delta 9cis,12cis,15cis}$ FA are present at the highest levels in the chloroplast membranes. Therefore, our data are generally consistent with this [37] and other previous works on chloroplasts that had been isolated from avocado fruits and cauliflower leaves [38], as well as on the callus of winter oilseed rape [39] and on the leaves of *Pisum sativum* L, winter wheat, and barley [13,40,41].

3.1.2. Carotenoid and Chlorophyll Content in the Chloroplast Membranes

Analysis of chlorophyll *a*, *b* and total chlorophylls (*a* + *b*) in the isolated chloroplasts showed no differences in the content of these pigments between the wild type and the mutant (Figure 2A,B,D). However, the leaf greenness measurements that were taken using a noninvasive method (chlorophyll meter) showed that for all four of the analysed leaves at all growth temperatures, the mutant was characterised by a more intense greenness (Figure 2E). Moreover, interestingly, according to our previous studies [42], a spectrophotochemical analysis of the chlorophyll content (especially chlorophyll *b*) in the leaf dry matter showed that 522DK had a lower content of chlorophyll than the Delisa cultivar, which was consistent with numerous studies that have proven that BR are important regulators of the chlorophyll accumulation because exogenous BR usually have caused an increase in chlorophyll content especially under stressful conditions [10]. In our current studies, the "greener" leaves of the 522DK mutant was rather a result of the mutant's semi-dwarfness (more compact cells with a higher density of chloroplasts/chlorophyll per leaf surface), but again an analysis of the pure chloroplast suspension revealed no difference between the wild type and the mutant. In this experiment, the content of chlorophyll in the chloroplasts was measured because these pigments also play an important role in membrane fluidity [6,43]. Because the chlorophyll contents in the chloroplast membranes of 522DK and Delisa were similar, we can assume that the chlorophylls regulated the fluidity of the chloroplast membranes in comparable manner.

Similar to chlorophyll, the contents of xanthophylls and carotenoids in the pure chloroplasts were also at the same level in the wild type and the mutant (Figure 2C). Interestingly, our earlier studies [42] showed that the BR-deficient mutant 522DK was characterised by a higher accumulation of carotenoids than Delisa when calculated per leaf dry mass. Contrary to these findings, Nie et al. [44] reported that carotenoid level increased in *Solanum lycopersicum*, which had a higher endogenous level of BR compared to its wild type. On the other hand, Koĉova et al. [45] found that exogenous brassinosteroids did not significantly affect the carotenoid content in maize leaves. Although the role of BR in the accumulation/biosynthesis of carotenoids seems to be ambiguous, it is certain that carotenoids have huge influence on the thylakoid membrane fluidity and they are also thought to be responsible for reducing the membrane fluidity of a membrane [6,43]. Because no differences in the carotenoid content in the chloroplasts of the mutant and wild type were observed in this study, we suspect that in the current experiment, the influence of carotenoids on the chloroplast membrane fluidity was comparable for Delisa and its mutant.

3.1.3. Brassinosteroid Content of the Chloroplasts

In the chloroplasts, we were able to determine the following BR: brassinolide, castasterone, 28-homocastasterone, 24-epibrassinolide, 28-norcastasterone, dolicholide, homodolicholide and homodolichosterone (Figure 3A–H).

Compared to the wild type, the BR-deficient mutant 522DK that had been cultured at 20 °C was characterised by a lower content of homocastasterone and 28-norcastasterone but higher content of homodolicholide and homodolichosterone in its chloroplasts. The other BR were at comparable level in the chloroplasts of both genotypes at this temperature.

In the mutant that had been grown at 5 °C, there was a higher content of homodolicholide, homodolichosterone and also dolicholide compared to the wild type (Figure 3D,G,H). The homodolichosterone level was even below detection limit in the wild type at 5 °C (Figure 3H). As for the other BR at 5 °C, there were no significant differences between the mutant and its wild type. Contrary to 20 °C, brassinolide was not detected at 5 °C in either the mutant or the wild type (Figure 3F).

As for the plants that had been acclimated at 27 °C, brassinolide was only detected in the chloroplasts of the mutant but not in the wild type (Figure 3F). While the chloroplasts of the 522DK plants at this temperature were characterised by a higher content of homodolicholide than the wild type, the level of dolicholide and homodolichosterone was below the detection limit in the chloroplasts of the mutant (Figure 3D,G,H). The other BR had a comparable concentration in the chloroplasts of both genotypes at this temperature.

As for the total amount of the detected BR (sum of all of the detected BR) (Figure 3I), the obtained results confirmed that the 522DK mutant was generally BR-deficient, but this was only observed at 20 °C for the chloroplasts. Interestingly, our earlier analysis of the BR content in the leaves of Delisa and 522DK [20] unambiguously showed that the mutants accumulated less BR in their leaf tissue than the wild type at all three temperatures (20 °C, 5 °C and 27 °C). Moreover, in the leaf material, only brassinolide, castasterone, and homocastasterone were found in that study, while in this study, many more BR were detected in the pure chloroplasts, including dolicholide, dolichosterone, homodolicholide, and also 28-norcastasterone and 24-epibrassinolide. Interestingly, dolicholide had already been detected by us in the barley leaves of other cultivars [46], but not in Delisa or its 522DK mutant [20]. In our opinion, the differences in the detectable BR profile in the Delisa and 522DK leaves and chloroplasts are due to the fact that in the case of the leaves, 300 mg of fresh leaf material was used for the BR extraction, while in the case of the chloroplast isolation, 40 g of the leaves (25 plants) were used to obtain 1.5 g of the chloroplasts (300 mg pure chloroplasts per sample for the BR analysis). For this reason, the presence of BR that were revealed in the chloroplast samples here was probably below the detection limit in the whole leaves in earlier studies.

The detection of BR in the barley chloroplasts confirms our earlier reports (research on wheat [27]) that these compounds accumulate in the chloroplasts. Based on the relatively limited knowledge of BR synthesis/decomposition sites in a cell, it is assumed that all BR biosynthetic enzymes belong to the family Cyt P450s and are located in the reticulum membrane [47]. The fact that BR accumulates in the chloroplasts (probably also in the membranes) of both the 522DK mutant and its wild type suggests that these hormones could be very important for the appropriate functioning of the chloroplasts. The phenomenon of the incorporation of BR in the chloroplast structure will require further research, even though some guidance regarding the role of BR was provided by Cai et al. [48], Zhang et al. [49] and Krumova et al. [50]. Exogenous BR prevented injuries to the chloroplast ultrastructure in plants that had been exposed to high and low temperatures [48,49]. However, according to Krumova et. al. [50], BR have an impact on the functioning and regulation of the photosynthetic apparatus and the architecture of the thylakoid membrane. The authors found that *Arabidopsis* BR mutants, which overexpress the BR receptor and are deficient in the BR biosynthesis, had increased thylakoid area compared to the wild type, which could be connected with changes in the chloroplast division mechanisms. On the other hand, at 20 °C, 5 °C, and 27 °C, the 522DK mutant had a better (than the wild type) efficiency of PSII (photosystem located in chloroplasts) [20]. Simultaneously (according to the current study), the mutant unambiguously accumulated only one BR—homodolicholide—in the chloroplasts at a higher amount than the wild type at all of the temperatures. Homodolicholide is a lesser-known BR and is present in relatively small amounts compared to

castasterone or homocastasterone (Figure 3A,D,E). Might it then be responsible for the better performance of the chloroplasts in the mutant that had been exposed to temperature stress? This will also require further studies.

As was mentioned above, the leaves of the mutant always had less BR than the leaves of the wild type [20]. In the case of mutant chloroplasts, however, we did observe: (1) the occurrence of lower amounts of BR, e.g., homocastasterone at 20 °C than in the wild type; (2) no differences in the BR content in the mutant and in the wild type (castasterone at all of the growth temperatures) and (3) a higher amount of BR in the mutant, (e.g., homodolicholide, independent of growth temperature).

In our opinion, this fact suggests that the presence of BR in the chloroplasts and perhaps their incorporation into the membranes is driven by accumulation mechanisms that do not have much in common with their biosynthesis process (and final BR production). Perhaps, the purely physicochemical mechanisms are involved (the membrane lipid environment was "beneficial" for the accumulation of specific steroids—especially homodolicholide in the mutant). Temperature also had an effect on the accumulation of BR in the chloroplasts. Moreover, as was mentioned above, previous studies have shown that the presence of mycotoxins, the application of exogenous brassinosteroids in a culture medium or the cultivar's tolerance to stress all influenced/modified the BR concentration in the chloroplasts in a different manner. A summary of the changes in the BR content in the chloroplast membranes of 522DK at different temperatures compared to its wild type is presented in Table S1.

3.2. Molecular Dynamics of the Barley Chloroplasts Measured Using EPR Spectroscopy

To study the molecular dynamics of the chloroplasts that had been isolated from the barley plants, two kinds of spin labels were incorporated into the membranes: SASL-5 and SASL-16. The first has a nitroxide group that is bound to the 5th carbon of the stearic acid and that is why it can provide information about the molecular dynamics processes that occur near the membrane surface. SASL-16 has a nitroxide group that is attached to the 16th carbon of the stearic acid and its incorporation into membranes provides information about the dynamic processes that occur inside the membrane [15]. The results of our experiment are presented in Figure 4A–F. It was found that the possibility to measure the spectrum of the spin labels (SASL-5 and SASL-16) was dependent on the ambient temperature of the plant from which the chloroplasts had been isolated. For example, for the chloroplasts that were isolated from the plants acclimated at 5 °C with incorporated SASL-16, it was possible to calculate the S parameter in a measurement of the temperature in the range of 0–17.5 °C (Figure 4E). On the other hand, for the chloroplasts that were isolated from the plants acclimated at 27 °C with incorporated SASL-16, it was possible to calculate the S parameter in the entire measurement temperature range that was used in this study, i.e., 0–45 °C. This shows that plant acclimation at 5 °C and 27 °C resulted in a good adaptation of the studied barley plants to low and high temperatures, respectively. Moreover, the dependence of the dynamic orientational order parameter on the temperature at which the plants were cultivated was also observed (Figure S2). As was mentioned earlier, according to our previous work [20], after acclimation at 5 °C and 27 °C, the 522DK mutant and its wild-type Delisa developed a tolerance to frost (up to −8 °C) and heat (38 °C and 45 °C). Additionally, in the study of Bojko et al. [51] on thylakoid membranes of *T. pseudonana* that were cultured at low and high temperatures (12 °C and 20 °C, respectively), a similar effect of efficient adaptation to acclimating temperatures was observed, which was connected, among others, with the molecular dynamics results.

The dynamic orientational order parameter (S) that was calculated from SASL-5 and SASL-16 spectra gradually decreased for the chloroplast membranes from both the wild-type Delisa and the mutant along with an increase in the measurement temperature (Figure 4; Figure S2). This effect was connected with an increase in the molecular dynamics of the membranes and a less ordered molecular environment of the spin labels during an increase in the measurement temperature [14]. The obtained values of S are in agreement

with the literature in which the dynamic orientational order parameter S varies between 1 (maximally ordered membranes) and 0 (maximally disordered membranes) [15,52]. An increase in the molecular dynamics is considered to represent an increase in membrane fluidity [53].

For the chloroplast membranes with an incorporated SASL-5 spin label in the measurement temperature range between 5 °C and 10 °C, the 522DK chloroplast membranes from the mutant that had been cultured at 20 °C were characterised by higher values of the S parameter than the Delisa's membranes (Figure 4A). There were no significant differences between the chloroplast membranes of Delisa and 522DK for the other measurement temperatures (Figure 4A). For the chloroplast membranes of the plants that had been acclimated at 5 °C and 27 °C, there were generally no noticeable differences between the wild-type Delisa and the mutant (Figure 4B,C); the only exception was at a measurement temperature of 10 °C, when the membranes of the mutant were characterised by a higher value of S (Figure 4B). To summarise, near the membrane surface, there were no differences between the Delisa cultivar and its mutant 522DK that had been acclimated at 5 °C and 27 °C. This is consistent with the fact that the surface area of membranes needs to be quickly ordered in order to support the proper interactions of the membranes with their neighbour area and it is also needed in order to avoid the uncontrolled diffusion of molecules such as oxygen or free radicals, which can cause destructive reactions inside membranes that are not fully adapted to the new conditions of environmental stimuli [14,52,54].

For the chloroplast membranes with an incorporated SASL-16 spin label, differences in the S parameter were observed between the mutant and the wild type that had been acclimated at 5 °C. The values of S parameter were significantly lower for the Delisa cultivar compared to its mutant in the entire tested temperature range and the differences were almost always statistically significant (Figure 4E). The same effect was observed for the membranes of the plants that were cultured at 20 °C, but only in the measurement temperature range between 25 °C and 30 °C (Figure 4D). In this case, interestingly, the S parameters were measurable at a temperature of 35 °C only for the chloroplast membranes that had been isolated from the Delisa cultured at 20 °C (but not for the mutant). No differences were observed for the membranes of the plants acclimated at 27 °C (Figure 4F). To conclude, for measurement of the chloroplasts with an incorporated SASL-16 spin label, we can say that the chloroplast membranes of the mutant 522DK that had been acclimated at 5 °C were more ordered (more rigid) than the membranes of the Delisa. Studies using a Langmuir bath led to similar conclusions (author's unpublished data). The monolayers were built from galactolipids (MGDG, DGDG—the main chloroplast lipid constituents [55]) and also from phospholipids that were isolated from the 522DK mutant and its wild type that had been acclimated at 5 °C. In the case of the mutant, the monolayers were characterised by statistically significantly lower (than Delisa) values of A_{lim}. A_{lim} represents the area that is occupied by a single molecule in a completely packed layer, provides information about membrane fluidity and decreases with a decrease in fluidity. In case of monolayers built from MGDG, values of A_{lim} [Å^2] were 65.6 ± 0.2 and 64.9 ± 0.3, respectively for Delisa and mutant. In case of monolayers built from DGDG values of A_{lim} were 55.2 ± 0.1 and 51.7 ± 0.2, respectively for Delisa and mutant. Usually, greater fluidity of cell membranes is considered as more favourable for a higher frost tolerance, which has been wider discussed in our earlier work [13]. The 522DK mutant, which has a slightly higher frost tolerance than the wild type Delisa [20], is, however, characterized by a greater rigidity of chloroplast membranes than Delisa, as shown in EPR studies. The question remains if/how fact of rigidification of chloroplast membranes is related to the frost tolerance of the mutant. Interestingly, as for the plasma membrane, it is known that the level of the expression of many cold-inducible genes increases two- to three-fold after the rigidification [56].

On the other hand, we have to mention that the 522DK mutant that was studied in the present experiment also had a better high-temperature tolerance than the wild-type Delisa [20], although there was no difference in the membrane molecular dynamics between

the mutant and the wild type after acclimation at 27 °C (Figure 4C,F). Interestingly, it is also worth noting here that most of the changes in the FA content were observed at 27 °C, but that no differences were observed in the molecular dynamics of the chloroplast membranes of plants that had been acclimated at 27 °C in either the hydrophilic and hydrophobic area.

Analysis of the other three parameters that were calculated from the SASL-16 spectra (ΔH_0 and rotational correlation times τ_{2B} and τ_{2C}) provided additional information about the movement of a spin label and at the same time about the movement of FA in the interior of the membrane. The ΔH_0 parameter provided information on the oscillation and rotation of a spin label and its rotation along the long axis of the molecule [15]. Lower values of ΔH_0 indicate a greater freedom of the spin label motion. Our results show that there were generally no differences in the motion of the molecules (ΔH_0 parameter) of the chloroplast membranes of the Delisa cultivar and its 522DK mutant at the tested temperatures (Figure 5A,B,C). For the other two parameters, τ_{2B} corresponded to the rotation of the spin label molecule along its long axis, while τ_{2C} provided information about the movement of the molecule in the direction perpendicular to the long axis. The rotational correlation times are assumed to reflect the local fluidity of a membrane—lower values indicate a greater motional freedom of the spin labelled FA and thus a higher fluidity of a membrane [14].

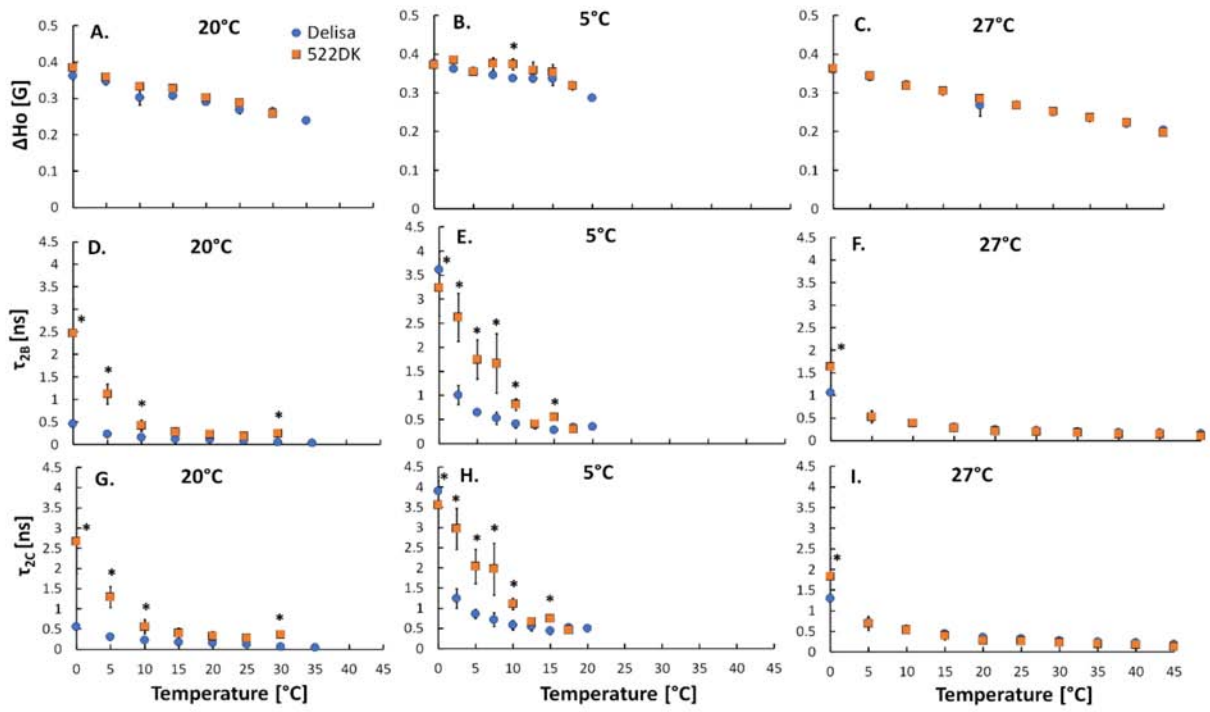

Figure 5. Dependence of parameters ΔH_0 (**A**–**C**), τ_{2B} (**D**–**F**) and τ_{2C} (**G**–**I**), which were calculated for the chloroplast membranes that were isolated from the barley wild-type Delisa and its mutant 522DK with incorporated SASL-16 on temperature. Plants had been cultivated at 20 °C and acclimated at 5 °C and 27 °C. Average data are given ±SE and significant differences between the wild type and mutant are indicated with an asterisk (*); comparisons were made separately for each EPR measurement temperature.

The values of τ_{2B} and τ_{2C} of 16-SASL in the chloroplast membranes from both Delisa and 522DK depended on the temperature of the measurement (Figure 5D–I). It was observed that within a temperature range between 0 °C and 7.5 °C, the chloroplast membranes of the mutant 522DK, which had been grown at 20 °C and acclimated at 5 °C, was characterised by significantly higher values of τ_{2B} than its wild type (Figure 5D,E). For the plants that had been acclimated at 27 °C, Delisa had a higher value of τ_{2B} than its mutant only around 0 °C (Figure 5F). Moreover, the values of τ_{2C} were noticeably higher for the

chloroplast membranes of the 522DK mutant than its wild type, which had been cultivated at 20 °C and acclimated at 5 °C, within a range of measurement temperatures between 0 °C and 10 °C (Figure 5G,H). For the chloroplast membranes of the plants that had been acclimated at 27 °C, there were significant differences between Delisa and the mutant only at a measurement temperature of 0 °C (Figure 5I). When the temperature of the measurement was higher, both parameters had lower values. This decrease in the rate of the molecule rotation along the long axis (τ_{2B}) is in line with an increased rigidity of membranes [14].

We can assume that the greater motional freedom had FA included in Delisa's chloroplast membranes compared to chloroplast membranes of 522DK. Therefore, all of the calculated parameters once again implied that the chloroplast membranes of 522DK at 20 °C and 5 °C were more ordered/less fluid than the chloroplast membranes of the Delisa cultivar.

The final matter to discuss is what caused the differences in the molecular dynamics of the membranes between the mutant and its wild type. Because the components that are important for membrane structure and properties, such as FA, carotenoids and chlorophylls were comparable in the chloroplasts of the wild type and mutant (Table 1, Table S1, Figure 2), it is likely that the differences in the membrane molecular dynamics might be connected with other components including BR. Among all of the BR found in current study, the concentration of castasterone and homocastasterone was usually at the highest level in the chloroplasts. In the chloroplasts of the cold-treated plants, these BRs were found in amounts of about 1000–6000 pg per g of F.W. The 522DK mutant that had been acclimated at 5 °C had more rigidified chloroplast membranes than the wild type, but also had a similar content of these two BR present in high content (Figure 3A,E). This may suggest that these two BR are not the cause of the differences in the membrane molecular dynamics. The chloroplast content of BR from the other groups such as homodolicholide, dolicholide, and homodolichosterone is a different matter. The cold-acclimated 522DK had a significantly higher content of these BR in the chloroplasts than the wild type (Figure 3D,G,H); however, their content was generally very low (not more than 30 pg per g of F.W.). Thus, it is difficult to say whether they can be responsible for the changes in the molecular dynamics. In turn, it should be remembered that the molecular dynamics of membranes was quite similar in the mutant and the wild type at 20 °C and 27 °C, although there were differences in the BR content. To conclude, the role of BR as membrane stabilisers remains an open question. We cannot totally reject the hypothesis about the role of BR in modifying the dynamics of membranes because there are other studies that suggest this. Li et al. [57] investigated the effect of exogenous BR on the fluidity of the plasma membrane in mango fruits that had been stored at 5 °C. Using the EPR method, the authors found that treatment with BR increased the membrane fluidity in mango fruit. In our earlier studies using the Langmuir bath, we showed that BR are incorporated into the structure of the membrane monolayer from lipids that were isolated from not-hardened and cold-hardened wheat. The changes in the physicochemical parameters of the monolayers such as compressibility were observed [41]. For further investigation of the role of BR as membrane stabilisers studies of EPR on model membranes would be helpful. We can also not exclude the fact that the amount of BR is not crucial here. Perhaps the proportions between each component of membranes including BR as well as sterols, which were not tested here, or some proteins are also important in regulating the chloroplast membrane fluidity. Theoretically, BR can also be located in so-called rafts such as cholesterol in animal cell membranes [58] or phytosterols in plant membranes [59], which affect the membrane properties differently because they are local. Rafts are specialised lipid domains and the role of phytosterols-rich rafts is considered not without merit in maintaining plant membranes in a state of dynamics that is less sensitive to temperature shocks [59]. However, this point of view regarding BR requires further studies.

4. Concluding Remarks

Based on our experiment, the following main findings can be pointed out:

(1) In the chloroplasts that were isolated from the barley wild-type Delisa and the mutant 522DK, regardless of the plant growth temperature, eight brassinosteroids at different concentrations were identified. Their diverse presence may indicate that they play some role in the functioning of these organelles.

(2) Mutant 522DK is a BR-deficient mutant that has a lower accumulation of BR compared to the wild type in its leaf tissue [18,20]. However, in the case of the chloroplasts, this regularity has not been proven in present studies. The mutant's chloroplasts had a higher level of three brassinosteroids: homodolicholide (regardless of the plant growth temperature), dolicholide (plants at 5 °C) and homodolichosterone (plants at 20 °C) than the wild-type Delisa. On the other hand, the content of homocastasterone was lower in the mutant's chloroplasts (plants at 20 °C), while castasterone was unchanged (regardless of the plant growth temperature). It is likely that the incorporation of BR into the chloroplast membranes is driven by accumulation mechanisms that do not have much in common with their biosynthesis process or the final BR production/concentration in cells.

(3) Although electron paramagnetic resonance has been used to study the cell membrane properties and the influence of different factors on those properties [60], knowledge about the influence of BR on the fluidity of cell membranes is still rudimentary. In the current work, it was shown that the main components that are important for the chloroplast membrane's structure/properties, such as FA, carotenoids and chlorophylls, were comparable in the chloroplasts of the wild type and mutant 522DK, but it cannot be unambiguously stated that the BR that were present in the chloroplasts were responsible for the differences in the chloroplast membrane molecular dynamics between the tested genotypes. However, in the hydrophobic (but not hydrophilic) area, the chloroplast membranes of the mutant 522DK that had been acclimated at 5 °C were more ordered (more rigid) than the membranes of the wild-type Delisa. Slight differences between the mutant and wild type were also observed after their growth at 20 °C. There was practically no difference in the membrane dynamics between the mutant and the wild type after acclimation at 27 °C in either the hydrophilic or hydrophobic area.

Supplementary Materials: The following are available online at https://www.mdpi.com/2218-273X/11/1/27/s1, Figure S1: Sample of the freshly isolated chloroplasts from fresh plant material taken to confirm the purity of the chloroplasts, seen under a Nikon Eclipse E600 microscope (Tokyo, Japan), Figure S2: Dependence of the dynamic orientational order parameter S calculated for the chloroplast membranes (with incorporated SASL-5 (A,B) and SASL-16 (C,D)) that were isolated from the barley wild-type Delisa (A,C) and its mutant 522DK (B,D) that had been cultivated at 20 °C and acclimated at 5 °C and 27 °C on temperature. Average data are given ±SE and significant differences between the temperatures of growth (20 °C, 5 °C, and 27 °C) are indicated with an asterisk (*); comparisons were made separately for each EPR measurement temperature, Table S1: Changes in the content of FA and BR in the barley chloroplasts of the BR-deficient mutant 522DK (mutation *HvDWARF*) compared to the wild-type Delisa (↑ increase compared to the wild type; ↓ decrease compared to the wild type; NC no change compared to the wild type).

Author Contributions: I.S. is the coordinator of the project 2018/31/N/NZ9/02430. I.S. and A.J. planned the research. D.L. provided valuable suggestions about the research and the interpretation of data in the discussions during the writing of manuscript; I.S. isolated the chloroplasts, prepared the samples for the BR analysis, analysed the protein content, performed the leaf greenness measurements, purified the brassinosteroids on the immunoaffinity columns; J.O. measured the brassinosteroid content on UHPLC–MS/MS. I.S. and D.L. performed the EPR measurements; I.S. analysed the data. D.G. provided the seeds of the mutant 522DK and cv. Delisa and characterised them genetically. D.L. and M.C. prepared the samples for the fatty acid analysis and measured the fatty acid content on GC. I.S. analysed the carotenoids content analysis under D.L. coordination; I.S. performed the statistical analysis and prepared the tables and figures. I.S. wrote the manuscript under the supervision of A.J. All authors have read and agreed to the published version of the manuscript.

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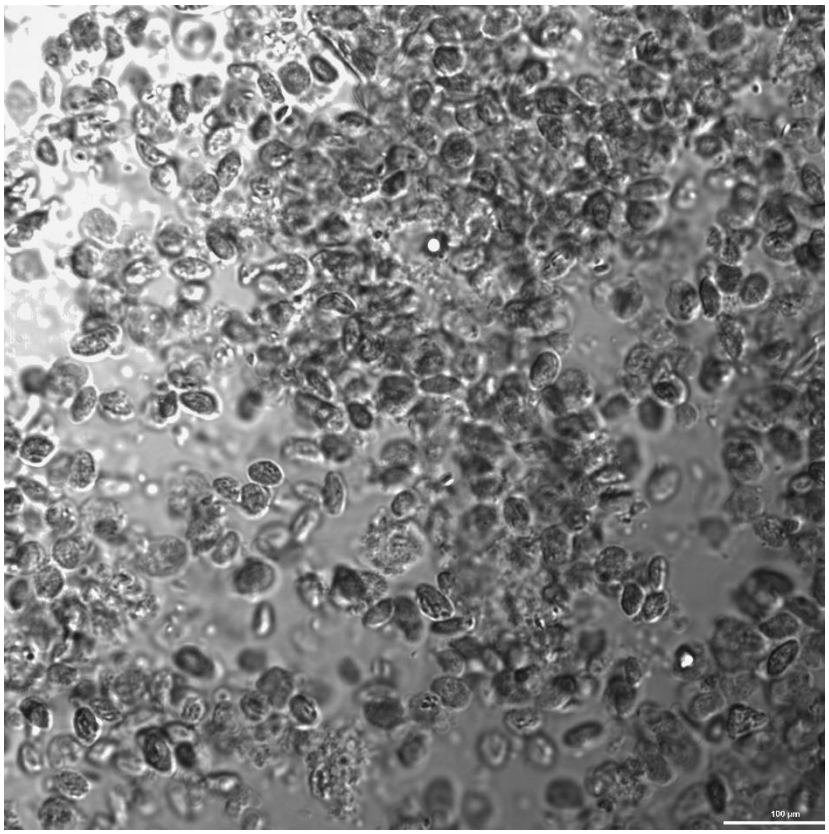

Figure S1: Sample of the freshly isolated chloroplasts from fresh plant material taken to confirm the purity of the chloroplasts, seen under a Nikon Eclipse E600 microscope (Tokyo, Japan).

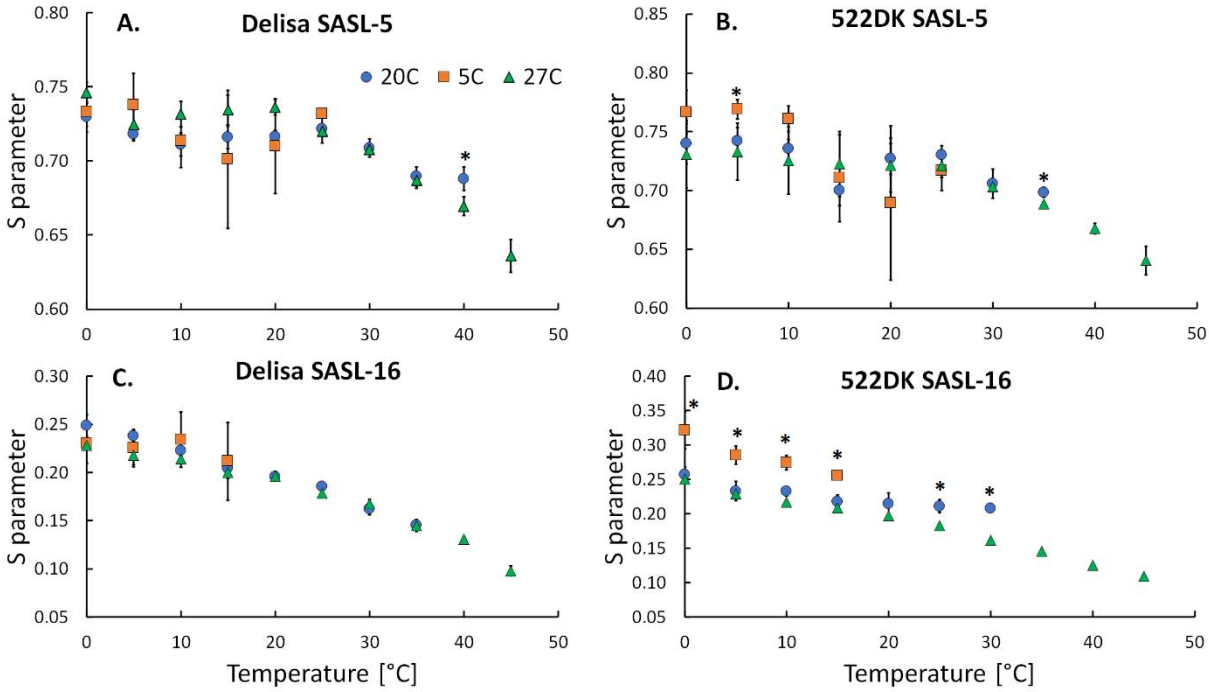

Figure S2: Dependence of the dynamic orientational order parameter S calculated for the chloroplast membranes (with incorporated SASL-5 (A,B) and SASL-16 (C,D)) that were isolated from the barley wild-type Delisa (A,C) and its mutant 522DK (B,D) that had been cultivated at 20°C and acclimated at 5°C and 27°C on temperature. Average data are given ±SE and significant differences between the temperatures of growth (20°C, 5°C, and 27°C) are indicated with an asterisk (*); comparisons were made separately for each EPR measurement temperature

Table S1. Changes in the content of FA and BR in the barley chloroplasts of the BR-deficient mutant 522DK (mutation *HvDWARF*) compared to the wild-type Delisa (↑ increase compared to the wild type; ↓ decrease compared to the wild type; NC no change compared to the wild type).

Compounds of chloroplast membranes	**Growth temperature**		
	20°C	**5°C**	**27°C**
FA			
10:0	↑	NC	↑
12:0	NC	NC	↑
14:0	NC	NC	↓
16:0	↑	NC	NC
16:1	NC	NC	NC
18:0	NC	NC	NC
18:1 $^{\Delta 9\ cis}$	↑	↑	NC
18:2 $^{\Delta 6\ cis}$	NC	NC	↑
18:3 (3)	NC	NC	NC
20:1	NC	NC	NC
BR			
Homocastasterone	↓	NC	NC
28-norcastasterone	↓	NC	NC
Castasterone	NC	NC	NC
Brassinolide	NC	NC	↑
24-epibrassinolide	NC	NC	NC
Dolicholide	NC	↑	↓
Homodolicholide	↑	↑	↑
Homodolichosterone	↑	↑	↓
Total BR content	↓	NC	NC

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Kraków, dn. 2.03.2021r.

OŚWIADCZENIE WSPÓŁAUTORA

Wyrażam zgodę na wykorzystanie przez Iwonę Sadura publikacji: Sadura Iwona, Latowski Dariusz, Oklestkova Jana, Gruszka Damian, Chyc Marek, Janeczko Anna (2021). Molecular dynamics of chloroplast membranes isolated from wild-type barley and a brassinosteroid-deficient mutant acclimated to low and high temperatures. [Biomolecules 11: 27] w przewodzie doktorskim opartym na zbiorze artykułów opatrzonych tytułem: „Rola brasinosteroidów w procesach aklimatyzacyjnych roślin jęczmienia do niskiej i wysokiej temperatury."

Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: udzieleniu wskazówek do modelu eksperymentu, nadzorowaniu pracy doktorantki (I. Sadura) przy wykonywaniu przez nią pomiarów dynamiki molekularnej membran chloroplastów za pomocą Elektronowego Rezonansu Paramagnetycznego (EPR), nadzorowaniu pracy doktorantki przy oznaczeniu zawartości karotenoidów w badanych próbkach, przygotowaniu próbek do analizy składu kwasów tłuszczowych, współpracy merytorycznej na etapie pisania manuskryptu.

Dariusz Latowski

.....

czytelny podpis

Dr Jana Oklestkova

Institute of Experimental Botany & Palacký University,
Laboratory of Growth Regulators, The Czech Academy of Sciences

Olomouc, 2.03.2021

STATEMENT OF THE CO-AUTHOR

I agree for the use of the publication: Sadura Iwona, Latowski Dariusz, Oklestkova Jana, Gruszka Damian, Chyc Marek, Janeczko Anna (2021). Molecular dynamics of chloroplast membranes isolated from wild-type barley and a brassinosteroid-deficient mutant acclimated to low and high temperatures. [Biomolecules 11: 27] in a doctoral dissertation based on a collection of articles. The title of dissertation: "The role of brassinosteroids in the acclimation processes to low and high temperatures of barley plants."

I declare that my participation in the above-mentioned work consisted in: UHPLC-MS/MS measurement of brassinosteroid content in barley.

Legible signature

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Katowice, dn. 2.03.2021r.

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: przygotowaniu materiału nasiennego (w tym nasion mutanta 522DK) do badań oraz współpracy przy pisaniu manuskryptu (analiza/dyskusja aspektów genetycznych).

Damian Gruszka

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Tarnów, dn. 2.03.2021r.

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: wykonaniu analizy kwasów tłuszczowych w badanym materiale za pomocą GC-MS.

Marek.Chyc Elektronicznie podpisany przez Marek.Chyc
Data: 2021.03.02 15:52:51 +01'00'

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Zakład Biologii Rozwoju

Kraków, dn. 2.03.2021r.

OŚWIADCZENIE WSPÓŁAUTORA

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Oświadczam, że mój udział w wyżej wymienionej pracy polegał na: współpracy z doktorantką (I.Sadura) przy planowaniu eksperymentu, interpretacji części wyników na potrzeby dyskusji oraz nadzorowaniu procesu tworzenia całego manuskryptu.

Anne Janeczko

.....

czytelny podpis

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Instytut Fizjologii Roślin im. Franciszka Górskiego PAN,

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Kraków, dn. 2.03.2021r.

OŚWIADCZENIE DOKTORANTA

W związku z wykorzystaniem poniżej wymienionych publikacji w rozprawie doktorskiej oświadczam, że mój udział w ich powstaniu polegał na:

Publikacja A

Sadura Iwona, Janeczko Anna (2018) Physiological and molecular mechanisms of brassinosteroid-induced tolerance to high and low temperature in plants. Biologia Plantarum 62: 601-616

Mój udział w tworzeniu publikacji polegał na wykonaniu przeglądu literatury dotyczącej działania brasinosteroidów w warunkach stresu temperaturowego; napisaniu manuskryptu (w tym przygotowaniu rycin) pod kierunkiem dr hab. inż. Anny Janeczko i edycji pracy zgodnie z wymaganiami czasopisma.

Publikacja B

Sadura Iwona, Pociecha Ewa, Dziurka Michał, Oklestkova Jana, Novak Ondrej, Gruszka Damian, Janeczko Anna (2019) Mutations in the *HvDWARF, HvCPD* and *HvBRI1* genes – involved in brassinosteroid biosynthesis/signalling: altered photosynthetic efficiency, hormonal homeostasis and tolerance to high/low temperatures in barley. Journal of Plant Growth Regulation 38: 1062-1081

Mój udział w tworzeniu publikacji obejmował: wykonanie testów tolerancji badanych roślin na mróz i wysoką temperaturę pod kierunkiem dr hab. inż. Ewy Pociechy; pomiar wydajności PSII; wykonanie ekstrakcji wszystkich fitohormonów z zebranego materiału liściowego oraz napisanie manuskryptu (w tym przegląd literatury, analizę statystyczną, wizualizację i interpretację otrzymanych wyników) pod kierunkiem dr hab. inż. Anny Janeczko; edycję końcową manuskryptu wg wymagań czasopisma.

Publikacja C

Sadura Iwona, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020) The HSP transcript and protein accumulation in brassinosteroid barley mutants acclimated to low and high temperature. International Journal of Molecular Sciences 21:1889

Mój udział w tworzeniu publikacji obejmował: wykonanie izolacji frakcji mikrosomalnej i cytoplazmatycznej z badanych roślin jęczmienia i zmierzenie w nich całkowitej zawartości białka; przygotowanie próbek ekstraktu liściowego i zmierzenie w nim całkowitej zawartości białka (prace pod kierunkiem dr hab. inż. A. Janeczko); wykonanie analizy akumulacji białek szoku cieplnego (w tym optymalizacji metody) pod nadzorem dr hab. Marty Libik-Konieczny; wykonanie densytometrycznej analizy wyników za pomocą programu ImageJ; wykonanie izolacji RNA i odwrotnej transkrypcji (do analizy *HSP90*, *HSP70*, *HSP18* i *HSP17*) pod nadzorem dr hab. inż. Barbary Jurczyk; wizualizację, analizę i interpretację wyników oraz napisanie manuskryptu (w tym przegląd literatury) pod nadzorem dr hab. inż. Anny Janeczko; edycję końcową manuskryptu wg wymagań czasopisma.

Publikacja D

Sadura Iwona, Libik-Konieczny Marta, Jurczyk Barbara, Gruszka Damian, Janeczko Anna (2020) Plasma membrane ATPase and the aquaporin HvPIP1 in barley brassinosteroid mutants acclimated to high and low temperature. Journal of Plant Physiology 244:153090

Mój udział w tworzeniu publikacji obejmował: wykonanie izolacji frakcji mikrosomalnej z badanych roślin jęczmienia i zmierzenie w niej całkowitej zawartości białka; przygotowanie próbek ekstraktu liściowego i zmierzenie w nim całkowitej zawartości białka (prace pod kierunkiem dr hab. inż. A. Janeczko); wykonanie analizy akumulacji białek HvPIP i H^+-ATPazy (w tym optymalizacji metody) pod nadzorem dr hab. Marty Libik-Konieczny; wykonanie densytometrycznej analizy wyników za pomocą programu ImageJ; wykonanie izolacji RNA i odwrotnej transkrypcji (do analiz *HvPIP* i *H^+-ATPazy*) pod nadzorem dr hab. inż. Barbary Jurczyk; wizualizację, analizę statystyczną i interpretację wyników oraz napisanie manuskryptu (w tym przegląd literatury) pod nadzorem dr hab. inż. Anny Janeczko; edycję końcową manuskryptu wg wymagań czasopisma.

Publikacja E

Rudolphi-Szydło Elżbieta, Sadura Iwona, Filek Maria, Gruszka Damian, Janeczko Anna (2020) The impact of mutations in the *HvCPD* and *HvBRI1* genes on the physicochemical properties of membranes from barley acclimated to low/high temperatures. Cells 9:1125

Mój udział w tworzeniu manuskryptu obejmował wykonanie izolacji lipidów z roślin jęczmienia, pod kierunkiem prof. dr hab. Marii Filek; pomocy dr hab. Elżbiecie Rudolphi-Szydło przy wykonaniu pomiarów na Wannie Langmuira; wykonaniu analizy statystycznej wyników oraz wizualizacji wyników pod nadzorem dr hab. inż. Anny Janeczko; edycji końcowej manuskryptu wg wymagań czasopisma.

Publikacja F

Sadura Iwona, Latowski Dariusz, Oklestkova Jana, Gruszka Damian, Chyc Marek, Janeczko Anna (2021). Molecular dynamics of chloroplast membranes isolated from wild-type barley and a brassinosteroid-deficient mutant acclimated to low and high temperatures. Biomolecules 11: 27

Mój udział w tworzeniu manuskryptu obejmował: udział w tworzeniu koncepcji pracy (modelu eksperymentu); uzyskanie finansowania (kierowanie projektem NCN nr 2018/31/N/NZ9/02430); izolację chloroplastów z badanych roślin jęczmienia (pod kierunkiem prof. dr hab. Marii Filek); przygotowanie próbek chloroplastów do analizy zawartości brasinosteroidów (ekstrakcja, oczyszczenie materiału – w tym oczyszczenie na kolumnach IA (Ołomuniec, Czechy)); pomiar całkowitej zawartości białka w próbkach chloroplastów; wykonanie pomiarów intensywności zieleni liści jęczmienia chlorofilomierzem; wykonanie pomiarów dynamiki molekularnej membran chloroplastów za pomocą EPR; analizę i interpretację danych otrzymanych z widm EPR; analizę zawartości karotenoidów pod nadzorem dr hab. Dariusza Latowskiego; wykonanie analizy statystycznej i wizualizację wyników; napisanie manuskryptu (w tym przegląd literatury) pod kierunkiem dr hab. inż. Anny Janeczko; edycję końcową manuskryptu wg wymagań czasopisma.

Iwona Sadura

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Czytelny podpis