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Identification of QTLs associated with albino plant formation and some new facts concerning green versus albino ratio determinants in triticale (×*Triticosecale* Wittm.) anther culture

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Abstract High frequency of albino haploids/doubled haploids (DHs), regenerated in androgenic cultures is one of the major obstacles that limit incorporation of DHs technology into cereal breeding programs. Therefore, quantitative trait loci (QTL) associated with albino plant production in triticale anther cultures were analyzed using the population of 90 DH lines derived from F1 cross 'Saka 3006' × 'Modus'. Composite interval mapping (CIM) and single marker analysis (SMA) in Windows QTL Cartographer ver 2.5 were used to localize the major QTLs. CIM method revealed seven QTLs with LOD scores between 2.9 and 5.6 on five chromosomes from B to R subgenomes (3B, 4B, 4R, 5R and 7R). Effects of all QTLs explained 8.3–17.6 % of the phenotypic variation and were confirmed by SMA analysis. Additionally SMA revealed another seven markers on chromosomes: 2AL.2BL, 3B, 2BS.6AL, 2RS.3R and 4R associated with QTL for albino plant regeneration (p < 0.01). The additional experiment with ten DH lines varied significantly in their androgenic responsiveness was conducted to analyze the changes in the level of oxidative stress, antioxidative system activity and endogenous hormonal balance associated with androgenesis-inducing low temperature stress treatment (3 weeks at 4 °C). The correlation analysis between albino/green plant regeneration ability and analyzed traits were performed by using Spearman Rank test (p ≤ 0.05). Revealed associations may suggest that some level of oxidative stress is necessary for transition from a non-photosynthetic proplastids to the functional chloroplasts. On the other hand, the efficient antioxidative enzyme system and endogenous hormonal balance are also very important.

Key message Fourteen chromosome regions were indicated to control albino plant formation during triticale anther culture. Additionally, reactive oxygen species (ROS) generation, antioxidative system activity and hormonal balance were discussed as determinants in androgenesis.

Keywords Anther culture · Albino plants · Composite interval mapping · QTL · Winter triticale

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Introduction

Microspore embryogenesis (ME), also called androgenesis, is defined as the reproduction of an individual with genetically exclusive male origin (Seguí-Simarro 2010). This remarkable process is considered as one of

the fastest and simplest route to obtain haploid and doubled haploid plants (DHs), which are very good tools for breeding programs, as well as for biotechnology and molecular studies. However, the androgenic effectiveness is expressed only under certain circumstances as a consequence of an environmental stress (Bonet et al. 1998) and it is controlled by an interaction of genetic and physiological factors.

One of very important problems in androgenic cultures of monocots is the formation of chlorophyll-deficient, so called 'albino' or 'albinotic' plants. This phenomenon is observed in majority of cereals like wheat (Andersen et al. 1987), barley (Knudsen et al. 1989), rice (Guiderdoni et al. 1992), rye (Immonen 1999) and oat (Kiviharju and Pehu 1998) with the frequency that may vary from 5 to 100 % of regenerants. Also in triticale, high frequencies of chlorophyll-defective regenerants have been usually reported (González et al. 1997; Ponitka et al. 1999; Schumann 1990) sometimes prevailing the frequency of properly developed plants (Pauk et al. 2000). Albinotic plants cannot survive in natural environment, outside in vitro culture, and have no agronomic value. Despite many efforts that have been made to bring the better understanding of the mechanisms leading to albino plant formation, the primary reasons are still unknown.

In order to elucidate the mechanism of albino plant formation researchers have focused on three areas: cytology, plastid genomics and the nuclear genome, identifying numerous factors as involved in control of the process (Makowska and Oleszczuk 2014). It has been revealed that the number of obtained albino plants depends on the pre-treatment used for androgenesis initiation, the composition of induction medium and in vitro culture conditions (Ćalić et al. 2013; Immonen and Robinson 2000; Jacquard et al. 2009; Lantos et al. 2013; Ślusarkiewicz-Jarzina and Ponitka 1997; Wojnarowiez et al. 2002). Some researches claim that origin of albinism in some cultivars is determined at the earliest phases of ME and obtaining green regenerants depends on the state of microspore plastids at the moment of sampling (Caredda et al. 2004, 2000; Muñoz-Amatriaín et al. 2009).

Molecular examinations on barley (Muñoz-Amatriaín et al. 2009) revealed that genes related to stress response, transcription and translation regulation, and degradation of pollen-specific proteins were associated with green plant production, while expression of genes related to plastid development was associated with albino plant regeneration. Albino plants showed an aberrant form of plastids and, frequently, deletions in their plastid DNA (Harada et al. 1991; Hofinger et al. 2000). It was proved that transcript levels of plastid encoded genes for photosynthetic proteins and ribosomal RNA were generally heavily reduced in albino plants in relation to green plants (Ankele et al. 2005; Dunford and Walden 1991; Hofinger et al. 2000). Translation deficiencies and the modified transcript pattern in androgenic albino plants could be explained by lack of functional plastid ribosomes (Hofinger et al. 2000). Genetic studies support the hypothesis that nuclear factors contribute to the formation of albino plants (Ankele et al. 2005; Zhou and Konzak 1992). As most of the proteins of photosynthesis-related complexes are encoded in the nuclear genome the participation of nuclear genes in this phenomenon seems to be dominant. Muñoz-Amatriaín et al. (2009) found that high number of chlorophyll-deficient plants in barley was associated with the expression of three genes that could be related to plastid development. One of them was gene with homology to *DAG,* which is essential for chloroplast development from proplastids, and acts very early in chloroplast development (Chatterjee et al. 1996). The second gene encoded a class B ankyrin repeat protein, which is involved in plastid differentiation (Garcion et al. 2006). The third one was a transcription factor, that encodes abscisic acid-insensitive 3 (ABI3), which is important for plastid identity and could influence on plastid ultrastructure (Rohde et al. 2000).

Statistical approach with the use of quantitative trait loci (QTL) analysis provide a new possibility for the identification of the molecular control over albinism. So far, QTLs for green plant percentage were mapped in wheat (Torp and Andersen 2009; Torp et al. 2001), rye (Grosse et al. 1996) barley (Muñoz-Amatriaín et al. 2008) and triticale (González et al. 2005; Krzewska et al. 2012) and many different genes affecting the trait have been recognized. However, little is known about the molecular and physiological mechanisms leading to the formation of albino plants and further studies are necessary to help in understanding this phenomenon.

In this study, a population of 90 DH lines derived from the cross between hexaploid winter triticale (×*Triticosecale* Wittm.) 'Saka 3006' and 'Modus'

was used for identification of QTLs associated with albino plant production by anther culture method. The same mapping population together with produced for it well-saturated genetic linkage map (Tyrka et al. 2011) was successfully used in several previous studies (Krzewska et al. 2012; Szechynska-Hebda et al. 2011; Żur et al. 2012). Among others, the QTLs associated with androgenic structure formation and green plant regeneration has been identified (Krzewska et al. 2012). In this report, the results of QTL analysis showing the association with albino plant formation supplement earlier published reports. Moreover, new statistical approach allows for acquisition of data showing possible associations between ROS generation, antioxidative system activity, endogenous hormones level and the effectiveness of androgenesis. These data has been received from additional experiments in which the anthers of highly responsive and highly recalcitrant DH lines selected out from the described above mapping population of triticale were used to analyze the changes induced by androgenesis—triggering low temperature stress treatment (3 weeks at 4 °C). As the analytical methods and majority of received data has been reported elsewhere (Żur et al. 2014, 2015), here only some results of correlation analysis between albino/green plant regeneration ability and analyzed traits are presented.

Materials and methods

Plant material and growth conditions

The mapping population of 90 DH lines derived from F1 generation of a cross between winter triticale inbred line 'Saka 3006' and cv. 'Modus' by crosses with maize method together with both DH parental lines were used in this study. The population was created by Dr Eva Bauer from the State Plant Breeding Institute, Hohenheim University in Stuttgart, Germany and kindly provided for this research. Several DH lines, namely: DH18, DH28, DH44, DH47, DH101 (1–5) and DH2, DH19, DH72, DH119, DH144 (6–10) selected out from 'Saka 3006' × 'Modus' mapping population and identified as highly responsive (1–5) and highly recalcitrant (6–10) were used in the additional experiments. In all experiments, the seeds were germinated in the dark for 2 days at room temperature, then placed in perlite pre-soaked with Hoagland's salt solution and vernalized (7 weeks at 4 °C with a photoperiod of 8/16 h day/night). Vernalized seedlings were planted in a mixture of soil, deacidified substrate peat and sand (2/2/1; v/v/v) and grown in a greenhouse at 20 ± 2 C with 16/8 h (day/night) photoperiod until the flowering.

Anther culture

Standard protocol described by Wędzony (2003) with several modifications according to Żur et al. (2012) was used for evaluation of the anther culture responsiveness. Tillers were collected when the majority of microspores were at the mid- to the late-uninucleate stage of development and low temperature pre-treated (3 weeks at 4 C in the dark; LT). Anthers were aseptically excised and cultured in C17 induction medium (Wang and Chen 1983) modified as described by Żur et al. (2012). Cultures were incubated in the dark at 28 ± 1 °C. About 6 weeks later androgenic structures (AS) of size >1 mm were transferred to regeneration medium 190-2 (Zhang and Xu 1983) with some modifications (Krzewska et al. 2012). Regeneration phase took place at 26 C, in the light (at about 30 µmol m^{-2} s^{-1} during the first week, then increased to 80–100 µmol m^{-2} s^{-1}) with 16 h/8 h (day/night) photoperiod.

In the additional experiment, anthers of ten selected DH lines isolated from freshly cut (FC) tillers collected at the phase of development optimal for androgenesis initiation were used as the control for anther cultures started according to standard procedure from low temperature pre-treated tillers (3 weeks at 4 °C; LT).

The androgenic responsiveness was evaluated for each DH line in three separate replication. The efficiency of androgenesis was expressed by several parameters from which GR/100AS—the number of green regenerants per 100 androgenic structures, AR/100AS—the number of albino regenerants per 100 androgenic structures, GR/100A—the number of green regenerants per 100 anthers, AR/100A—the number of albino regenerants per 100 anthers, GR/AR (AS)—the ratio between GR and AR frequency calculated per 100AS, GR/AR (A)—the ratio between GR and AR frequency calculated per 100 A are presented in this study. The parameters were

Table 1 Effect of tested variables on androgenic effectiveness in anthers culture of 92 DH lines of winter triticale mapping population 'Saka 3006' × 'Modus'

Sources of variation	SS	df	MS	F	P
Green plants regeneration (GR/100AS)					
(1) Genotype	19,220	91	211	3.29	***
(2) Experiment replication	3804	2	1902	29.66	***
(1) * (2)	13,727	182	75	1.18	NS
Albino plants regeneration (AR/100AS)					
(1) Genotype	12,300	91	135.7	2.83	***
(2) Experiment replication	1715	2	857.5	17.9	***
(1) * (2)	8367	182	46	0.96	NS

SS sum of squares, df degrees of freedom, MS mean square, F statistic in ANOVA, p probability, NS not statistically significant

*** $p \leq 0.001$

Table 2 Mean efficiency of regeneration in anther cultures of parental DH lines (DH 'Saka 3006', DH 'Modus') and derived from their F1 cross hybrid population of 90 DH lines. Offspring DH population is characterized also by the extremes range (min–max)

	GR/100AS	AR/100AS	GR/AR (AS)	GR/100A	AR/100A	GR/AR (A)
DH 'Saka 3006'	4.4 ± 1.5	6.2 ± 1.6	0.7	1.6 ± 0.5	1.9 ± 0.4	0.8
DH 'Modus'	7.0 ± 2.1	7.4 ± 1.1	0.9	6.7 ± 1.5	8.1 ± 1.8	0.8
Mean for DHs progeny	7.9 ± 0.2	7.6 ± 0.2	1.0	5.0 ± 0.2	4.4 ± 0.1	1.1
Max–min range for DHs progeny	0–31	0–31	0–10	0–38	0–19	0–11

The data are the mean ± SD of three separate experimental replications with 10 biological replications (plates containing 100 anthers) each. GR/100AS, number of green regenerants (GR) per 100 androgenic structures (AS); AR/100AS, the number of albino regenerants (AR) per 100 androgenic structures (AS); GR/100A, number of green regenerants (GR) per 100 anthers (A); AR/100A, the number of albino regenerants (AR) per 100 anthers (A); GR/AR (AS), the ratio between GR and AR frequency calculated per 100AS; GR/AR (A), the ratio between GR and AR frequency calculated per 100 A

calculated as the mean from at least ten replications, with a 60 × 15 mm Petri dishes containing 100 anthers from one spike considered as one replication.
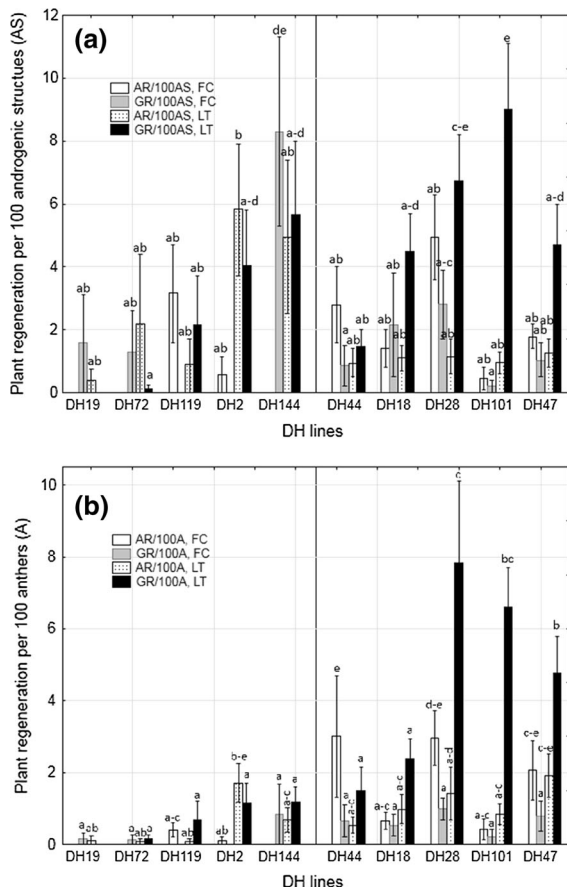
Statistical and QTL analysis

All statistical analyses were performed using STATISTICA version 10.0 (Stat Soft Inc., USA, 2011) package. For each tested variable the normal distribution of scores has been verified by Shapiro–Wilk test to validate the use of the parametric tests. The effect of tested variables was examined by multi-factor analysis of variance (ANOVA). Variables with non-normal distributed data were analyzed with non-parametric Kruskal–Wallis tests. Non-parametric Spearman's Rank-Order Correlation coefficients (R) were used to analyze the association between variables with non-normal distribution of scores.

Genetic map for used in the study triticale population was constructed by Tyrka et al. (2011) and consists of 1568 markers (155 SSRs, 28 AFLPs, 1385 DArTs) distributed within 21 linkage groups. The map covers 2397 cM and the average distance between markers is 4.1 cM.

QTL analysis was performed with Windows QTL Cartographer version 2.5 (Wang et al. 2012) by using two methods: single marker analysis (SMA) and composite interval mapping (CIM). A LOD threshold of 3.0 was used to detect QTL; however, in the case of consistent detections a LOD score beyond 2.0 was considered significant. The percentage of phenotypic variation was calculated with a single factor regression (R^2).

Fig. 1 The effect of low temperature tillers pre-treatment on plant regeneration in anther cultures of ten DH lines of triticale **a** calculated per 100 androgenic structures (AS), **b** calculated per 100 anthers. DH lines are lined according to an increasing androgenic responsiveness from recalcitrant (DH19–DH144) to responsive (DH44–DH47). Presented data are the mean ± SD of ten biological replications for each DH line. Mean values marked with the same letter within the group do not differ significantly according to Duncan's multiple range test (p ≤ 0.05). *AR/100AS* the number of albino regenerated plants per 100 androgenic structures, *GR/100AS* the number of green regenerants per 100 androgenic structures, *AR/100A* the number of albino regenerated plants per 100 anthers, *GR/100A* the number of green regenerants per 100 anthers, *FC* freshly cut tillers, *LT* cold treated tillers (3 weeks at 4 °C)

Results

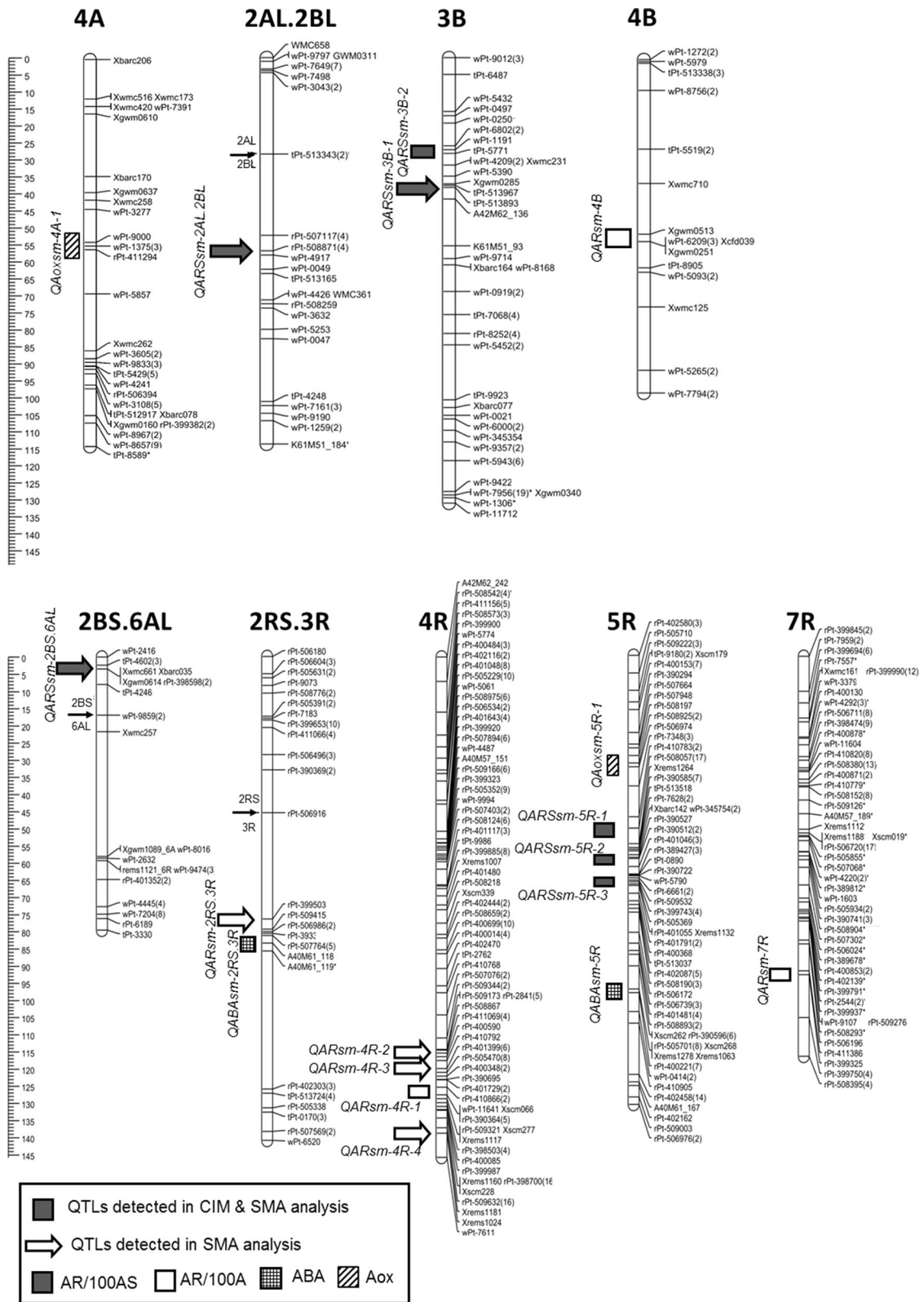### Green/albino plant production in triticale anther cultures

In all experimental replicates of the main study circa 53 % of regenerated plants were albinotic. Variation analysis (Table 1) showed significant influence

(p ≤ 0.001) of both donor plant genotype and experimental replication on regeneration effectiveness. The effect of interaction between variables was not significant. To characterize precisely the regeneration ability of produced androgenic structures, six parameters were recorded and analyzed (Table 2). Received data indicate that parental lines were similar in respect of albino regenerants frequency (AR/100AS) whereas the number of regenerated green plants (GR/100AS) was almost 2-fold higher in the case of DH line 'Modus' (Table 2). The significant differences were also found in parameters characterizing final androgenic efficiency (GR/100A, AR/100A), where both green and albino plant production were more than 4-fold higher for paternal genotype 'Modus'. However, very similar GR/AR ratios suggest that this difference is the result of variation in the number of produced AS and their total regeneration ability. In the studied DH progeny a much wider variation with extremes significantly exceeding characteristics of parental genotypes was observed (Table 2). Nevertheless, mean effectiveness of green and albino plant formation was very similar.

The results of plant regeneration in anther cultures of ten selected DH lines significantly different in their androgenic potential are presented on Fig. 1a, b. It could be seen that both highly recalcitrant and highly responsive DH lines of triticale were characterized by rather low regeneration ability as only up to 9 and 6 % of AS regenerated green and albino plants, respectively. The final maximal effectiveness of androgenesis calculated per 100 anthers, what correspond with an average-sized triticale spike, gained maximally 8 green and 3 albino plants. Regeneration effectiveness was extremely low in control anther cultures started without LT tillers pre-treatment, both in the case of recalcitrant and responsive DH lines (Table 3). Similarly low level of regeneration concerned also standard anther cultures (LT) of recalcitrant DH lines. In contrast, in anther cultures of responsive DH lines low temperature increased 3-fold the frequency of green regenerants. The albino plant production remained not influenced what resulted in 7-fold increase of GR/AR (AS) ratio (Table 3).

Mapping QTL for albino plants

The QTL analyses were performed separately for each replication but only consistent results are presented in

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4A

2AL.2BL

3B

4B

2BS.6AL

2RS.3R

4R

5R

7R

QTLs detected in CIM & SMA analysis

QTLs detected in SMA analysis

AR/100AS ☐ AR/100A ▦ ABA ▨ Aox

◀**Fig. 2** Linkage map 'Saka 3006' × 'Modus' and location of QTLs for albino plant regeneration. Results of composite interval mapping for each studied traits (*AR/100AS* the number of albino regenerated plants per 100 androgenic structures, *AR/100A* the number of albino regenerated plants per 100 anthers, *ABA* abscisic acid accumulation, *Aox* antioxidative activity). *Boxes* indicate significant QTLs identified with both methods: SMA and CIM. The *arrows* indicate significant markers revealed only in SMA. Distances between markers are in centiMorgans (cM)

this paper (QTLs identified at least in two out of three experimental replications).

The CIM analysis revealed seven QTLs localized on five chromosomes from B and R subgenomes associated with albino plants regeneration (Table 4; Fig. 2). All of them were confirmed by SMA analysis as regions containing the markers significantly linked to the studied trait. Additionally, the results of SMA analysis indicted seven statistically significant (p < 0.01) markers on chromosomes 2AL.2BL, 3B, 2BS.6AL, 2RS.3R and 4R (Table 5).

The most significant QTLs associated with AR/100AS were located on chromosome 5R with high LOD scores from 3.1 to 5.6. The highest percentage of phenotypic variance, almost 17 %, was explained by the locus *QARSsm-5R-1*. All of these QTLs show a positive effect when the allele comes from cv. 'Modus'. The LOD value of the third QTL

Table 3 Mean efficiency of regeneration in anther cultures of ten DH lines significantly varied in respect of anther culture responsiveness initiated with (LT) or without (FC) low temperature tillers pretreatment. Data are the mean ± SD for five DH lines with 10 biological replications for each DH line (petri dish containing 100 anthers isolated from individual spike)

	PreT	GR/100AS	AR/100AS	GR/AR (AS)	GR/100A	AR/100A	GR/AR (A)
Mean for 'recalcitrant' DH lines	FC	1.0 ± 0.6	1.1 ± 0.6	0.9	0.1 ± 0.1	0.2 ± 0.1	0.5
	LT	2.3 ± 0.7	2.8 ± 0.9	0.8	0.6 ± 0.2	0.5 ± 0.1	1.2
Mean for 'responsive' DH lines	FC	1.7 ± 0.6	2.4 ± 0.4	0.7	0.7 ± 0.2	1.7 ± 0.3	0.4
	LT	5.1 ± 0.7*	1.1 ± 0.2	4.9	4.2 ± 0.5*	1.1 ± 0.2	3.8

GR/100AS, number of green regenerants (GR) per 100 androgenic structures (AS); AR/100AS, the number of albino regenerants (AR) per 100 androgenic structures (AS); GR/100A, number of green regenerants (GR) per 100 anthers (A); AR/100A, the number of albino regenerants (AR) per 100 anthers (A); GR/AR (AS), the ratio between GR and AR frequency calculated per 100AS; GR/AR (A), the ratio between GR and AR frequency calculated per 100 A

* Data marked with asterisk are significantly different according to non-parametric Kruskal–Wallis tests (p ≤ 0.05)

Table 4 Main characteristics of significant QTLs for the studied traits in triticale mapping population of 90 DH lines of winter triticale mapping population 'Saka 3006' × 'Modus'

Trait	QTL	Flanking markers (cM)[a]	LOD	R^2 (%)	Add	Marker linked to a QTL	
						Marker name[b]	p
AR/100AS	*QARSsm-3B-1*	wPt-1191 (26)–tPt-5771 (28)	2.9	8.28	1.24	tPt-5771	**
	QARSsm-5R-1	tPt-7245 (50)–Xrems1264 (54)	5.6	16.85	−1.75	rPt-508057	****
	QARSsm-5R-2	Xbarc142 (58)–rPt-509048 (59)	3.1	11.22	−1.46	wPt-345754	****
	QARSsm-5R-3	rPt-509532 (65)–rPt-505369 (66)	4.3	12.98	−1.57	rPt-399743	****
AR/100A	*QARsm-4B*	Xgwm0513 (51)–Xgwm0251 (53)	4.8	17.56	1.16	Xgwm0251	**
	QARsm-4R-1	wPt-6160 (125)–wPt-11641 (127)	3.5	11.76	−1.14	wPt-11641	***
	QARsm-7R	rPt-411386 (91)–rPt-399325 (92)	5.6	17.34	−1.44	rPt-399325	***

AR/100AS, the number of albino regenerated plants per 100 androgenic structures; AR/100A, the number of albino regenerated plants per 100 anthers; LOD, logarithm of the odds for peaked marker; R^2 (%), % of phenotypic variance explained by the QTL, Add additive effect of the 'Saka 3006' allele; p, significance in SMA analysis **, ***, **** p < 0.01, 0.001, 0.0001, respectively

[a] cM position of the marker on the genetic map of a given chromosome

[b] The closest marker to LOD peak

Table 5 Additional significant markers associated with studied traits revealed in single marker analysis

Trait	QTL	Marker (cM)	p
AR/100AS	*QARSsm-2AL.2BL*	rPt-508871 (56)	**
	QARSsm-3B-2	Xgwm0285 (37)	**
	QARSsm-2BS.6AL	tPt-4602 (2)	***
AR/100A	*QARsm-2RS.3R*	rPt-399503 (76)	**
	QARsm-4R-2	rPt-411069 (115)	****
	QARsm-4R-3	rPt-401399 (119)	****
	QARsm-4R-4	Xrems1024 (138)	***

AR/100AS, the number of albino regenerated plants per 100 androgenic structures; AR/100A, the number of albino regenerated plants per 100 anthers; p, significance in SMA analysis **, ***, **** p < 0.01, 0.001, 0.0001, respectively

(*QARSsm-3B-1*) was 2.9 and peaked at DArT marker tPt-5771. Moreover, localized close to described QTL connected with AR/100AS. Although, the most significant marker (p < 0.001) was found in the telomeric region of the short arm of the chromosome 2BS.6AL (Table 5; Fig. 2).

The major QTLs associated with the second studied variable AR/100A were localized on four chromosomes: 4B, 2RS.3R, 4R and 7R (Fig. 2). It seems that one of the most important genomic region for this parameter is interval between rPt-411386 (91 cM) and rPt-399325 (92 cM) markers on chromosome 7R. This QTL explained over 17 % of phenotypic variation with values of LOD 5.6. The most QTLs were detected on chromosome 4R. One of them was revealed by CIM method and other three markers were found in SMA analysis (Tables 4, 5). Moreover, for all these QTLs the positive effect was inherited from 'Modus'. The next QTL, which peaked at SSR marker Xgwm0251, was detected on chromosome 4B and only in this case the additive effect comes from 'Saka 3006' allele.

Nonparametric Spearman Rank Correlation

Correlation analysis was performed with the use of Spearman Rank test (p ≤ 0.05) for the data received in additional experiments with ten DH line of triticale used as the object of the study.

Presented here (Table 6), are the results concerning the associations between parameters of plant regeneration effectiveness (GR/100AS, AR/100AS, GR/AR (AS)), final androgenesis effectiveness (GR/100A,

AR/100A, GR/AR (A)), endogenous level of plant growth regulators (IAA, IBA, *trans* and *cis* isomers of zeatin (*t*Z, *c*Z) and zeatin riboside (*t*ZR, *c*ZR), kinetin (Kin) and abscisic acid (ABA), generation of ROS (superoxide anion ($O^{\cdot-}$), hydrogen peroxide (H_2O_2)) as well as the activity of antioxidative enzymes (superoxide dismutase (SOD), catalase (CAT), peroxidase (POX)) and total activity of low molecular weight antioxidants (AntiOx).

Due to the fact that recalcitrant DH lines were much more varied in respect of regeneration ability in comparison with responsive DH lines, higher number of significant associations has been found when the statistical analysis was done separately for each group of DH lines.

Correlation analysis for responsive DH lines in the control conditions (FC, Table 6) detected significant associations between GR/AR (AS) and CAT activity, AR/100A and AntiOx, and GR/AR (A) and H_2O_2 generation suggesting negative effect of oxidative stress and positive influence of more effective antioxidative system. On the contrary, in the group of recalcitrant DH lines, higher activity of POX was negatively correlated with green plant regeneration (GR/100AS) and final green plant production (GR/100A) and simultaneously positively correlated with albino plant regeneration/final effectiveness (AR/100AS, AR/100A). Significant correlations were found also between *c*Z, *c*ZR and both green and albino plant regeneration/final production (GR/100AS, AR/100AS, GR/100A, AR/100A). The level of *cis* isomers of Z and ZR correlated negatively with green plants regeneration/final effectiveness (GR/100AS, GR/100A) and positively with albino plant regeneration/final effectiveness (AR/100AS, AR/100A). Negative correlation was found also between ABA level and albino plant regeneration/final effectiveness (AR/100AS, AR/100A).

For responsive DH lines after LT tillers pretreatment, the effectiveness of green plant regeneration (GR/100AS) was positively correlated with AntiOx and negatively correlated with ABA concentration. Albino plant regeneration/final effectiveness (AR/100AS, AR/100A) looks to be negatively influenced by higher level of IAA whereas GR/AR (AS) negatively correlated with the activity of SOD. Regeneration effectiveness of recalcitrant genotypes could be determined mainly by hormonal background. Green plant regeneration (GR/100AS) was positively

Table 6 Spearman Rank Correlation test ($p \leq 0.05$) for the data received with ten DH line of triticale significantly different in androgenic responsiveness

Parameter	Endogenous hormone levels								Oxidative stress		Antioxidative activity			
	ABA	IAA	IBA	tZ	cZ	tZR	cZR	Kin	$O^{\cdot-}$	H_2O_2	SOD	CAT	POX	AntiOx
Responsive DH lines—control														
GR/100AS														
AR/100AS														
GR/AR (AS)												0.90		
GR/100A														
AR/100A														−0.90
GR/AR (A)										−0.90				
Recalcitrant DH lines—control														
GR/100AS					−0.97		−0.97						−0.97	
AR/100AS	−0.89				0.89		0.89						0.89	
GR/AR (AS)														
GR/100A					−0.97		−0.97						−0.97	
AR/100A	−0.89				0.89		0.89						0.89	
GR/AR (A)														
Responsive DH lines—cold treated														
GR/100AS	−0.90													0.90
AR/100AS		−0.97												
GR/AR (AS)											−0.90			
GR/100A														
AR/100A		−0.90												
GR/AR (A)														
Recalcitrant DH lines – cold treated														
GR/100AS		−0.90			0.90		0.90	−0.90						
AR/100AS						0.90		−0.90						
GR/AR (AS)			−0.95							0.90				
GR/100A		−0.90			0.90		0.90	−0.90						
AR/100A				0.89	0.89			−0.89			−0.89	−0.89		−0.89
GR/AR (A)			−0.95											

GR/100AS, the number of green regenerants per 100 androgenic structures; AR/100AS, the number of albino regenerants per 100 androgenic structures; GR/AR (AS), the ratio between GR and AR frequency calculated per 100 AS; GR/100A, the number of green regenerants per 100 anthers; AR/100A, the number of albino regenerants per 100 anthers; GR/AR (A), the ratio between GR and AR frequency calculated per 100 A; *ABA* abscisic acid; *IAA* indole-3-acetic-acid; *IBA* indole-3-butyric acid; *tZ* trans zeatin; *cZ* cis zeatin; *tZR* trans zeatinriboside; *cZR* cis zeatinriboside; *Kin* kinetin; $O^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; *SOD* superoxide dismutase; *CAT* catalase; *POX* peroxidise; *AntiOx* low molecular weight antioxidants

correlated with *cZ* and *cZR* levels and negatively correlated with IAA. Higher concentration of Kin seems to diminish both green and albino plant regeneration (GR/100AS, AR/100AS). The content of IBA correlated negatively with GR/AR (AS) whereas *tZR* positively correlated with AR/100AS. Quite unexpectedly, the final effectiveness of albino plant production (AR/100A) was correlated with both hormonal balance and the capacity of antioxidative system. Additionally to the parameters that seems to be significantly associated with albino plant regeneration (*tZR*, Kin), positive correlation with *tZ* and negative assoc iations with SOD, CAT and AntiOx activities were also detected.

Generally, no correlation was found between parameters, which describe albino and green plant regeneration/final production ability.

Discussion

Factors determining the efficiency of green and albino plant regeneration

The interest in DHs as the models for basic research and its practical exploitation in breeding programs is still growing. Due to that, a lot of effort has been put in an identification of the most important factors controlling androgenic responsiveness and effective production of DH lines. Although, numerous research papers, review articles, and books covering DH techniques have been published over the last decades, there are still some limitations in deployment of this technology for many important crop species. To overcome this problem, new molecular methods, which use marker genes associated with a specific trait, could be very helpful. One of the most important advantage of QTLs analysis is the possibility to identify genes of relatively small effects that do not produce individually recognizable phenotypes (Thomas et al. 2003). Another benefit comes from the ability to distinguish between stable QTLs detected in different genotypes/environments/seasons from those which reveals high level of variation resulting from QTL × environment × genotype interactions. This, seems to be very important as the quality of donor plant and conditions of its growth could significantly influence the final effectiveness of DH production (Datta 2005).

In triticale, low regeneration ability and high frequency of albino regenerants are the main limitations to incorporation of DHs into breeding programs (González et al. 1997; Mozgova et al. 2012; Pauk et al. 2000; Ponitka et al. 1999). Without chlorophyll, the primary pigment involved in light harvesting and its transformation into chemical energy, albinotic plants cannot survive in natural environment, and do not represent any agronomic value. Tuvesson et al. (2003) reported that albino plantlets have outnumbered green regenerants 2.6-fold, whilst in other studies, albinos have represented 42 % of all regenerated plantlets (Warzecha et al. 2005). In the main experiment

presented, the mean number of regenerating AS gained 18.5 % with the frequency of albino plants at about 53 %. Even worse results were received in additional experiment performed on ten selected DH lines (6.3 % of regenerating AS among which 40 % regenerated albino plants).

In some cases, QTLs connected with albino plants were significantly conjugated with loci associated with green regenerants what explain strong correlations detected between studied variables by (Muñoz-Amatriaín et al. 2008). In our study however, the correlation between green and albino plants formation was not found suggesting that the cellular mechanisms that control these processes are different. Similarly, González et al. (2005) and He et al. (1998) also revealed no correlation between green and albino plant regeneration in triticale and rice anther cultures, respectively.

Rather low variation between responsive DH lines in respect of regeneration parameters explain the fact that the majority of detected associations concerns recalcitrant genotypes. However, as the number of produced AS was in this group of genotypes usually very low, high variation in regeneration parameters does not mean the same in respect of absolute values. Another question is if found correlations signalize a real causal link between tested parameters. Despite all these concerns some interesting conclusions can be drawn from received data.

First, drastic change in detected associations observed as result of LT tillers treatment confirmed earlier hypothesis (Żur et al. 2014, 2015) that this stress factor stimulate androgenesis by affecting cellular redox status and hormonal balance (Baek and Skinner 2012; Bonnecarrere et al. 2011; Guo et al. 2006). Among ROS, H_2O_2 seems to be directly involved in androgenesis initiation (Żur et al. 2014), proper AS formation and plant regeneration. Without LT, osmotic stress induced by anther isolation acts as the signal stimulating microspore reprogramming. However, too high H_2O_2 generation diminish cell viability, so microspore survival and further proper development depends on the antioxidative system efficiency. Correlation analysis suggests also distinct difference in the role of two H_2O_2-decomposing enzymes: positive effect of CAT, stimulating GR frequency in anther cultures of responsive DH lines and negative effect of POX stimulating albino and detrimental for green plant regeneration efficiency in

recalcitrant DH lines. This effect could be explained by different properties of these enzymes (Dat et al. 2000; Mittler 2002). It is supposed that CAT is responsible for scavenging high amounts of H_2O_2 especially during stress whereas POX enabling the fine modulation of its level in specific locations, probably for signalling purposes. Despite the facts that exogenous stress can drastically reduce the number of plastids in microspores (Caredda et al. 2000) and that in plants chilling temperatures can reduce up to 90 % of chlorophyll content (Tewari and Tripathy 1998) all presented data together with significantly higher CAT activity detected in anthers isolated from freshly cut tillers of recalcitrant DH lines in comparison with responsive DH lines (Żur et al. 2014) suggest that some level of H_2O_2 is important not only for microspore reprogramming but also for successful transition from a non-photosynthetic proplastid to a functional chloroplast. Similarly, Makowska and Oleszczuk (2014) concluded that stress is capable of reprogramming a microspores themselves but not necessarily the plastids. This may lead to the production of androgenic embryos containing plastids that follow the gametophytic development, what leads directly to regeneration of chlorophyll-deficient plants.

The situation changes after LT tillers treatment, which stimulated antioxidative system activity (SOD, CAT, POX) in responsive DH lines whereas in recalcitrant genotypes increased SOD activity was accompanied with diminished CAT and POX activity (Żur et al. 2014). In this case positive correlation found between GR regeneration and low molecular weight antioxidant activities in cultures of responsive DH lines seems to be in agreement with data published by Asif et al. (2013). They reported positive effect of glutathione, one of the most effective, especially in plastids, non-enzymatic ROS scavengers, on plant regeneration in isolated microspore cultures of triticale and wheat. In this experiment, negative correlation of GR/AR (AS) with SOD activity in responsive DH lines and positive correlation with H_2O_2 in recalcitrant genotypes suggest that higher generation of H_2O_2 stimulate green at the cost of albino plant regeneration. Generally however, LT treatment seems to change the main importance for effective androgenesis induction from redox status to endogenous hormonal composition of the anthers.

It is well known that chloroplast biogenesis is controlled by cytokinins (CKs) and exogenously applied CKs can stimulate development of chloroplasts from proplastids, amyloplasts, and etioplasts (after Polanska et al. 2007). It has been revealed that this effect was mediated by up-regulation of the expression of some plastid-related genes, both of plastid and nuclear origin. Among them were the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPCO) and chlorophyll *a*/*b* binding protein (Parthier 1989). This group of hormones is involved also in almost all aspects of chloroplasts function: ultrastructure remodelling, enzyme activities, accumulation of photosynthetic pigments and photosynthetic activity (Zubo et al. 2008). In this experiment however, in anthers of recalcitrant DH lines isolated without LT treatment, GR regeneration and final production effectiveness were negatively correlated with prevailing form of cytokinins (cZ, cZR) detected in triticale anthers (Żur et al. 2015). On the contrary these plant hormones positively correlated with AR regeneration/final production effectiveness. Similarly, the presence of various anomalies in the ultrastructure of chloroplasts, from transgenic tobacco was the result of overproduction of endogenous CKs (Synkova et al. 2006). Surprisingly, after LT the same type of CKs positively correlated with GR regeneration/final production, whereas *trans* isomers of Z and ZR seems to have positive effect on AR regeneration/final production effectiveness. As LT increased endogenous content of these phytohormones in anthers of studied genotypes of triticale (Żur et al. 2015) some distinct changes in hormone reception/signaling pathways could be suspected.

In anthers of responsive DH lines isolated from freshly cut tillers (FC), no correlation was found between hormone concentration and the regeneration ability of produced AS. Disturbed hormone balanced induced by LT resulted in negative associations between ABA and GR regeneration and IAA and albino plant regeneration/final production effectiveness. It is well known that in plants, ABA plays a key role in stress response and also together with cytokinins take part in the development of the photosynthetic apparatus (Kravtsov et al. 2011). Just recently, trying to overcome the problem of albinism in microspores and anther culture of horse chestnut Calic et al. (2013) found that addition of low

concentration (0.01 mg l^{-1}) of ABA to media reduced this obstacle. On the other hand, high concentration of ABA (8 mg l^{-1}) in media decreased the induction and regeneration ability of calluses obtained from wheat mature embryos (Fazeli-nasab et al. 2012). Previous results, where weak/moderate but significant negative correlation between the concentration of ABA in triticale anthers and parameters of regeneration efficiency was found (Żur et al. 2012) were confirmed only fragmentary in presented study as ABA seems to diminished albino plant regeneration. As in response to exogenous ABA application increased level of hydrogen peroxide (H$_2$O$_2$) and increased antioxidative system activity were observed (Agarwal et al. 2005; Jiang and Zhang 2001) it could be the mechanism of indirect ABA involvement in triticale microspore embryogenesis.

Localization of genomic regions controlling albino plant regeneration

One way to overcome the problem of albinism is the localization of the regions and identification of genes which control the formation of chlorophyll-deficient plants both in plastid and nuclear genome. Although, large defects in the ptDNA structure are often detected among albino plants regenerated, it cannot be treated as the primary cause of albinism. Changes were also identified in the transcription levels of the nucleus-coded chloroplast-localized proteins (Dunford and Walden 1991) what indicates the important role of the nuclear genome in proper plastid function. Based on crosses between albinism-susceptible and albinism-resistant cultivars, it has been concluded that the genes responsible for this character are inherited in a Mendelian fashion and hence, they must be nuclear encoded (Larsen et al. 1991). Moreover, recent studies confirmed that the phenomenon of albinism is genetically determined (Clément et al. 2005; Kravtsov et al. 2011; Muñoz-Amatriaín et al. 2008) and that the chloroplast development in hybrid genotypes was mostly influenced by nuclear factors (Kumari et al. 2011). Therefore, studies focused on nuclear genome, for example on the identification of genetic loci associated with the ratio of green to albino regenerants obtained via androgenesis seems to be very reasonable.

Majority of studies aimed at the identification of QTLs controlling the regeneration phase of androgenesis have been focused on the ability to form green plants. The works on wheat, barley and rice have identified many different genome regions affecting this trait (Chen et al. 2007; Grosse et al. 1996; Manninen 2000; Torp et al. 2001). Localization of genomic regions associated with green plant regeneration ability in triticale has been reported by González et al. (2005) and Krzewska et al. (2012). Only limited number of papers focused on QTLs associate with albino plant regeneration and in all of them barley have been used as the plant model (Bregitzer and Campbell 2001; Muñoz-Amatriaín et al. 2008).

In presented study, QTL analysis by using both methods—SMA and CIM identified 14 chromosomal regions among subgenome B (4 QTLs) and R (10 QTLs) involved in albino plants formation during anther culture in winter triticale (Fig. 2). The biggest number of OTLs connected with AR/100A were localized in subgenome R and the positive effect originated from 'Modus' allele. It could be connected with general recalcitrance in regard to the in vitro culture response in rye (Ma et al. 2003; Ma and Pulli 2004; Targońska et al. 2013). Generally problems associated with rye anther and microspore culture are poor embryogenic callus induction, low green plant regeneration and high proportion of albinos (Ma and Pulli 2004). The lack of response was found to be controlled by at least two interacting genes (Rakoczy-Trojanowska and Malepszy 1995). Bolibok et al. (2007) reported that nine putative QTLs for rye tissue culture response have been mapped on chromosomes 1R, 4R, 5R, 6R and 7R. In our study four of QTLs connected with albino plants formation were found on chromosome 4R. Moreover, one QTL *QARsm-4R-4* was located in the same region as QTL associated with final green plant regeneration ability—*QGRsm4R-2* (Krzewska et al. 2012). Three of them: *QARsm-4R-1*, *QARsm-4R-2*, *QARsm-4R-3* were conjugated with QTLs controlling final regeneration ability (Krzewska et al. 2012). Similar results were received by González et al. (2005), who also detected QTLs for final efficiency of green plants regeneration on chromosome 4R on triticale genome. Moreover, callus induction and somatic embryogenesis ability in rye tissue cultures are also determined by loci located on this chromosome (Bolibok et al. 2007).

Three regions on chromosome 5R had a major effect on albino plant regeneration ability. One of them *QARSsm-5R-1* with high LOD value (5.6) explains almost 17 % of phenotypic variation. Earlier

reports revealed that QTLs controlling green plant formation are located on chromosome 5R in rye (Grosse BA, Deimling S, Geiger HH Mapping of genes for anther culture ability in rye by molecular markers. In: Vortr P?anzenzeuchtg 1996), 5B in wheat (Torp et al. 2001) or 5H in barley (Bregitzer and Campbell 2001; Muñoz-Amatriaín et al. 2008). This could indicate a possible role of cereal group 5 chromosomes in the control of green and albino plant regeneration in tissue culture.

According to our research, the triticale chromosome 7R seems to be connected with albino plant regeneration as well as with final efficiency of androgenesis (González et al. 2005; Krzewska et al. 2012). QTL *QARsm-7R* was mapped in the same chromosome region as *QRsm-7R-1* controlling the total number of regenerants per 100 anthers (Krzewska et al. 2012). Bolibok et al. (2007) found on this chromosome locus influencing callus induction in the culture of rye immature inflorescences. What is more, chromosomes: 3RS, 4RL and 5RL in rye are identified as carrying the QTLs affecting chlorophyll content (Milczarski and Masojc 2002). The locus *QChc-3R.1* had approximate position as QTL *QARsm-2RS.3R* connected with albino plant regeneration in our study.

SMA revealed that one of DArT markers (rPt-399503) on chromosome 2RS.3R was significantly associated with QTL for AR/100A. Other authors have reported the involvement of loci on this chromosome in the green plant regeneration capacity from microspores in triticale (González et al. 2005), the photosynthetic viability and general androgenesis process in rye (Grosse et al. 1996).

A few regions in subgenome B in triticale seem to control the regeneration yield during androgenesis. However, QTLs located in wheat subgenome had minor effects on studied traits and all positive alleles came from maternal genotype 'Saka 3006'. Torp et al. (2001) identified three QTLs for green plant percentage on chromosomes: 2AL, 2BL and 5BL in DH mapping population of wheat. Other studies on wheat also revealed QTLs associated with green plant regeneration on chromosome 5BL (Zhang et al. 2003) and on chromosomes 2A and 2B (Anca et al. 2007).

Our result did not confirm the existence of QTL on chromosome 5B responsible for green or albino plant regeneration, but we found one QTL on chromosome:

2AL.2BL and two QTLs on chromosome 3B associated with albino plant formation ability (AR/100AS). Moreover, QTL analysis identified one chromosomal region for AR/100A with high LOD score (4.8), explaining up to 17.6 % of the phenotypic variance on chromosome 4B.

QTL analysis seems to prove ROS and ABA involvement in androgenesis regulation (Fig. 2). It occurred that one chromosomal region controlling AntiOx activity localized on chromosome 4A (data not published) was conjugated with locus *QRASsm-4A-1* associated with total regeneration ability in triticale (Krzewska et al. 2012) and another QTL on chromosome 5R connected with antioxidative activity (data not published) was mapped close to *QARSsm-5R-1* responsible for albino plants regeneration.

Additionally, some QTLs associated with ABA accumulation in triticale anthers (Żur et al. 2012) were localized on the same chromosomes as QTLs connected with albino plant formation (Fig. 2). The example could be QTL *QARsm-2RS.3R* was conjugated with marker rPt-509415 significantly associated with QTL for endogenous level of ABA whereas *QAchsm-5R* was mapped on chromosome 5R the same as loci *QARSsm-5R-3*. It could be supposed that close localization of QTLs associated with in vitro embryogenesis, ABA and antioxidative system activity is not meaningless and suggests a complex network of interactions.

In conclusion, the major QTLs controlling albino plant creation are located on rye chromosomes, where the positive effect was originated from 'Modus' allele. QTLs mapped on chromosomes B had minor effect on studied trait and show a positive effect when the allele comes from maternal genotype.

The analysis of correlations found between albino/green plant regeneration ability, hormonal balance and antioxidative system activity confirmed earlier hypothesis (Żur et al. 2014, 2015) that LT stimulate androgenesis by affecting cellular redox status and hormonal balance. It seems that in anthers isolated without LT treatment, some critical level of H_2O_2 generation was a prerequisite for successful transition from a non-photosynthetic proplastid to a functional chloroplast. LT treatment seems to change the main importance for effective androgenesis induction from redox status to endogenous hormonal composition of the anthers.

The presented results made the next step in widening the knowledge of molecular background of albino plant formation during androgenesis in winter triticale. Markers identified as linked with QTLs controlling studied traits could help breeders in the selection of best genotypes and optimizing the inputs needed for DH production. Nonetheless, obtained results should be confirmed and validated in different genetic backgrounds, by testing the reliability of markers associated with QTLs to predict phenotype.

Author contribution MK co-performed the experiments, co-analyzed the data and co-wrote the manuscript. ICz-M, ED, GG-P performed the experiments and collected the raw data. IŻ designed the experiments, co-analyzed the data and co-wrote the manuscript.

Compliance with Ethical Standards

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