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Plastid differentiation during microgametogenesis determines green plant regeneration in barley microspore culture

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ABSTRACT

Developing plants from *in vitro* culture of microspores or immature pollen grains (androgenesis) is a highly genotype-dependent process whose effectiveness in cereals is significantly reduced by occurrence of albino regenerants. Here, we examined a hypothesis that the molecular differentiation of plastids in barley microspores prior to *in vitro* culture affects the genotype ability to regenerate green plants in culture. At the mid-to-late uninucleate (ML) stage, routinely used to initiate microspore culture, the expression of most genes involved in plastid transcription, translation and starch synthesis was significantly higher in microspores of barley cv. 'Mercada' producing 90% albino regenerants, than in cv. 'Jersey' that developed 90% green regenerants. The ML microspores of cv. 'Mercada' contained a large proportion of amyloplasts filled with starch, while in cv. 'Jersey' there were only proplastids. Using additional spring barley genotypes that differed in their ability to regenerate green plants we confirmed the correlation between plastid differentiation prior to culture and albino regeneration in culture. The expression of *GBSSI* gene (*Granule-bound starch synthaseI*) in early-mid (EM) microspores was a good marker of a genotype potential to produce green regenerants during androgenesis. Initiating culture from EM microspores that significantly improved regeneration of green plants may overcome the problem of albinism.

1. Introduction

The microspores released from the tetrad undergo microgametogenesis to form mature pollen grains [1]. First, the vacuolisation of microspores and the nucleus displacement to an acentric position occur, indicating the stages of the microspore progress from the early uninucleate microspores in which the nucleus is localised in the centre to the late uninucleate microspores with a peripheral position of enlarged nucleus [2]. Microspore development is followed by the first pollen mitosis, which results in the formation of two unequal-sized daughter cells – a vegetative and a generative cell [3]. The mature pollen grain consists of two sperm cells enclosed in a vegetative cell that accumulates starch deposited in the starch grains [1].

The microspores of cereals contain proplastids – undifferentiated, colourless, small plastids that do not play any metabolic function but they give rise to all types of plastids [4]. During the progression of pollen development, the plastids in the vegetative cell differentiate into amyloplasts and serve as storage for starch needed for the germination of pollen grains and pollen tube growth [5,6]. In cereals, the differentiation of proplastids into amyloplasts occurs in the vacuolated microspores [7,8], however, little is known about the molecular mechanisms that underlie this process. The molecular aspects of amyloplast formation, including gene expression and proteomic analyses, have only been reported for the endosperm tissue of rice and wheat [9–12] and potato tubers [13–15]. Initiation of amyloplast differentiation requires transcriptional and translational activity within the plastids, which serve as a checkpoint of proplastid to amyloplast transition and a plastid-to-nucleus retrograde signal [16]. The retrograde signalling controls the induction of amyloplast development by activating the nuclear-located genes encoding the starch biosynthesis enzymes such as glucose-1-phosphate adenylyltransferase (Agp), granule-bound starch synthase (GBSS), starch synthase (SS) and starch-branching enzyme (Sbe) [17–19]. However detailed mechanisms of the signal transduction and the stage of plastid differentiation during pollen development remain unclear.

By treating vacuolated microspores with stress factors such as high

Abbreviations: E, early; BN, immature binucleate pollen; EM, early-mid; ML, mid-to-late; P, mature pollen grain; ptDNA, plastid DNA; TEM, transmission electron microscope

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or low temperatures or starvation, it is possible to change their developmental pathway from a gametophytic into a sporophytic one and to induce microspore embryogenesis [20]. Developing plants from *in vitro* culture of male gametophytes (androgenesis) is the most effective method of producing doubled haploids (DH) [21–23]. Doubled haploidy is the essential technology utilised to accelerate the development of new cultivars in breeding programmes [24–26]. Therefore, an effective protocol for producing DH is the main concern of androgenetic studies.

Among many factors that affect the efficiency of androgenesis, the stage of microspore development is crucial because of the narrow reprogramming window [27]. It was demonstrated that the mid-to-late uninucleate (ML) stage of microspore development is the most effective stage to induce androgenesis in most crops [21]. However, androgenesis is a highly genotype-dependent process whose effectiveness in cereals is severely limited by the regeneration of albino plants that lack the chlorophyll in normally green tissues. The high level of albino plants among androgenic regenerants, sometimes reaching 100%, has been reported for all major cereals including barley [28,29], triticale [30], wheat [31,32] and rice [33]. For this reason, albinism often limits the use of agronomically important genotypes in the breeding programmes that utilise doubled haploidy.

Deletions in the plastid DNA of albino plants have been suggested as the main cause of altered plastid formation in wheat [34–36], rice [37,38], barley [35,39,40] and triticale [36]. Studies of chloroplast development in anther culture of winter barley cv. ‘Igri’ that mostly regenerates green plants and spring cultivars that mostly produce albino plants indicated that the degradation of the plastid genome was the cause of albino formation [41–43]. However, in wheat anther cultures only a part of the examined albino regenerants contained altered plastid DNA, although all of them showed changed transcriptome profiles compared to the green plants [44]. In barley, a microarray analysis of pretreated anthers derived from DH lines that differed in their green/albino plant ratio identified 21 genes related to albino formation [45]. Additionally, the QTLs for the level of green regenerants have been identified in barley [46,47] and triticale [48].

The possible impact of plastid differentiation during pollen development *in vivo* on the formation of albino plants *in vitro* has not yet been the subject of androgenic studies. Despite the many ultrastructural and molecular aspects that have been covered by research, the primary mechanism that underlies the formation of albino plants during androgenesis has not yet been revealed. Additionally, the genotype-dependence of the albino plant phenomenon remains unexplained.

In this study, we addressed the question of whether the genotype-dependent frequency of albino regenerants in barley microspore cultures *in vitro* is the consequence of plastid differentiation during pollen development *in vivo*. To verify this hypothesis we performed a detailed analysis of plastid differentiation during the successive stages of pollen development in spring barley cultivars that differed in their ability to regenerate green plants during androgenesis. Based on the analysis of expression profiles of genes involved in transcription, translation and starch biosynthesis in microspore plastids, together with observations of the plastid morphology using light microscopy and TEM, we demonstrate that the early activation of starch biosynthesis genes during microspore development *in vivo* is associated with the high rate of albino plants among *in vitro* regenerants. Our results show that initiating cultures from an earlier stage of microspore development, before amyloplast formation, might increase the contribution of green plants in isolated microspore cultures and thus overcome the problem of albinism in barley.

2. Material and methods

2.1. Plant material and growth conditions

Ten spring barley cultivars with divergent pedigrees were used: ‘Argento’ (Denmark), ‘Bordo’ (Poland), ‘Bruce’ (Canada), ‘Chevallier’ (Great Britain), ‘Jersey’ (Netherlands), ‘Justina’ (Germany), ‘Larker’ (USA), ‘Loosdorfer’ (Austria), ‘Mercada’ (Germany) and ‘Tamparkorn’ (Great Britain). Except for the six-row cvs. ‘Bruce’, ‘Larker’ and ‘Tamparkorn’, the rest of cultivars are two-row. Among these genotypes, ‘Argento’, ‘Bruce’, ‘Justina’ and ‘Mercada’ are used as fodder, whereas ‘Bordo’, ‘Chevallier’, ‘Jersey’, ‘Larker’, ‘Loosdorfer’, and ‘Tamparkorn’ are malting cultivars.

The donor plants for the *in vitro* culture and *in vivo* analyses were sown and grown at 18/16 °C for three weeks in a growth room with light intensity of 200 μM s^{-1} m^{-2} and then transferred to a growth chamber under controlled conditions at a day/night temperature of 17/14 °C, illumination 480-500 μM s^{-1} m^{-2} photon flux density and 16/8 h photoperiod. To initiate the *in vitro* culture, spikes containing microspores at the mid-to-late uninucleate (ML) stage of development were collected. To perform the experiments during the *in vivo* pollen development, microspores at the stages: early uninucleate (E), early-mid uninucleate (EM) and mid-to-late uninucleate (ML) as well as immature binucleate pollen (BN) and mature pollen grains (P) were used (Supplementary Data Fig. S1).

2.2. Isolated microspore culture

Tillers with spikes containing microspores at the ML developmental stage were collected when the distance between the flag and the penultimate leaf was 6 − 8 cm, depending on the genotype. The distance was determined experimentally and confirmed for each batch of spikes by acetocarmine staining of anthers excised from the middle part of one spike. Tillers were surface sterilised with 70% ethanol. The microspores were freshly isolated according to Coronado et al. [49]. Briefly, eight to ten spikes were homogenised in 20 ml 0.4 M mannitol using a Waring Variable-Speed Laboratory Blender (Waring Laboratory Science) twice for 20 s. The homogenate was filtered through 100 μm nylon mesh and the remaining spike tissue was re-blended and then re-filtered. The microspores were collected *via* centrifugation (110 × *g*; 10 min; 4 °C), resuspended in 5 ml 0.55 M maltose overlaid with 2 ml 0.4 M mannitol and centrifuged (110 × *g*; 10 min; 4 °C) once more. The microspores present in the interphase were collected and pretreated in SMB1 medium at 25 °C for 48 h, after adjustment of their density to 100,000 microspores per 1 ml in Petri dish. All media (Supplementary Data Table S1) were prepared according to Coronado et al. [49] and Kumlehn et al. [50]. The medium was then exchanged for KBP induction medium and cultures were incubated at 25 °C in the dark for seven days, followed by the addition of another 1 ml of fresh KBP medium. The incubation of the culture was then continued under the same conditions on a rotary shaker at 65 rpm. The developed multicellular structures were transferred onto KBPD differentiation medium for two weeks, then the microspore-derived embryos were placed on K4NB regeneration medium and cultured at 25 °C in the dark for five days, after which they were exposed to 100 μM s^{-1} m^{-2} of light with a 16/8 h photoperiod. The number of green and albino plants were counted after four weeks and the number of both types of regenerants per 100,000 microspores (one induction Petri dish) was estimated. At least three independent isolations of microspores were performed to initiate the *in vitro* culture for each cultivar.

For isolation of EM microspores, tillers showing 2 − 3 cm distance between the flag and the penultimate leaf were collected. The EM microspores for the *in vitro* cultures were isolated with some modifications because of their higher sensitivity to mechanical damage. The spikes were blended twice in 20 ml 0.4 M mannitol at a very low speed (Waring blender speed set between two and three on a ten-speed scale) for 30 s. The microspores were collected *via* centrifugation at 120 × *g*, and the viable microspores were collected at 130 × *g*.

2.3. Preparation of samples and extracting the nucleic acids

Microspores (E, EM, ML) from five-six spikes were isolated by blending (Waring Laboratory Science) them twice for 30 s at a low speed in 0.4 M mannitol followed by filtering through 100 μm nylon mesh. To isolate immature binucleate (BN) and mature pollen (P) grains, the florets and anthers, respectively, were excised and slowly blended in 0.4 M mannitol and then filtered. The suspension was centrifuged at 110 × g for 10 min in order to collect the microspores and pollen grains. The total RNA was isolated by grinding samples in a frozen mortar using a RNAqueous™ Total RNA Isolation Kit (Invitrogen) according to the manufacturer's instructions, in three independent biological repetitions. One biological repetition was represented by microspores isolated from approx. six spikes. To extract DNA, samples containing freshly isolated microspores, immature and mature pollen grains were homogenised in liquid nitrogen with glass beads (Sigma Aldrich) using a FastPrep Instrument (MP Biochemicals). DNA from freshly isolated microspores at the E, EM and ML stages and immature pollen grains (BN) were extracted using the C-TAB method [51]. The DNA from mature pollen grains was isolated according to Torres et al. [52]. The isolated DNA was treated with 10 μg of RNase at 37 °C for 45 min. The concentration and purity of the isolated samples (three biological repetitions, with microspores from approx. six spikes per repetition) were evaluated using an ND-1000 spectrophotometer (Thermo Fisher Scientific).

2.4. RT-qPCR and qPCR

One μg of total RNA per sample was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed in a 20 μl reaction volume using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) with random primers, according to the manufacturer's instructions. The obtained cDNA was diluted five-fold with water and used at a volume of 2.5 μl in RT-qPCR. In qPCR, 50 ng of DNA was used as the template to quantify the gene copy number. Both analyses were carried out in a 10 μl volume using a LightCycler® 480 SYBR Green I Master (Roche) in two technical repeats. The primers used in the analyses were designed with Primer3 [53] and are listed in Supplementary Data Table S2 (RT-qPCR) and Supplementary Data Table S3 (qPCR). Analyses were performed using a LightCycler 480 (Roche) under the following reaction conditions: initial denaturation 5 min at 95 °C, followed by 10 s at 95 °C, 20 s at a temperature specific for the primers, 10 s at 72 °C, repeated in 40 cycles. Denaturation for the melt curve analysis was conducted for 5 s at 95 °C, followed by 1 min at 65 °C and heating up to 98 °C (0.1 °C/s for the fluorescence measurement). The Ct values and the value of the qPCR efficiency were obtained from LinRegPCR [54].

2.5. Determining the relative expression level

The relative expression level was calculated using the ΔΔCt method [55] and calibrated to the early uninucleate stage (E) of microspore development of cv. 'Jersey'. As an internal control, two genes, *TMA7* and *SERF* were used in the experiments with the cultivars 'Jersey' and 'Mercada', while the genes *SERF* and *H2A* were used as the references for the experiments with all ten cultivars (Supplementary Data Table S2). The stability of expression of the reference genes was evaluated using NormFinder [56] and BestKeeper [57] and was found to be adequate for all of them.

2.6. Semi-quantitative RT-PCR

The validation of the *GBSSI* gene expression level using a semi-quantitative RT-PCR with the *H2A* gene as the internal control was performed for ten barley cultivars listed above. RNA from the EM microspores (500 ng/sample) was used to synthesise cDNA as previously described. The reaction was prepared in a 20 μl volume with 1 × Pol Buffer B (EURx); 0.25 mM of each dNTP (Promega); 0.5 μM forward and 0.5 μM reverse primer; 0.5 U Color Taq DNA Polymerase (EURx) and 3 μl of cDNA. The amplifications were performed as follows: 95 °C for 5 min followed by 94 °C for 40 s, 58 °C for 30 s and 72 °C for 30 s and repeated in 30 cycles, followed by a final elongation for 5 min at 72 °C. The products of the reactions were visualised in 1.8% agarose gel.

2.7. Plastome copy number

The plastid DNA copy number was evaluated as the number of plastid genes per haploid genome using the qPCR method, according to Lutz et al. [58]. The plastid genes were quantified in relation to two single copy nuclear genes: *ARF1* and *EF1* (Supplementary Data Table S2). The plastome copy number was presented as the average value of the quantification of the plastid genes that were localised within a plastid genome as followed: *psbA*, *matK*, *psbD*, *atpI*, *clpP*, *infA* in LSC; *ndhB*, *16S*, *ndhH* in IR and *ndhF* in SSC (Supplementary Data Table S2). LSC and SSC cover the long and short single copy region, respectively, whereas IR refers to the inverted repeat region in a plastome that contains two copies of the plastome genes.

2.8. Light and transmission electron microscope (TEM) analysis

Anthers containing microspores at various developmental stages were removed from freshly harvested spikes, followed by fixation and preparation according to Schumann et al. [59]. An electron microscopic analysis using a Tecnai Sphera G2 (FEI Company) was performed based on a previously described protocol [60]. Briefly, the plant material was fixed by immersion for 6 h at RT in a 50 mM cacodylate buffer (pH 7.2) containing 0.5% (v/v) glutaraldehyde and 2.0% (v/v) formaldehyde, washed in a cacodylate buffer and twice in distilled water. Next, the samples were fixed in 1.0% (v/v) osmium tetroxide for 1 h at RT, washed twice in distilled water, dehydrated by passage through an acetone series (20–100%) and infiltrated with Spurr resin (Sigma Aldrich) initially 33%, then 66% and finally 100%. Semi-thin (2 μm) cross sections were cut from the embedded samples and stained for 2 min at 60 °C with 1% (w/v) methylene blue and 1% (w/v) Azur II in 1% (w/v) aqueous borax, followed by Lugol's iodine treatment for 10 min at RT. After washing and drying, the prepared cross sections were examined using a Zeiss Axiocam camera in a Zeiss Axiovert 135 microscope (Zeiss).

The analysis was performed in three independent biological repetitions with at least 100 microspores (at E, EM, ML stage) per repetition. The types of plastids were recognised according to the common description as proplastids and amyloplasts [5]. However, we also distinguished two different phases of proplastids that occurred in the microspores: initial undifferentiated proplastids and differentiating proplastids.

2.9. Statistical analysis

To estimate the significant differences (at $P < 0.05$) between the compared samples, the One Way Analysis of Variance followed by Tukey's HSD test was applied unless otherwise noted. The relationship between the different features was estimated using Pearson's correlation coefficient, followed by determining the *P*-value to verify the statistical significance of a correlation.

3. Results

3.1. The androgenic response of the tested cultivars

First, we evaluated ten spring barley cultivars of different origins for their ability to regenerate green plants from isolated microspore culture. All of the tested cultivars showed a high induction and

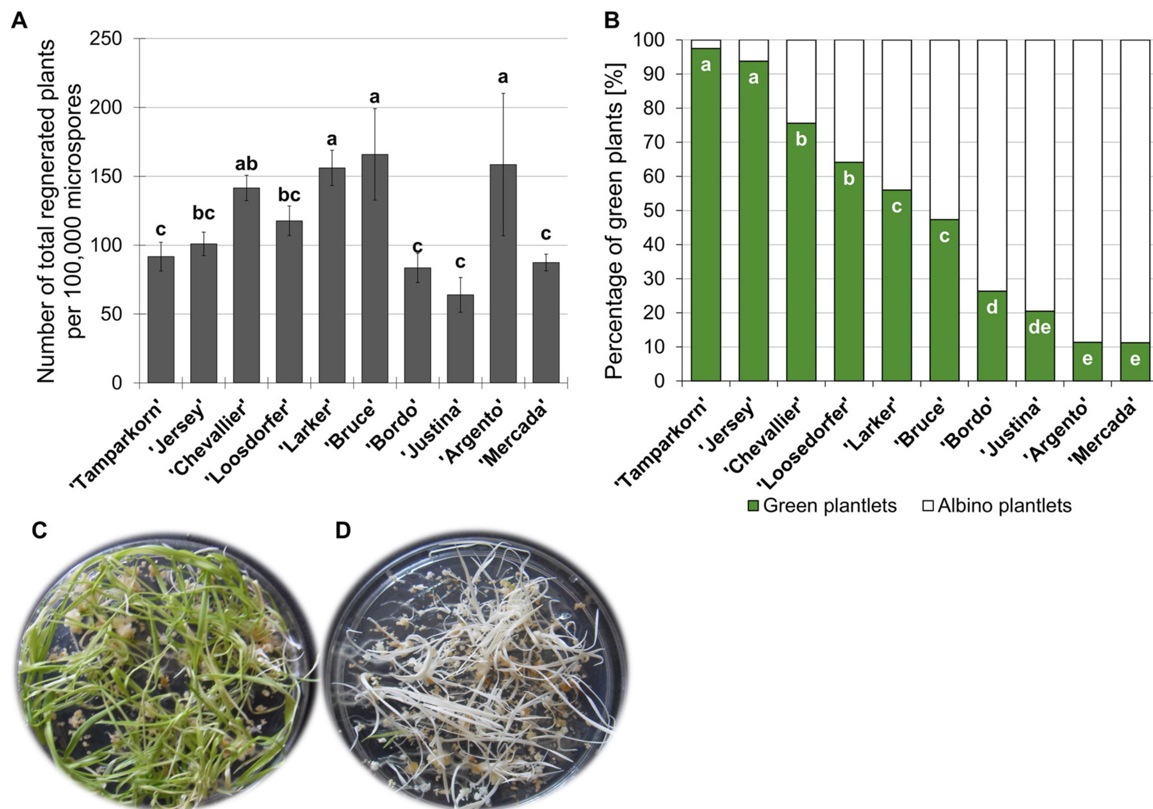


Fig. 1. The androgenic response of cultivars in isolated microspore culture.
(A) The total number of all regenerated plants per 100,000 isolated microspores. (B) The percentage of green plants among regenerants. (C–D) Petri dishes of cv. 'Jersey' (C) and 'Mercada' (D) after 21 days in regeneration medium. Given values present mean of n ≥ 3 with SD. Different letters indicate a significant difference between cultivars according to Tukey's HSD test ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regeneration potential in microspore culture with an average rate of 116.7 plants regenerated per 100,000 cultured microspores and a range of 63.9–165.9 regenerants (Fig. 1A). However, the cultivars revealed a much higher variation in the proportion of green regenerants among the regenerated plants, which differed from 11.0%–97.5% of the green regenerants (Fig. 1B). Based on the Tukey's HSD test ($P < 0.05$), five groups of cultivars could be distinguished in regards to their ability to regenerate green plants, with the cvs. 'Tamparkorn' and 'Jersey' expressing the highest green plant rate and the cvs. 'Argento' and 'Mercada' the lowest. Among these four cultivars, 'Jersey' and 'Mercada' had a similar regeneration efficiency (85–100 regenerated plants per 100,000 microspores), while they showed an extreme difference in the ratio of green to albino regenerants (Fig. 1C,D). The cv. 'Tamparkorn' had a similar regeneration capacity and green/albino ratio as 'Jersey', but it was a six-row cultivar, while 'Jersey' and 'Mercada' were two-row. Taking all the above into consideration, these two genotypes were selected for further studies whose aim was to characterise plastid differentiation at the molecular and ultrastructure levels during pollen development *in vivo*.

3.2. Expression profiles of genes involved in plastid biogenesis during pollen development in barley cultivars 'Jersey' and 'Mercada'

To evaluate whether the cultivars that displayed different green plant regeneration levels during androgenesis exhibited any differences in plastid development, we analysed the expression of the genes related to transcription and translation in the microspore and pollen plastids (Supplementary Data Table S2). At the early (E) stage of microspore development, the analysed genes (except for *RpoTp*) exhibited a similar expression level in both cultivars (Fig. 2; Supplementary Data Fig. S2). However, as pollen development progressed, divergent profiles of gene expression were observed between the cultivars. In cv. 'Jersey', which produced mostly green regenerants, the plastid-localised genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, encoding the subunits of RNA polymerase PEP (plastid-encoded RNA polymerase) and the translation-related genes (*16S*, *23S rRNA*, *rps2*, *rps7*, *rps8*, *rps15*, *rpl2*, *rpl16*, *infA*) encoding rRNA, plastid-specific ribosomal proteins and translation initiation factor showed a common expression profile: the highest level at the early-mid (EM) stage, a rapid decline at the mid-to-late (ML) stage and an increase at the binucleate (BN) stage. Conversely, the expression of the majority of the analysed genes remained at the same level throughout the E to ML stages in cv. 'Mercada', which regenerated mostly albino plants. At the ML stage, which is the stage of initiating microspore culture, the expression level of the plastome-encoded genes was significantly higher (2 to 16x) in the microspores of cv. 'Mercada' than in cv. 'Jersey'. Moreover, at this stage, a significantly higher expression of many nuclear-encoded genes engaged in plastid transcription and translation, was observed in cv. 'Mercada' compared to cv. 'Jersey'. Among these genes were two sigma factor encoding genes that are essential for the PEP activity (Supplementary Data Fig. S2A). The genes encoding factors involved in the initiation, elongation or termination of translation had the most heterogeneous expression, although they still had divergent patterns between the compared cultivars and a higher expression level at the ML stage in the 'Mercada' microspores (Supplementary Data Fig. S2B). The differences in the expression profiles of the examined genes suggested various dynamics of plastid differentiation during the early stages of *in vivo* pollen development in both cultivars. During pollen maturation, however, both genotypes had a similar, strong reduction in the expression of all of the analysed genes (Fig. 2; Supplementary Data Fig. S2).

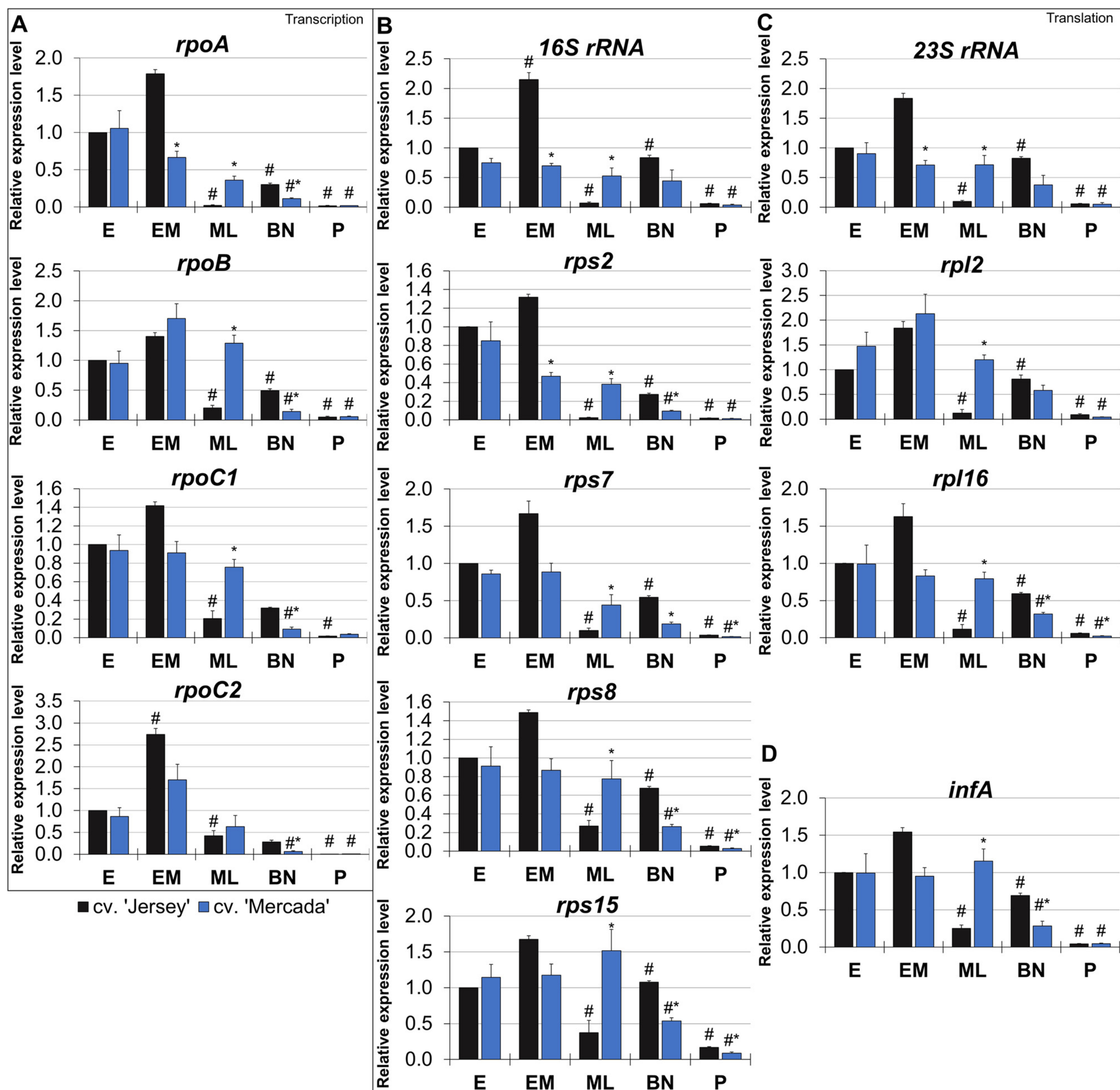
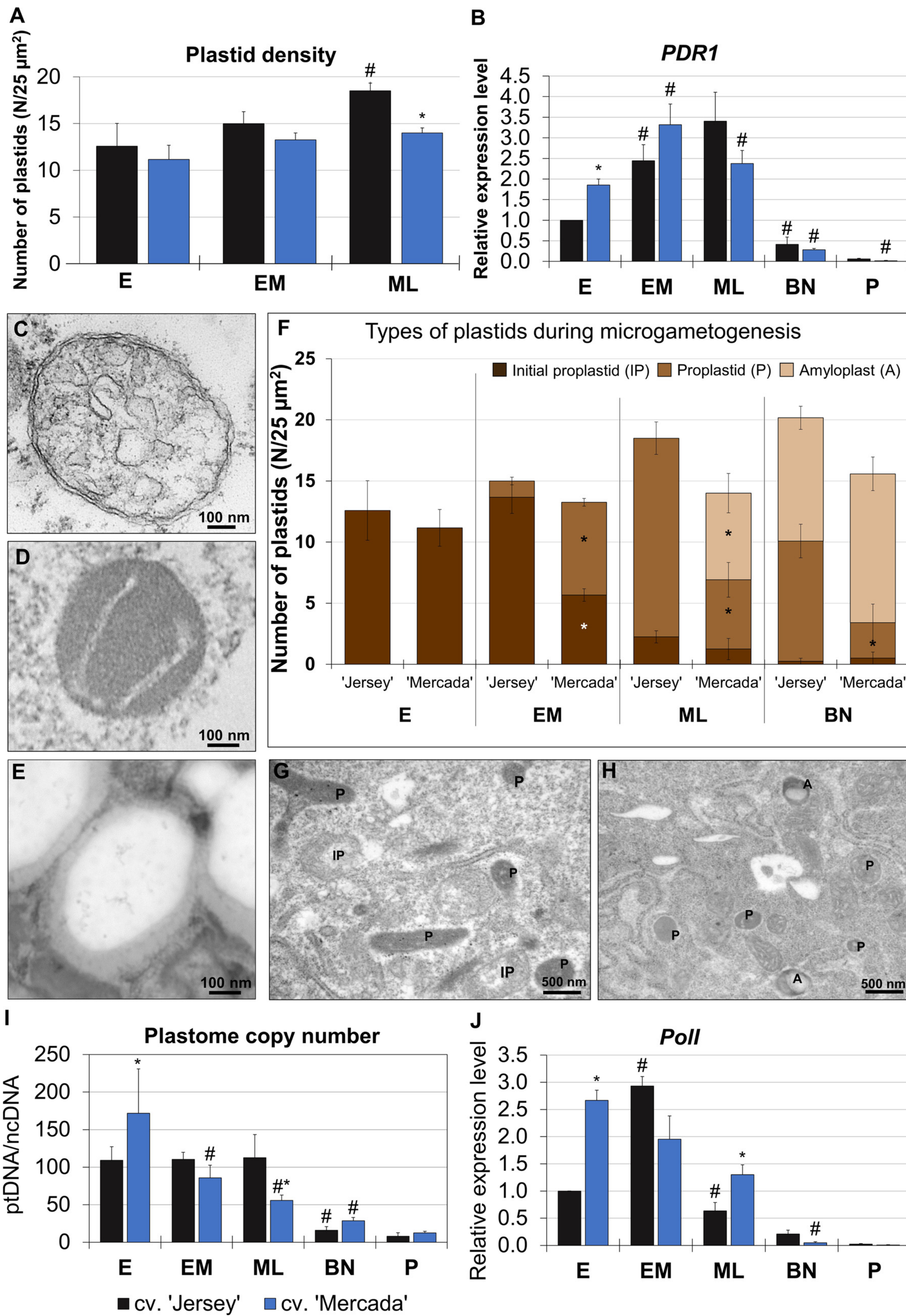


Fig. 2. Expression profiles of plastid-localised genes involved in plastid biogenesis during pollen development in 'Jersey' and 'Mercada' cultivars.
(A) Genes involved in transcription in plastids: *rpoA*, *rpoB*, *rpoC1*, *rpoC2* encoding subunits of PEP (plastid-encoded polymerase). (B–D) Genes involved in translation, encoding: (B) components of small ribosomal subunit: *16S rRNA*, *rps2*, *rps8*, *rps7*, *rps15,* (C) large ribosomal subunit: *23S rRNA*, *rpl2*, *rpl16* and (D) *infA* encoding translation initiation factor in plastids. Graphs show mean values of n ≥ 3 with SEM, normalised to early (E) microspores of cv. 'Jersey'. An asterisk presents a significant difference between cultivars at a certain stage of pollen development. A hash indicates a value significantly different from the preceding stage of development within each cultivar (Tukey's test, $P < 0.05$). Stages of pollen development: E – early uninucleate, EM – early-mid uninucleate, ML – mid-to-late uninucleate, BN – immature binucleate pollen, P – mature pollen grain.

In addition to the gene expression analysis, we counted the density of the plastids in the microspores at different stages of development based on the microscopic observations. Both cultivars had a similar density of plastids in the E and EM microspores (Fig. 3A). In cv. 'Jersey', however, the number of plastids significantly increased at the ML stage, whereas it remained unchanged in cv. 'Mercada'. At the ML stage, when plant material is usually collected for culture, the density of plastids in the 'Jersey' microspores was 32% higher than in the microspores of 'Mercada'. In accordance with this data, the expression of the *PDR1* gene encoding a plastid-dividing ring1 protein involved in plastid divisions increased gradually from the E to ML stage in cv. 'Jersey' and reached the highest level in the ML microspores. The transcript level of *PDR1* in cv. 'Mercada' reached the highest level at the EM stage and decreased by 30% at the ML stage (Fig. 3B). In both cultivars, the *PDR1* expression declined by 90% in the immature BN pollen grains.

3.3. Amyloplast formation during pollen development in 'Jersey' and 'Mercada' cultivars

Considering the differences in plastid biogenesis between 'Jersey' and 'Mercada', we performed a detailed analysis of plastid differentiation during pollen development. Microscopic observations in TEM enabled three types of plastids to be distinguished during microspore and pollen development: initial undifferentiated proplastids, differentiating proplastids (referred to proplastids) and amyloplasts accumulating starch (Fig. 3C–E). The undifferentiated proplastids were characterised



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by a low electron density and the presence of single invaginations from the inner membrane (Fig. 3C), whereas the electron density of the differentiating proplastids was much higher (Fig. 3D). At the early stage (E), only undifferentiated initial proplastids were observed in both cultivars (Fig. 3F). The microspores of cv. 'Mercada' had a faster differentiation of proplastids, as in the EM microspores the initial

Fig. 3. Plastid development in 'Jersey' and 'Mercada' cultivars during microgametogenesis.
(A) Density of plastids in microspores at the succeeding developmental stages. (B) The relative expression level of *PDR1* gene (*Plastid-dividing ring1*). (C–E) Ultrastructure of different types of plastids identified during pollen development including (C) initial proplastid, (D) proplastid and (E) mature amyloplast. (F) Density and types of plastids observed in both cultivars during successive stages of microspore development. (G) Overview of plastids present on 25 μm^2 of cytoplasm in cv. 'Jersey' and (H) 'Mercada' in microspore at the ML stage. (I) Plastome copy number per cell. (J) The relative profile of *PolI* (*Organellar DNA polymeraseI*) expression during pollen development. Values in (A) and (F) present mean of at least $n \geq 300$ microspores with SD. Graphs in (B), (I) and (J) show mean values of $n \geq 3$ with SD in (I) and SEM in (B) and (J). Relative expression level normalised to E microspores of cv. 'Jersey'. An asterisk presents a value significantly different between cultivars at a certain stage of pollen development. A hash indicates a value significantly different from the preceding stage within cultivar (Tukey's test, $P < 0.05$). IP – initial proplastid, P – proplastid, A- amyloplast. Stages of pollen development: E – early uninucleate, EM – early-mid uninucleate, ML – mid-to-late uninucleate, BN – immature binucleate pollen, P – mature pollen grain.

proplastids and proplastids were present in similar numbers. Moreover, during the progression of microspore development, at the ML stage a high number of amyloplasts was observed in addition to an equal amount of proplastids and only a few initial proplastids. Conversely, no differentiation of proplastids occurred at the EM stage in cv. 'Jersey', which resulted in the initial proplastids being the main group of plastids in the EM microspores. Hence, at the next stage of development (ML), the microspores of cv. 'Jersey' contained mostly differentiating proplastids, a few initial proplastids and no amyloplasts. This analysis showed that at the ML stage, cv. 'Mercada' contained amyloplasts that were filled with starch grains, while in cv. 'Jersey' there were only undifferentiated and differentiating proplastids. In cv. 'Jersey', amyloplasts occurred in the BN pollen grains in a comparable numbers with proplastids, whereas at this stage we observed four times more amyloplasts than proplastids in cv. 'Mercada'.

Together with the observations of the progression of plastid differentiation, the plastome copy number was assessed using qPCR (Fig. 3I). In cv. 'Jersey', the plastome copy number was unchanged from the E to ML stage and significantly decreased in the immature BN pollen. In cv. 'Mercada', a gradual decrease in the plastome copy number was observed throughout all stages of pollen development. At the E stage, cv. 'Mercada' had ca. 40% more plastid genomes than 'Jersey', while at the ML the results were reverse – the average plastome copy number in 'Mercada' was two-fold lower than in 'Jersey' (Fig. 3I). This data indicates a faster degradation of the plastid genomes in cv. 'Mercada'. The significant deviation between the copy numbers of individual plastid genes that was observed in the 'Mercada' microspores as early as the E stage supports this hypothesis (Supplementary Data Fig. S3). Additionally, the expression profile of the *PolI* gene encoding the DNA polymerase responsible for plastome replication differed between the cultivars (Fig. 3J). At the E stage of microspore development, the *PolI* expression was two-fold higher in cv. 'Mercada' than in 'Jersey', after which it decreased progressively, similar to the plastome copy number in this genotype. The observed difference in plastome copy number between 'Jersey' and 'Mercada' at the time of initiating *in vitro* culture (ML stage) may indicate a relationship between the stability of plastid DNA and the progress of amyloplast formation.

In parallel to cytological observations, we determined the relative expression level of six genes located in the nuclear genome (Supplementary Data Table S2), which are engaged in starch biosynthesis during microspore and pollen development *in vivo*. At the early stage (E), the expression of the starch biosynthesis genes was very low in both cultivars. At the next stage (EM), most of the analysed genes showed the same expression level as earlier in cv. 'Jersey', while in cv. 'Mercada', their transcription increased significantly and was from 4 to 150 times higher than in 'Jersey' (Fig. 4). In both cultivars, the expression of all genes increased even further until the BN pollen stage; however, in 'Mercada', most genes (except for *AgpL* and *Dpe2*) reached the highest transcriptional activity as early as the ML stage. It should be noted that at this stage, the relative transcription level of all starch synthesis-related genes was three to nine times higher in the microspores of 'Mercada' compared to 'Jersey'. These results, together with the microscopic observations of the high number of amyloplasts in the ML 'Mercada' microspores indicate an earlier activation of the starch synthesis genes in this genotype. The most pronounced increase in transcription activity between the E and ML microspores was observed for the *GBSSI* gene encoding an enzyme involved in the elongation of the amylose chain (280x for 'Jersey' and 2500x for 'Mercada'). The expression level of this gene in the EM and ML microspores can be used as an indicator of plastid differentiation in developing microspores.

3.4. Plastid differentiation during pollen development in other spring barley cultivars

To confirm the hypothesis that early amyloplast formation during microspore development *in vivo* is associated with the high ratio of albino regenerants during androgenesis, we analysed the expression profiles of the starch synthesis-related genes and plastid differentiation among other barley cultivars. The ten tested genotypes represented different abilities for regenerating green plants in microspore culture, which ranged from 10% to almost 100% (Fig. 1). Of the starch synthesis genes that were analysed in 'Jersey' and 'Mercada' cultivars, we selected *Sbe1, Dpe2* and *GBSSI* due to their significantly increased expression during microspore development. The expression level of *Sbe1* (encoding starch branching enzyme1) increased as early as in the EM microspores of all genotypes compared to the preceding E stage (Fig. 5A). Moreover, an analysis of the *Dpe2* and *GBSSI* expression in the EM microspores permitted two groups of genotypes to be distinguished: 1) the cultivars, which, similar to 'Jersey', had the same *Dpe2* and *GBSSI* transcription level in the E and EM microspores and 2) the group of cultivars that had a highly increased expression of these genes in the EM microspores, similar to 'Mercada'. The first group of genotypes demonstrated a high ability to regenerate green plants in microspore culture (more than 60% green regenerants), while in the second group, albino plants comprised the majority of regenerants (Fig. 1).

An even more clear difference between these two groups of genotypes was revealed by the cytological observations of the plastids in the microspores at the ML stage, which are used to initiate the cultures. In the ML microspores of cultivars that produced a low percentage of green plants, the presence of starch-accumulating plastids was detected during the microscopic observations (Fig. 6). The majority of those cultivars had a similar number of amyloplasts and differentiating proplastids, whereas in the cultivars with a high ability to regenerate green plants, no amyloplasts were detected in the ML microspores. Additionally, an increase in the number of plastids between the EM and ML stages was detected in cvs. 'Jersey', 'Tamparkorn', 'Chevallier' and 'Loosdorfer', which produced more than 60% green regenerants during androgenesis (Supplementary Data Fig. S4). A simple staining of starch with Lugol's solution permitted the starch grains in the ML microspores of these cultivars to be visualised (Fig. 6B), which supported the previous observations. The level of albino plants regenerated from microspore culture and the amyloplast number in the ML microspores had a very strong positive correlation (*r*-value = 0.94). Additionally, the number of differentiating proplastids was strongly negatively correlated with the regeneration of albino plants (Fig. 7A).

The activation of the *Dpe2* and *GBSSI* genes involved in starch synthesis was detected as early as in the EM microspores of the group of cultivars that produced mostly albino regenerants. A statistical analysis revealed a very strong and strong positive correlation between the expression level of the *Dpe2* (*r*-value = 0.85), *GBSSI* (*r*-value = 0.86),

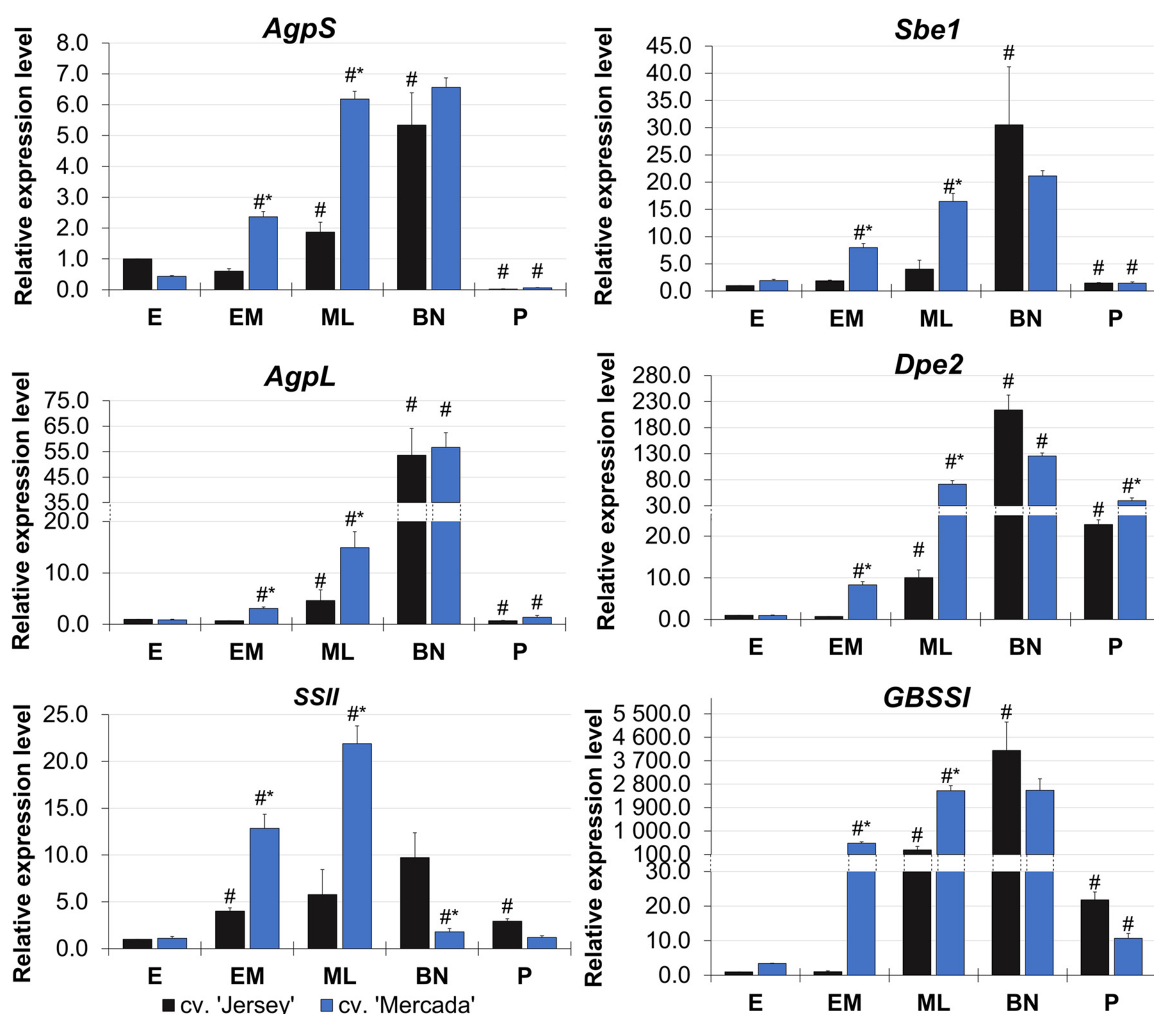

Fig. 4. The expression profiles of genes involved in starch biosynthesis during microgametogenesis of 'Jersey' and 'Mercada' cultivars.
Graphs show mean values of n ≥ 3 with SEM for relative expression level normalised to E microspores of cv. 'Jersey'. An asterisk presents a value significantly different between cultivars at a certain stage of pollen development. A hash indicates a value significantly different from the preceding stage within cultivar (Tukey's test, $P < 0.05$). Stages of pollen development: E – early uninucleate, EM – early-mid uninucleate, ML – mid-to-late uninucleate, BN – immature binucleate pollen, P – mature pollen grain.

Sbe1 (*r*-value = 0.76) genes in the EM microspores and the density of the starch-accumulating plastids in the cytoplasm of ML microspores (Fig. 7B–D). The analysis also showed a very strong and strong positive correlation between the expression level of the *Dpe2, GBSSI* and *Sbe1* genes in the EM microspores and the rate of regenerated albino plants in the *in vitro* culture (*r*-value = 0.82, 0.85 and 0.67, respectively, Fig. 7B–D). Taking into account the obtained data, we assume that the faster conversion of proplastids into amyloplasts, which results in a high proportion of amyloplasts in the ML microspores, leads to the formation of albino plants during androgenesis.

The expression level of the *GBSSI* gene in the EM microspores was also observed in a semi-quantitative PCR, in which only the cultivars that produced less than 60% of green plants had a strong visible amplification of the *GBSSI* transcript (Fig. 5B). Therefore, we consider that the expression of this gene in EM microspores can serve as a marker of amyloplast differentiation, and as a result, as an indicator of the genotypes that will produce mostly albino regenerants in isolated microspore culture.

3.5. The microspore developmental stage at the initiation of culture influences the regeneration of green plants

Based on the positive correlation between the amyloplast differentiation and albino plant regeneration, we assumed that using an earlier stage of microspore development for *in vitro* culture should influence the number of green regenerants. Therefore, we performed a preliminary study to compare the green plant regeneration in cultures that had been initiated from microspores at the early-mid uninucleate (EM) and mid-to-late uninucleate (ML) developmental stages. We used five cultivars that had a differential level of green regenerants in a microspore culture initiated from ML microspores. The 'Jersey' and 'Loosdorfer' cultivars produced more than 60%, whereas 'Bordo', 'Justina' and 'Mercada' regenerated less than 25% green plants.

In the isolated microspore cultures of cvs. 'Bordo', 'Justina' and 'Mercada', the stage of microspore development did not influence the overall number of regenerants (Fig. 8A). However, in the 'Mercada' culture that had been initiated from EM microspores, the number of green plants increased more than four-fold compared to the ML stage, which enabled ca. 60 plants per 100,000 microspores to be obtained (Fig. 8B). The proportion of green plants among all regenerants increased from 12.6% to 46.6%. An even more striking improvement was achieved for the 'Bordo' and 'Justina' cultivars in which the percentage of green regenerants increased from ca. 20% to 84% of the total number of regenerated plants. In cvs. 'Jersey' and 'Loosdorfer', the regeneration capacity decreased in the cultures that had been initiated from EM microspores compared to the ML stage (Fig. 8A). Nevertheless, the

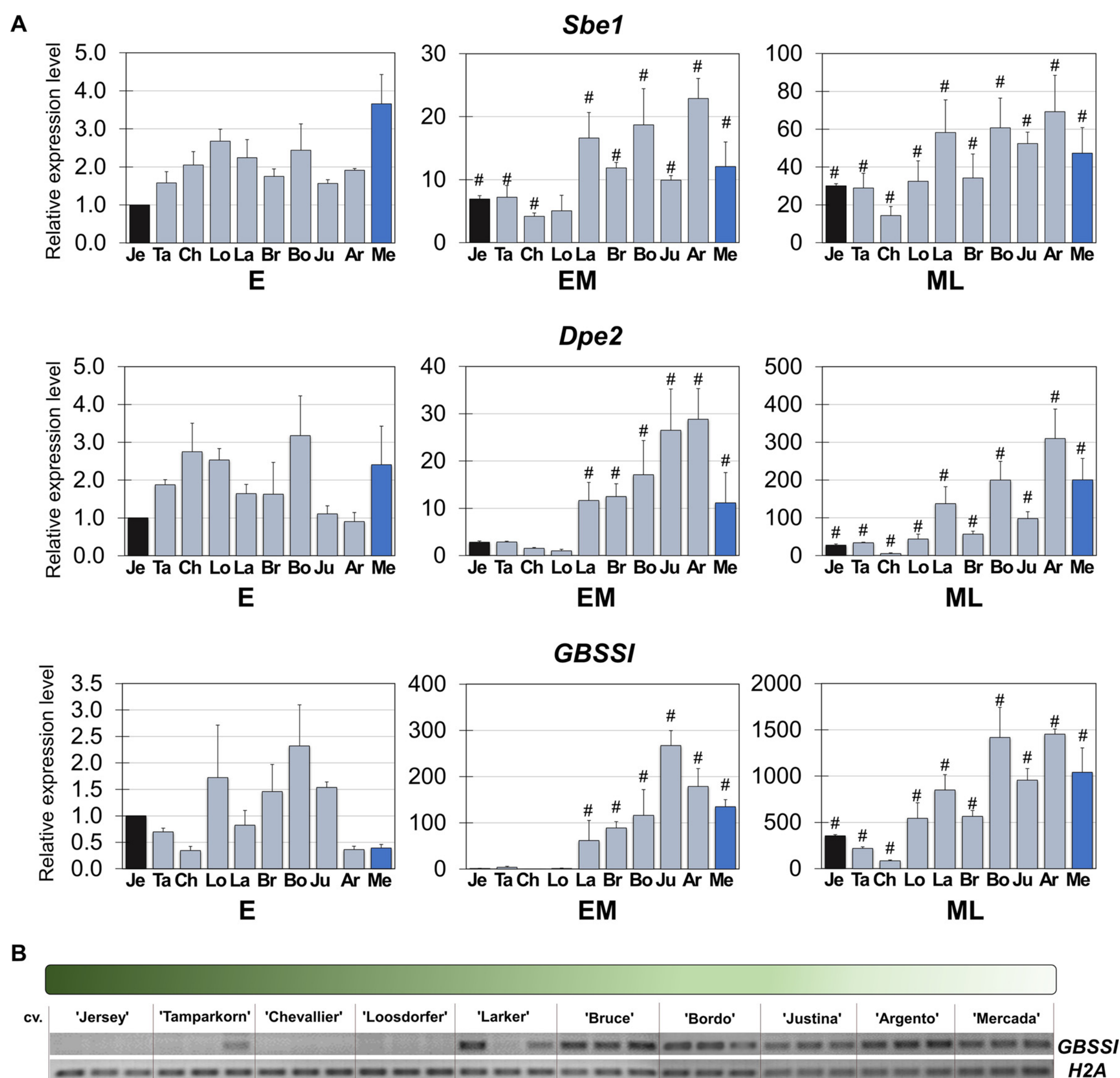

Fig. 5. The expression profiles of genes involved in starch biosynthesis in barley cultivars varying in the percentage of green plants regenerated in isolated microspore culture.
(A) The relative expression level of genes during successive stages of microspores development. Graphs show mean values of n = 3 with SEM of relative expression level normalised to E microspores of cv. 'Jersey'. A hash demonstrates a value significantly different from the preceding stage within cultivar (Tukey's test, $P < 0.05$). Stages of pollen development: E – early uninucleate, EM – early-mid uninucleate, ML – mid-to-late uninucleate. (B) Semi-quantitative analysis of *GBSSI* gene expression in EM microspores of 10 cultivars, with gradient chart of green to albino plants ratio. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

contribution of green plants among the regenerants did not differ between the cultures initiated using EM or ML microspores for these genotypes (Fig. 8B).

These results indicate that initiating culture from microspores at an earlier stage of development, prior to amyloplast differentiation, significantly improves the regeneration of green plants and overcomes the problem of albinism during barley androgenesis.

4. Discussion

Androgenesis, which is the most effective method of DH production, is widely used in barley breeding programmes. Although barley is an easily inducible species, its genotype-dependent response, which results in a large number of albino regenerants, often limits the effective application of this DH system [61,62]. To date, studies on the mechanisms that lead to the formation of albino plants in cereal androgenesis have primarily focused on plastid development during *in vitro* cultures. In barley, ultrastructural analyses of plastids in microspore-derived embryos and regenerated plants have been performed for several spring cultivars that produce mostly albino regenerants and for the winter cv. 'Igri', which regenerates almost exclusively green plants [42,43]. The green regenerants of cv. 'Igri' contained regular chloroplasts with well-developed grana and thylakoids, and a low content of accumulated starch grains. These plants originated from microspore-derived embryos whose plastids had intensified divisions and a high DNA content. Conversely, the plastids of albino regenerants of the spring cultivars displayed few divisions, a high content of starch grains and a limited thylakoid and DNA content. The authors concluded that the development of albino plants was not initiated at the time of regeneration but that it had begun earlier during the androgenetic process [43].

Here, we demonstrate that the ratio of green to albino regenerants in isolated microspore culture of spring barley cultivars is determined by the state of plastid differentiation in the microspores at the stage of culture initiation. The cultivars that produced mostly albino

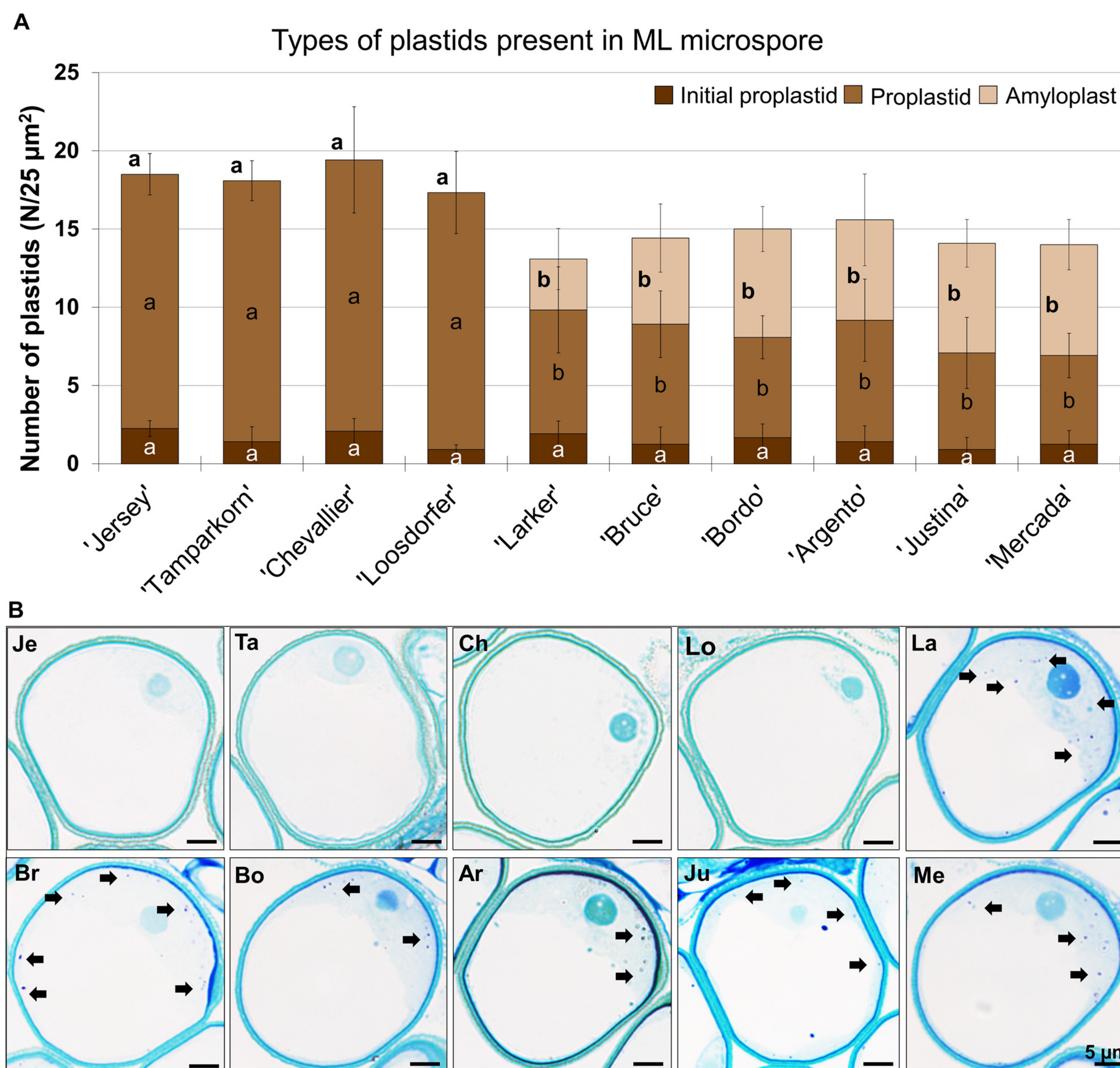

Fig. 6. Types of plastids present in ML microspores of barley cultivars that differ in green to albino plant ratio in isolated microspore culture.
(A) Density of particular types of plastids in the ML microspores of barley cultivars. The given values present mean of n ≥ 300 microspores with SD. Different letters indicate significant differences between cultivars in regards to each plastid type, according to Tukey's test ($P < 0.05$). (B) Representative pictures of microspores at the ML stage with starch grains visualised by Lugol's solution of 10 cultivars: (Je) 'Jersey', (Ta) 'Tamparkorn', (Ch) 'Chevallier', (Lo) 'Loosdorfer', (La) 'Larker', (Br) 'Bruce', (Bo) 'Bordo', (Ar) 'Argento', (Ju) 'Justina' and (Me) 'Mercada'. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

regenerants showed a high activation of the starch-biosynthesis genes as early as in the EM stage, which preceded the collection of microspores for an *in vitro* culture, and the formation of amyloplasts in the ML microspores that are used to initiate a culture. Conversely, the cultivars that produced mostly green plants had differentiating proplastids in their ML microspores, which were characterised by a few internal membranes and a dense matrix. The Pearson's correlation coefficient indicated a very strong positive association between the number of amyloplasts in the ML microspores and the level of regenerated albino plants. An increase in the expression level of the *GBSSI*, *Dpe2* and *Sbe1* genes was positively associated with the presence of amyloplasts in the ML microspores and with the regeneration rate of albino plants. Studies on Bright Yellow-2 (BY-2) cultured tobacco cells exhibiting non-photosynthetic, highly prolific properties indicated that the activation of the expression of *GBSSI*, *Dpe2* and *Sbe1* genes is required for starch biosynthesis and the deposition of starch granules within a plastid is required to form an amyloplast [16,19]. It should be pointed out that plastid differentiation at the early stages of microspore development in barley has not previously been described.

The expression level of the *GBSSI* gene can easily be detected in semi-quantitative PCR and may serve as a system to identify the genotypes that predominantly produce albino regenerants prior to initiating *in vitro* culture. Furthermore, a qPCR analysis permits the cultivars that produce a moderate (30–60%) and high (75–90%) number of albino plants to be distinguished. The possibility of indicating the genotypes that have a low potential for regenerating green plants enables them to be excluded from breeding programmes in which the production of a high number of DH lines from F_1 hybrids is required. Alternatively, when such genotypes are the source of desirable genes for breeding programmes, the information about their possible

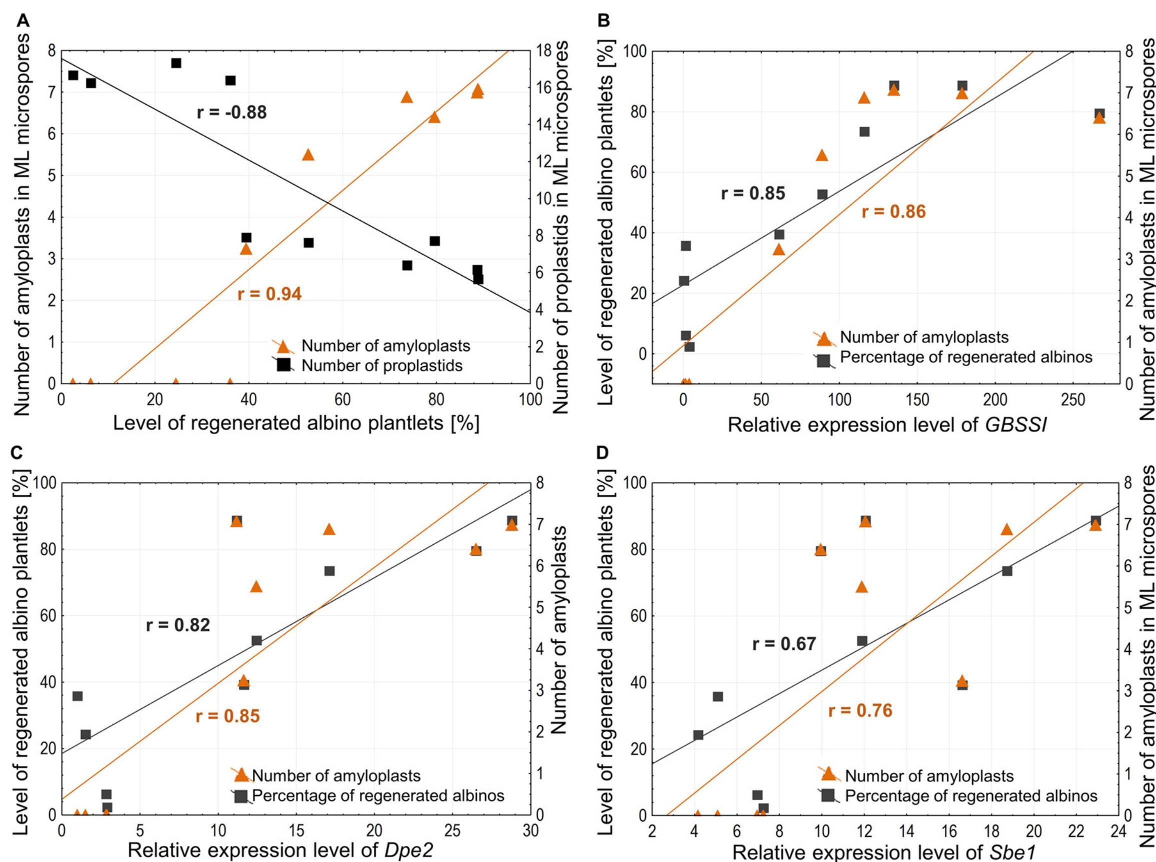

Fig. 7. Correlation between the microspore plastid differentiation and albino plant regeneration among barley cultivars exhibiting different ratio of green/albino regenerants in isolated microspore culture.
(A) Correlation between the density of amyloplasts or proplastids (number/25 μm^2) in ML microspores and the level of albino plant (number/100,000 microspores) in 10 barley cultivars. (B–D) Correlation between the amyloplast density in ML microspores, regeneration level of albino plant and expression level of (B) *GBSSI* (C) *Dpe2* (D) *Sbe1* genes in EM microspores of 10 barley cultivars. Pearson's correlation coefficient was used to test the relationship between the average values obtained for cultivars. *P*-value, determined for each *r*-value, lower than 0.05 was considered as statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

performance in androgenesis would be very useful for determining a sufficient number of donor plants to initiate culture and to produce the required number of DH lines.

During the progression of pollen development, we observed a significant decrease in the plastome copy number estimated by qPCR. The degradation of plastomes during starch deposition is a well-documented process in the vegetative cell of pollen grains [63,64]. In our experiments, a decrease in plastid DNA copy number occurred as early

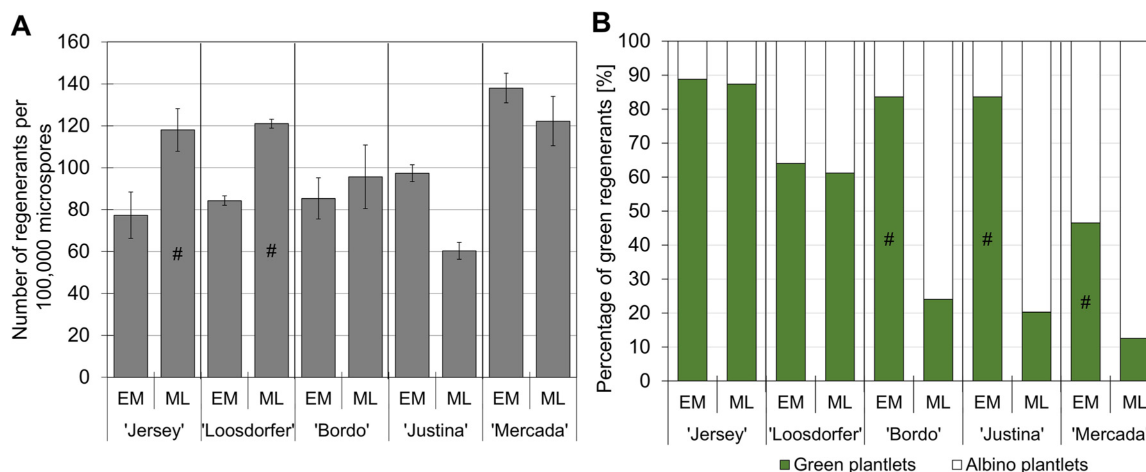

Fig. 8. The efficiency of overall regeneration and green plant production in isolated microspore culture initiated from EM and ML microspores.
(A) The level of total regenerated plants and (B) the percentage of green plants among regenerants in isolated microspore culture of five barley cultivars initiated from EM and ML microspores. Given values present mean of n ≥ 3 with SD. A hash indicates a significant difference in the number of total (A) and green regenerants (B) between stages used to initiate the *in vitro* culture according to Tukey's test ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

as in the EM microspores in the cultivar that produced more albino plants. This indicates that the rate of plastid differentiation during pollen development has an impact on the plastome copy number, associated with the progression of amyloplast formation.

Many studies have attempted to increase the frequency of the regeneration of green plants by changing the culture conditions such as pretreatment [65–69] or media composition [70–73]. However, the influence of pretreatment, media and culture conditions modifications remain highly genotype-dependent. Moreover, studies on the effects of external factors on the efficiency of androgenesis indicate that the regeneration capacity does not correlate with the occurrence of green and albino plants. Apart from expanding the overall plant regeneration rate, no external factor that has been introduced into *in vitro* culture has overcome the problem of albinism [65,66,69]. The high number of albino plants produced by the cultivars that contained amyloplasts in the ML microspores suggests that modifications of the *in vitro* culture procedure, including pretreatment, cannot reverse the differentiation of proplastids into amyloplasts that occurs during microgametogenesis. Analysis of plastid development *in vivo* enables the true capability of different cultivars to regenerate green plants during androgenesis to be revealed. Moreover, the proposed marker (*GBSSI* expression) can be used to investigate and verify the potential of genotypes before initiating a culture. We showed that using microspores at a stage of development earlier than ML, which is usually recommended for culture initiating, while technically more demanding, can increase the number of green plants among the regenerants from isolated microspore culture in barley. This improvement is clearly connected with a lack of proplastid differentiation into amyloplasts in the early microspores of the high albino-producing genotypes.

5. Conclusions

The study provides new insights into the phenomenon of genotype-dependent regeneration of albino plants during androgenesis in barley. Genotypes that have a similar regeneration potential, but differ in the ratio of green to albino regenerants, exhibit divergent expression profiles of genes involved in transcription, translation and starch biosynthesis in microspore plastids. Cultivars producing mostly albino regenerants in androgenesis show early activation of starch synthesis genes, differentiation of proplastids into amyloplasts and degradation of plastomes during microspore development *in vivo*. The expression of the *GBSSI* gene (encoding a granule-bound starch synthaseI) may serve as a marker of genotype potential to produce green regenerants during androgenesis. We demonstrated that initiating culture from microspores at the early-mid stage of development, before amyloplast differentiation, significantly improves the regeneration of green plants and may overcome the problem of albinism in barley microspore embryogenesis.

Authors' contribution

IS conceived the study; IS and MG designed the experiments and analysed the data; MG performed the gene expression and plastome copy number experiments and analysis; MM performed the cytological and ultrastructure studies; MG, BC, JJ and JZ handled the isolated microspore culture; MG, MM and IS wrote the manuscript. All of the authors read and approved the manuscript.

Declaration of Competing Interest

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

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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.plantsci.2019.110321.

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