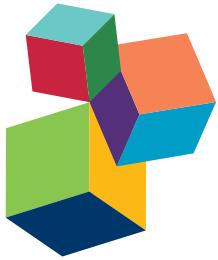


DOUBLED HAPLOIDY IN MODEL AND RECALCITRANT SPECIES

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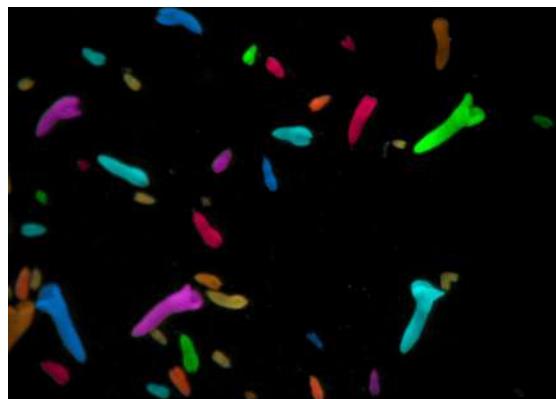
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DOUBLED HAPLOIDY IN MODEL AND RECALCITRANT SPECIES

Topic Editor:

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This image shows different *Brassica napus* embryos at different developmental stages from heart-shaped to torpedo, derived from isolated and in vitro cultured microspores. Embryos have been artificially colored using software.

should be in order to obtain DHs on a routine basis. Indeed, many of them are still considered as low-responding or recalcitrant to these treatments, including many of the most important crops worldwide. Although many groups are making significant progresses in the understanding of these intriguing experimental pathways, little is known about the origin, causes and ways to overcome recalcitrancy. It would be very important to shed light on the particularities of recalcitrant species and the special conditions they need to be induced. In parallel, the knowledge gained from the study of basic aspects in model species could also be beneficial to overcome recalcitrancy. In this e-book, we present a compilation of different approaches leading to the generation of DHs in model and in recalcitrant species, and different studies on new and relevant aspects of this process, useful to extract common traits and features, to know better these processes, and eventually, to elucidate how to make DH technology more efficient.

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Doubled haploids (DHs) are powerful tools to reduce the time and costs needed to produce pure lines to be used in breeding programs. DHs are also useful for genetic mapping of complex qualitative traits, to avoid transgenic hemizygotes, for studies of linkage and estimation of recombination fractions, for screening of recessive mutants. These are just some of the advantages that make DH technology one of the most exciting fields of present and future plant biotechnology.

All of the DH methods have model species where these technologies have been developed, or that respond every efficiently to their corresponding induction treatment. However, not all the species of economical/agronomical interest respond to these methodologies as they

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Editorial: Doubled Haploidy in Model and Recalcitrant Species

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Keywords: plant breeding, haploidy, doubled haploidy, gynogenesis, androgenesis

The Editorial on the Research Topic

Doubled Haploidy in Model and Recalcitrant Species

Doubled haploid (DH) technology is a powerful tool in plant breeding to reduce the time and costs needed to produce pure lines, the cornerstone of hybrid seed production. This biotechnological alternative to classic methods allows for a reduction of the typical 7–8 inbreeding generations needed to fix a hybrid genotype to only one *in vitro* generation. It is therefore much faster and cheaper, being the principal advantage of DH technology in plant breeding, but not the only. Indeed, DHs are also useful for genetic mapping of complex qualitative traits, for linkage studies and estimation of recombination fractions, to unmask recessive mutants, to avoid transgenic hemizygotes, or for reverse breeding, among others (Forster et al., 2007; Dunwell, 2010; Dwivedi et al., 2015). These are some of the advantages that make DH technology one of the most exciting fields of present and future plant biotechnology.

At present, there are several ways to produce haploids and eventually DHs (after a process of chromosome doubling), involving both female and male gametophytes. From the female gametophyte, haploids may be produced by uniparental genome elimination and by induction of gynogenesis. Uniparental genome elimination is typically achieved by crossing two sexually incompatible species, in some intraspecific crosses when one genitor carries specific mutation(s), or through genetic manipulation of CENH3, a centromeric variant of the H3 histone (Ravi and Chan, 2010, 2013; Karimi-Ashtiyani et al., 2015). Gynogenesis is a route through which unfertilized ovules, ovaries or even entire flowers are cultured *in vitro* to induce the development of a haploid embryo, generally from the egg cell (Bohanec, 2009). From the male gametophyte, haploids may be obtained through androgenesis (Seguí-Simarro, 2010). The most common and useful androgenic pathway is microspore/pollen embryogenesis, through which microspores/pollen are reprogrammed toward embryogenesis. Discovered more than 40 years ago (Guha and Maheshwari, 1964), this process has become of great practical importance for the agronomic industry due to its convenience for producing DH lines much faster, cheaper, and in more species than the other methods above mentioned (Forster et al., 2007; Dunwell, 2010). This is why when possible, microspore embryogenesis is the method of choice to produce DHs.

For all these methods, there are species where they are most efficient. This is why these species are used as experimental models to study basic aspects of the process. This is the case of onion for gynogenesis, and of barley and rapeseed for microspore embryogenesis in monocots and dicots, respectively. This Research Topic includes examples of research focused on different aspects of gynogenesis and microspore embryogenesis in these species. For example, Fayos et al. compare the performance of different onion germplasms under different experimental conditions to induce gynogenesis, regenerate gynogenic plants, and promote chromosome doubling. As to microspore embryogenesis, several articles use barley to study it. Daghma et al. develop a time-lapse imaging system to track the first embryogenic divisions, finding that most embryogenic structures come from symmetrically divided vacuolated

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microspores, with very few coming from asymmetric divisions. Lippmann et al. develop a micro-culture system whereby they demonstrate that co-cultivated wheat pistils release a low molecular weight signal that increases considerably the production of embryogenic structures. They postulate that the use of cut pistils as sources of this feeder substance might be extended to other species. Solís et al. use 5-azacytidine, a non-methylable base analog, to study the effects of DNA hypomethylation during microspore embryogenesis. They find that hypomethylation promotes the developmental switch toward proliferation, but prevents further differentiation into true embryos, both in barley and rapeseed. Also in rapeseed, the use of the most advanced sample preservation techniques allowed for the discovery of new processes associated to the embryogenic switch. In Parra-Vega et al., and Parra-Vega et al., the authors report the occurrence of plastolysomes (autophagic plastids) that engulf and digest cytoplasm regions, being finally released to the apoplast. They also describe the parallel formation of a callosic layer beneath the microspore intine, and the *de novo* formation of abnormal cell walls with altered callose and cellulose composition. All these events appear to have a dramatic impact in the developmental fate of the embryogenic microspore, including genome duplication by nuclear fusion.

However, not all the species respond well enough to DH technology. Indeed, many of them are still considered as recalcitrant to these treatments, including many of the most important crops worldwide. Despite the work of many groups, little is still known about how to overcome recalcitrancy. This is why it is also important to shed light on the particularities of recalcitrant species and the special conditions they need to be induced. In this Research Topic, Castillo et al. show

that preconditioning or coculture with ovaries increases the efficiency of DH production and chromosome doubling in different bread wheat cultivars, being the increases higher in those most recalcitrant. Interestingly, these results are in line with those from Lippmann et al. about the role of the female parts in helping the development of microspore-derived embryos in an *in vitro* environment, devoid of the complex crosstalk between embryo, endosperm and seed tissues that takes place during zygotic embryogenesis. In this crosstalk, hormones play a key role. This is why Žur et al. present a review focused on the current knowledge of hormonal regulation during microspore embryogenesis. Besides the role described for the principal hormones, either when they act endogenously or when applied exogenously, this review presents new and interesting notions about their involvement in this process. A remarkable example of the application of this kind of knowledge is the study brought by Chiancone et al., which achieves an important milestone inducing for the first time the development of microspore-derived embryos in different cultivars of *Citrus*, a very recalcitrant fruit crop, through the use of meta-topolin, a plant hormone rarely used in microspore embryogenesis.

Together, the papers of this Research Topic show some relevant advances in the understanding of the processes that lead to the formation of DH plants, and in their application to improve its performance in recalcitrant genotypes.

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Doubled haploid production from Spanish onion (*Allium cepa* L.) germplasm: embryogenesis induction, plant regeneration and chromosome doubling

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The use of doubled haploids in onion breeding is limited due to the low gynogenesis efficiency of this species. Gynogenesis capacity from Spanish germplasm, including the sweet cultivar Fuentes de Ebro, the highly pungent landrace BGHZ1354 and the two Valenciana type commercial varieties Recas and Rita, was evaluated and optimized in this study. The OH-1 population, characterized by a high gynogenesis induction, was used as control. Growing conditions of the donor plants were tested with a one-step protocol and field plants produced a slightly higher percentage of embryogenesis induction than growth chamber plants. A one-step protocol was compared with a two-step protocol for embryogenesis induction. Spanish germplasm produced a 2–3 times higher percentage of embryogenesis with the two-step protocol, Recas showing the highest percentage (2.09%) and Fuentes de Ebro the lowest (0.53%). These percentages were significantly lower than those from the OH-1 population, with an average of 15% independently of the protocol used. The effect of different containers on plant regeneration was tested using both protocols. The highest percentage of acclimated plants was obtained with the two-step protocol in combination with Eco2box (70%), whereas the lowest percentage was observed with glass tubes in the two protocols (20–23%). Different amiprofos-methyl (APM) treatments were applied to embryos for chromosome doubling. A similar number of doubled haploid plants were recovered with 25 or 50 µM APM in liquid medium. However, the application of 25 µM in solid medium for 24 h produced the highest number of doubled haploid plants. Somatic regeneration from flower buds of haploid and mixoploid plants proved to be a successful approach for chromosome doubling, since diploid plants were obtained from the four regenerated lines. In this study, doubled haploid plants were produced from the four Spanish cultivars, however further improvements are needed to increase their gynogenesis efficiency.

Keywords: onion, gynogenesis, Spanish germplasm, flower bud, embryogenesis, Eco2box, chromosome doubling

Introduction

Onion (*Allium cepa* L.) is a valuable crop for food and medicinal purposes, ranking second after tomato in the list of vegetables cultivated worldwide, with production of over 90 million tons on 4.7 million ha (FAO, 2013). Onion is an important crop in Spain, which is the third largest producer in Europe, after Russia and Netherlands. The onion production in Spain is over 1.2 million tons, ranking second among crop vegetables. Onion is an allogamous species and therefore both open pollinated cultivars and hybrids are cultivated. Hybrids have many advantages, including higher productivity, genetic uniformity and seed production for commercial use (Campion et al., 1995; Foschi et al., 2009). Uniform highly inbred lines are needed for hybrid production, but they are difficult to obtain through conventional methods of plant breeding (between 10 and 12 years) due to severe inbreeding depression and their biennial cycle, (Jakše et al., 2010). Haploid onion plant production and subsequent chromosome doubling offers a time-saving approach to obtain pure inbred lines (Dunwell, 2010; Chen et al., 2011). Onion breeding programs based on DH are being conducted at different public institutions such as Cornell University (Hyde et al., 2012), Wisconsin University in collaboration with Ljubljana University (Duangjit et al., 2013), Texas A&M University (Walker et al., 2006), INTA (Dr. Galmarini, personal communication), the Agricultural University of Kraków in collaboration with private companies (Adamus, personal communication), and Pamukale University (Alan et al., 2014; Celebi-Toprak et al., 2015).

Of the different methods for *in vitro* onion haploid production, only gynogenesis has been reported to be successful. Haploid onion plants have been produced from ovules, ovaries or whole flower buds (Muren, 1989; Campion and Alloni, 1990; Keller, 1990; Campion et al., 1992; Bohanec et al., 1995; Geoffriau et al., 1997; Michalik et al., 2000). Of the three aforementioned *in vitro* techniques, ovule culture was the least efficient. Ovary or flower bud culture showed similar results concerning embryo induction, but flower bud culture was less laborious (Bohanec et al., 1995; Bohanec and Jakše, 1999).

The main bottlenecks of gynogenesis in onion are the low rates of embryogenesis induction, plant survival and chromosome doubling from most of the materials (Geoffriau et al., 1997). Several aspects, including genotype and growing conditions of donor plants, culture medium and chromosome doubling procedure, need to be considered to achieve successful rates of gynogenesis (Bohanec, 2009; Chen et al., 2011). Material genotype and genetic structure are the most important factors (Jakše et al., 2010). Thus, low rates of gynogenesis induction have been reported in open-pollinated populations (0–3%) (Geoffriau et al., 1997; Bohanec and Jakše, 1999). Nevertheless, higher rates were achieved in specific synthetic populations, hybrid F1s, and inbred lines (10–33%) (Geoffriau et al., 1997; Bohanec and Jakše, 1999; Michalik et al., 2000; Bohanec et al., 2003).

Temperature stress treatment of donor plants, inflorescences, flowers or isolated ovules can trigger the switch from the gametophytic to the sporophytic pathway in different species (for review see Chen et al., 2011). In onion, the growth of donor plants at low temperatures with high illumination increased

embryogenesis percentages (Puddephat et al., 1999; Michalik et al., 2001). However, the application of temperature stress treatment to flower buds or pre-growth on starvation medium did not enhance the rate of gynogenesis (Bohanec, 1998).

The first studies on onion gynogenesis were performed with ovary or ovule culture. In most cases, a two-step protocol was used including a pre-culture of the flower buds before ovary or ovule isolation. In these reports the basal media B5 (Gamborg et al., 1968), MS (Murashige and Skoog, 1962) and BDS (Dunstan and Short, 1977) were used (Muren, 1989; Campion and Alloni, 1990; Keller, 1990; Campion et al., 1992). Afterwards, flower bud culture protocols were developed based at first on those used for ovary and ovule culture with some modifications of the culture media, including growth regulators (Bohanec et al., 1995; Martínez et al., 2000; Michalik et al., 2000), and later on a simplified one-step protocol, consisting of culturing the whole flower bud in an induction medium until the embryo stage (Bohanec and Jakše, 1999; Jakše and Bohanec, 2003).

A low rate of spontaneous chromosome doubling has been described in onion gynogenesis and is below 10% in most cases (Alan et al., 2003; Jakše et al., 2010). Therefore, anti-mitotic agents have been applied to different explants to increase this rate, including small bulbs (Campion et al., 1995), plantlets during micropropagation (Geoffriau et al., 1997; Alan et al., 2004, 2007), and embryos (Jakše and Bohanec, 2000; Grzebelus and Adamus, 2004). Embryos have several advantages over other explants, including shortening the time needed for plant acclimation and avoiding the step of excision and regrowth of the bulb and/or plant (Jakše and Bohanec, 2000). A comparison of colchicine, trifluralin, orizalin and amiprofos-methyl (APM) treatments showed that colchicine was the least efficient in chromosome doubling and trifluralin and orizalin resulted in higher hyperhydricity (Grzebelus and Adamus, 2004). Alan et al. (2007) compared different strategies for ploidy level manipulation in onion gynogenesis, reporting that somatic regeneration of spontaneous DH plants from flower buds of haploid and mixoploid plants was the most reliable. This strategy was applied later by Jakše et al. (2010).

The main objective of this study was to evaluate the gynogenesis capacity of Spanish onion germplasm using flower bud culture and to optimize the percentage of acclimated doubled haploid (DH) plants. Two gynogenesis induction protocols, previously described in the literature, different plant containers for plant regeneration, and APM treatments for chromosome doubling, were assayed. Somatic regeneration was also tested as an alternative approach for chromosome doubling of haploid (H) gynogenetic plants.

Materials and Methods

Material

Research was carried out from 2012 to 2014 in experimental plots in a shade house located at 41°39'N latitude. In 2012, two Valenciana type commercial varieties Recas (Veronsa) and Rita (kindly provided by Ramiro Arnedo S.A.) were used, as well as a half-sib family from the breeding program carried out

with the cultivar Fuentes de Ebro, a landrace known for its mild and sweet flavor (Mallor et al., 2011a; Mallor and Sales, 2012). Fuentes de Ebro has a high commercial value due to its differentiated quality, provided by the Protected Designation of Origin (PDO)¹ label, according to Regulation (EEC) 1146/2013 of the European Union. In 2013, the cultivars Fuentes de Ebro, Recas, and BGHZ1354, and the population OH-1 were used. BGHZ1354 is a landrace provided by the Vegetable Germplasm Bank of Zaragoza (BGHZ, Zaragoza, Spain) characterized by a high level of pungency (Mallor et al., 2011b). OH-1 is a synthetic population specially designed for high gynogenesis induction, obtained with inbred lines B2923B and B0223B (Havey and Bohanec, 2007).

Growing Conditions of Donor Plants

In February 2011, seeds of donor plants (Fuentes de Ebro, Rita and Recas were sown in polystyrene trays in a greenhouse with a substrate mixture of peat (50%), coconut (30%), sand (20%), and N:P:K (14:16:18) with micronutrient fertilizer (Projar S.A., Valencia, Spain). In April, the plantlets were transplanted directly to soil in the field under natural conditions and bulbs were collected for repose in September. In order to obtain flower heads, the bulbs were planted in November in plastic pots (4 l) and grown in the field under natural conditions. One month before bolting, some bulbs were transferred to a growth chamber exposed to 16 h photoperiod at a continuous temperature of 15–18°C and an illumination of 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Philips Master SON-T, PIA Hg Free 150 W and Phillips Master TL_D 58 W/865. The rest of the bulbs were kept in the field under natural conditions.

In February 2012, seeds of donor plants from Fuentes de Ebro, BGHZ1354, Recas, and OH-1 were sown, grown and harvested as described previously. In November 2012, the bulbs were transplanted directly to soil in the field under natural conditions for inflorescence development in 2013. Whole umbels were harvested from mid-May to the end of June.

Sterilization

The whole umbel was harvested when 30% of the flowers were at three to 4 days before anthesis (Figure 1A). Flowers of 3.5–4.5 mm in length were selected (Figure 1B) from each umbel and sterilized in ethanol 70% for 2 min and 16.5 g l^{-1} dichloroisocyanuric acid disodium salt with two drops of Tween 80 for 10–12 min and followed by 4–5 rinsed with sterile distilled water.

Culture Media

Two different protocols were assayed for gynogenesis induction. In 2012, the protocol described by Jakše and Bohanec (2003) (Protocol A) was followed. Flower buds were cultured on an induction medium consisting of BDS medium (Dunstan and Short, 1977) with some modifications (Supplemental Table S1) and were kept in the same medium until embryo production (one-step protocol). In 2013, a two-step protocol described by

Michalik et al. (2000) (Protocol B) was also used. In this protocol, flower buds were plated on A₁ medium (Muren, 1989) for 1 month and later transferred to R₁ medium (Michalik et al., 2000) (Supplemental Table S1) until embryo production. For protocol comparison, flower buds from the same umbel were randomly distributed in each protocol. Thirty flowers were inoculated in each 90 mm Petri dish.

Chromosome Doubling

Embryos were used as explants for chromosome doubling. The duplication agent amiprofos-methyl (APM) was applied in liquid or solid elongation medium and consisted of BDS (x1/2) with 15 g l^{-1} glucose (Supplemental Table S1). In 2012, 25 μM APM was applied for 24–48 h in liquid medium. In 2013, concentrations of 25 and 50 μM APM were applied for 24 h in liquid elongation medium in a first experiment. In a second experiment, APM (25 μM) was applied in liquid medium for 24 h or in solid medium for 24 or 72 h. Good quality embryos from the “OH-1” population were randomly distributed in different treatments. APM treatment was performed in the dark and afterwards embryos were rinsed with liquid elongation medium.

Plant Regeneration, Acclimation and Bulb and Seed Production

In 2013, two experiments were performed. In the first, glass tubes were sealed with plastic or cellulose plugs (Steristophen Typ 20, Carl Roth GmBH+Co). In the second, glass tubes with plastic plugs were compared with Magenta boxes and polypropylene boxes with an aeration filter (Eco2box-green filter-Model 80MM H, Duchefa). Two to three plants were plated in each Magenta box and 4–6 plants in the Eco2box. Good quality embryos from the OH-1 population were randomly distributed in different containers.

In 2012, APM treated embryos were cultured in solid elongation medium in individual glass tubes with plastic plugs (150 × 25 mm). In 2013, glass tubes with plastic glass and Eco2boxes were used for plant regeneration from Spanish germplasm.

Culture Conditions

Flower buds and plantlets were incubated in a growth chamber at 24°C with 16 h photoperiod and 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by Phillips Master TLD Super 80 58 W/840 and OSRAM 30 W/2700 K.

After 6–10 weeks in elongation medium, rooted, healthy plants were transferred to 110 mm Ø pots containing soil (3:2 peat:vermiculite), covered with a plastic glass for 10 days and planted in a greenhouse. Plants were watered with Hoagland nutrient solution (Hoagland and Arnon, 1950) for the first month. For bulb and seed production, all H, mixoploid and DH plants were transplanted to 50 l plastic pots in the shade house with the same substrate mixture that had been used for donor plant growing.

Somatic Regeneration

Flower buds from H and mixoploid lines obtained in 2013 that bolted in July 2014 were harvested at the same stage as

¹Commission Implementing Regulation (EU) No. 1146/2013 of 5 November 2013 on entering a name in the register of protected designations of origin and protected geographical indications [Cebolla Fuentes de Ebro (PDO)].



FIGURE 1 | Production of doubled haploid onion plants by gynogenesis. **(A)** Onion umbels at the time of harvest. **(B)** Optimal stage of flower bud development for culture (3.5–4.5 mm length, flowers tagged with an asterisk). **(C)** Flower after 7 days of culture. **(D)** Flowers from cultivar Recas after 45 days of culture. **(E)** Gynogenetic embryo from

Fuentes de Ebro emerging from the ovary at 90 days of culture. **(F)** Isolated embryo from Rita. **(G)** Onion embryo treated with APM in liquid medium. **(H)** OH-1 plant regeneration in Eco2box. **(I)** Plants during acclimation in greenhouse. **(J)** Bulb formation in a shade house. **(K)** Seed production from Rita.

for gynogenesis induction, and cultured following the protocol described by Luthar and Bohanec (1999). Flower buds were inoculated in induction medium (I) for 7–8 days and then transferred to differentiation medium (R₂) (Supplemental Table S1). Shoot clumps were transferred to Magenta boxes containing solid elongation medium.

Ploidy Analysis

Ploidy was estimated by flow cytometry from acclimated plants. Young leaves were chopped in 2 ml Cystain UV ploidy solution (Partec) and filtered through a 30 µm nylon filter. Samples were analyzed in a PAS cytometer (Partec). Leaves from young seedlings were used as control.

Statistical Analysis

The following variables were calculated: percentage of gynogenesis induction (number of embryos or calli/100 flowers), percentage of embryogenesis induction (number of embryos/100 flowers), percentage of acclimated plants

(number of acclimated plants/100 embryos), percentage of hyperhydryicity (number of hyperhydrated plants/100 embryos), percentage of H, mixoploid (n/2n), DH and tetraploid as the number of plants/100 acclimated plants. All experiments were established in a completely randomized design. Percentages of gynogenesis induction, embryogenesis induction, acclimated plants, hyperhydryicity, ploidy levels and globular structures were analyzed using the Chi Square test by the FREQ procedure.

Results

Effect of Donor Plant Growing Conditions on Gynogenesis Induction and Plant Production

Bulbs from Fuentes de Ebro, Recas, and Rita were grown in the field and in a growth chamber. A total number of 9934 flower buds from the three cultivars were grown in 2012 following the protocol described by Jakše and Bohanec (2003) (Protocol A) (Table 1). Flowers opened after 3–6 days of culture (Figure 1C),

TABLE 1 | Spanish germplasm gynogenesis following the protocol described by Jakše and Bohanec (2003) (Protocol A) in 2012.

Cultivar	Growing conditions	Flowers	Gynogenesis induction		Embryogenesis induction		Acclimated plants	
			Calli + Embryos	Calli+Embryos/Flowers (%)	Embryos	Embryos/Flowers (%)	Plants	Plants/Embryos (%)
Fuentes de Ebro	F*	1206	9	0.75a***	8	0.66a***	1	12.5a**
	GC**	1688	7	0.41a	6	0.36a	0	0.0b
Recas	F	1998	30	1.50a	28	1.40a	0	0.0b
	GC	1062	16	1.51a	14	1.32a	1	7.1a
Rita	F	2966	49	1.65a	42	1.42a	2	4.8b
	GC	1014	11	1.08a	8	0.79a	2	25.0a

*F, Field; **GC, Growth Chamber; ***Values followed by the same letter within the cultivar are not significantly different ($P < 0.05$).

stayed green during the first month of culture and progressively turned yellow as the culture progressed. Some flowers from Recas and Fuentes de Ebro developed small calli on the flower base after 30–40 days of culture (Figure 1D). Gynogenetic embryos emerged directly from the ovules rupturing the ovary wall after 70–150 days of culture (Figure 1E). The percentage of gynogenesis induction and the percentage of embryogenesis were quite similar, as calli were rarely formed (Table 1). Both variables depended on the cultivar and the growing conditions of the donor plants. The highest percentage of embryogenesis induction was produced from Recas, followed by Rita and Fuentes de Ebro. No statistically significant differences were found in percentages of gynogenesis and embryogenesis induction between the two growing conditions for all cultivars. However, field plants of Fuentes de Ebro and Rita rendered an 80% higher percentage of embryogenesis than growth chamber plants.

Well-developed gynogenetic embryos (Figure 1F) were treated with APM in elongation medium (Figure 1G). Then embryos were transferred to a solid elongation medium for plant development (Figure 1H), and afterwards the plants were transplanted to plastic pots into the greenhouse for acclimation (Figure 1I) and bulb and seed production (Figures 1J,K). A high proportion of embryos developed abnormal or hyperhydrated plants, up to 50% in Fuentes de Ebro and 21% in Recas (data not shown), leading to a low percentage of acclimated plants. A higher percentage of acclimated plants was obtained from field plants in Fuentes de Ebro (12.5%) and from growth chamber plants in Recas and Rita (7.14 and 25%, respectively). Two plants obtained from Fuentes de Ebro and Recas were DH and H, respectively, and those from Rita were one DH and two mixoploids ($n/2n$) (data not shown).

Effect of Culture Protocol on Gynogenesis Induction and Plant Production

Since low percentages of embryogenesis induction and acclimated plants were obtained in 2012, the two-step protocol described by Michalik et al. (2000) (protocol B) was compared with protocol A. The OH-1 population was included in order to determine whether the frequencies of gynogenesis induction obtained were within the predicted range. The gynogenesis capacity of landrace BGHZ1354, was also evaluated. Plants were grown in the field, since in 2012 the percentage of embryogenesis

induction was slightly higher in 2 out of the 3 cultivars in this growing condition, and also the number of plants that can be grown is not limited by the growth chamber capacity. A total number of 10,767 flowers from Fuentes de Ebro, Recas, BGHZ1354, and OH-1 was cultured (Table 2). Different rates of embryogenesis induction were obtained with the two protocols, depending on the material. Spanish germplasm produced 2–3 times higher percentages of embryogenesis with protocol B, Recas showing the highest percentage (2.09%), followed by BGHZ1354 with 1.28% and Fuentes de Ebro with 0.53%. Recas and BGHZ1354 had statistically significant differences between protocols but not the lowest responding cultivar. The percentage of embryogenesis from Spanish germplasm was significantly lower than those obtained from the OH-1 population, where no differences for percentage of embryogenesis between protocols were observed (around 15%).

The percentage of acclimated plants from both protocols also varied depending on the cultivar (Table 2). In BGHZ1354 and Recas, protocol B gave rise to a 2- and 3-fold increase, respectively, in comparison to protocol A. However, no differences in percentage of acclimated plants between protocols were observed in Fuentes de Ebro. Although the percentage of acclimated plants from OH-1 rose from 34 to 47% with protocol B, these differences were not statistically significant.

Furthermore, differences in percentages of embryogenesis induction and plant acclimation were also observed between plants from the same cultivar. Only 2 out of 9 plants from Fuentes de Ebro produced embryos, the highest percentage of embryogenesis being 3.3% with protocol B and 2.0% with protocol A. In OH-1, this percentage varied from 0 up to 93% with protocol B and from 0 to 76% with protocol A (data not shown).

Effect of the Container During Plant Regeneration

In order to reduce plant hyperhydricity, two experiments were performed to assay containers with different levels of ventilation, using OH-1 embryos obtained with both induction protocols (Table 3). In the first, glass tubes sealed with plastic and cellulose plugs were assayed. No statistically significant differences in percentages of hyperhydricity and acclimated plants were observed between plastic and cellulose plugs, independently of

TABLE 2 | Spanish germplasm gynogenesis following protocol described by Jakše and Bohanec (2003) (Protocol A) and described by Michalik et al. (2000) (Protocol B) and the population OH-1 (Havey and Bohanec, 2007).

Cultivar	Protocol	Flowers	Embryogenesis induction		Acclimated plants		Ploidy level of acclimated plants		
			Embryos	Embryos/Flowers (%)	Plants	Plants/Embryos (%)	n	n/2n	2n
Fuentes de Ebro	A	1125	3	0.27a*	1	33.3a*	1	—	—
	B	1127	6	0.53a	2	33.3a	1	—	1
Recas	A	1149	10	0.87b	1	10.0b	1	—	—
	B	1149	24	2.09a	8	33.3a	3	3	1
BGHZ1354	A	1486	6	0.40b	1	16.7b	—	1	—
	B	1481	19	1.28a	6	31.5a	4	1	1
OH-1	A	1624	244	15.00a	84	34.5a	ND	ND	ND
	B	1626	250	15.42a	118	47.2a	ND	ND	ND

*Values followed by the same letter within the cultivar are not significantly different ($P < 0.05$).

TABLE 3 | Effect of plant container during plant regeneration in gynogenesis of OH-1 population.

Experiment	Protocol	Treatment	Embryos	Hyperhydricity		Acclimated plants	
				Plants	Plants/Embryos (%)	Plants	Plants/Embryos (%)
Glass tube plugs	A	Plastic	40	5	12.5a*	12	30.0a*
		Cellulose	43	5	11.6a	16	37.2a
	B	Plastic	30	7	23.3a	13	43.3a
		Cellulose	24	5	20.8a	8	33.3a
Container	A	Glass+plastic	35	6	17.1a	7	20.0b
		Eco2box	28	3	10.7a	7	25.0ab
		Magenta	16	3	18.8a	6	37.5a
	B	Glass+plastic	26	9	34.6b	6	23.1c
		Eco2box	24	1	4.2a	17	70.8a
		Magenta	23	8	34.8b	12	52.2b

*Values followed by the same letter within the cultivar are not significantly different ($P < 0.05$).

the protocol used. The percentage of hyperhydricity almost doubled in plants obtained with protocol B than with protocol A.

In the second experiment, different types of plant containers were compared; glass tube with plastic plug, Eco2box and Magenta box (Table 3). An interaction between container and protocol was observed for percentages of hyperhydricity and acclimated plants. The lowest percentage of hyperhydricity (4.17%) and the highest percentage of acclimated plants (up to 70%) were obtained with Eco2box and protocol B. In protocol A, no significant differences for hyperhydricity percentages were observed between the different types of container, but Magenta boxes produced the highest percentage of acclimated plants (37.5%). In both protocols glass tubes rendered the lowest percentage of acclimated plants (20–23%). As in the previous experiment, protocol B rendered higher percentages of hyperhydricity and acclimated plants than protocol A, except when Eco2 boxes were used.

Effect of APM Application on Chromosome Doubling

Two concentrations of APM were applied in liquid medium to OH-1 embryos to optimize the percentage of chromosome doubling (Table 4). No significant differences in the percentages of acclimated plants and diploid plants were observed between

the two concentrations. However, the percentage of acclimated plants decreased from 55 to 37% when the concentration of APM was raised from 25 to 50 μ M, and the percentage of DH plants increased up to 38%. As a consequence, a similar number of DH plants were produced with both concentrations. Two plants with higher ploidy levels (4n) were also obtained with the lower concentration of APM.

In a second experiment, APM application was compared in solid and liquid media. No significant differences in the percentage of acclimated plants were observed between the application of APM for 24 h in solid and liquid media, but there was a slight increase in the solid medium (from 27 to 40%). APM application in solid medium for 72 h slightly decreased plant survival and produced the lowest percentage of haploid and diploid plants, but up to 57% of mixoploid plants. The highest percentage (35%) and number of DH plants was reached when 25 μ M APM was applied in solid medium for 24 h.

Plant Production from Spanish Germplasm in 2013

Plant production for the onion breeding program was carried out in parallel with experiments described above for the optimization of DH plant production. Protocol A was followed and embryos were treated with 25 μ M APM in liquid medium for 24 h as

TABLE 4 | Effect of APM application on chromosome doubling in OH-1 embryos.

Experiment	Treatment	Embryos	Acclimated plants		Ploidy level of acclimated plants			
			Plants	Plants/Embryos (%)	n (%)	n/2n (%)	2n (%)	4n (%)
APM concentration	25 µM APM	69	38	55.1a*	17 (44.7a*)	8 (21.1a*)	11 (28.9a*)	2 (5.3*)
	50 µM APM	70	26	37.1a	9 (34.6a)	6 (23.1a)	10 (38.5a)	0 (0.0)
Medium support	25 µM APM liq (24 h)	44	12	27.2a	6 (50.0a)	3 (25.0b)	3 (25.0ab)	0 (0.0)
Aplication time	25 µM APM sol (24 h)	42	17	40.5a	10 (58.8a)	1 (5.9c)	6 (35.3a)	0 (0.0)
	25 µM APM sol (72 h)	41	14	34.2a	3 (21.4b)	8 (57.1a)	2 (14.3b)	1 (7.1)

*Values followed by the same letter within the cultivar are not significantly different ($P < 0.05$).

TABLE 5 | Gynogenesis induction from Spanish germplasm in 2013 following Protocol (A) from Jakše and Bohanec (2003) with some modifications.

Cultivar	Flowers	Embryogenesis induction		Acclimated plants		Ploidy level of acclimated plants		
		Embryos	Embryos/Flowers (%)	Plants	Plants/Embryos (%)	n (%)	n/2n (%)	2n (%)
Fuentes de Ebro	6998	18	0.25d*	3	16.67b*	2 (66.7a*)	0 (0.0b*)	1 (33.3b*)
Recas	3227	24	0.74bc	4	16.67b	2 (50.0b)	0 (0.0b)	2 (50.0a)
BGHZ1354	3819	40	1.05b	13	32.50ab	5 (50.0b)	3 (30.0a)	2 (20.0c)
OH-1	4533	826	18.22a	302	36.60 a	—	—	—

*Values followed by the same letter within the cultivar are not significantly different ($P < 0.05$).

performed in 2012, but embryos were transferred to Eco2boxes or glass tubes for plant elongation. A total number of 15,064 flower buds from plants grown in the field from Fuentes de Ebro, Recas, BGHZ1354 and OH-1 were inoculated (Table 5). Great differences were observed between cultivars in the percentage of embryogenesis induction. As expected, OH-1 produced the highest percentage of embryogenesis (18.2%), obtaining a significantly lower rate with the Spanish germplasm. BGHZ1354 showed the highest percentage of embryogenesis (1.05%), and Fuentes de Ebro the lowest (0.52%). These percentages are higher than those obtained with a smaller number of flowers in the experiment to compare protocols (Tables 2, 5). However, similar percentages of embryogenesis were produced from the Recas cultivar.

As for the Spanish germplasm, BGHZ1354 showed the highest percentage of acclimated plants (32.5%), with close rates to those obtained from OH-1. Fuentes de Ebro and Recas reached almost 17%. These percentages were significantly higher than those obtained in 2012 (Tables 1, 5). Different rates of DH plants were obtained from each cultivar: 50, 33, and 20% from Recas, Fuentes de Ebro, and BGHZ1354, respectively. A level of 30% mixoploid (n/2n) plants was obtained from BHGZ1354 (Table 5).

Chromosome Doubling by Flower Bud Somatic Regeneration

Somatic regeneration from flower buds following the protocol described by Luthar and Bohanec (1999) was performed as an alternative approach for chromosome doubling from haploid and mixoploid (n/2n) plants. Five out of the 33 acclimated plants from Spanish germplasm (Tables 2, 5), and 2 out of the 21 plants from the OH-1 population flowered in July 2014 and were used for somatic regeneration (Table 6). After 3–4 weeks

of culture, globular embryogenic structures developed on the bases of the flower buds. Large differences in percentages of embryogenic structures and acclimated plants were observed among cultivars and lines. The haploid line from Fuentes de Ebro produced a medium percentage of embryogenic structures (33%), and a low rate of acclimated plants (2.5%). Of the lines from BGHZ1354, a high percentage of embryogenesis was observed from lines 13.P.045 (72%) and 13.P.074 (53%). The other lines from BGHZ1354 and the 2 lines from OH-1 never or rarely developed embryogenic structures. Shoots were regenerated from lines 13.P.041, 13.P.045, and 13.P.074 that had a high percentage of globular structures, and even from the low embryogenic line 13.P.042. No acclimated plants were produced from OH-1.

Differences in the ploidy level of somatic regenerated plants were observed between H lines. The two plants from 13.P.042 were diploid, whereas similar percentages of H and diploid plants were obtained from 13.P.045. The mixoploid line that produced embryogenic structures rendered up to 88% of diploid plants. Mixoploid (2n/4n) and tetraploid plants were also produced from H and mixoploid plants.

Discussion

The availability of protocols for onion DH production represents a unique opportunity to have completely homozygous and stable inbred lines. Gynogenesis capacity from Spanish germplasm has been evaluated and different protocols have been assayed to increase the number of acclimated plants, in order to introduce pure inbred lines in an onion breeding program, to improve bulb size, uniformity and storability (Mallor and Sales, 2012). Spain is the third largest producer in Europe with over 1.2 million tons. We should remark that the cultivar “Fuentes de

TABLE 6 | Chromosome doubling of gynogenetic haploid and mixoploid plants by flower bud somatic regeneration.

Origin	Gynogenic line	Initial ploidy level	Flowers	Globular structures (%)	Acclimated plants		Ploidy level of acclimated plants			
					Plants	Plants/100 Flowers	n (%)	2n (%)	2n/4n (%)	4n (%)
Fuentes de Ebro-17	13.P.041	N	122	32.8c*	2	1.6bc*	1 (50.0a*)	1 (50.0c*)	0	0
BHGZ1354-16	13.P.045	n	255	71.8a	16	6.3b	7 (43.8a)	8 (50.0c)	1 (6.0a*)	0
BHGZ1354-1	13.P.042	n	134	3.0d	2	1.5bc	0	2 (100.0a)	0	0
BHGZ1354-14	13.P.213	n/2n	182	0.0d	0	0.0c	0	0	0	0
BHGZ1354-17	13.P.074	n/2n	309	52.8b	50	16.2a	0	44 (88.0b)	3 (6.0a)	3 (6.0)
OH-1-8	13.E2.034	n	55	0.0d	0	0.0c	0	0	0	0
OH-1-8	13.E2.040	n	160	2.5d	0	0.0c	0	0	0	0

*Values followed by the same letter within lines are not significantly different ($P < 0.05$).

Ebro” used in this study has a high commercial value due to its differentiated quality. Genetic factors, including cultivar, donor plant genotype, geographic origin and genetic structure are thought to be the most important for the success of gynogenesis induction (Campion and Alloni, 1990; Geoffriau et al., 1997; Bohanec and Jakše, 1999; Michalik et al., 2000; Chen et al., 2011). In Spanish germplasm, cultivar and plant genotype also has a strong effect on gynogenesis induction. The two Valencia-type cultivars Rita and Recas showed the highest embryogenesis percentage (0.87–2.09%), followed by the highly pungent landrace BGHZ1354 (0.40–1.28%), and finally the sweet cultivar Fuentes de Ebro (0.27–0.75%). A similar percentage of embryogenesis induction was described in the unique Spanish cultivar Morada de Amposta evaluated previously (2.1%) and in 5 Portuguese cultivars (average 0.98%) (Bohanec and Jakše, 1999). However, gynogenesis capacity of Spanish germplasm used in this study was lower than that from the American OH-1 population, and other American inbred and F1s reported by Bohanec and Jakše (1999). It has also been reported that American materials were on average 5 times more responsive than European cultivars (Bohanec et al., 2003), and northern European cultivars were more responsive than southern- and eastern European material (Geoffriau et al., 1997; Bohanec and Jakše, 1999). On the other hand, open pollinated cultivars, such as the ones used in this study, had shown a lower percentage of embryogenesis than inbred lines or F1s (Geoffriau et al., 1997; Bohanec and Jakše, 1999). Deleterious genes regulating vegetative growth are responsible for hampering gynogenic embryo development and plant regeneration and are eliminated during inbreeding (Geoffriau et al., 1997; Hyde et al., 2012).

A great variation in gynogenesis induction was also reported even between plants from the same inbred line. By way of example, B2923B showed a percentage of embryogenesis from 2.1 to 54.7% (Bohanec et al., 2003). Similar results were obtained in this study among individual plants from the same cultivar or population, since each plant was a different genotype. In this study, plant response from the synthetic population OH-1, characterized by its high embryogenesis induction and obtained from inbreds B2923B and B0223B (Prof Havey, University of Wisconsin), varied from 0 to 93%. As for the Spanish germplasm, the highest variation, from 0 to 11%, was found in Recas.

The environmental conditions under which the donor plants are grown are also a key factor influencing embryogenesis

induction (Campion et al., 1992; Chen et al., 2011). Onion plants in the growth chamber during bolting at 14–15 °C produced higher percentages of embryogenesis than plants in the greenhouse (13–25°C) (Puddephat et al., 1999) or plants in growth chamber at 4°C or 18°C or in the field (Michalik et al., 2001). In this study, no statistically significant differences were obtained between the two growing conditions, probably due to the low number of embryos produced. However, Fuentes de Ebro and Rita plants grown in the field rendered a slightly higher percentage of embryogenesis than those in the growth chamber at 15–18°C.

The differences in the percentage of gynogenesis induction from plants grown under natural conditions observed between years could be due to environmental, climatic or physiological conditions, as they were grown in pots or in soil under natural conditions, as well as genotype effects. A variation in the percentage of gynogenesis induction between the same bulbs over 2 years (23.2–32.9%) has been described in an onion inbred line (Bohanec et al., 2003).

Gynogenesis response of onion material depended on the culture media used (Campion et al., 1992; Jakše et al., 1996; Michalik et al., 2000). Thus, local cultivars from Argentina produced more haploid plants in media containing polyamines than without growth regulators (Martínez et al., 2000). In order to improve the percentage of embryogenesis obtained with our material in 2012 with the one-step protocol (protocol A) described by Jakše and Bohanec (2003), a two-step protocol (protocol B), that had been proved to induce gynogenesis efficiently in Polish cultivars (Michalik et al., 2000), was also assayed. A real comparison of the two protocols was possible since the same cultivars have been used. The low-responding Spanish material produced 2–3 times higher percentages of embryogenesis and plant acclimation with the two-step protocol described by Michalik et al. (2000). No significant differences were obtained in Fuentes de Ebro, probably due to the low number of embryos produced. The high gynogenetic population OH-1 produced similar percentages of embryogenesis, an average of 15%, independently of the protocol used. It had been reported previously that inbred lines B2923B and B0223B, used for the creation of the OH-1 synthetic population, reached 18–33% of gynogenesis induction (Bohanec et al., 2003; Jakše and Bohanec, 2003). Therefore, our results are comparable to those described by these authors.

The protocols differed in culture medium composition, vitamins, amount of ammonium and growth regulators. Regarding the latter, 2iP and NAA in protocol B, instead of 2,4-D and BA (protocol A) could account for the higher gynogenesis efficiency. Higher percentages of embryogenesis were also reported with Polish onion cultivars, with media containing 2iP and NAA (Michalik et al., 2000). The effect of growth regulators on plant acclimation confirms the results described by Bohanec et al. (1995). Hence, protocol B will be adopted for the development of onion haploids in our laboratory.

The presence of an unbalanced gaseous environment in the culture container could lead to plant hyperhydricity (Kozai and Smith, 1995; Lai et al., 2005). The use of different containers and/or container closure could cause different rates of gas exchange. In this study, a high percentage of hyperhydricity was observed during the first year, especially in Fuentes de Ebro (up to 50%) and Recas (21%). Different types of plug for glass tubes and containers were tested to decrease hyperhydricity and even though cellulose plugs were expected to reduce hyperhydricity and favor a better gas exchange than plastic plugs, a similar percentage of hyperhydricity was obtained with both types of plug in this study. Nevertheless, hyperhydricity was reduced to 4% and the percentage of acclimated plants was increased up to 70% with Eco2box in protocol B. This could be due to the presence of an aeration filter in the lid, favoring gas exchange. Our results agree with those reported in other crops in which hyperhydricity was reduced or even completely eliminated in ventilated cultures (Zobayed et al., 1999; Lai et al., 2005; Casanova et al., 2008; Ivanova and Van Staden, 2010). As far as we know, Eco2boxes have not been used previously for plant regeneration in onion. The differences observed from Spanish germplasm in the percentage of acclimated plants between years could be due to the use of both Eco2boxes and glass tubes for plant regeneration in the second year, but exclusively glass tubes in the first year. Other important factors associated with hyperhydricity are the amount of ammonium and the type of cytokinin in the medium (Ivanova and Van Staden, 2008). Since ammonium was only used in the first month of culture in the two-step protocol, the higher percentage of hyperhydricity produced with this protocol could be related to the use of the cytokinin 2iP instead of BA. However, BA has been reported to produce higher rates of hyperhydricity than other cytokinins such as kinetin, zeatin or thidiazuron (Chukwujekwu et al., 2002; Chen et al., 2006; Ivanova and Van Staden, 2011).

Several duplication agents and different strategies have been used for chromosome doubling of onion haploid embryos/plants (Campion et al., 1995; Alan et al., 2004; Grzebelus and Adamus, 2004). In general, the optimal concentration of the duplication agent and the duration of treatments must always be determined in relation to the percentage of doubled plants and the percentage of plant survival (Bohanec, 2009; Castillo et al., 2009). Although a relatively low number of embryos was used for each treatment, an increase in the concentration of APM from 25 to 50 μM in a liquid medium significantly increased the percentage of chromosome doubling. However, the same number of DH plants was obtained in both concentrations, since the high concentration reduced the percentage of plant survival. Similar results were reported previously in onion by Jakše et al. (2003).

A higher percentage of diploid plants were produced when duplication agents were incorporated in liquid rather than in solid media (Jakše et al., 2003; Alan et al., 2004). However, in our study APM in a solid medium produced a higher survival rate (40%) and a higher percentage of doubling (35%) than in a liquid medium, resulting in 14% of DH/embryos treated. This percentage was higher than the 4% obtained by Jakše et al. (2003) with the same concentration of APM and application time. The differences between these two studies could be due to the use of different genotypes, since a genotype effect has been reported (Alan et al., 2004). In this study, increasing the time of application from 24 to 72 h in a solid medium significantly increased the percentage of mixoploid ($n+2n$) plants up to 57%, confirming the results reported by Jakše et al. (2003).

An alternative approach for chromosome doubling in onion is *in vitro* adventitious somatic regeneration (Alan et al., 2007; Jakše et al., 2010). This method has two advantages over the application of antimitotic agents: no potentially damaging chemicals are used and a higher percentage of doubling efficiency can be achieved (Alan et al., 2007; Jakše et al., 2010). When the protocol described by Luthar and Bohanec (1999) was applied, a large variation in somatic regeneration response was observed between different germplasms. The landrace BGHZ1354 showed the highest percentage of globular embryogenic structures (up to 72%), followed by "Fuentes de Ebro." No correlation between somatic and gynogenetic embryogenesis was observed, since DH lines 13.E2.034 and 13.E2.040 derived from OH-1-8 plant produced a low percentage of embryogenic structures (0–2.5%), whereas OH-1-8 showed the highest percentage of embryogenesis induction (76–98%). Similar results were observed from line 13.P.042, indicating that genetic control for somatic regeneration and gynogenesis induction are inherited independently.

In the first study using this strategy for chromosome doubling 60% of the somatic plants regenerated from haploid flowers were spontaneously doubled (Alan et al., 2007). Jakše et al. (2010) produced diploid plants from 83 and 100% of regenerated haploid and mixoploid lines, respectively, and the average percentages of diploid plants among the regenerants was 55.8% from mixoploid lines and 9.7% from haploid lines. In this study, 4 out of the 7 lines that bolted the first year were regenerated, and diploid plants were produced from all of them (100%). The percentage of spontaneous doubling was 88% from the mixoploid line and between 47 and 100% from the haploid lines. Our results confirm that somatic regeneration is an alternative approach for chromosome doubling in onion. As described by Alan et al. (2007), the use of different strategies offers an integrated approach to recover diploid onion gynogenetic plants. In this study, both the application of APM to embryos, and diploidization of haploids and mixoploid lines by somatic regeneration were used successfully.

The gynogenesis capacity of Spanish germplasm has been evaluated. This germplasm showed a low capacity for embryogenesis induction, the sweet cultivar Fuentes de Ebro being the most recalcitrant. For the first time comparison of two protocols has been addressed, the protocol described by Michalik et al. (2000) being more efficient for embryogenesis induction and acclimated plants than the one described by Jakše

and Bohanec (2003). A new generation of tissue culture vessel, the Eco2box container, significantly reduced the percentage of hyperhydricity leading to a higher percentage of plant survival. Two strategies for chromosome doubling, the application of 25 µM APM in solid medium for 24 h and somatic regeneration from flower buds of haploid and mixoploid plants, were successfully used. Although gynogenetic plants were obtained from all the Spanish cultivars, further improvements of the protocol using different strategies should be performed in order to produce a high number of DH plants that could be used efficiently in onion breeding programs.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00384/abstract>

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Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging

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Plants display a remarkable capacity for cellular totipotency. An intriguing and useful example is that immature pollen cultured *in vitro* can pass through embryogenic development to form haploid or doubled haploid plants. However, a lack of understanding the initial mechanisms of pollen embryogenesis hampers the improvement and more effective and widespread employment of haploid technology in plant research and breeding. To investigate the cellular dynamics during the onset of pollen embryogenesis, we used time-lapse imaging along with transgenic barley expressing nuclear localized Green Fluorescent Protein. The results enabled us to identify nine distinct embryogenic and non-embryogenic types of pollen response to the culture conditions. Cell proliferation in embryogenic pollen normally started *via* a first symmetric mitosis (54.3% of pollen observed) and only rarely did so *via* asymmetric pollen mitosis I (4.3% of pollen observed). In the latter case, proliferation generally originated from the vegetative-like cell, albeit the division of the generative-like cell was observed in few types of pollen. Under the culture conditions used, fusion of cell nuclei was the only mechanism of genome duplication observed.

Keywords: barley, pollen, embryogenesis, live-cell, imaging

INTRODUCTION

The life cycle of higher plants involves the alteration of sporophytic and gametophytic generations. Whereas the sporophyte constitutes the plant in its apparent form, the female and male gametophytes are reduced in size and have become depending on the sporophyte.

Under natural conditions, spontaneous formation of haploid embryos and plants in angiosperms can arise from female gametophytic cells but not from male gametophytic cells (McKone and Halpern, 2003). However, male gametophytes (i.e., immature pollen) cultivated *in vitro*, can be induced to become embryogenic and form sporophyte-like haploid embryos and plants. Through spontaneous or artificially triggered genome duplication, doubled haploids can arise that themselves are genuine sporophytes.

The value of haploid technology in plant research and breeding lies in the fact that the founder cells of doubled haploids are products of meiosis, and resultant plants constitute pools of diverse recombinant, yet genetically fixed individuals. Their recombinant genome is fixed through homozygosity of the doubled haploid plants produced. The employment of haploid technology has become widely used in breeding programs of many crop species (Germanà, 2011).

Doubled haploids are also widely used in genetic studies, such as QTL, gene mapping and marker-trait association (Murovec and Bohanec, 2012). Genetic transformation of embryogenic

pollen allows for the production of instantly true-breeding transgenic plants (Kumlehn et al., 2006; Eudes and Chugh, 2008; Chauhan and Khurana, 2011). A novel approach for haploid induction has recently been developed by the genetic engineering of the centromeric region (Ravi and Chan, 2010).

Although distinct pathways of pollen embryogenesis have been proposed (Sunderland and Evans, 1980; Hu and Kasha, 1999), recent evidence hints at the presence of multiple pathways within one culture (Hu and Kasha, 1999; Kasha et al., 2001). Maraschin et al. (2005), using time-lapse studies, identified three such pathways within immature barley pollen cultures.

Spontaneous genome doubling can result in completely fertile doubled haploids. In barley and wheat, spontaneous doubling frequencies between 18 and 85% have been observed, (Jähne and Lörz, 1995; Hu and Kasha, 1999). Several mechanisms for plant genome doubling have been proposed: (i) endoreduplication, (ii) nuclear fusion, (iii) endomitosis, and (iv) c-mitosis (Jensen, 1974; d'Amato, 1989; González-Melendi et al., 2005; Kasha, 2005; Seguí-Simarro and Nuez, 2008). However, none of the proposed genome doubling mechanisms has ever been observed in living cells.

Despite the great value of pollen embryogenesis, very little is known about the underlying cellular mechanisms. In vacuolated immature pollen, embryogenesis can be induced by various treatments both *in vivo* and *in vitro* (Touraev et al., 1997). Due to the

high amenability to pollen embryogenesis, barley has become a model species to study this phenomenon in temperate cereal crop species (Sunderland et al., 1974; Kasha, 2007).

This present paper is the first to give a full account of the initial cellular dynamics in the pollen embryogenesis process until the formation of growing multicellular structures. The approach relies on multi-dimensional (4D) live-cell imaging of transgenic pollen expressing the *Green Fluorescence Protein (GFP)* gene with nuclear localization signal using a temporal resolution of 3 min.

RESULTS

GENERATION OF TRANSGENIC BARLEY EXPRESSING SV40-NLS:GFP

A total of 71 primary transgenic (T_0) plants were generated by co-culture of embryogenic pollen with *Agrobacterium* strain LBA4404/pSB1. This strain harbored a *GFP* gene fused to the Simian virus SV40 nuclear localization signal under the control of the maize *UBIQUITIN1* promoter with first intron (Figure 1C). Ploidy and presence of the selectable marker gene *HPT* were checked by flow cytometry and PCR, respectively. Most regenerants were haploid (43.7%) or diploid (47.9%), while the remaining ones were tetra- (5.6%) or mixoploid (2.8%). Five spontaneously doubled haploid and 20 colchicine-treated plants were grown to maturity. Three colchicine-treated haploids did not set grain. The remaining 22 fertile doubled haploids contained the *HPT* selectable marker gene. Confocal Laser Scanning Microscopy (CLSM) analysis showed only a single line to accumulate appreciable amounts of GFP in its nuclei. This line was used to produce embryogenic pollen cultures from which 27 T_1 regenerants were generated including 7 tetraploid, 5 triploid, and 15 diploid plants. Sexual progeny of 7 randomly selected diploid T_1 -lines were grown. To confirm integration of *HPT* and *NLS:GFP*, four T_2 -families were selected at random for DNA gel blot analysis (Figure 1A) and PCR (Figure 1B). Primer pairs and specific probe were selected to cover most functional parts of the T-DNA (Figure 1C). All plants contained both *HPT* and *NLS:GFP* (Figure 1B) and showed an identical T-DNA integration pattern (Figure 1A), confirming that the doubled haploid T_1 -lines were homozygous for the transgene. Nuclear localization of GFP in T_0 transgenic plants was confirmed by CLSM (Figures 1D–F).

LIVE-CELL IMAGING OF CULTURED POLLEN OVER TIME

In eight separate experiments, the development of a total of 71 immature pollen was followed over a time span of up to 15 days. Of these pollen, 70 were (uni-nucleate) microspores and a single one bi-cellular. With a diameter of about 40 μm , the bi-cellular pollen was distinctly larger than the microspores, which were about 30 μm . Over time, the bi-nucleate pollen increased in size without showing any mitotic activity and started to accumulate starch (Supplementary Figures 1C,D) before dying on the second day of culture (Supplementary Figures 1E,F; see Supplementary movie 1). The small generative-like nucleus of this bi-cellular pollen remained spherical (Supplementary Figures 1A–D; see Supplementary movie 1). The remaining 70 immature pollen that were at the late microspore stage at the onset of observation showed various developmental patterns with regards to pollen mitosis I and to their final fate, so that nine

different developmental pathways could be discerned (Table 1). The majority of pollen (61.1%; types I, II, and III) started the development with a symmetrical cell division, whereas types IV, V, and VI had in common to undergo asymmetric pollen mitosis I. In contrast to types I–VI, no mitotic activity was observed in types VII–IX.

The nucleus of type I pollen (54.3% overall) contained a single nucleolus (Figure 2A). Prior to mitosis, the nucleus migrated from a strictly peripheral to a more central position and began moving rapidly while increasing in size, with cytoplasmic strands radiating from the nuclear periphery (Figures 2B,C) (Supplementary movie 2). After symmetric first mitosis (Figure 2D), the two resulting daughter cells remained mitotically active. Despite numerous rounds of synchronized mitoses (Figures 2F,G), the pollen did temporarily not increase in size; during this period of time, the cytoplasmic proportion of the individual cells increased while the vacuoles accordingly dwindled in size (Figures 2A–H). This type of pollen did not show any detectable starch accumulation. Type I development represents the major pathway of pollen embryogenesis under the conditions used in this study.

Type II showed an early difference to type I development (2.9% overall; see Supplementary movie 3). Here the nucleus prior to mitosis did not move to a central position and remained opposite of the pollen aperture. Furthermore, the cytoplasm remained largely peripheral and did not form cytoplasmic strands upon mitosis (Supplementary Figures 2B–F). Despite the formation of two nuclei of equal size and shape, a cell wall remained absent as judged by Differential Interference Contrast (DIC) microscopy (Supplementary Figures 2B–G) an unopposed movement of the two daughter nuclei throughout the cytoplasm was observed (Supplementary Figures 2B,C). Later this pollen started to deposit starch from day 3 onwards (Supplementary Figure 2D) and shortly died as indicated by cell shrinkage and the loss of GFP signal in the nucleus (Supplementary Figures 2F–H).

In type III development (4.3% overall; see Supplementary movie 4), the nucleus remained opposite of the pollen aperture at the time of symmetric cell division (Figures 3A,B). In contrast to type II development, the two daughter nuclei moved to the center of the cell (Figure 3C) with cytoplasmic strands radiating from their surface (Figure 3D), but showed no further mitotic activity. Type III pollen gradually accumulated starch without increasing in size over time (Figures 3D–H). In contrast to type II, type III pollen remained viable for more than 2 weeks, i.e., during the entire time of observation as shown by GFP signal in their nuclei (Figure 3H).

Of the pollen whose first mitosis was asymmetrical (types IV, V, and VI), only type IV followed an embryogenic pathway (4.3% overall; Figure 4, Supplementary movie 5). Type IV pollen significantly increased in size and cytoplasmic strands appeared prior to the first mitosis (Figure 4C). The nucleus, however, remained residing opposite of the pollen aperture (Figures 4A–D). The first mitosis was followed by an asymmetric cell division resulting in a large vegetative-like cell and a much smaller generative-like cell (Figure 4E). Whereas the latter remained inactive and opposite of the pollen aperture, the former showed active movements and the appearance of cytoplasmic strands that preceded

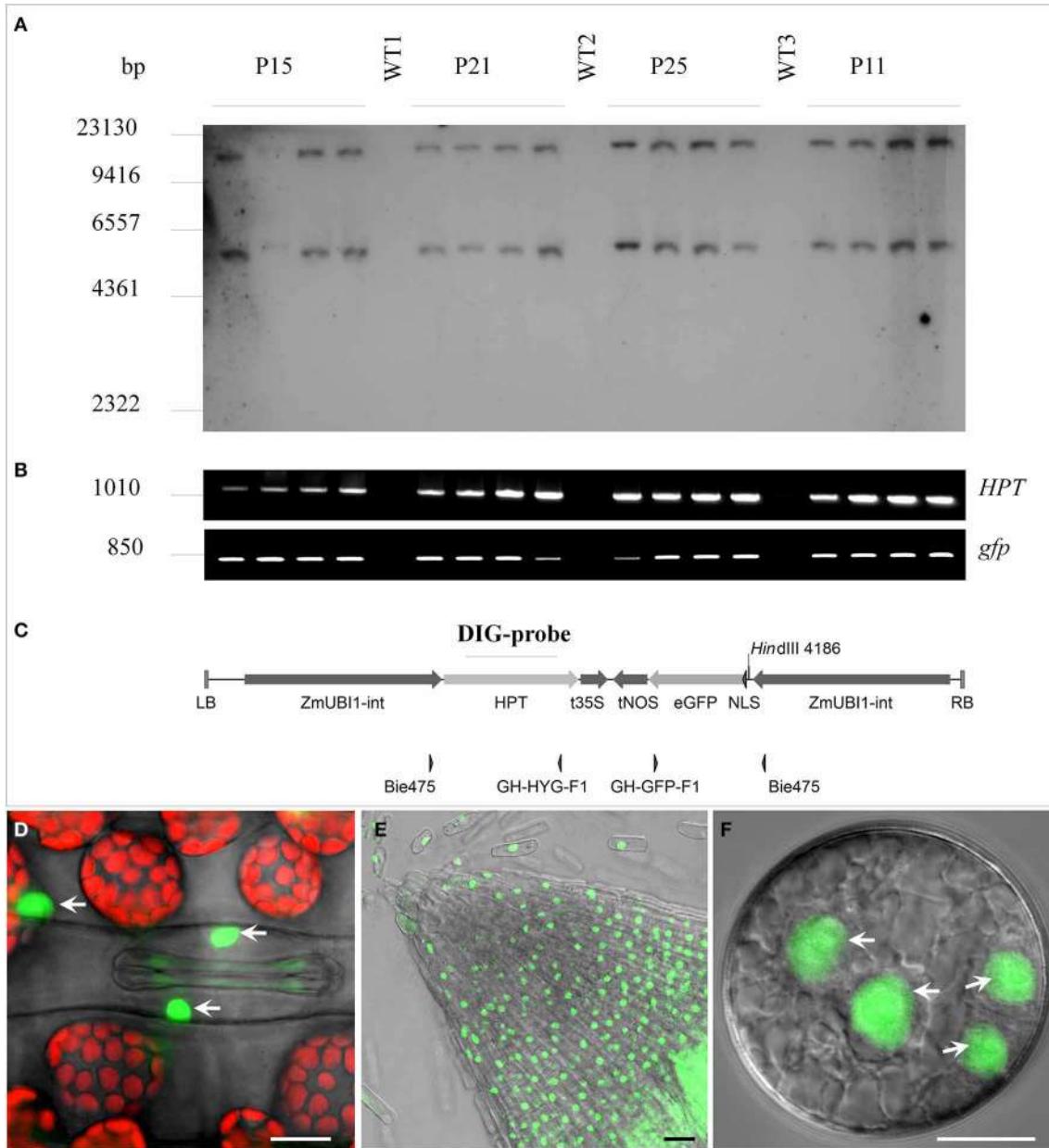


FIGURE 1 | Characterization of the doubled haploid SV40-NLS:GFP transgenic barley line displaying nucleus-specific accumulation of GFP. Randomly chosen T₂-siblings (four per family) derived from four T₁-plants (P11, P15, P21, P25) were analyzed for genomic T-DNA integration and transgene zygosity. **(A)** DNA gel blot analysis of HindIII-digested genomic DNA hybridized with an HPT-specific probe. The two bands seen per lane indicate genomic integration of two T-DNA copies. **(B)** PCR analysis with primer pairs specific for the HPT (upper band) and the GFP (lower band) genes. **(C)** Map of T-DNA with primer pairs and hybridization probe positions indicated. WT1, 2 and 3,

wild type individuals of cv. "Igri"; LB, left border; ZmUBI1-int, maize *UBI/QUITIN1* promoter with first intron; HPT, hygromycin B phosphotransferase protein coding region; t35S, *CaMV 35S* gene terminator; tNOS, *NOPALINE SYNTHASE* gene terminator; eGFP, synthetic S65T green fluorescent protein coding region; NLS, SV40 Simian virus 40 nuclear localization signal; RB, right border. **(D)** Leaf tissue with GFP in the nuclei of guard and other epidermis cells. Chlorophyll autofluorescence shown in red. **(E)** GFP accumulation in the nuclei of a root tip. **(F)** GFP accumulation in nuclei of immature pollen after the second embryogenic pollen mitosis. Bar = 30 µm.

a further mitosis (**Figures 4F,G**) resulting in two similar daughter nuclei (**Figure 4H**). Further synchronized divisions produced a multicellular structure that showed no sign of amyloplast formation and starch accumulation (**Figures 4I–L**). Compared to type I development, cell proliferation in type IV was delayed by

several days. Over the time of observation, the generative-like cell degenerated in most cases; and although it remained viable in some cases, (**Figures 4E–L**).

Developmental type V (2.9% overall) was very similar to developmental type II in terms of cell size, thin-layered cytoplasm,

Table 1 | Types of pollen development as observed in live-cell imaging experiments during the initial 2 weeks of culture under conditions supportive for pollen embryogenesis.

Developmental type	No. of pollen	% of total pollen
SYMMETRIC MITOSIS I		
Embryogenic	I	38
Bi-nucleate, starchy, collapsed	II	2
Bi-nucleate, starchy, survived	III	3
ASYMMETRIC MITOSIS I		
Embryogenic	IV	3
Division of generative cell, no starch, survived	V	2
Bi-cellular, starchy, collapsed	VI	5
FAILED MITOSIS I		
Micronuclei formation	VII	1
Pollen expansion	VIII	2
No development	IX	14
		20.0

starch accumulation and ultimate cell degeneration, except for the fact that here the first pollen mitosis was asymmetric.

Type VI pollen (7.1% overall; Supplementary movie 6) was characterized by forming a larger than usual, spherical, generative-like cell (**Figures 5A,B**) that was capable of undergoing two successive symmetric and synchronized divisions (**Figures 5C–H**). The nuclei produced by the mitotic activity of the generative-like cell remained within the original boundaries as defined after the first asymmetric division (**Figures 5B–H**). Of particular note is the small size of nuclei originating from the generative-like cell as compared to those derived from the vegetative-like cell (**Figures 5F–H**).

Of the pollen that failed to undergo mitosis, developmental type VII (1.4% overall; Supplementary movie 7) showed a remarkable increase in nuclear size over time (Supplementary Figures 3C–E). The pollen itself, however, did not expand and neither did form amyloplasts for starch accumulation. Cytoplasmic strands were not visible and the nucleus eventually disintegrated into multiple micronuclei (Supplementary Figures 3G–H). Despite fragmentation of the nucleus (on end of day 2 × of culture), the cell was still alive when observation ended.

Developmental type VIII (2.8% overall) pollen became exceptionally large (diameter 60 μm) but did not show any particular signs of development before dying within the first 2–3 days of culture.

Immature pollen of developmental type IX (20.0% overall) did not show any developmental changes during the time of observation; this pollen died sooner or later.

SPONTANEOUS GENOME DOUBLING DURING POLLEN EMBRYOGENESIS

Live-cell imaging revealed that nuclear fusion is a common process observed in more than 40% of the multicellular pollen and occurring throughout pollen embryogenesis (**Figures 6–8**; Supplementary movie 8) rather than being limited to a certain stage. Notably, polyploid products of multiple consecutive nuclear fusions were also frequently observed (**Figures 6B,G,O**,

8). In some extreme cases, such processes resulted in large tube-shaped nuclei (**Figure 8E**).

More detailed information on the nuclear fusion process was obtained from electron microscopy studies. Nuclear fusion starts with a close alignment of two nuclei (**Figures 7A, 8A–C**), followed by fusion of the nuclear envelopes (**Figures 7B,C**). In some cases, cell wall was partially present at the site of the assumed nuclear fusion (**Figures 7D,E**). Nuclear fusion may account for the often occurring irregular shape of nuclei and the recurrent presence of cytoplasmic pockets within the nucleoplasm (**Figures 7E,G**). An intriguing observation was that of an elongated nucleus featuring a median invagination and unusual distribution of heterochromatin, which appeared to be absent from a narrow median band in the plane of the median constriction (**Figure 7G**).

The information gained from live-cell imaging and electron microscopy studies suggests that mitosis is not always followed by cell wall formation (**Figures 6–8**). Failure of cell wall formation can occur at any stage of pollen embryogenesis and so can nuclear fusion. This would explain the chimeric ploidy level often observed within individual multicellular structures (**Figure 8E**).

DISCUSSION

Only a few previous studies have followed the embryogenic development of isolated pollen in culture, e.g., Indrianto et al. (2001) in wheat as well as Kumlehn and Lörz (1999) and Maraschin et al. (2005) in barley. In these pioneering observations, time intervals of several hours or days were used, thus allowing only for a sketchy outline of the process. We here present a detailed monitoring of pollen embryogenesis from the vacuolated immature uni-nucleate barley pollen (the pre-mitotic microspore) until the formation of growing multicellular structures using a temporal resolution of only 3 min. The use of transgenic barley expressing a GFP-construct with nuclear-localization signal further greatly improved the accuracy of observations.

POLLEN DEVELOPMENT *IN VITRO*

Populations of immature pollen *in vitro* always show variability in the developmental state of individuals. Reasons for this heterogeneity include that pollen is typically isolated using a number of different spikes at once, that the florets of a spike show a developmental gradient along the rachis, and that nutrient provision and signal perception even of each individual pollen grain may well depend on the particular position within the anther. In addition, pollen of hybrid plants (that are typically used to produce doubled haploids in breeding practice) or of outbreeding species are genetically diverse due to meiotic segregation. In our experiments, 58.6% of pollen analyzed eventually formed multicellular structures (**Table 1**). This agrees with observations by Maraschin et al. (2005) who identified only three developmental pathways; whereas the present study enabled us to distinguish nine different types of pollen response to the given culture conditions (**Table 1**).

Indrianto et al. (2001) in wheat and Maraschin et al. (2005) in barley showed that multicellular structures were only obtained from immature pollen that were enlarged after induction treatment. In the present study, however, the type I pollen grains that underwent cell proliferation most efficiently did temporarily not increase in size during the initiation of cell proliferation. A very

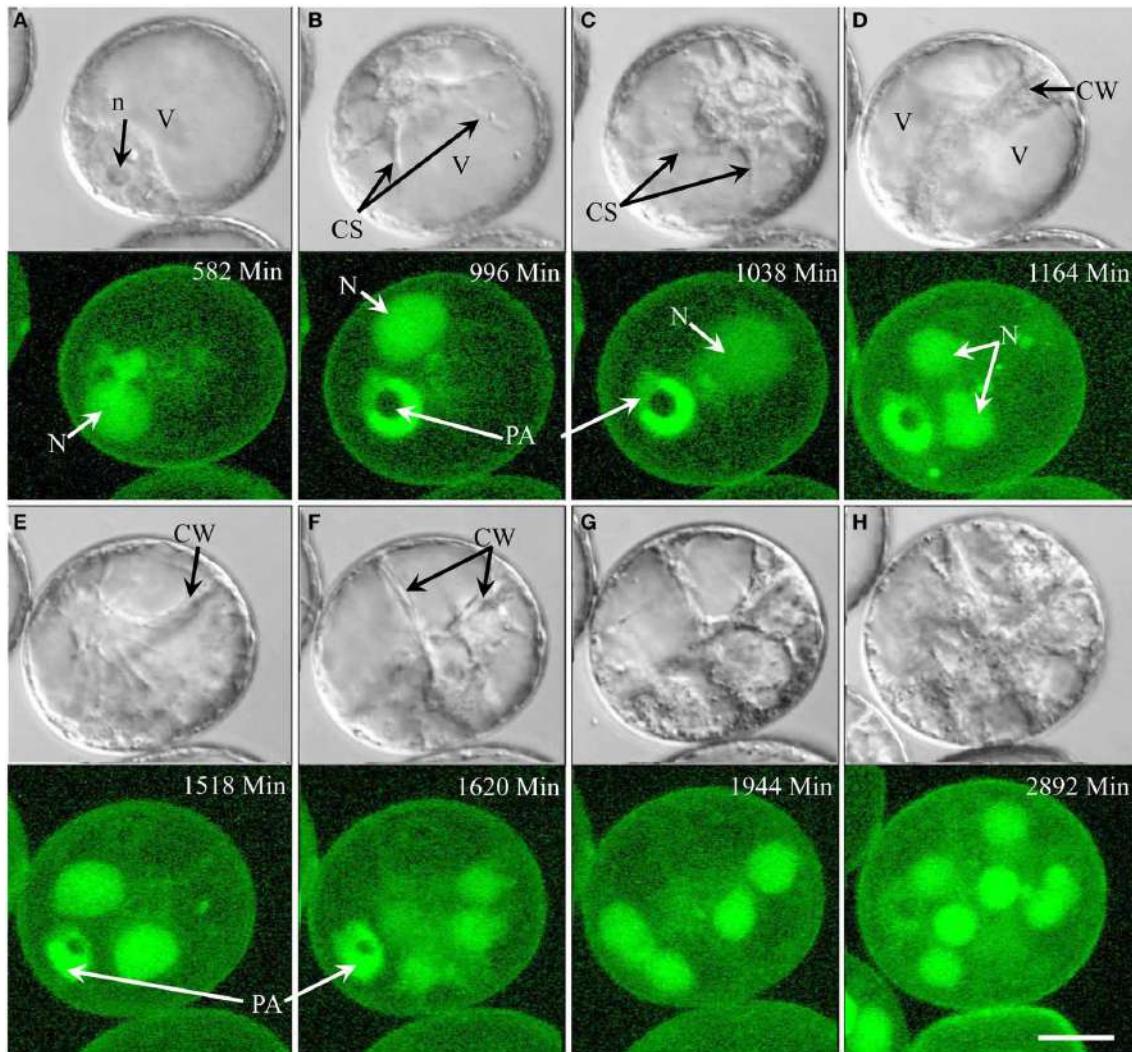


FIGURE 2 | Time-lapse of type I development (embryogenic pollen) shown by synchronously acquired DIC and fluorescence images. **(A)** Uni-nucleate pollen (microspore) with nucleus close to pollen aperture. **(B,C)** The nucleus has migrated away from the pollen periphery and cytoplasmic strands are formed. The blurred fluorescence signal indicates the break-down of the nuclear envelope prior to mitosis. **(D)** Newly formed cell wall (DIC) and

a pair of daughter nuclei (GFP) after mitosis. **(E)** Appearance of cytoplasmic strands indicating imminent second mitosis. **(F,G)** Newly formed intermediate cell wall (DIC) separates four cells (GFP) contained within the pollen envelope. **(H)** Additional cycles of mitosis create a multicellular structure. CS, cytoplasmic strand; CW, cell wall; N, nucleus; n, nucleolus; PA, pollen aperture. Bar = 20 μ m.

similar scenario has been shown in cultures of isolated wheat zygotes (Kumlein et al., 1999; Figure 2I), where an initial series of cell divisions result in a step-wise decrease in cell size without substantial enlargement of the whole proembryo prior to the onset of its exponential growth based upon proliferation of small, cytoplasmic rich cells that are characteristic for the proper of globular zygotic embryos. Likewise, the formation and ongoing proliferation of small cells is indicative of the embryogenic nature of type I pollen. Type IV pollen did temporarily increase in size, which was associated with a remarkable delay in first mitosis. The resultant vegetative-like cell then behaved like the microspore cell of type I pollen in undergoing embryogenic development involving a successive decrease in cell size over an initial series of mitoses followed by continued proliferation of small cells. By

contrast, other pollen grains that increased in size shortly after inductive treatment (types II, V, and VIII) eventually accumulated starch in amyloplasts and did not show continuous cell proliferation. Since amyloplasts are not unambiguously recognized by differential interference contrast microscopy used in this study, we have investigated samples of comparable developmental stages of barley pollen embryogenesis also by transmission electron microscopy and thereby proved that these structures are indeed starch-containing granules (data not shown).

Successful induction of embryogenesis in uni-nucleate pollen was usually associated with a first symmetric mitosis (92.7% of embryogenic pollen; type I) and only rarely with a first asymmetric mitosis (7.3% of embryogenic pollen; type IV). In contrast to pollen response type IV, both microspore daughter

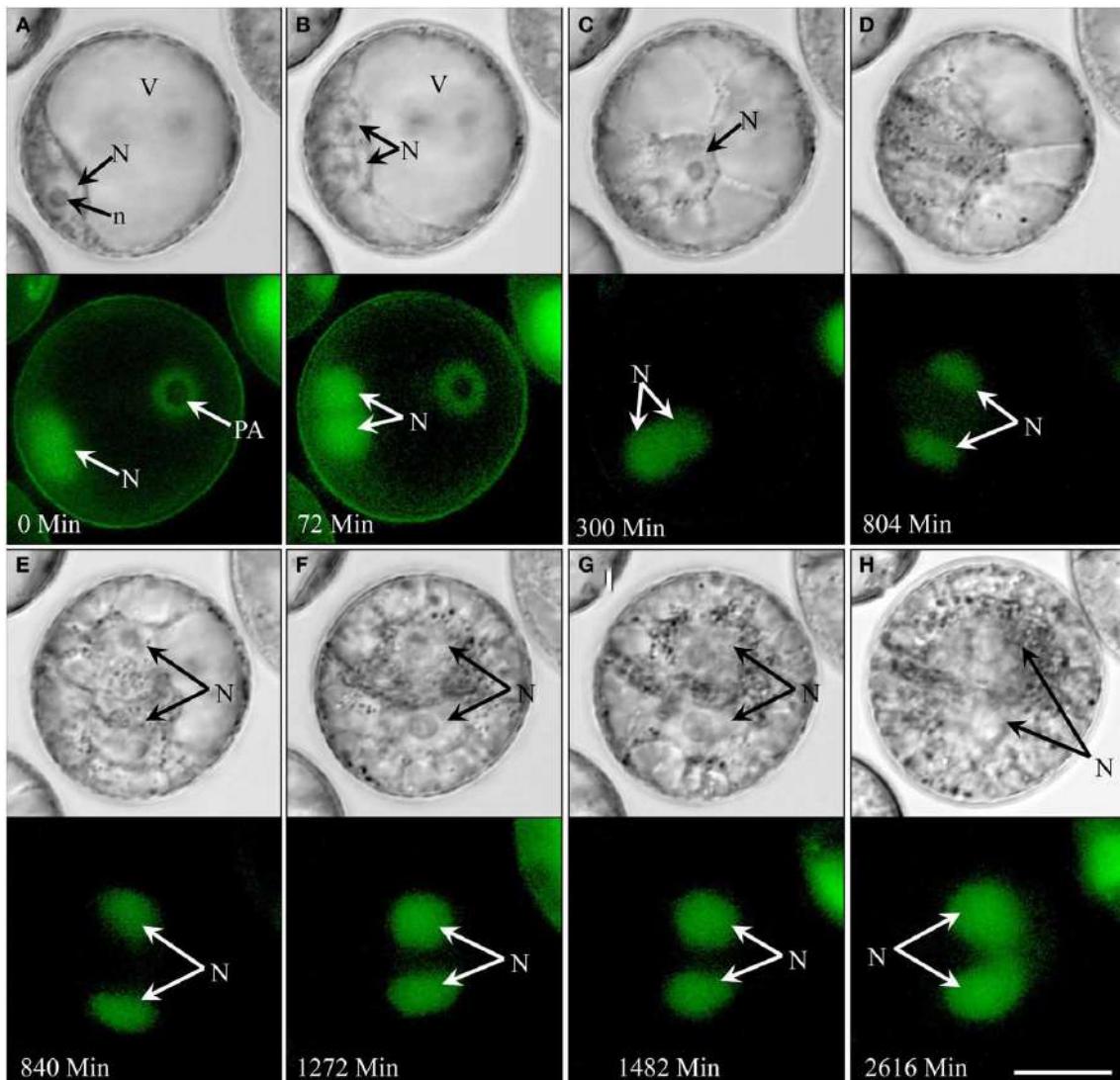


FIGURE 3 | Type III development over time (non-embryogenic pollen) shown by synchronously acquired DIC and fluorescence images. After the initial symmetric division, the pollen remains bi-cellular, showing no further cell divisions but gradually accumulating starch while remaining viable throughout the observation period (14 days). **(A)** Uni-nucleate pollen with

large vacuole and the nucleus residing opposite to the pollen aperture. **(B,C)** Symmetrical cell division. **(D,E)** Cell with two similar sized daughter nuclei and large vacuoles. **(F–H)** Increase of the cytoplasmic volume and starch accumulation. N, Nucleus; n, nucleolus; PA, pollen aperture; V, vacuole. Bar = 20 μ m.

cells contributed to the cell proliferation following a first symmetric pollen mitosis (type I).

A comparison between regular pollen maturation and pollen embryogenesis is shown in **Figure 9**. The developmental pathways identified show three main routes based on the type of the first pollen mitosis (**Figures 9H,L,U**). In the most common route, first mitosis was symmetric and the two daughter cells proliferated synchronously to eventually form an embryo-like structure (**Figures 9H–K**). Starch was not detected in early stages, though some amyloplasts appeared later on (**Figures 9J,K**). In the less common second route, pollen firstly divided asymmetrically to produce generative and vegetative-like cells (**Figure 9L**). Depending on the fate of the generative-like cell, two sub-routes

were identified. In the first sub-route, only the vegetative-like cell proliferated (**Figures 9M–O**) while the generative-like cell remained opposite to the cell aperture and often degenerated within the period of observation (**Figures 9M–P**). In the second sub-route both generative and vegetative-like cells underwent mitosis (**Figures 9Q–S**). In the third route, pollen failed to undergo first mitosis and in some case the nucleus was degenerated into small micronuclei (**Figure 9U**).

NUCLEAR FUSION LEADS TO GENOME DOUBLING

Spontaneous genome doubling during pollen embryogenesis can produce doubled haploid plants that, in contrast to haploids, show normal fertility.

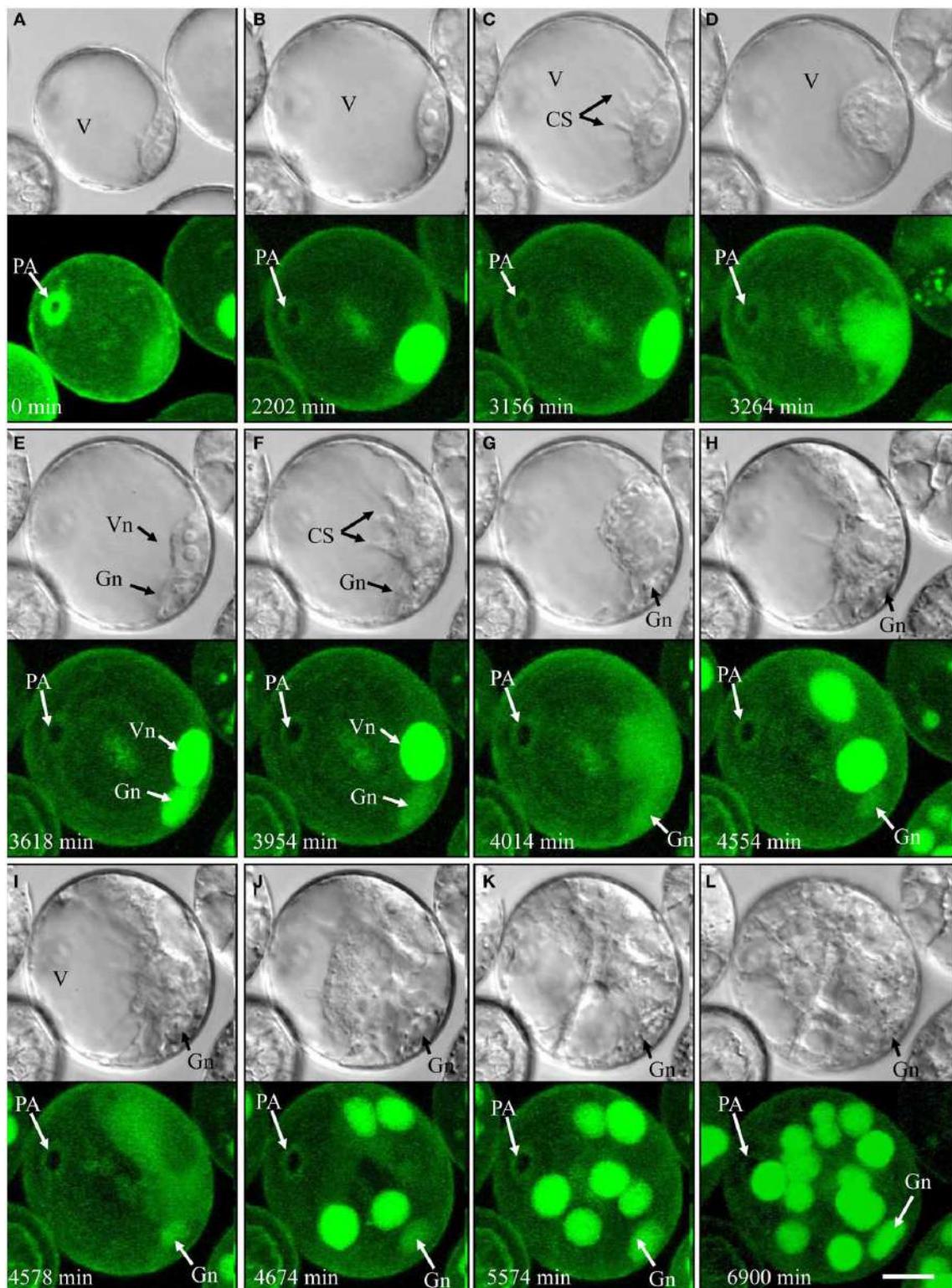


FIGURE 4 | Time-lapse of type IV development (embryogenic pollen) shown by synchronously acquired DIC and fluorescence images. **(A)** Uni-nucleate pollen with large vacuole and thin layer of peripheral cytoplasm. **(B)** Uni-nucleate pollen increases in size. **(C)** Cytoplasmic strands appear prior to pollen mitosis I. **(D–F)** Large spherical vegetative-like nucleus and smaller

ellipsoid generative-like nucleus after asymmetric division. **(G–L)** Synchronized mitotic events originate from the vegetative-like cell; note that the generative-like cell does not show any mitotic activity. CS, cytoplasmic strand; Gn, generative-like nucleus; PA, pollen aperture; V, vacuole; Vn, vegetative-like nucleus. Bar = 20 μ m.

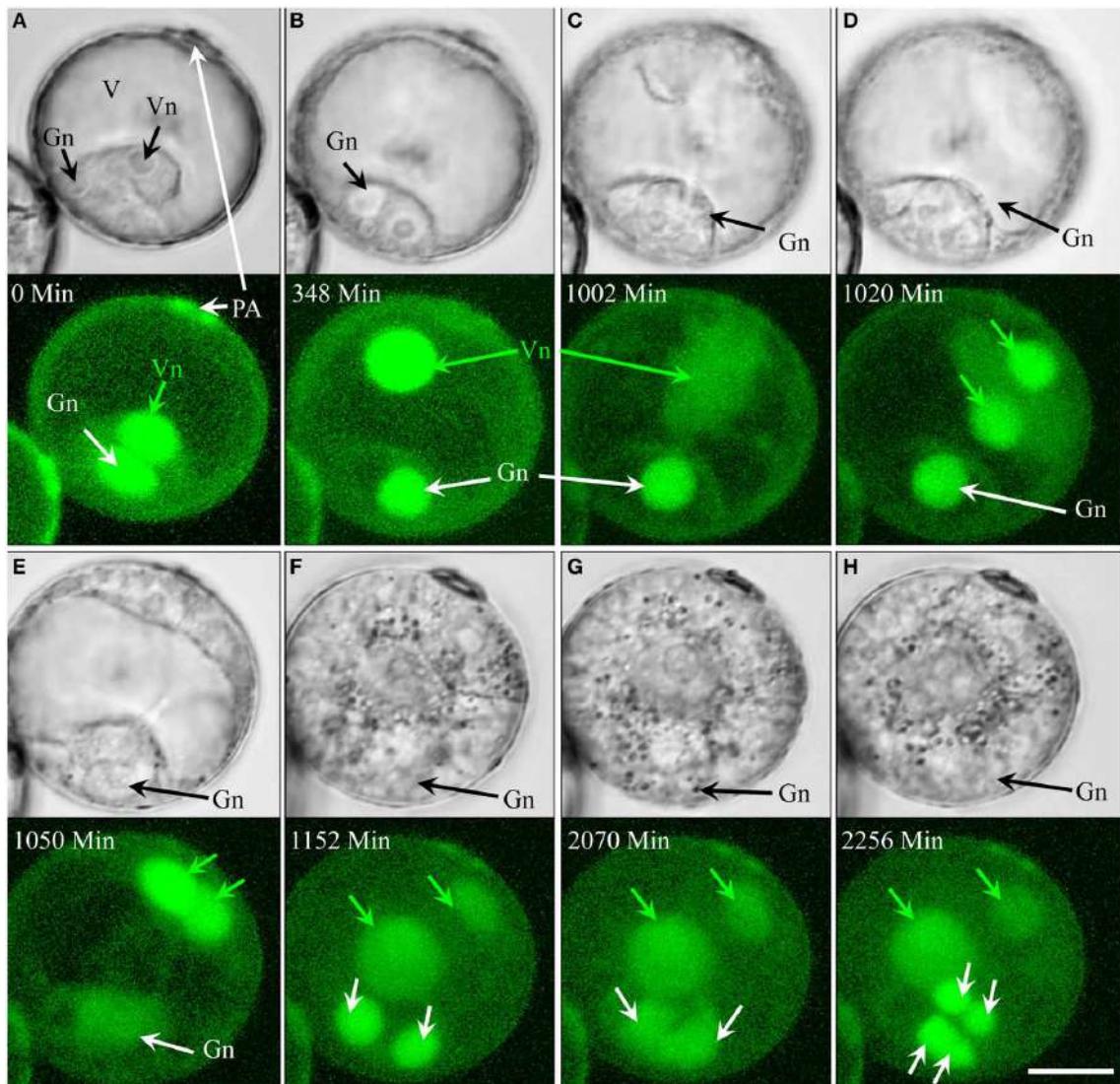


FIGURE 5 | Time-lapse of type VI (non-embryogenic pollen). Development shown by synchronously acquired DIC and fluorescence images of GFP. Green arrows indicate vegetative-like nucleus and its daughter nuclei. White arrows indicate generative-like nucleus and its daughter nuclei (**A,B**). Asymmetric division resulted in a large vegetative-like and a small generative-like cell. (**C,D**) First mitosis of the vegetative nucleus; the spherical generative-like nucleus

remains fixed opposite to the pollen aperture. (**E,F**) First mitosis of the generative-like cell. (**G,H**) Second symmetric and synchronized mitosis of the nuclei originated from the generative-like cell. Note that the nuclei derived from the generative-like nucleus are much smaller than those derived from the vegetative-like nucleus. Gn, generative-like nucleus; PA, pollen aperture; V, vacuole; Vn, vegetative-like nucleus. Bar = 20 μ m.

Sunderland et al. (1974) proposed the fusion of mitotic nuclei as an explanation for genome duplication in embryogenic pollen grains of *Datura*. An alternative mechanism was published by Seguí-Simarro and Nuez (2008) who observed that karyokinesis is followed by a disrupted cytokinesis, which allows the daughter nuclei to fuse within the same cytoplasm. Lee and Chen (1987) and Kasha (2005) claimed the fusion of generative and vegetative nuclei in cultures of barley pollen after the degradation or incomplete assembly of the separating cell wall.

Our observations revealed that mitosis is not always followed by cytokinesis which allows mitotic daughter nuclei to fuse. Indeed, nuclear fusion turned out to be the only means of

genome doubling in the cultures analyzed in the present study. It could occur at any time during pollen embryogenesis, which explains the chimeric ploidy status of individual multicellular structures (Figures 6, 8) and plants. González-Melendi et al. (2005) showed that when nuclei coexist within the same cytoplasm, their envelopes may fuse. However, the same authors also argued that the absence of cell wall is not sufficient to explain nuclear fusion. There are indeed many examples of stable multi-nucleate cells that occur naturally, e.g., bi-nucleate tapetal cells, coenocytic endosperm and the female gametophyte, or can be experimentally-induced (Risueño et al., 1968; Nishihama et al., 2001; Park and Twell, 2001; Olsen, 2004). Consequently, hitherto

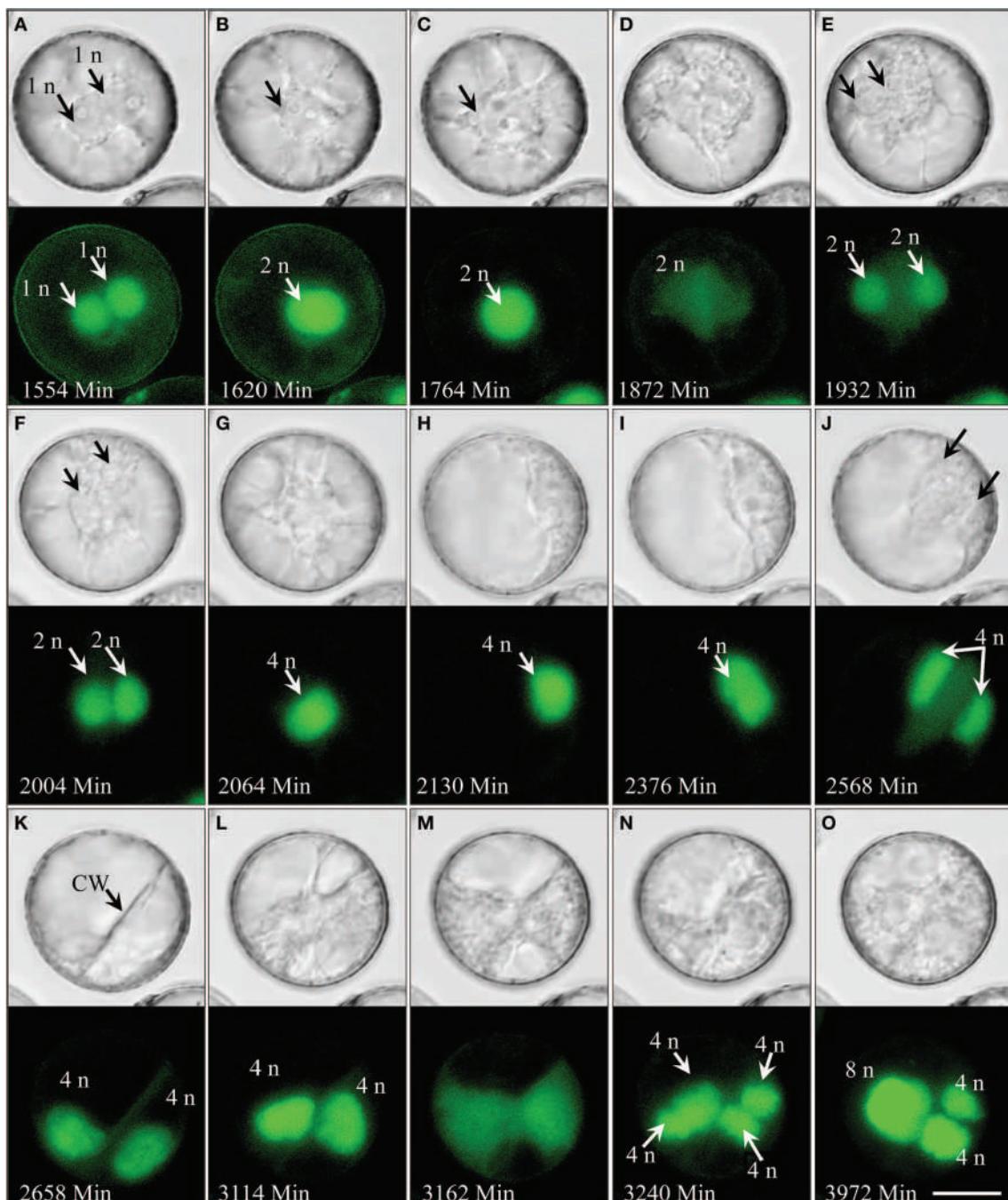


FIGURE 6 | Spontaneous genome doubling during pollen embryogenesis shown by synchronously acquired DIC and fluorescence images of GFP. **(A)** Two haploid nuclei after the first pollen mitosis. **(B,C)** Nuclei adhere to one another and eventually fuse to form a diploid nucleus. **(D–F)** Second mitosis producing a pair of diploid sister

nuclei. **(G–I)** The two diploid nuclei adhere to one another and fuse to a tetraploid nucleus. **(J,K)** Third mitosis resulted in two tetraploid nuclei. **(L–O)** Fourth synchronized mitosis resulting in four tetraploid nuclei, two of which later fuse to a single octaploid nucleus. CW, cell wall; n, haploid genome. Bar = 20 μ m.

unknown factors must exist, that stimulate attachment and fusion of nuclear envelopes (Chen et al., 1984; González-Melendi et al., 2005; Seguí-Simarro and Nuez, 2007).

Fusion between vegetative and generative nuclei was never observed in the present study. Every asymmetric pollen mitosis

I ended with a physical barrier between vegetative and generative-like cells, which effectively precluded their respective nuclei from fusing. The integrity of the separating cell wall is also supported by the observation that in cases where generative-like cells were able to undergo symmetric divisions, all daughter cells

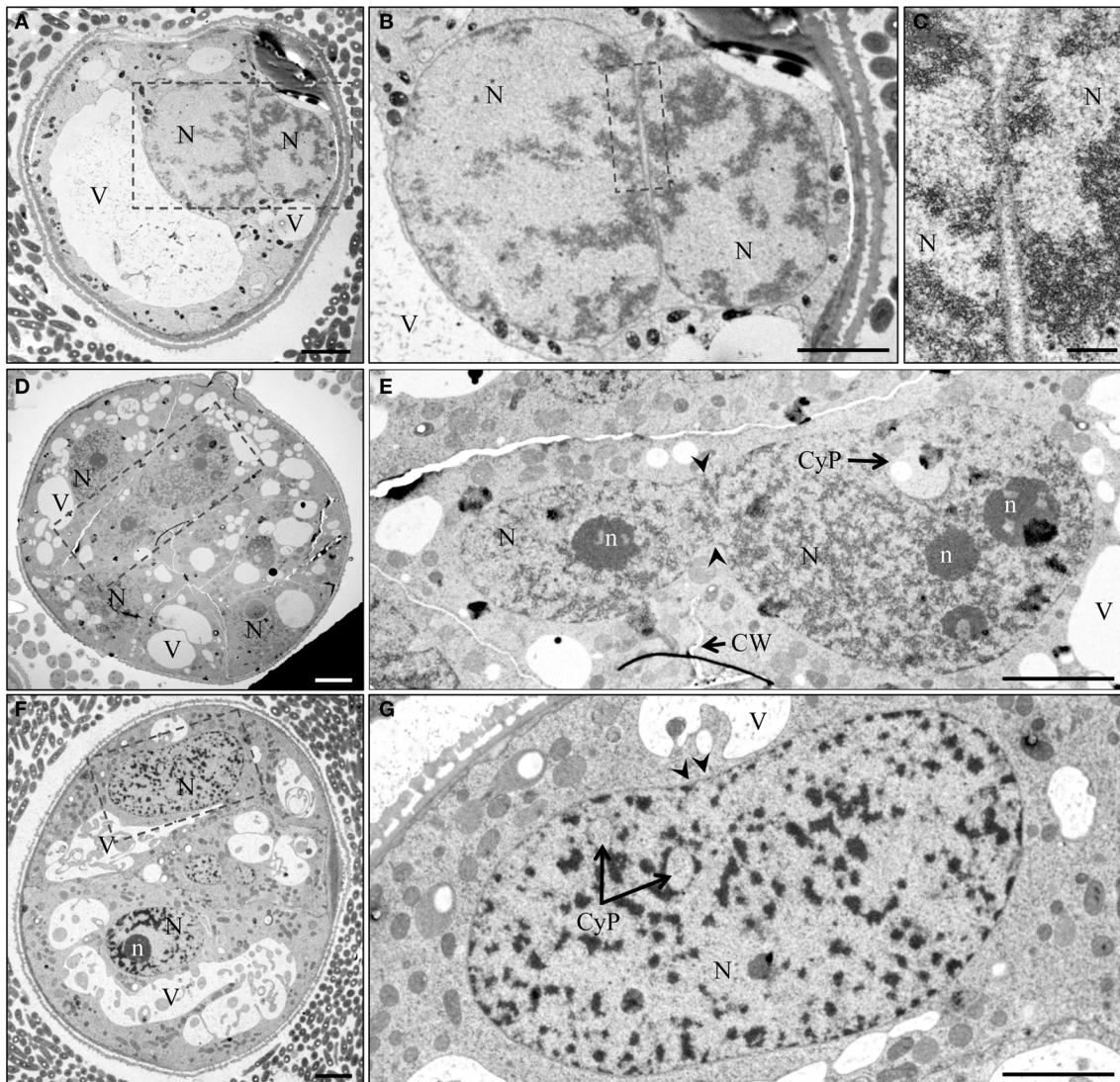


FIGURE 7 | TEM micrographs of nuclear fusion at different stages of pollen embryogenesis. (A) Induced immature pollen during first day of culture with two nuclei in close vicinity. **(B,C)** Detail of **(A)** shows the absence of cell wall and the close proximity of the nuclear envelopes. Arrow indicates region of assumed membrane fusion. **(D)** Multicellular structure 7 days after initiation of pollen embryogenesis. **(E)** Two nuclei

after fusion with incomplete cell wall formation near the site of nuclear fusion. **(F)** Multicellular structure 7 days after initiation of pollen embryogenesis. **(G)** Elongated nucleus with clear median constriction (arrowheads), cytoplasmic pockets and a narrow median band marking the site of fusion. CyP, cytoplasmic pocket; CW, cell wall; N, nucleus; n, nucleolus; V, vacuole. Bar = 20 μ m.

remained within the boundary of the original generative-like cell (**Figure 5**).

The high frequency of whole genome duplication events and resultant polyploid nuclei observed is not associated with a corresponding proportion of high polyploidy amongst the regenerants, as was shown for example in the present study by the flow-cytometric analysis of the *gfp*-transgenic plants generated via agroinoculation of embryogenic pollen cultures. It is well conceivable that a ploidy larger than twice the normal somatic value can lead to disadvantages in further embryogenic development and regeneration which effects selection in favor of viable individuals in the ploidy range between 1 and 4 n with a further

preference of diploids. Also in the ploidy-chimeric structures frequently found in this study, the same selection principle may effect a preferential development of cells and tissue domains having a ploidy within this tolerable range.

SUMMARY AND PERSPECTIVE

Pollen embryogenesis can be followed after symmetric or asymmetric mitosis. The appearance of starch granules or pollen expansion prior to or right after pollen mitosis I is associated with failure of pollen embryogenesis under the conditions used in this study. Under the culture conditions used, nuclear fusion was the only mechanism of genome doubling and could occur

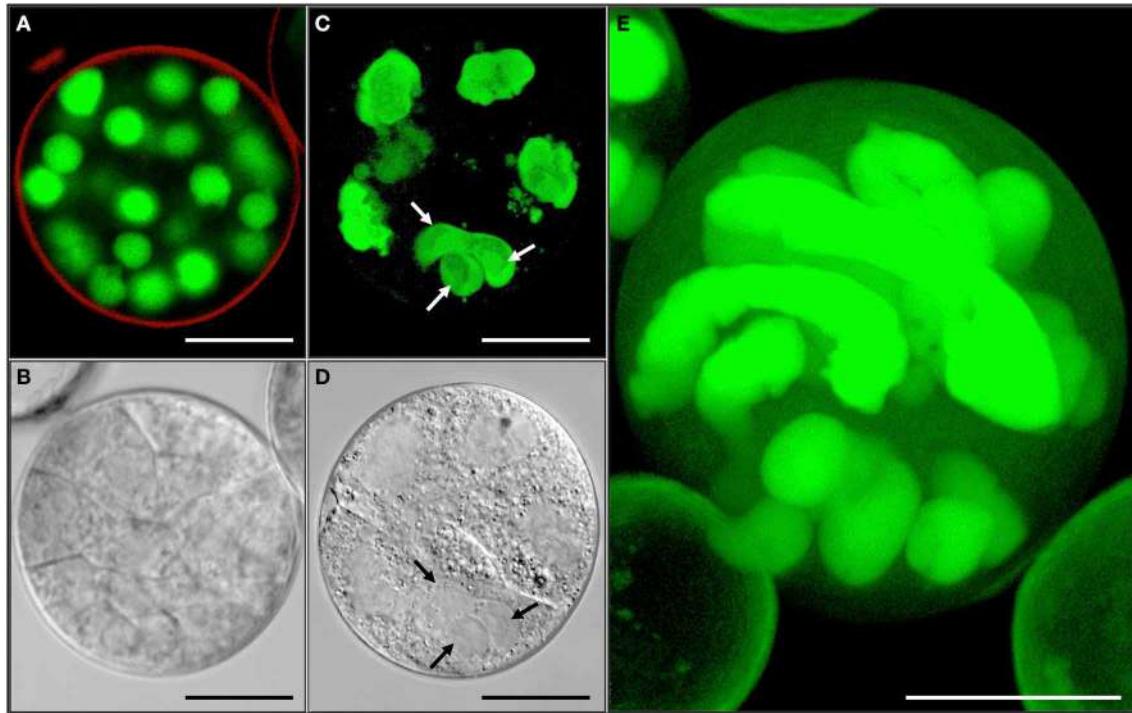


FIGURE 8 | Variable ploidy level in a multicellular structure shown by synchronously acquired DIC and fluorescence images. (A,C,E) GFP. (B,D) DIC. (A,B) Haploid multicellular structure with cell walls and spherical nuclei. (C,D) Chimeric polyploid multicellular structure with irregular shaped nuclei

often not separated by cell wall. Note the difference of nuclear sizes. Arrows refer to a possible triple fusion. (E) Multicellular structure with highly polyploid nuclei next to small spherical, likely haploid, nuclei. Bar = A,B,D–E = 10 μ m; C = 1 μ m.

at any developmental stage during pollen embryogenesis, provided cell wall formation had failed entirely or locally. In the rare occasion of a generative-like cell showing mitotic activity, the nuclei remained significantly smaller than those of normal embryonic cells. This makes the contribution of these cells to embryogenesis highly doubtful. Because cultures of immature pollen are highly heterogenic, it was necessary to follow the fate of individual pollen, in order to unambiguously identify and validate those developmental types that truly account for pollen embryogenesis.

The descriptive information provided here will be a valuable source for the evaluation of pollen cultures used to produce doubled haploid plants. This will especially apply for the establishment and improvement of protocols for recalcitrant species (e.g., rye and oats) or genotypes that have so far been hardly amenable to pollen embryogenesis. The thorough characterization of two embryogenic pathways and their unambiguous discrimination from seven non-embryogenic types of response as was performed in the present study we also consider as a vital prerequisite for future transcriptomics and metabolomics approaches relying on the collection of individually selected pollen, which may help to cope with the unavoidable heterogeneity in pollen populations. Also, the findings presented here on the mechanism and temporal occurrence of whole genome duplication events are likely to have implications on the utilization of embryogenic pollen cultures in induced mutagenesis, genetic transformation and genome engineering approaches with

regards to zygosity and chimerism of the genetic alterations obtained in doubled haploids. In addition, the novel technical opportunities provided by the experimental setup established and utilized in the present study may facilitate the elucidation of the still unknown molecular triggers of pollen embryogenesis by over-time observation of fluorescence-tagged subcellular structures or candidate proteins essentially involved in this process.

METHODS

GENETIC TRANSFORMATION OF BARLEY USING EMBRYOGENIC POLLEN CULTURES

Barley transformation of the winter type cv. Igri using hygromycin as selective agent in the culture media was performed as previously described (Kumlehn et al., 2006). The hypervirulent *A. tumefaciens* strain LBA4404/pSB1 (Komari et al., 1996) carrying the binary vector pGH252n was used to inoculate embryogenic pollen. The binary vector was cloned followed standard procedures described in detail in the Supplementary materials and methods section, and its introduction into agrobacteria was performed by electroporation.

MOLECULAR ANALYSIS OF TRANSGENIC PLANTS

Genomic DNA prepared from leaf material (Pallotta et al., 2000) was analyzed by standard PCR using primers for the coding sequence of the *HPT* gene GH-HYG-F1 (5'-GATCGGACGATTGCGTCGCA-3') and GH-HYG-R2 (5'-T

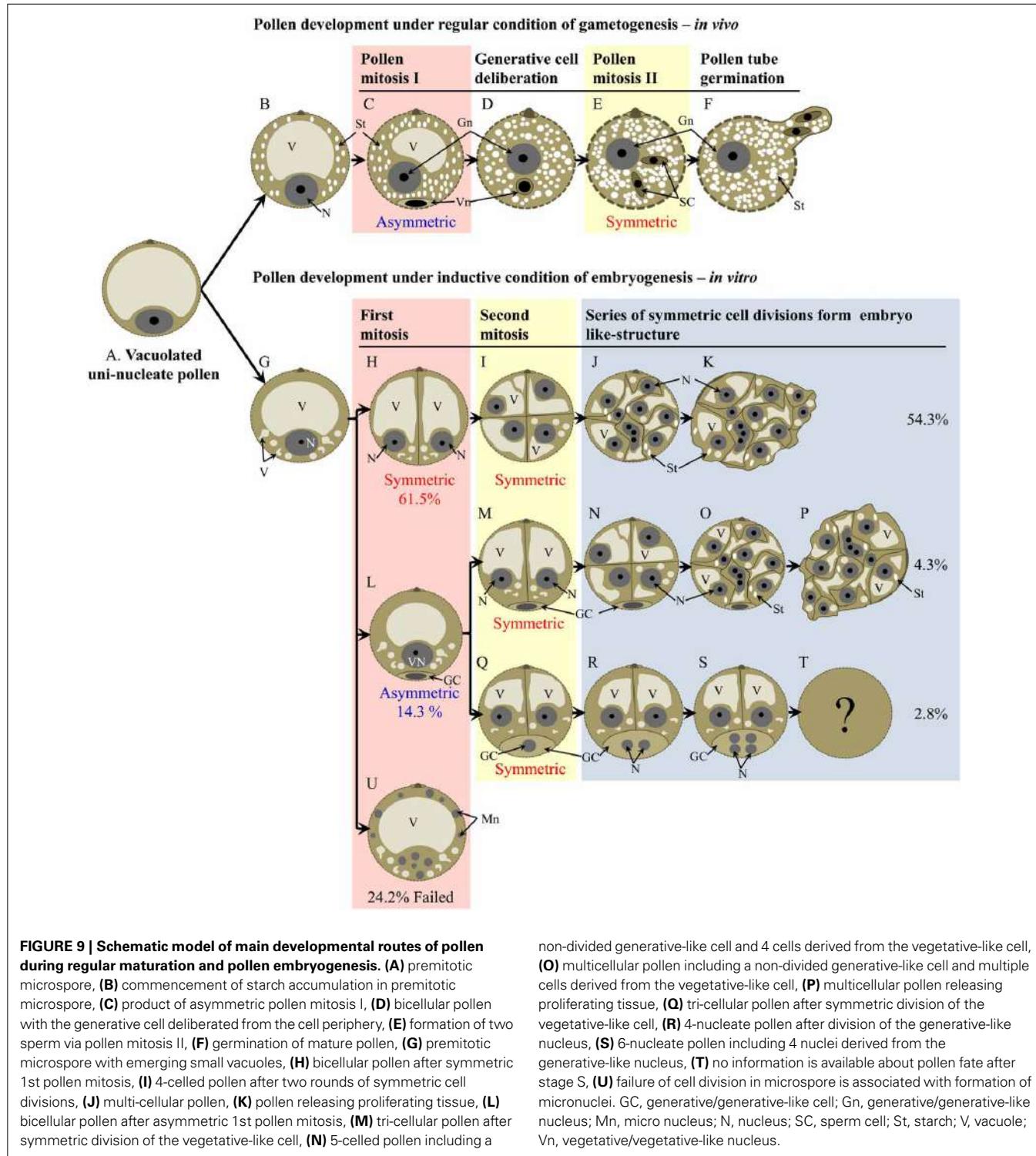


FIGURE 9 | Schematic model of main developmental routes of pollen during regular maturation and pollen embryogenesis. (A) premitotic microspore, (B) commencement of starch accumulation in premitotic microspore, (C) product of asymmetric pollen mitosis I, (D) bicellular pollen with the generative cell deliberated from the cell periphery, (E) formation of two sperm via pollen mitosis II, (F) germination of mature pollen, (G) premitotic microspore with emerging small vacuoles, (H) bicellular pollen after symmetric 1st pollen mitosis, (I) 4-celled pollen after two rounds of symmetric cell divisions, (J) multi-cellular pollen, (K) pollen releasing proliferating tissue, (L) bicellular pollen after asymmetric 1st pollen mitosis, (M) tri-cellular pollen after symmetric division of the vegetative-like cell, (N) 5-celled pollen including a

non-divided generative-like cell and 4 cells derived from the vegetative-like cell, (O) multicellular pollen including a non-divided generative-like cell and multiple cells derived from the vegetative-like cell, (P) multicellular pollen releasing proliferating tissue, (Q) tri-cellular pollen after symmetric division of the vegetative-like cell, (R) 4-nucleate pollen after division of the generative-like nucleus, (S) 6-nucleate pollen including 4 nuclei derived from the generative-like nucleus, (T) no information is available about pollen fate after stage S, (U) failure of cell division in microspore is associated with formation of micronuclei. GC, generative/generative-like cell; Gn, generative/generative-like nucleus; Mn, micro nucleus; N, nucleus; SC, sperm cell; St, starch; V, vacuole; Vn, vegetative/vegetative-like nucleus.

ATCGGCACTTGCATCGGC-3'), or GFP GH-GFP-F1 (5'-G GTCACGAACCTCCAGCAGGA-3') and GH-GFP-R2 (5'-TACGGCAAGCTGACCCTGAA-3'). For DNA gel blot analysis, genomic DNA was digested with *Hind*III, separated in 0.8% (w/v) agarose gel (30 µg per lane) and blotted onto Hybond N+ membrane (Amersham, Braunschweig, Germany) by

capillary transfer under alkaline conditions according to the manufacturer's instructions. Membranes were hybridized with DIG-labeled HPT according to the manufacturer's instructions (Roche, Mannheim, Germany). Hybridization and detection was performed according to the protocol for non-radioactive DNA gel blot experiments (Roche, Mannheim, Germany).

ANALYSIS OF REPORTER GENE EXPRESSION USING CONFOCAL LASER SCANNING MICROSCOPY

Leaves, roots and pollen from T₀ transgenic plants were analyzed for the presence of GFP with a Zeiss LSM 510 META CLSM (Carl Zeiss Microscopy GmbH, Jena, Germany) using a 488 nm laser line for excitation. GFP signals were detected with a 505–530 nm bandpass filter. Auto-fluorescence of chlorophyll was detected with a 650 nm long-pass filter.

PLOIDY ANALYSIS AND COLCHICINE TREATMENT

Ploidy level of regenerants was assessed using a Ploidy Analyser PA I (Partec GmbH, Münster, Germany). Nuclei were stained with CyStain UV (Partec GmbH, Münster, Germany) according to the manufacturer's instructions. Haploid regenerants were treated with colchicine to induce genome doubling (Takamura and Miyajima, 1996). A detailed description is provided in the Supplementary materials and methods section.

LIVE-CELL IMAGING

Live-cell imaging was performed as described by Daghma (2011). Cultures of transgenic immature pollen were observed over a period of up to 15 days. Pollen was subjected to inductive treatment involving incubation of dissected anthers in 0.4 M mannitol at 25°C for 1 day followed by 4°C for 1 day before isolation and culturing in SMB medium for 1 day at 25°C in the dark. The capture of images was started immediately after pollen had been transferred into KBP medium. GFP was excited with a 488 nm argon-krypton laser line. DIC images were acquired with a HeNe 633 laser line. Z-stacks of 9 images were taken every three min with a spacing of 1.5–4 μm depending on expansion of pollen during the development. Scanning of every Z-stack took 80 s. To reduce the risk of bleaching, laser intensity was kept below 4% emission. The developmental progress of a total of 71 individual immature pollen grains was analyzed in five separate experiments.

TRANSMISSION ELECTRON MICROSCOPY

For transmission electron microscopy, pollen was prepared using high pressure freezing and freeze substitution as described by Daghma et al. (2011).

AUTHOR CONTRIBUTIONS

Diaa Eldin S. Daghma, Jochen Kumlehn, and Michael Melzer designed the research. Diaa Eldin S. Daghma and Goetz Hensel performed the experiments and analyzed the data. Twan Rutten contributed to time-lapse imaging. Diaa Eldin S. Daghma, Goetz Hensel, and Jochen Kumlehn wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00675/abstract>

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The low molecular weight fraction of compounds released from immature wheat pistils supports barley pollen embryogenesis

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Pollen embryogenesis provides a useful means of generating haploid plants for plant breeding and basic research. Although it is well-established that the efficacy of the process can be enhanced by the provision of immature pistils as a nurse tissue, the origin and compound class of the signal molecule(s) involved is still elusive. Here, a micro-culture system was established to enable the culturing of populations of barley pollen at a density too low to allow unaided embryogenesis to occur, and this was then exploited to assess the effect of using various parts of the pistil as nurse tissue. A five-fold increase in the number of embryogenic calli formed was obtained by simply cutting the pistils in half. The effectiveness of the pistil-conditioned medium was transitory, since it needed replacement at least every 4 days to measurably ensure embryogenic development. The differential effect of various size classes of compounds present in the pistil-conditioned medium showed that the relevant molecule(s) was of molecular weight below 3 kDa. This work narrows down possible feeder molecules to lower molecular weight compounds and showed that the cellular origin of the active compound(s) is not specific to any tested part of the pistil. Furthermore, the increased recovery of calli during treatment with cut pistils may provide a useful tool for plant breeders and researchers using haploid technology in barley and other plant species.

Keywords: co-culture, feeder signals, haploid technology, microspore, nurse culture, plant-regeneration

Introduction

Cell-to-cell communication is a fundamental requirement for cell proliferation in all multicellular organisms. Some signaling mechanisms involve adjacent cells, but others act over substantial distances. Plant compounds reminiscent of mitogens (a well-researched set of animal long-distance signaling compounds), or at least displaying signal transducer activity, have been identified. While some of these signaling molecules have proven to be small metabolites, others are polypeptides and other complex molecules. The role of phytohormones (e.g., auxins and cytokinins) in the regulation of cell division and differentiation has been exhaustively researched (Lindsey et al., 2002), while certain sugars have also been shown to participate in the control of cell division (Riou-Khamlichi et al., 1999; Eveland and Jackson, 2012). Various proteins like chitinases, peroxidases, or arabinogalactan proteins have all been shown to have a signaling function during embryogenesis, pollen tube differentiation and root development (De Jong et al., 1992; Van Engelen and de Vries, 1992; Wink, 1994; Willats and Knox, 1996). In the last decade however, peptides have

been increasingly recognized to act as growth factors and as cell-to-cell signaling molecules (Lindsey et al., 2002). An example is the pentapeptide phytosulfokine alpha, which has been shown to promote cell division in asparagus cell suspension and rice protoplast cultures, somatic embryogenesis in carrot (Matsubayashi et al., 2001) as well as pollen embryogenic development in *Triticeae* species (Asif et al., 2014). Triggering plant cell division clearly requires the perception of certain extracellular signals (Stuart and Street, 1971; McCabe et al., 1997), as does embryo formation in *Fucus* sp. (Berger et al., 1994). Auxin, in conjunction with particular cell wall components, provides the signaling required to co-ordinate polarity establishment (Souter and Lindsey, 2000). The full nature of the plant signaling network however remains far from being fully understood.

The general importance of cell-to-cell communication is well-illustrated by the observation that in a suspension cell culture, a threshold population density (commonly at least 10,000 per mL) exists, below which cell division is not sustained (Spangenberg and Koop, 1992). Vrinten et al. (1999) demonstrated that embryogenic development is significantly reduced when a density of 10^4 isolated barley microspores per mL is used instead of 10^5 . In some situations, achieving a certain threshold is sufficient to maintain cell division and development (Shillito et al., 1983; Hoekstra et al., 1993), but in others, there is a further requirement to supply heterologous materials as a nurse tissue (Zheng et al., 2002). The use of nurse tissue has proven to be particularly beneficial in attempts to culture isolated somatic or gametophytic cells and protoplasts. Some research effort has been devoted to establishing culture conditions in which cell density dependence can be removed; typically, this has involved the formulation of complex media, the co-cultivation of nurse material, or the use of micro-cultures (reviewed by Spangenberg and Koop, 1992).

Haploid technology provides a rapid means of fixing meiotic recombination, and is used in a growing number of crop improvement programs. One of the major bottlenecks which has been encountered is species and genotypic variation with respect to the efficiency of inducing *in vitro* cultured pollen to become embryogenic. Embryogenic development of immature barley pollen can be triggered by a temporary impact of stress conditions such as low or high temperatures or starvation (Daghma et al., 2012). The process is then initiated by a symmetric cell division of either the microspore or the vegetative cell of bicellular pollen. This deviation from regular pollen development is followed by successive rounds of cell divisions initially taking place within the pollen envelope (González-Melendi et al., 2005; Daghma et al., 2014). Right after pollen wall rupture that is caused by the exponentially proliferating cells, the calli do typically not show any tissue differentiation. At this developmental stage, some particularly spherical individuals could at best be interpreted as embryo proper-like structures. Yet soon thereafter, the majority of calli form a smooth, periderm-like surface and some assume a pear-shaped form reminiscent of immature zygotic embryos, which reveals the highly embryogenic nature of pollen-derived calli (Oleszczuk et al., 2006). The typical eventual outcome of further development is that multiple embryo-like structures are

formed at the callus surface, much like somatic embryos that can be produced from the scutellum of cultured immature zygotic embryos.

The provision of dissected immature barley pistils was shown by Köhler and Wenzel (1985) to enhance the success rate of pollen embryogenesis, and this finding has since been widely exploited in the production of doubled haploids in barley (Li and Devaux, 2001; Lu et al., 2008), wheat (Zheng et al., 2002), oilseed rape (Huang et al., 1990), and maize (Szarka et al., 2001). A comparison of the benefit of including various immature flower explants in wheat has shown that it is the pistil which is the most effective in stimulating embryogenic development (Puolimatka and Pauk, 1999). As wheat pollen embryogenesis appears to be scarcely possible without recourse to nurse tissue, the inclusion of dissected pistils has long become the standard practice for embryogenic pollen cultures (EPCs) of this species.

In a previous attempt to identify substances responsible for the stimulatory effect of conditioned media, Köhler and Wenzel (1985) used a culture method that allows pollen to be released from cultivated immature anthers into liquid culture medium pre-conditioned by dissected barley pistils. Whereas, proteins, amino acids, and sugars were experimentally excluded to possess a conditioning effect, a lower-molecular weight organic substance was demonstrated to stimulate microspore-derived callus production. Its retardation in thin-layer chromatography was very similar to that of indoleacetic acid, however, the substance was shown not to contain an indole component and the true nature of this substance eventually remained elusive to date.

Vrinten et al. (1999) identified genes specifically expressed at the onset of embryogenic development of isolated barley microspores. Two of these genes (*HvECGST* and *HvECLTP*) encode secreted proteins that may be involved in the protection of cells from damage by reactive oxygen species which are assumed to excessively occur as a consequence of stress treatment required to induce embryogenic pollen development. However, the most interesting candidate gene found in the same study was named *ECA1* (early culture abundant 1) and codes for another secreted protein with similarity to the protein component of a hydroxyproline-deficient arabinogalactan protein (AGP) previously detected in suspension-cultured cells of carrot (Baldwin et al., 1993). In addition, *HvECA1* expression was shown to be significantly reduced in low-density cultures in which embryogenic development is hampered. By analysing molecules secreted from EPCs of barley and maize, respectively, Paire et al. (2003) and Borderies et al. (2004) confirmed the presence of AGPs, which further supported the concept that these glycoproteins may belong to the substances essentially involved in the requirement of a cell culture density threshold as well as in the beneficial effect of conditioned media. Later, Letarte et al. (2006) demonstrated that the addition of the arabinogalactan Larcoll or the AGP gum arabicum to EPCs of wheat reduced microspore mortality and increased the number of embryogenic structures produced.

Here, we have developed a barley pollen micro-culture system in an attempt to identify the origin of the stimulatory molecule(s)

secreted by the immature wheat pistil and parts thereof and reduce possible compound classes. The density of pollen was reduced below the normal threshold for embryogenesis to be initiated in the absence of co-cultivated wheat pistils, and the nurse tissue itself was dissected to determine which part of the pistil produced the stimulatory effect. A pistil-conditioned medium was then used to specify both the size and stability of the stimulating molecule(s).

Results

The Effect of Various Pistil Parts on Pollen Embryogenic Development

The minimum culture density of immature pollen grains of cv. "Igri" required to permit embryogenic development without the need of any nurse tissue was 3200 per mL (Figure 1); lower densities than this supported only a few rounds of cell division, insufficient to produce any regenerable callus. In assessing the stimulatory effect of the nurse tissue therefore, the culture density was reduced by more than one order of magnitude to 75 grains per mL. The nurse tissues tested consisted of either entire or specific parts of wheat pistils (Figure 2), and all of these induced the formation of small calli (100–500 μm) to a similar extent (Figure 2A), while the negative control (no nurse tissue) did not develop any callus (Supplemental Table S1). Isolated ovules (both longitudinally bisected and uncut; lOvu, Ovu) had much the weakest stimulatory effect on callus growth, which was diagnosable already after 7 days of culture due to the comparatively low proportion of enlarged pollen (Supplemental Figure S1). After 2 weeks of culture, the pollen co-cultivated with stigmas (St), ovules, whole ovaries (Ovr; pistil with the stigma being cut off) or the basal, micropylar section thereof (mOvr) remarkably lagged behind in development as compared to those supported by the other tested nurse materials that had already stimulated the formation of larger individuals reminiscent of immature embryos (Supplemental Figure S1). A callus diameter of at least 500 μm is generally considered as being large enough to be capable of whole plant regeneration. Supporting the test pollen with an EPC at a density of 50,000 per mL induced the formation of only a small number of calli $\geq 500 \mu\text{m}$; nonetheless, this positive control treatment was significantly superior to the co-cultivation of ovules or to pollen cultured without nurse tissue (Figure 2B, red line). While the stigma or the micropylar section of the ovary could satisfactorily substitute for the whole pistil (P) as a nurse tissue, longitudinally bisected pistils (IP) were superior to perpendicularly (cross-)bisected ones (cP), which were in turn superior to the chalazal part of the ovary (cOvr); and the latter was superior to the whole ovary (Figures 2B,C). Taken together, bisected pistils were consistently superior to other tested nurse tissues in supporting the formation of embryogenic calli $\geq 500 \mu\text{m}$, whereas the behavior of longitudinally bisected pistils, either with (IP) and without (IP⁻Ovu) ovarian tissue attached, showed that the presence of gametophytic tissue made no positive contribution to the stimulatory effect of the pistil. The relevant raw data are presented as Supplemental Table S1, and a

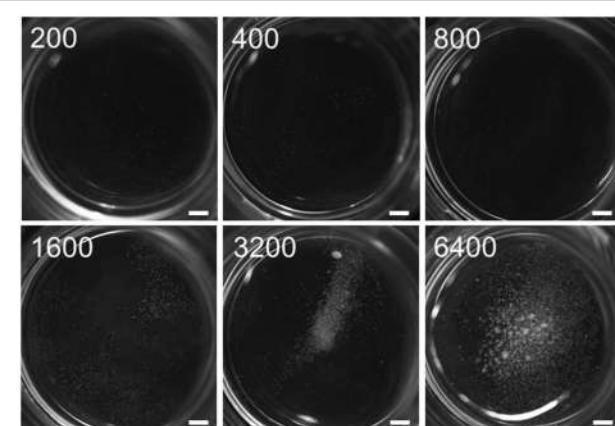


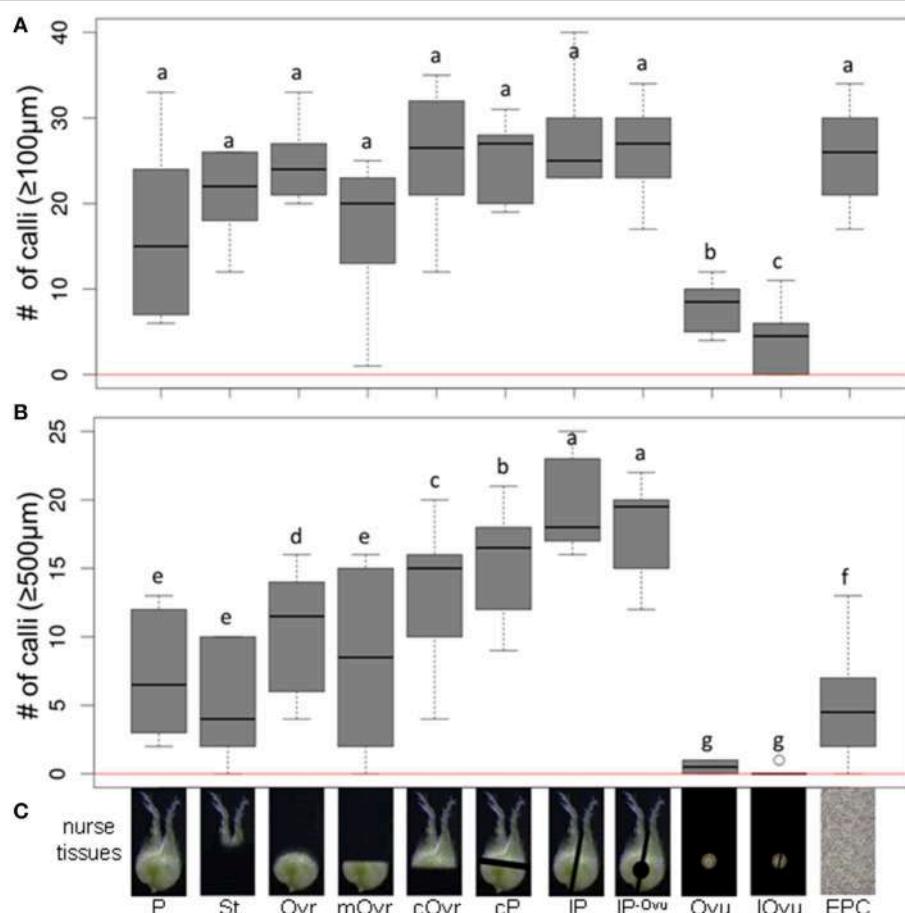
FIGURE 1 | Threshold of self-feeder effect in embryogenic pollen culture. Isolated microspores of cv. "Igri" were cultured at various densities (numbers per mL are given on the individual photographs recorded after 4 weeks of culture) in the absence of nurse tissue to determine the minimum culture density required to permit embryogenic development. The microspores were kept in Millicell inserts to facilitate the observation of their response at low culture density, while the inserts were positioned in 3.5 cm Petri dishes containing a total volume of 2 mL medium. Callus formation was completely inhibited at a culture density below the threshold level of ca. 1000 microspores per mL medium, whereas embryogenic calli developed only in cultures with a density of 3200 mL or higher. Bar size = 1.5 mm.

representative image set during the cultivation of embryogenic pollen is given as Supplemental Figure S1.

Since the presence as a nurse tissue of longitudinally bisected pistils gave the strongest stimulatory effect on embryogenic pollen development, an attempt was then made to optimize the number of pistils to be used. To this end, low-density cultures (75 isolated microspores of cv. "Igri" per mL medium) involving the co-cultivation of 0.5–6 bisected pistils and EPCs at standard density as positive control were compared. The inclusion of between one and six longitudinally bisected pistils per mL medium was the most effective treatment (Figure 3; raw data given in Supplemental Table S2). The median values of callus formation $\geq 500 \mu\text{m}$ in size increased step-wise with the number of co-cultivated pistil halves, while the use of only half a pistil per mL resulted in a significantly reduced pollen-derived callus formation as compared with all treatments involving one to six bisected pistils. The response across the tested range of numbers of pistils added to the cultures indicated that just one bisected pistil is capable of almost entirely satisfying the demand for stimulating signal molecules under the given circumstances.

Pollen Embryogenesis and Plant Regeneration in cv. "Golden Promise"

Barley cv. "Golden Promise" is recalcitrant with respect to pollen embryogenesis (Coronado et al., 2005). When microspores isolated from cold-treated spikes were cultivated at a density of 5000 per mL without any nurse tissue, no callus formation ensued, but in the presence of uncut pistils, a small number of embryogenic calli of diameter $\geq 500 \mu\text{m}$ did form (Figure 4A).



In all (across three biological replicates), 10 plantlets (0/4/6) were regenerated, of which only one (10%) was non-albino. When the nurse tissue consisted of longitudinally bisected pistils, the number of calli of diameter $\geq 100\mu\text{m}$ formed was 229 ± 12 (six-fold increased), of which over half had a diameter of $\geq 500\mu\text{m}$. This treatment yielded a mean of 10–11 plantlets per replicate, of which 15.6% were green. The latter were successfully established in soil, constituting a five-fold improvement over the use of uncut pistils (Figure 4B).

The Identification of the Molecular Size Range of the Stimulatory Compound(s) and its Stability

Pistil-preconditioned nutrient medium was only able to promote barley pollen embryogenesis when regularly refreshed. When an inductive medium was replaced only every 7 days, embryogenic development was not supported, whereas replacement every

4 days was effective (Figure 5A). As expected, however, embryogenic pollen development was much reduced as compared to continuous co-cultivation of bisected pistils. The composition of inductive medium was fractionated into two molecular size fractions, which were then added one by one to a non-inductive (low density, no nurse tissue) pollen culture. Stimulation of pollen embryogenesis was only induced by the presence of the smaller sized fraction which passed the molecular weight cut-off of $\leq 10\text{ kDa}$. Further fractionation showed that the effect was due to a compound(s) of size below the molecular weight cut-off of $\leq 3\text{ kDa}$ (Figure 5B). The stimulatory effect of adding this size-fraction was lower than that directly provided by the cut pistils, conditioned medium or the $\leq 10\text{ kDa}$ fraction, which can be attributed to the expected loss of a substantial proportion of the effective signal molecules owing to the fractionation procedure.

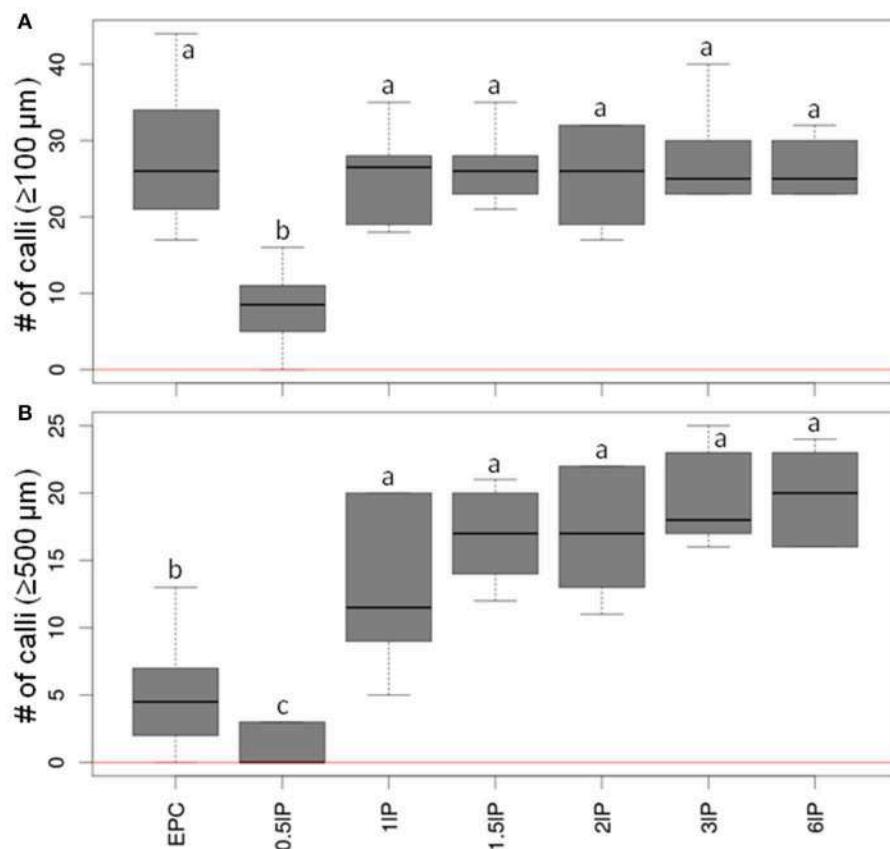


FIGURE 3 | The effect of varying the number of co-cultivated, longitudinally bisected pistils on embryogenic pollen development. Isolated microspores at an initial density of 75 per mL were co-cultivated with various numbers of bisected wheat pistils. The microspores were kept within Millicell inserts to facilitate the observation of their response at low culture density and without being obscured by nurse tissue. The inserts were positioned in 3.5 cm Petri dishes containing a total volume of 2 mL medium, while the nurse

tissue was kept in the medium portion outside the inserts. The diagrams show the formation of embryogenic calli of diameter **(A)** $\geq 100 \mu\text{m}$ and **(B)** $\geq 500 \mu\text{m}$ after 4 weeks of culture. EPC, embryogenic pollen culture of cv. "Igri" precultured for 1–2 weeks and used as nurse tissue at a density of 50,000 pollen grains per mL; IP, longitudinally bisected pistil (numbers of bisected pistils co-cultivated per mL medium are given). The red horizontal lines represent the responses of respective cultures in the absence of nurse tissue.

Discussion

The presence of immature pistils as a nurse material is known to stimulate *in vitro* embryogenic development of immature pollen in barley (Köhler and Wenzel, 1985; Li and Devaux, 2001; Lu et al., 2008), wheat (Puolimatka et al., 1996; Broughton, 2008), and other monocotyledonous (Szarka et al., 2001) and also dicotyledonous crops (Huang et al., 1990). Particularly in wheat, the co-cultivation of pistils has proved to be a critical component for doubled haploid production from isolated microspores (Hu and Kasha, 1997; Zheng et al., 2002).

The principle of pistil co-cultivation was subjected to a detailed investigation in the present study. A considerable challenge in doing so has been that not only dissected pistils, but also embryogenic barley pollen themselves have the capability of serving as highly effective nurse material. For instance, the co-cultivation of embryogenic barley pollen facilitated the establishment of embryogenesis and plant regeneration from isolated barley and wheat zygotes (Holm et al., 1994; Kumlehn

et al., 1998) as well as follow-on investigations on the individual developmental fate of isolated zygotes and parthenogenetic egg cells of wheat (Kumlehn et al., 1999, 2001). In the present study, the stimulation induced by the nurse material was decoupled from that generated endogenously within the population of pollen grains by a careful manipulation of the culture density of the immature pollen grains. The microculture system developed for this purpose can be used to readily assess the benefit of different nurse tissues, extracts or specific compounds.

One of the drivers of the notion of developing such a microculture system was the suggestion that the stigma and style contain plentiful arabinogalactan proteins (Gane et al., 1995), a class of compounds which has been shown to enhance the processes of somatic embryogenesis in carrot (Van Engelen and de Vries, 1992; Van Hengel et al., 2001) and embryogenic pollen development in wheat (Letarte et al., 2006). However, the provision of stigma was less beneficial than that of bisected pistils or other parts thereof (Figure 2). As a nurse tissue, the chalazal

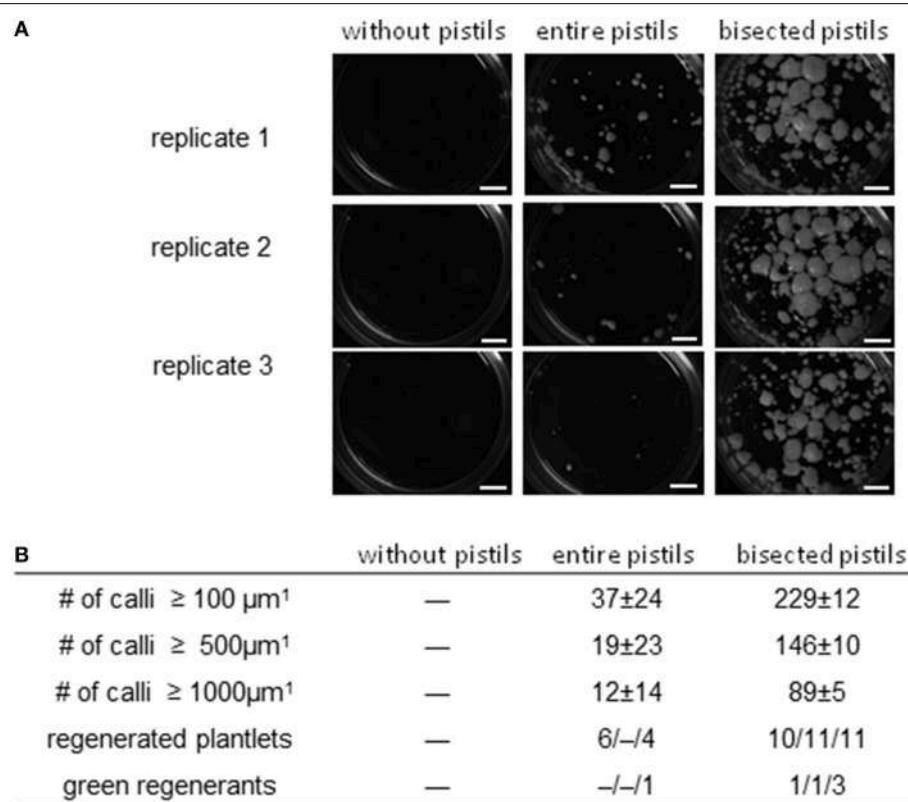


FIGURE 4 | The effect of entire and bisected pistils on embryogenic pollen development in the recalcitrant cv. “Golden Promise.” Isolated microspores at an initial density of 5000 per mL medium were co-cultivated with either entire or bisected wheat pistils as compared to a negative control without nurse tissue. The microspores were kept in Millipore inserts to facilitate the observation of their response without being obscured by nurse tissue. The inserts were positioned in 3.5 cm Petri dishes containing a total volume

of 2 mL medium, while the nurse tissue was kept in the medium portion outside the inserts. Three entire pistils or parts thereof were used per mL medium. **(A)** The formation of embryogenic calli after 4 weeks of pollen culture. Bar size = 2 mm. **(B)** Numbers of embryogenic calli obtained in three size classes (data shown in the form mean \pm SD) and plantlet regeneration (data shown for total and green regenerants as single values from three different biological replicates).

part of the pistil has proved to be significantly superior to the basal, micropylar part of the pistil, implying that the relevant signaling molecules are more abundant in the former. The order of efficacy observed (bisected pistil < chalazal part of the pistil < ovary) suggests that the release of these signals is in some way dependent on if the pistil is cut, rather than on the particular tissue of pistils provided as a nurse tissue. The ineffectiveness of the ovules may reflect their inability to survive for an extended time under the given conditions *in vitro*. Although it was not possible to identify which specific part of the pistil was the source of the stimulatory signal (Figure 2B), embryogenic callus formation was considerably enhanced by cutting the pistils. The most likely explanation of this somewhat surprising finding is that damaging the pistil accelerated the secretion of the signaling molecules, although it is also conceivable that an upsurge in the production of signaling molecules forms part of the wounding response.

Although the supply of pistil-preconditioned medium was effective in stimulating pollen embryogenesis, the medium did need refreshing at least every 4 days, suggesting that the effective compound(s) was either rather labile or was consumed

by the pollen. In contrast, according to Köhler and Wenzel (1985), the one-off provision of pistil-conditioned medium produces a detectable enhancement. Given however that in the latter study, the population density of the pollen was sufficient to self-induce embryogenesis, it is considered likely that even a comparatively short period of extra stimulation could have generated a detectable enhancement in embryogenic development. The stimulatory effect of the nurse pistils must have been exerted by a compound(s) below the molecular weight cut-off of 3 kDa, thereby excluding a number of potential candidates. These include both arabinogalactan proteins as well as chitinases that have been suggested to stimulate embryogenic development (De Jong et al., 1992; Letarte et al., 2006). What remain as potential viable candidates are various peptides such as the phytosulfokines or those identified in the nutrient medium of EPCs of rapeseed (Boutilier et al., 2005), phytohormones as suggested by Köhler and Wenzel (1985), polyamines, sugar derivatives and other metabolites. Identification of the signaling molecule(s) may help to define the identity of the relevant signaling pathway, but even if not, it could conveniently be used in pure form as an additive to promote whole plant regeneration

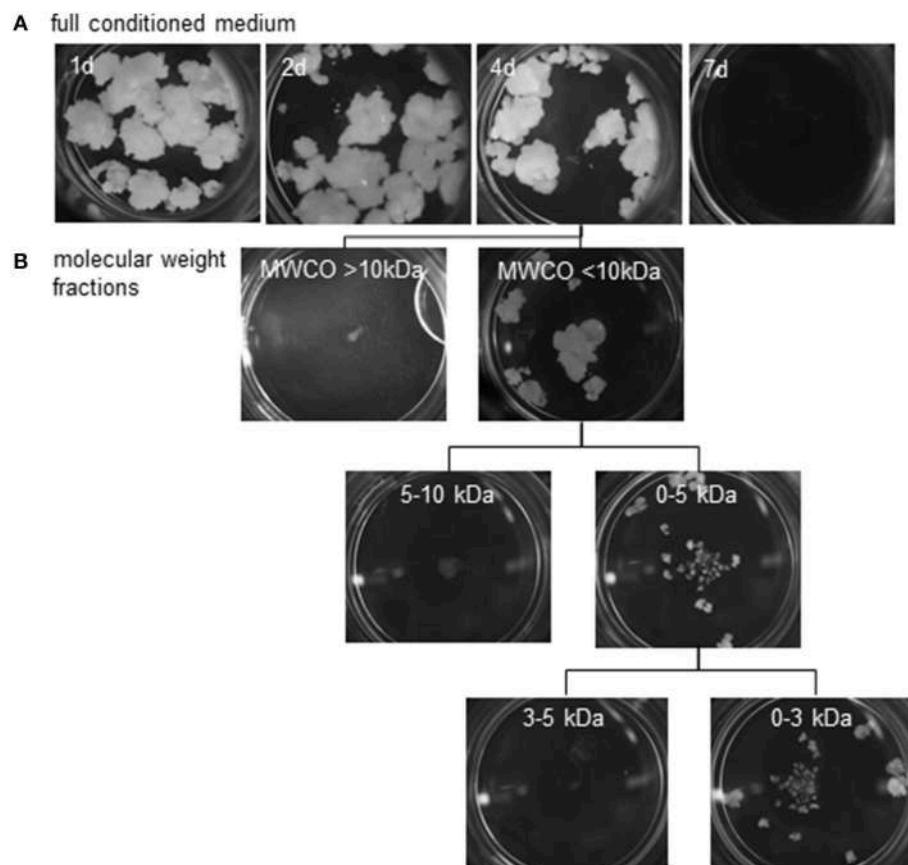


FIGURE 5 | Reduction of possible feeder compounds using size exclusion. Embryogenic callus formation from populations of ca.150 immature pollen grains of cv. "Igri" supplemented at various intervals by (A) fresh pistil-preconditioned medium (intervals of medium exchange are given on the individual photographs recorded after 4

weeks of culture), (B) various size fractions of an extract of pistil-conditioned medium. A 4 day interval was sufficient to stimulate embryogenic development. The feeder effect is shown to be due to a compound(s) of size below the molecular weight cut-off of <3kDa. MWCO: Molecular weight cut-off.

from *in vitro* cultured immature pollen grains, and perhaps from other types of plant cell culture as well. Specifying the functionality of particular fractions or candidate molecules will be materially facilitated by the micro-culture system described in this communication.

A beneficial outcome of testing various pistil parts was that a practical level of embryogenic development and green plant regeneration was achievable from the pollen of the recalcitrant cv. "Golden Promise." Extrapolating from an admittedly small-scale experiment suggests that ca. 15 non-albino regenerant plants could be derived from the pollen harvested from a single barley spike (ca. 10^5 grains); such a level of yield would be well-acceptable in the context of doubled haploid based research and breeding. For comparison, after optimization of culture conditions for seven recalcitrant barley cultivars involving the co-cultivation of (uncut) wheat pistils, Li and Devaux (2001) obtained about 15 regenerant plants per spike on average, out of which ca. 16% were green, which is well-comparable with the result of the present study in cv. "Golden Promise" when uncut pistils were used as nurse tissue. In addition, these results are on

a par with what was previously achieved in cv. "Golden Promise" under the same inductive conditions (4 weeks cold treatment of spikes) as in the present study along with standard culture density and co-culture of uncut wheat pistils (Coronado et al., 2005). However, the efficiency of total and green plant regeneration achieved here using bisected pistils was not only superior to the directly compared use of uncut pistils, but also exceeded the result obtained by Li and Devaux (2001) also with regards to the best performing one amongst the recalcitrant cultivars tested in their study.

The occurrence of albino plantlets is a particular problem in EPCs of grass species and is thought to be associated with the impact of stress required to induce pollen embryogenesis (Ankele et al., 2005). Owing to the lack of chlorophyll, such individuals are not capable of being photosynthetically active and thus cannot be established in soil. Whereas, the efficiency of embryogenic development and total plant regeneration was improved by the principle of bisecting the pistils used for co-cultivation, the ratio of green plant formation was at best slightly enhanced as compared to the use of uncut pistils.

Summary and Perspective

Signal molecules secreted into the nutrient medium either by the cultivated pollen themselves or by co-cultivated barley or wheat pistils are indispensable for the development of EPCs of barley, which holds undoubtedly also true for any other plant species. In the absence of heterologous nurse tissue, there is no embryogenic development at a culture density of ≤ 1600 immature barley pollen grains per mL medium. While the cellular origin of the effective compound(s) secreted from co-cultivated wheat pistils is not specific to any tested pistil part, the increased recovery of calli during treatment with bisected pistils indicates improved release of those molecules from the explants and may provide a useful tool for plant breeding and research using haploid technology in barley, as was exemplified in the present study using the cv. "Golden Promise," and probably in other plant species as well. In addition, effective nurse systems will be particularly useful in EPCs subjected to genetic manipulations such as induced mutagenesis and transformation, since the cultured pollen grains often show a high mortality owing to the impact of the applied chemical, radiation, particle bombardment or agro-inoculation. As a consequence, only a subpopulation of at best intermediate culture density is left behind. In such situations, a nurse system may compensate for the lost self-feeding effect which is only ensured at higher culture density.

This study unambiguously narrowed down the size of candidate compounds to a molecular weight cut-off of 3 kDa, and we demonstrate that the effectiveness of the secreted molecules was only transitory. This information is valuable for future studies aiming to identify particular molecules that support embryogenic development in plant cell cultures. In the same context, the micro-culture system developed in this study for low-density cultures holds great promise for the assessment of stimulatory effects of other nurse tissues, extracts, knowledge-based candidate compounds or those contained in large molecule libraries. Moreover, the highly effective nurse system established may greatly facilitate the culture, observation and manipulation of particularly sensitive cells and tissues, protoplasts as well as single cells.

Methods

Donor Plants, Stress Treatment, and the Isolation of Immature Pollen

Grains of the winter barley cultivar "Igri" and the spring barley cultivar "Golden Promise" were germinated under a 16 h photoperiod and a day/night temperature of 14/12°C. Two week-old seedlings of cv. "Igri" were then vernalized for 8 weeks by exposure to a 9 h photoperiod at 2°C; finally, the seedlings of both cultivars were further grown under 16 h photoperiod and a day/night temperature of 18/14°C. Immature pistils were harvested from the wheat cultivar "Bob White" at a stage when the pollen was bi-cellular. Immature barley spikes were detached when the majority of pollen was right before entering pollen mitosis I, held at 4°C for about 4 weeks, then chopped into 2–3 cm long segments prior to homogenization in 20 mL cold 0.4 M mannitol for 20 s. The homogenate was filtered through

100 µm nylon mesh and the particulate matter re-blended in 10 mL 0.4 M mannitol and re-filtered. The combined filtrate was centrifuged (100 × g, 4°C, 10 min), the supernatant was discarded and the pellet re-suspended in 5 mL 0.55 M maltose, which was then over-layered with 1.5 mL 0.4 M mannitol. After a second centrifugation, the interphase, in which most of the immature pollen grains had been concentrated, was withdrawn and suspended in 20 mL 0.4 M mannitol. The density of pollen grains present was estimated using a haemocytometer and the suspension centrifuged once more as above. The supernatant was discarded and the pellet re-suspended in sufficient KBP medium (Kumlein et al., 2006) to obtain a final density of approximately 10,000 pollen grains per mL, if not specified otherwise. The exact density was estimated from a direct count of the number of pollen grains present in five separate 1 µL aliquots prior to the establishment of cultures described below.

In Vitro Pollen Culture

A 12 mm Millicell Culture Plate Insert (Merck Millipore Darmstadt, Germany) with a hydrophilic, permeable and transparent PTFE membrane (0.4 µM pore size) in place of a solid bottom plate was positioned inside a 3.5 cm Petri dish containing 2 mL KBP medium, of which 200 µL was pipetted into the insert. Using this culture system, test cells can be grown separated from nurse tissue which itself is co-cultivated in the same medium but outside the insert, while signal molecules are allowed to freely move through the membrane between both partial volumes of the culture medium.

Adequate aliquots of the immature pollen suspension of cv. "Igri" were added to the inserts to establish culture densities of 75–6400 immature pollen grains per mL with respect to the total volume of medium in the Petri dish. The nurse material was added to the Petri dish outside the Millicell insert, the dishes were sealed with Parafilm and then held at 24°C in the dark.

EPCs of cv. "Igri" to be used as nurse tissue were initially prepared at a culture density of 100,000 microspores per 3.5 cm Petri dish containing 1 mL KBP medium. After 1–2 weeks of preculture, the medium was replaced by 1.8 mL fresh medium and a Millicell insert containing another 200 µL medium along with the test pollen was positioned into the dish. The resultant density of the EPC used as nurse tissue was 50,000 per mL with respect to the total volume of medium in the dish.

Immature pollen grains extracted from cv. "Golden Promise" were cultured at a density of 5000 per mL KBP medium. This was achieved by culturing 10,000 isolated microspores per Millicell insert, while the total volume of medium per 3.5 cm Petri dish was 2 mL (as detailed above). After 4 weeks of culture on a shaker at 65 rpm, any calli which had developed were placed on K4NB medium (Kumlein et al., 2006) in the dark at 26°C for 1 week, then brought into the light (16 h per day). After a further 3 weeks, the differentiated plantlets were counted and individually transferred to 6-cm pots containing soil substrate. These pots were placed in a tray covered by a transparent hood to maintain saturated humidity environment for about 2 weeks. Further growth conditions were as described above for the donor plants.

For the statistical analysis of embryogenic pollen development, a Student-Newman-Keuls test was performed across six biological replicates per treatment using SigmaStat 3.0 software (Systat Software GmbH, Germany).

Use of Conditioned Medium and the Identification of Maximum Molecular Weight of the Stimulating Molecule(s)

KBP medium (Kumlehn et al., 2006) conditioned by cultivation of 10 bisected wheat pistils per mL for 7 days was used as a supplement to support embryogenic pollen development without direct co-cultivation of nurse tissue. In EPCs without nurse tissue, conditioned media were refreshed at intervals of 1–7 days over a period of 28 days. In addition, media pre-conditioned by pistil cultivation were subjected to molecular size fractionation, which was effected using polyethersulfon Vivaspin 6 size exclusion concentrators with molecular weight cut-off levels of 10, 5, and 3 kDa (Sartorius AG, Germany). The retentate was solubilized in fresh KPB medium. The filtrate and solubilized retentate fractions were filter-sterilized through a 0.2 μm filter before being tested for their effect on embryogenic pollen development in cv. "Igri" using a culture density of 75 per mL.

Author Contributions

JK, HM, and RL designed the research, RL performed the experiments, JK, RL, HM, and SF analyzed the data, RL and JK wrote and SF and HM critically edited the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00498>

Figure S1 | The effect of using whole or partial wheat pistils as a nurse material on embryogenic pollen development in cv. "Igri" after 7, 14, and 24 days of culture. Representative examples of cultures involving as nurse material. P, pistil; St, stigma; Ovr, ovary; mOvr, micropylar ovary half; cOvr, chalazal ovary half; cP, cross-bisected pistil; IP, longitudinally bisected pistil; IP-Ovu, longitudinally bisected pistil without ovule; Ovu, ovule; IOvu, longitudinally bisected ovule; EPC, embryogenic pollen culture of cv. "Igri" precultured for 1–2 weeks and used as nurse tissue at a density of 50,000 pollen grains per mL as compared with noNT: no nurse tissue as negative control. Bar size for seven and 14 days old cultures: 100 μm; bar size for 24 days old cultures: 2 mm.

Table S1 | The effect of using whole or partial wheat pistils as a nurse tissue on embryogenic pollen development in cv. "Igri" at low culture density (75 isolated microspores per mL) as assessed after 4 weeks of culture. Embryogenic calli of diameter **(A)** ≥100 μm, **(B)** ≥500 μm, and **(C)** ≥1 mm. noNT, no nurse tissue; P, pistil; St, stigma; Ovr, ovary; mOvr, micropylar ovary half; cOvr, chalazal ovary half; cP, cross-bisected pistil; IP, longitudinally bisected pistil; IP-Ovu, longitudinally bisected pistil without ovule; Ovu, ovule; IOvu, longitudinally bisected ovule; EPC, embryogenic pollen culture of cv. "Igri" precultured for 1–2 weeks and used as nurse tissue at a density of 50,000 pollen grains per mL.

Table S2 | The effect on embryogenic pollen development in cv. "Igri" of varying the number of co-cultivated, longitudinally bisected pistils (IP, numbers used per mL medium are given). An embryogenic pollen culture (EPC) of cv. "Igri" was used as a positive control at a population density of 50,000 pollen grains per mL. The numbers of embryogenic calli of diameter **(A)** ≥100 μm, **(B)** ≥500 μm, **(C)** ≥1 mm were assessed following 4 weeks of culture. noNT: no nurse tissue.

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5-azacytidine promotes microspore embryogenesis initiation by decreasing global DNA methylation, but prevents subsequent embryo development in rapeseed and barley

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Microspores are reprogrammed by stress *in vitro* toward embryogenesis. This process is an important tool in breeding to obtain double-haploid plants. DNA methylation is a major epigenetic modification that changes in differentiation and proliferation. We have shown changes in global DNA methylation during microspore reprogramming. 5-Azacytidine (AzaC) cannot be methylated and leads to DNA hypomethylation. AzaC is a useful demethylating agent to study DNA dynamics, with a potential application in microspore embryogenesis. This work analyzes the effects of short and long AzaC treatments on microspore embryogenesis initiation and progression in two species, the dicot *Brassica napus* and the monocot *Hordeum vulgare*. This involved the quantitative analyses of proembryo and embryo production, the quantification of DNA methylation, 5-methyl-deoxy-cytidine (5mdC) immunofluorescence and confocal microscopy, and the analysis of chromatin organization (condensation/decondensation) by light and electron microscopy. Four days of AzaC treatments (2.5 μM) increased embryo induction, response associated with a decrease of DNA methylation, modified 5mdC, and heterochromatin patterns compared to untreated embryos. By contrast, longer AzaC treatments diminished embryo production. Similar effects were found in both species, indicating that DNA demethylation promotes microspore reprogramming, totipotency acquisition, and embryogenesis initiation, while embryo differentiation requires *de novo* DNA methylation and is prevented by AzaC. This suggests a role for DNA methylation in the repression of microspore reprogramming and possibly totipotency acquisition. Results provide new insights into the role of epigenetic modifications in microspore embryogenesis and suggest a potential benefit of inhibitors, such as AzaC, to improve the process efficiency in biotechnology and breeding programs.

Keywords: microspore culture, epigenetic inhibitors, demethylating agents, totipotency, microspore reprogramming, *Hordeum vulgare*, *Brassica napus*

Introduction

Microspore embryogenesis is a fascinating process of cellular reprogramming and totipotency acquisition. In this process, a differentiating cell, the microspore, abandons its gametophytic developmental program in response to the application of a stress treatment *in vitro*, producing a complete embryo capable of germinating and regenerating a haploid or double-haploid mature plant. Microspore embryogenesis has been set up through isolated microspore cultures in several different plant species (Touraev et al., 1997; Massonneau et al., 2005; Forster et al., 2007; Testillano and Risueño, 2009). Microspore embryogenesis is also a powerful biotechnological tool in plant breeding as a method for the rapid production of isogenic lines, generation of new genetic variability and new genotypes, but this technique has had limited efficiency in many crops that are of particular interest (Maluszynski et al., 2003; Germana, 2011). Despite recent advances, there is still little known about the mechanisms that promote reprogramming of differentiating cells and their conversion, in response to stress, into totipotent cells capable of forming an embryo and a plant, without the fusion of the gametes (Grafi et al., 2011).

Stress-induced plant cell reprogramming and acquisition of cellular totipotency involves repression and/or activation of numerous genes associated with the new development program as well as changes in global genome organization (Finnegan et al., 2000). Epigenetic marks are involved in the regulation of global gene expression programs in the genome (Kohler and Villar, 2008). DNA methylation, by DNA methyltransferases, constitutes a prominent epigenetic modification of the chromatin fiber which is associated with gene silencing. This epigenetic mark changes during plant cell differentiation and proliferation processes, and regulates gene expression (Finnegan et al., 2000; Meijón et al., 2010). Recently, work by our group has shown modifications in global DNA methylation that accompanied the change of developmental program of the microspore toward embryogenesis, indicating an epigenetic reprogramming after microspore induction to a totipotent state and embryogenesis initiation. This epigenetic reprogramming involved a global DNA methylation decrease with the activation of cell proliferation, and a subsequent DNA methylation increase with embryo differentiation, in very different plant species, like *Brassica napus* (Solís et al., 2012; Testillano et al., 2013), *Hordeum vulgare* (El-Tantawy et al., 2014), and *Quercus suber* (Rodríguez-Sanz et al., 2014a).

In eukaryotic cells, 5-Azacytidine (AzaC), a known analog of 5-cytosine, inhibits DNA methyl transferase activity leading to genomic DNA hypomethylation (Friedman, 1981). AzaC has been used as a demethylating agent in several different plant systems, leading to a wide range of effects on development depending on the dose, time, and process (Loschiavo et al., 1989; Li et al., 2001; Pedrali-Noy et al., 2001; Santos and Fevereiro, 2002; Yamamoto et al., 2005; Yang et al., 2010; Fraga et al., 2012; Pecinka and Liu, 2014; Teyssier et al., 2014). Treatments with AzaC have also been reported to affect chromosome behavior and structure in root cells (Castilho et al., 1999; Vorontsova et al., 2004). In addition AzaC has been shown to shorten

nucleologenesis by early NOR replication, and may possibly lead to early entry of root meristematic cells in the next cell cycle (De-La-Torre et al., 1991; Mergudich et al., 1992). However, there have been no studies with AzaC treatments in isolated microspore cultures and its effects on microspore embryogenesis initiation and progression, in correlation with changes in DNA methylation levels and distribution patterns.

In this work, the effects of AzaC on microspore embryogenesis induction and progression, as well as on global DNA methylation levels, nuclear distribution of methylated DNA and chromatin organization have been analyzed in two plant species, the dicot *B. napus* (rapeseed) and the monocot *H. vulgare* (barley).

Material and Methods

Plant Material and Growth Conditions

Brassica napus L. cv. Topas (rapeseed) and *Hordeum vulgare* L. cv. Igri (barley) were used as donor plants. Barley seeds were germinated in soil for 1 month at 4°C. After that, they were grown at 12°C with a 12/12 light/dark cycle (10,000–16,000 lx) for 1 month in a plant growth chamber (Sanyo; relative humidity about 70%), and then in a greenhouse under a controlled temperature of 18°C. Rapeseed seeds were sown in soil and plants were grown under controlled conditions at 15/10°C in a 16/8 h light/dark cycle in a plant growth chamber (Sanyo) with 60% relative humidity.

Microspore Isolation and Culture

Rapeseed microspore culture was performed as previously described (Prem et al., 2012). Selected flower buds containing microspores at the vacuolated stage [the most responsive stage for embryogenesis induction (González-Melendi et al., 1995)] were surface-sterilized in 5% commercial bleach for 20 min and then rinsed 6–7 times with sterile distilled water. Ten to fifteen buds were crushed using a cold mortar and pestle in 5 ml of cold NLN-13 medium (Lichter, 1982; Duchefa) containing 13% sucrose (w/v). The suspension was filtered through a 48 µm nylon mesh and the filtrate collected in 15 ml falcon centrifuge tubes. The crushed buds were rinsed with 5 ml NLN-13 to make up the volume to 10 mL and the filtrate was then centrifuged at 185 × g for 5 min at 4°C. The pellet was resuspended in 10 mL of cold NLN-13 and centrifuged as mentioned above. This process was repeated three times for washing of the microspores. The final pellet was suspended in the NLN-13, and the cell density was adjusted to 10,000 cells per mL. After isolation, cultures were subjected to 32°C temperature for embryogenesis induction and checked every 2 days under the stereomicroscope till development of globular embryos was observed, around 10 days after culture initiation. Thereafter, cultures were shifted to 25°C on an orbital shaker at 60 rpm (amplitude of rotation: 20 mm) until complete development and maturation of the embryos was observed, around 30 days after culture initiation, as previously described (Prem et al., 2012).

Barley microspore culture was performed as previously described (Rodríguez-Serrano et al., 2012). Spikes containing

microspores at the vacuolated stage were collected and surface sterilized by immersion in bleach at 5% for 20 min, followed by 3–4 washes with sterile distilled water. The sterilized spikes were then pre-treated at 4°C for 23–24 days as stress treatment to induce embryogenic development. The isolation and culture of the microspores were performed as previously described (Rodríguez-Serrano et al., 2012) with final density of 1.1×10^5 cell per mL in an appropriate volume of KBP medium (Kumlehn et al., 2006). To isolate the microspores, the spikes were blended in 20 mL of precooled 0.4 M mannitol using a Waring Blender (Eberbach, Ann Arbor, MI, USA) precooled in a refrigerator, and the extract was filtered through a 100 µm nylon mesh (Wilson, Nottingham, UK) into a vessel at 4°C. The microspore suspension collected was transferred into a 50 ml tube and centrifuged at $100 \times g$ for 10 min at 4°C. After removing the supernatant, the pellet was resuspended in 8 mL of ice-cold 0.55 M maltose. This volume was distributed between two 15 mL tubes and each aliquot cautiously over layered with 1.5 mL of mannitol solution. After gradient centrifugation at $100 \times g$ for 10 min at 4°C, the interphase band consisting of an almost pure population of vacuolated microspores was resuspended in mannitol solution giving a final volume of 20 mL. The pelleted microspores were diluted in an appropriate volume of KBP medium to obtain a cell density of 1.1×10^5 cells per mL. The microspores were incubated at 25°C in the dark. Embryos were observed after around 30 days.

Treatments of Microspore Cultures with AzaC

The demethylating agent 5-AzaC (Sigma) was added to the culture plates at the culture initiation from a freshly prepared concentrated solution of 500 µM in culture media, after filtering with a sterile Ministart filter (Sartorius Biotech). In a first experiment, this solution was added to rapeseed microspore cultures at three different concentrations, 2.5, 5, and 10 µM, keeping parallel plates without the drug as control. The rest of treatments were performed at the selected concentration of 2.5 µM.

Short AzaC treatments were performed from culture initiation during 4 days, time of the proembryo formation stage in both *in vitro* microspore cultures, rapeseed (Prem et al., 2012) and barley (Rodríguez-Serrano et al., 2012).

Long AzaC treatments were carried out from culture initiation until the stage of embryo formation (cotyledonar embryos in rapeseed and coleoptilar embryos in barley), during 30 days in both systems (Prem et al., 2012; Rodríguez-Serrano et al., 2012).

Quantification of the number of three types of structures, “proembryos,” “developing embryos,” and “embryos” was performed at defined time points of the cultures. Quantifications were carried out using stereomicroscope micrographs randomly obtained from control and AzaC-treated microspore culture plates. “Proembryos” were rounded multicellular structures, still surrounded by the exine, which displayed higher size and density than microspores. “Developing embryos” were structures formed after the exine breakdown and much larger than proembryos; these term “developing embryos” included embryos at different developmental stages of the two pathways

(monocot and dicot species). Mean percentages of “proembryos” and “developing embryos,” and total number of “embryos” (fully developed) per Petri dish were calculated from random samples of two independent experiments and 10–15 different culture plates per each *in vitro* system. A total of 100–140 micrographs and 1000–1800 embryo structures were evaluated for each culture time point, each treatment, and each plant species. The results were shown in histograms in which columns represented mean values and bars represented SEM. Significant differences between non-treated (control) cultures and AzaC-treated cultures were tested by Student’s *t*-test at $P \leq 0.05$.

Cell Death Detection and Quantification

To determine changes in viability of cells, detection of dead cells in microspore cultures was performed by Evans blue staining (Rodríguez-Serrano et al., 2012) in control and AzaC-treated cultures. Culture samples were incubated with a 0.25% (w/v) aqueous solution of Evans Blue for 30 min and observed with a light microscope under bright field. The number of dead (stained by Evans Blue) and live (unstained by Evans Blue) cells were quantified on random micrographs from two replicas (Evans blue-stained preparations) and three independent samples of each culture treatment; mean percentages of dead cells were calculated. A total of 150–200 micrographs and 2000–2500 structures were evaluated per culture treatment. The results were shown in histograms in which columns represented mean values and bars represented SEM. Significant differences in the percentage of dead cells between non-treated (control) cultures and AzaC-treated cultures at different concentrations were tested by Student’s *t*-test at $P \leq 0.05$.

Quantification of Global DNA Methylation

Genomic DNA was extracted from samples of microspore cultures of rapeseed and barley at the stage of proembryo formation (4 days), in non-treated conditions and after short treatments with 2.5 µM AzaC. The DNA extraction was performed using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) as previously described (Solís et al., 2014). A MethylFlash Methylated DNA Quantification Kit (Colorimetric; Epigentek, Farmingdale, NY, USA) was used for the quantification of the global DNA methylation according to the manufacturer’s instruction, using 200 ng of genomic DNA (Testillano et al., 2013) collected from various culture plates of each sample (for barley: 20–25 plates of 50 mm diameter and 1.5 mL of culture medium each; for rapeseed: 8–10 plates of 90 mm diameter and 15 mL of culture medium each). Three biological (independent culture experiments) and two analytical (DNA methylation colorimetric assays) replicates per sample were taken and mean percentages of 5-methyl-deoxy-cytidine (5mdC) of total DNA were calculated. The results were shown in histograms in which columns represented mean values and bars represented SEM. Significant differences between non-treated (control) cultures and AzaC-treated cultures were tested by Student’s *t*-test at $P \leq 0.05$.

Fixation and Processing for Light Microscopy Analysis

Samples from different culture times were collected and fixed overnight at 4°C with 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.3. Culture samples of the first stages contained isolated microspores and small multicellular proembryos, they were previously embedded in gelatine. After fixation, samples were washed in PBS, dehydrated in an acetone series, embedded in Historesin Plus at 4°C and sectioned at 2 µm thickness using an ultramicrotome (Ultradex E Reichert). Some semithin resin sections were stained with 1% toluidine blue, for structural analysis, mounted with Eukitt, and observed under bright field microscopy. Other sections were stained with 1 mg mL⁻¹ DAPI (4',6-diamidino-2-phenylindole), specific staining for DNA, for 10 min, for observation of the nuclei under UV excitation and epifluorescence microscopy.

5mdC Immunofluorescence and Confocal Microscopy

Immunolocalization of 5mdC was performed as previously described (Solís et al., 2012; Testillano et al., 2013). Historesin semithin sections were mounted on 3-aminopropyltriethoxysilane-coated slides, denatured with 2N HCl for 45 min, washed in PBS and treated with 5% bovine serum albumin (BSA) in PBS for 10 min, incubated with anti-5mdC mouse antibody (Eurogentec) diluted 1/50 in 1% BSA and Alexa-Fluor-488 anti-mouse IgG antibody (Molecular Probes) diluted 1/25. As negative controls, either DNA denaturation step or first antibody was omitted. Sections were counterstained with 1 mg mL⁻¹ DAPI (4',6-diamidino-2-phenylindole) for 10 min and analyzed by confocal laser microscopy (TCS-SP5, Leica). Images of maximum projections were obtained with software running in conjunction with the confocal microscope (Leica software LCS version 2.5). Confocal microscopy analysis was performed using the same laser excitation and sample emission capture settings in all immunofluorescence preparations of each species, rapeseed or barley, allowing an accurate comparison between signals of control and AzaC-treated cells.

Electron Microscopy and Ultrastructural Analysis

Samples to be observed for transmission electron microscopy (TEM) were processed and embedded in Epon 812 or K4M Lowicryl resin, as previously described (Testillano et al., 2005; Solís et al., 2014). Samples to be embedded in Epon resin were fixed in Karnovsky fixative (4% formaldehyde + 5% glutaraldehyde in 0.025M cacodylate buffer, pH 6.7), dehydrated in a methanol series for 3 days and slowly embedded in Epon resin for 2 days. Epon blocks were polymerized at 60°C for 2 days. Samples to be embedded in K4M Lowicryl were fixed in 4% formaldehyde in PBS at 4°C, overnight, dehydrated in a methanol series by Progressive Lowering of Temperature (PLT) and embedded in K4M Lowicryl at -30°C, in an Automatic Freeze-Substitution unit (AFS, Leica, Vienna). 80 nm thick ultrathin sections were collected on 75 mesh copper grids,

counterstained with uranyl acetate and lead citrate and observed in a JEOL 1010 TEM operating at 80 kV.

5mdC Immunogold Labeling for Electron Microscopy

Immunogold labeling for 5mdC ultrastructural localization was performed as previously described (Solís et al., 2014). Lowicryl ultrathin sections were obtained and collected on 200 mesh nickel grids with a carbon-coated Formvar supporting film. Ultrathin sections were floated on drops of distilled water, denatured with 2N HCl for 45 min and washed in PBS before incubation in 5% BSA. For immunogold labeling, they were incubated with anti-5mdC antibody (diluted 1:50) for 1 h at room temperature. After washing with PBS, the sections were incubated with anti-mouse secondary antibody conjugated to 10 nm gold particles (BioCell) diluted 1:25 in PBS for 45 min. Then, the grids were washed in PBS, rinsed in distilled water and air-dried. Negative controls were performed by omitting either the DNA denaturation step or the first antibody. Finally, the grids were counterstained with 5% uranyl acetate and 1% lead citrate, and observed with a JEOL 1010 microscope operating at 80 kV.

Results

Effects of Short AzaC Treatments on Microspore Embryogenesis Initiation

Isolated microspore *in vitro* cultures were set up and embryogenesis induction performed, both according to previously described protocols in *B. napus* (Prem et al., 2012) and *H. vulgare* (Rodríguez-Serrano et al., 2012), as described in the “Materials and Methods” section. Vacuolated microspores (**Figures 1A,B** and **2A,B**), the most responsive developmental stage for embryogenesis induction in both monocot and dicot species (González-Melendi et al., 1995; Testillano et al., 2002, 2005), were subjected to the corresponding inductive stress treatment for each system, i.e., 32°C for *B. napus* and 4°C for *H. vulgare*. Four days after induction and culture initiation, responsive microspores that initiated the embryogenesis pathway had divided and produced multicellular structures still surrounded by the exine, the so-called microspore-derived “proembryos” (**Figures 1C,D** and **2C,D**). These proembryos (arrows in **Figures 1E** and **2E**) were clearly distinguished from the non-responsive microspores present in the culture, they were rounded structures displaying higher size and density than microspores, in both *in vitro* systems, rapeseed and barley. Over the following days in culture, microspore embryogenesis progressed; the exine broke down, and embryos developed following a pathway similar to the zygotic embryogenesis in monocot and dicot species. In the case of rapeseed, globular (**Figures 1F,G**), heart, torpedo (**Figure 1H**), and cotyledonary embryos (**Figure 1I**) were formed (Prem et al., 2012), while in barley microspore cultures globular, transitional, scutellar, and coleoptilar monocot embryos (**Figures 2F–H**) were developed (Rodríguez-Serrano et al., 2012).

Firstly, different concentrations of AzaC, 2.5, 5.0, and 10 µM, were tested during short treatments (4 days) on rapeseed

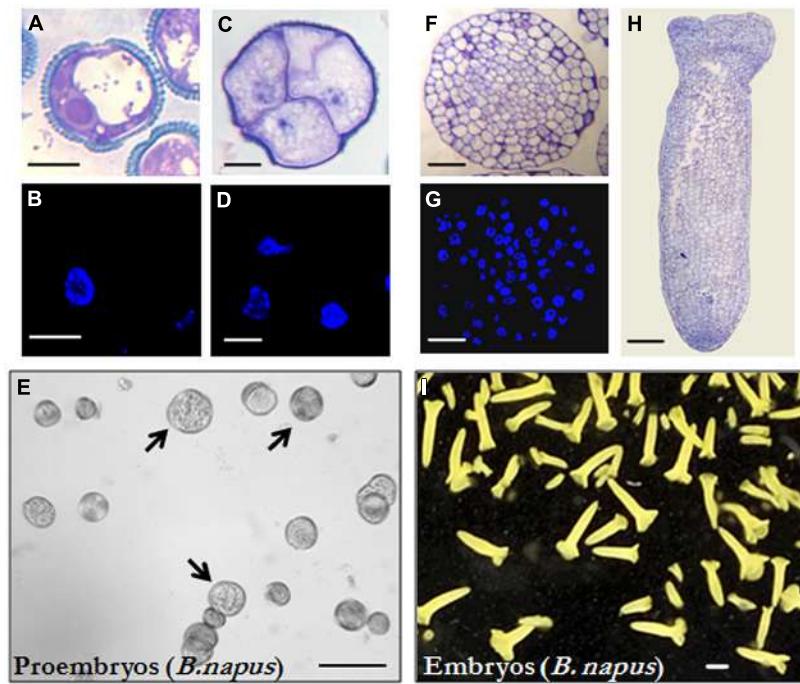


FIGURE 1 | Microspore embryogenesis in *Brassica napus*. **(A,B)** Vacuolated microspores at the beginning of the culture. **(C,D)** Proembryos formed by four cells, still surrounded by the exine (the microspore wall). **(E)** *In vitro* culture at the proembryo formation stage (4 days), proembryos are pointed by arrows. **(F,G)** Globular embryos. **(H)** Torpedo embryo. **(I)** *In vitro* culture at the embryo production stage (30 days), most embryos show the typical morphology

of cotyledonary embryos of the dicot embryogenesis pathway, some embryos at earlier developmental stages (heart and torpedo embryos) are also present. **(A,C,F,H)** Micrographs of toluidine blue-stained sections for general structure visualization. **(B,D,G)** DAPI staining for nuclei visualization (blue). **(E,I)** General views of cultures observed under the stereomicroscope. Bars represent, in **(A–D)** 10 μ m, in **(E)** 250 μ m, in **(F,G)** 50 μ m, in **(H)** 100 μ m, in **(I)** 1mm.

microspore cultures, and their effects on both, cell death, and microspore embryogenesis initiation efficiency (proembryo formation) were evaluated. The percentage of dead cells, identified by positive Evans blue staining (**Figure 3A**), present in cultures at the proembryo formation stage (**Figure 1E**) were quantified. Results showed a high level of dead cells in control cultures at the proembryo formation stage. Cell death may be contributed by both the isolation and *in vitro* culture procedures and by the application of the stress treatment on non-responsive microspores (**Figure 3B**). Microspore cultures treated with 2.5 and 5 μ M AzaC showed a small but statistically significant reduction in cell death, in comparison with control cultures (**Figure 3B**).

Quantifications of proembryos at the same culture time point showed significant higher proportion of these multicellular structures upon 2.5 μ M AzaC treatment compared to control cultures (**Figure 3C**). By contrast, higher AzaC concentrations (5 and 10 μ M) reduced the proportion of proembryos. Therefore, the concentration of 2.5 μ M was selected for the subsequent AzaC treatments in microspore cultures.

Short AzaC treatments were also applied to barley microspore cultures, at the concentration of 2.5 μ M, by adding the drug to the culture medium from the beginning of the culture until the proembryo formation stage (4 days). The quantification of the proembryos formed in untreated and AzaC-treated microspore

cultures of barley revealed that short AzaC treatments also produced a significantly higher proportion of proembryos in comparison with non-treated cultures (**Figure 3D**) in barley, like in rapeseed.

Effects of Short AzaC Treatments on Global DNA Methylation Levels and Distribution Patterns of Methylated DNA

To evaluate whether the presence of AzaC at a concentration of 2.5 μ M affected the DNA methylation of cells in microspore embryogenesis cultures, global DNA methylation levels were quantified in control and treated cultures of rapeseed and barley after short AzaC treatments (4 days), from the beginning of the culture until the proembryo formation stage (**Figures 1E** and **2E**). Results showed significant decreases in global DNA methylation after the AzaC treatments in both plant species (**Figure 4**). In *B. napus* microspore cultures treated by AzaC, DNA methylation levels reached only half of that in control cultures (**Figure 4A**). In barley microspore cultures, the level of methylated DNA also diminished after AzaC treatment (**Figure 4B**), but to a lesser extent than in rapeseed cells.

Immunofluorescence assays with 5mdC antibodies and confocal laser scanning microscopy analysis were performed to analyze the effects of short AzaC treatments on the nuclear localization pattern of methylated DNA. Immunofluorescence images of treated samples were obtained in the confocal

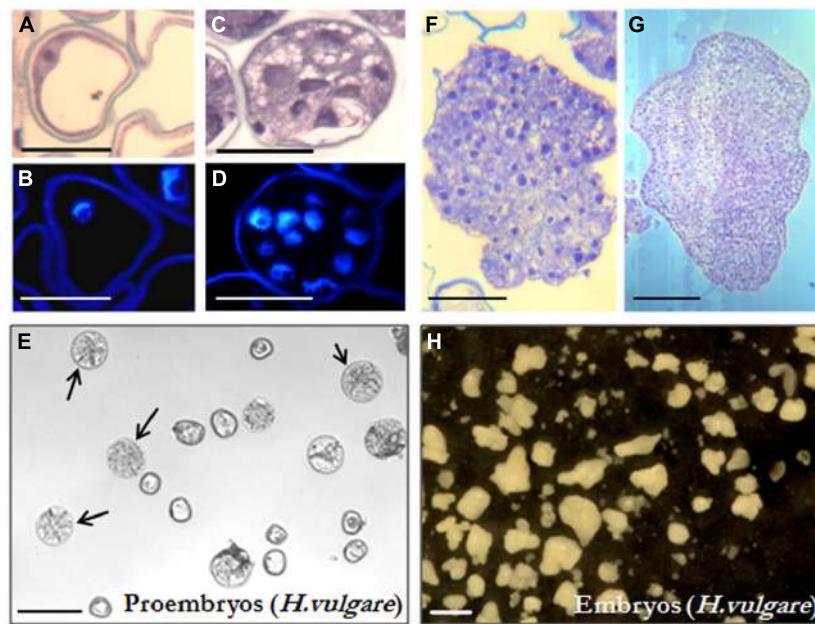


FIGURE 2 | Microspore embryogenesis in *Hordeum vulgare*.

(A,B) Vacuolated microspores at the beginning of the culture. (C,D) Proembryos formed by several cells, still surrounded by the exine (the microspore wall). (E) In vitro culture at the proembryo formation stage (4 days), proembryos are pointed by arrows. (F,G) Early and late transitional embryos. (H) In vitro culture at the embryo production stage (30 days), embryos show the typical morphology of coleoptilar embryos of

the monocot embryogenesis pathway, some embryos at earlier developmental stages (globular, early, and late transitional and scutellar embryos) are also present. (A,C,F,G) Micrographs of toluidine blue-stained sections for general structure visualization. (B,D) DAPI staining for nuclei visualization (blue). (E,H) General views of cultures observed under the stereomicroscope. Bars represent, in (A,B) 20 μ m, in (C,D) 50 μ m, in (E) 250 μ m, in (F,G) 100 μ m, in (H) 1 mm.

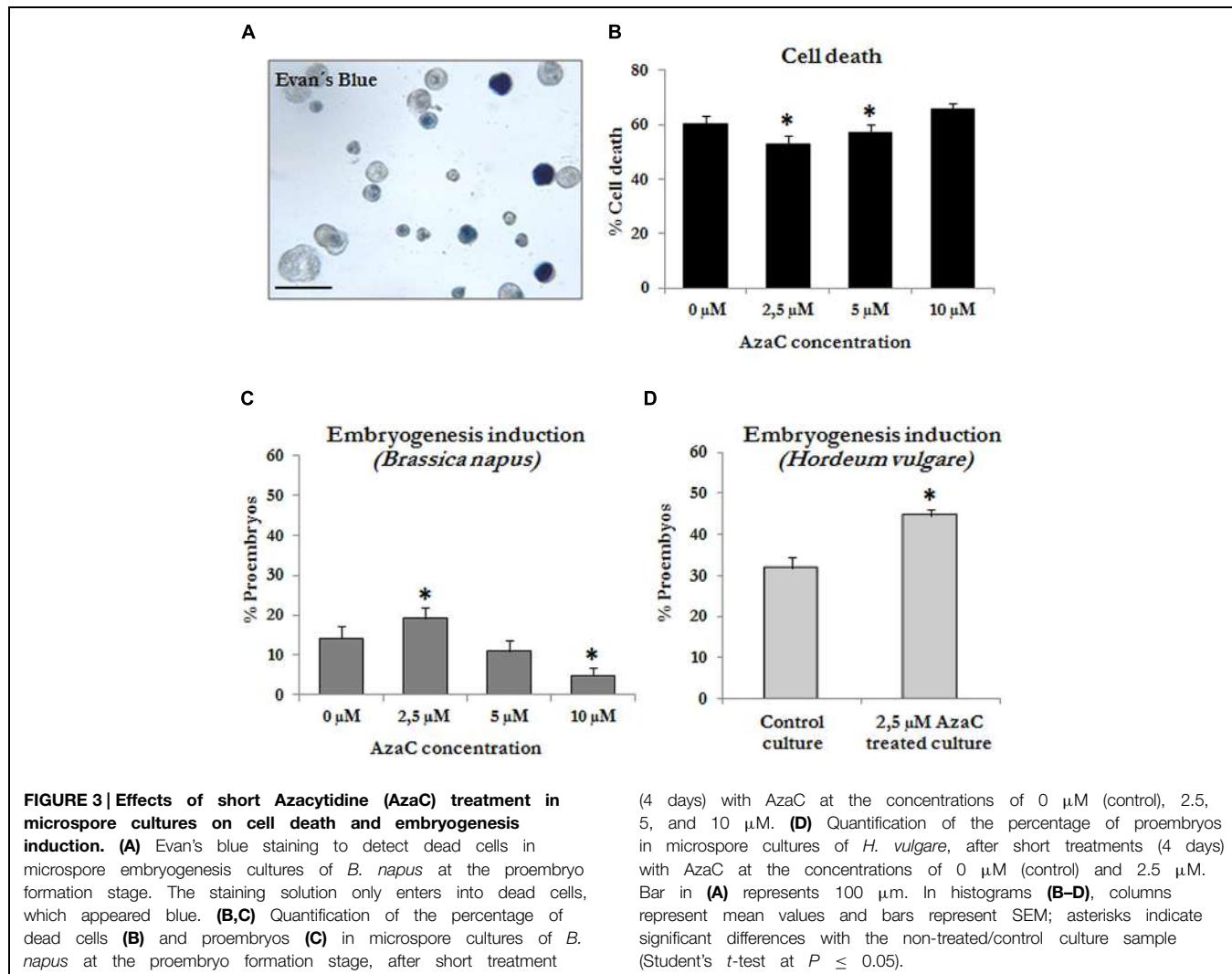
microscope under the same excitation intensity and emission capture settings than the non-treated samples, allowing an accurate comparison between signals. In non-treated cultures of rapeseed, microspore-derived proembryos were formed by several cells with a central rounded nucleus each, separated by straight cell walls and surrounded by the microspore wall, the exine (**Figure 5A**). The 5mdC immunofluorescence signal was concentrated in 4-to-6 conspicuous foci preferentially at the nuclear periphery and associated with heterochromatin foci (condensed chromatin masses), which were also revealed by the DAPI specific staining of DNA (**Figures 5A,A'**). In microspore cultures treated with 2.5 μ M AzaC, proembryos exhibited a cellular organization similar to that in control cultures (**Figure 5B**). Nevertheless, the immunofluorescence assays showed a different nuclear pattern of 5mdC distribution with very low or no 5mdC signal concentrated in 1-to-2 small foci per nucleus (**Figures 5B,B'**).

Barley microspore-derived proembryos, still surrounded by the exine, displayed numerous small cells with large nuclei and wavy cell walls (**Figure 5C**), which is the typical organization of microspore proembryos in monocot species like barley (Ramírez et al., 2001) and maize (Testillano et al., 2002). No significant differences on the structural organization of proembryos were observed in AzaC-treated cultures (**Figure 5D**). In control cultures, the 5mdC immunofluorescence signal was intense, covering the whole nucleus (**Figures 5C,C'**) which also exhibited an intense fluorescence intensity by DAPI

(**Figure 5C'**). In proembryos developed in the presence of AzaC, the 5mdC immunofluorescence signal was less intense and was distributed over the entire nucleus (**Figures 5D,D'**). Negative controls avoiding either the DNA denaturation step or the first antibody did not provide any labeling in the nucleus or any subcellular compartment, in any of the plant species analyzed.

Effects of Short AzaC Treatments on Chromatin Condensation Patterns

Changes in the chromatin condensation degree/pattern of proembryo cells after short AzaC treatments were analyzed in relation to the distribution of methylated DNA, by light and electron microscopy (**Figures 6 and 7**). After toluidine blue staining, nuclei of rapeseed proembryos appeared very clear, with several dark regions, mainly located at the nuclear periphery, as revealed by light microscopy (**Figure 6A**). High magnification fluorescence images of DAPI-stained samples showed a discrete number of brightly stained heterochromatin foci of variable size dispersed in euchromatin, which exhibited lower fluorescence (**Figure 6B**). The 5mdC immunofluorescence signal was intense in the heterochromatin regions while not excluded from euchromatin, which showed a faint 5mdC immunofluorescence signal throughout the nucleus (**Figure 6B'**). After the treatment with AzaC, proembryo nuclei showed a homogeneous chromatin distribution in both toluidine blue (**Figure 6C**) and DAPI (**Figure 6D**) staining with no or little apparent heterochromatin



foci. Concomitantly, the 5mdC immunofluorescence signal was very low and occasionally accumulated at one or two bright nuclear foci (**Figure 6D'**).

Transmission electron microscopy analysis revealed the chromatin ultrastructural organization of rapeseed proembryo nuclei, which exhibited a very low condensed chromatin pattern (**Figure 6E**) with a few isolated and electron dense condensed chromatin masses (arrows in **Figure 6E**), which occupied a low fraction of the nuclear volume and were mainly located at the nuclear periphery. These condensed chromatin masses most likely corresponded to the dark spots of heterochromatin observed at light microscopy, in toluidine blue-stained preparations. A large fraction of the nuclear volume was occupied by a wide interchromatin region (Ir) that displayed abundant fibrillo-granular ribonucleoprotein structures (RNPs), which are typical of this nuclear domain (Testillano et al., 2000, 2005; Seguí-Simarro et al., 2011). Together with the RNPs, decondensed chromatin fibers of different thicknesses (euchromatin) were localized (**Figure 6E**). 5mdC immunogold labeling revealed the ultrastructural distribution of methylated

(4 days) with AzaC at the concentrations of 0 µM (control), 2.5, 5, and 10 µM. **(D)** Quantification of the percentage of proembryos in microspore cultures of *H. vulgare*, after short treatments (4 days) with AzaC at the concentrations of 0 µM (control) and 2.5 µM. Bar in **(A)** represents 100 µm. In histograms **(B–D)**, columns represent mean values and bars represent SEM; asterisks indicate significant differences with the non-treated/control culture sample (Student's *t*-test at $P \leq 0.05$).

DNA; numerous gold particles were found decorating the large condensed chromatin masses, while no labeling was observed in decondensed chromatin (**Figure 6F**). Much less 5mdC immunogold labeling was found in the rest of the nucleus, with only a few gold particles observed as clusters on the very small masses of condensed chromatin, and as isolated particles (**Figure 6G**). The results of the 5mdC immunogold labeling correlated with the distribution of the 5mdC immunofluorescence on the heterochromatin. Negative controls avoiding either the denaturation step or the first antibody did not provide gold labeling on the nucleus or any subcellular compartment.

In barley proembryos, a completely different chromatin organization was found. In control cultures, nuclei of barley proembryos appeared densely stained by toluidine blue (**Figure 7A**); this staining revealed a dense chromatin pattern distributed throughout the entire nuclear area. By contrast, barley proembryos of AzaC-treated cultures showed lower toluidine blue staining density in their nuclei (**Figure 7C**), indicating a less condensed chromatin pattern than in control

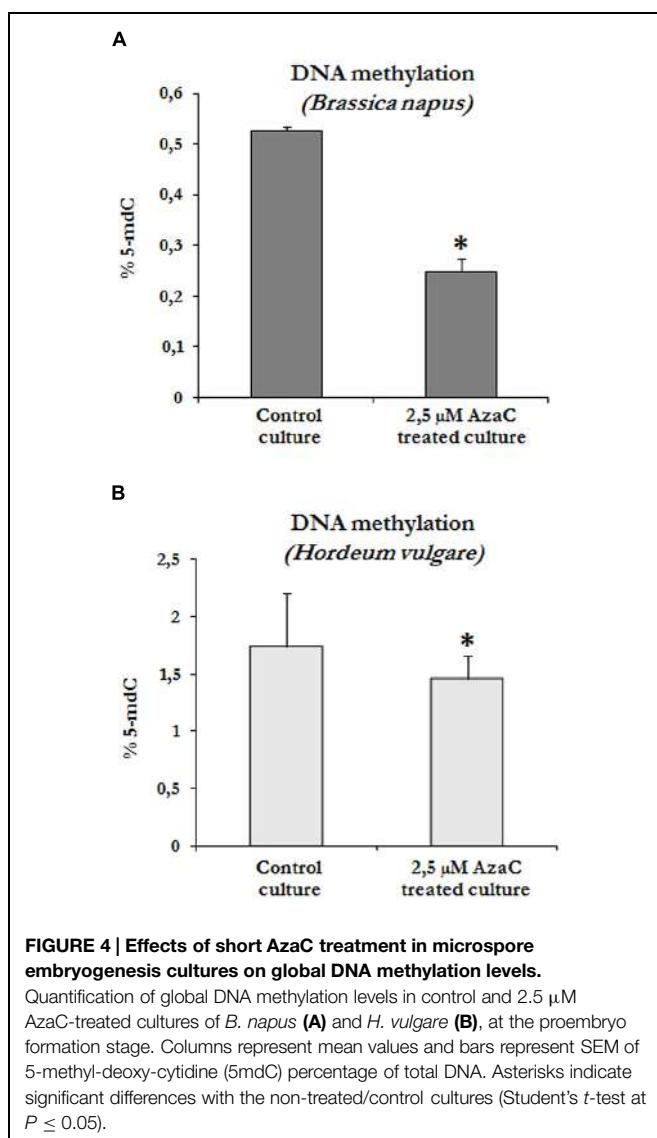


FIGURE 4 | Effects of short AzaC treatment in microspore embryogenesis cultures on global DNA methylation levels.

Quantification of global DNA methylation levels in control and 2.5 μ M AzaC-treated cultures of *B. napus* (A) and *H. vulgare* (B), at the proembryo formation stage. Columns represent mean values and bars represent SEM of 5-methyl-deoxy-cytidine (5mdC) percentage of total DNA. Asterisks indicate significant differences with the non-treated/control cultures (Student's *t*-test at $P \leq 0.05$).

samples. DAPI staining provided an intense fluorescence to proembryo nuclei of non-treated cultures (Figure 7B) while nuclei of AzaC-treated proembryos showed less intense DAPI fluorescence (Figure 7C), revealing a less condensed chromatin pattern in treated nuclei. In control proembryos, the signal of 5mdC immunofluorescence was intense and distributed in a reticular pattern (Figure 7B'). AzaC-treated nuclei showed a less intense distribution pattern of 5mdC immunofluorescence (Figure 7D'), when observed under the confocal microscope with the same excitation and capture settings as those used in non-treated nuclei. These observations suggested a decrease in the degree of chromatin condensation in AzaC-treated nuclei. Nucleoli appeared as non-stained (dark) rounded regions inside the nucleus in both DAPI and immunofluorescence images (Figures 7B,B',D,D').

Ultrastructural analysis by TEM showed the pattern of chromatin condensation in barley proembryo nuclei (Figure 7E). High magnification electron micrographs showed

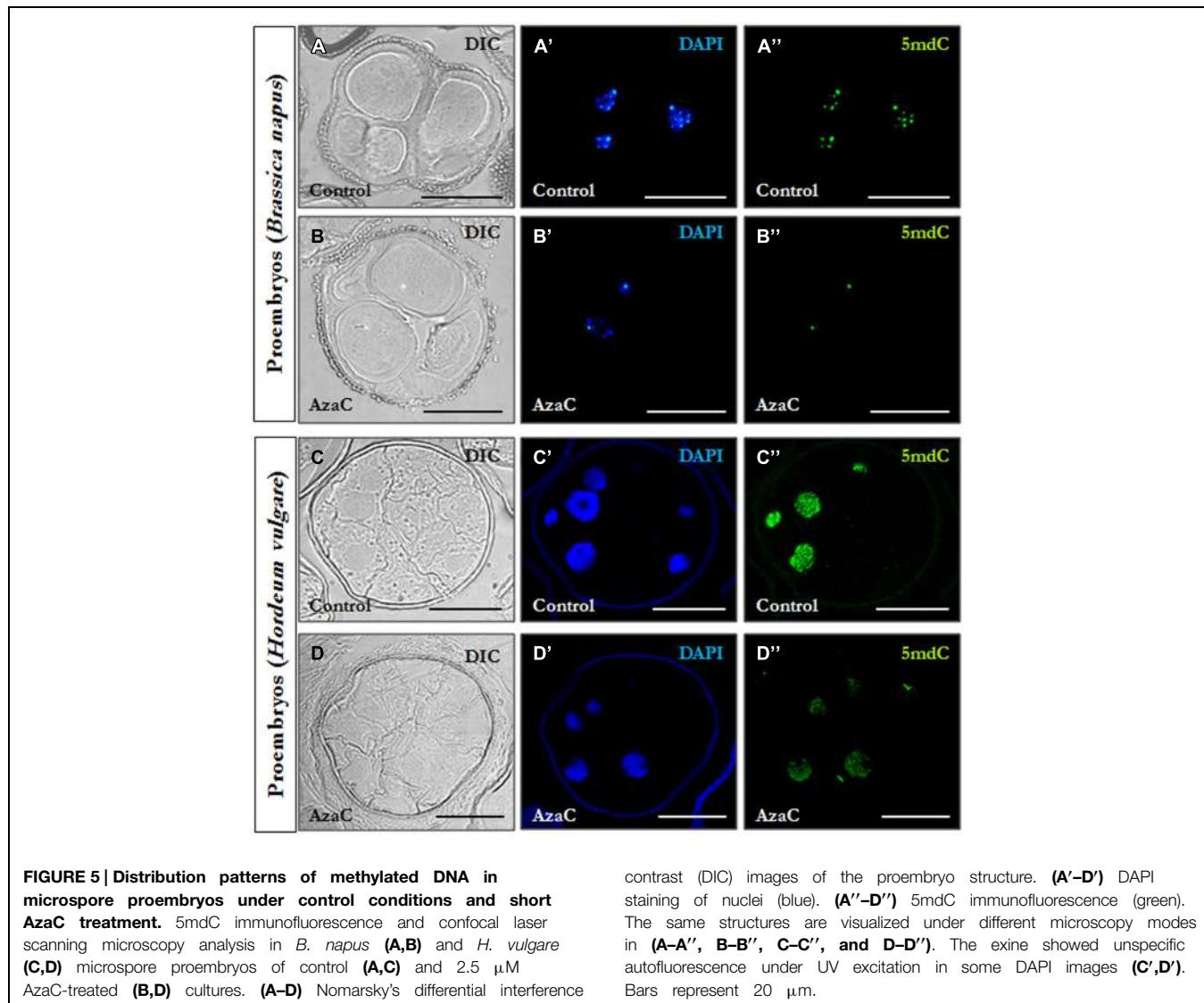
heterochromatin patches distributed throughout the whole nucleus, connected by chromatin threads of different thicknesses (Figure 7F). In this species, the abundant condensed chromatin masses (heterochromatin) occupied a significant proportion of the nucleus in comparison with the euchromatin (decondensed chromatin). The Ir that typically contained fibrillo-granular RNPs was less abundant in barley than in rapeseed proembryo nuclei (compare Figures 6E and 7F). The ultrastructural analysis of the condensed chromatin pattern of barley proembryo nuclei revealed that the distribution pattern of the heterochromatin corresponded to that of the methylated DNA revealed by 5mdC immunolocalization assays.

Effects of Long AzaC Treatments on Microspore-Derived Embryo Development

Long treatments with AzaC (30 days from culture initiation, the period in which most embryos finished their development) were carried out to evaluate the effects of the drug on embryo production, in the two stress-induced microspore embryogenesis systems, rapeseed and barley. Parallel cultures were performed in the presence and absence of the drug and the production of embryos were analyzed in the two *in vitro* systems at the embryo production stage, after 30 days of culture initiation. The embryos found were late torpedo and cotyledonary embryos in rapeseed (Figure 1I) and late scutellar and coleoptilar embryos in barley (Figure 2H). The results showed a very marked reduction of embryo production in 2.5 μ M AzaC-treated cultures in which only very few embryos were found in both species, in contrast with control cultures which exhibited numerous embryos (Figures 8A–D). The quantification of embryos in control and AzaC-treated cultures demonstrated a large decrease in the level of embryo production induced by the drug, in both systems (Figures 8E,F).

To assess the effects of AzaC on the progression of microspore embryogenesis after the proembryo stage, in barley microspore cultures, treated and non-treated-cultures were monitored under the microscope every few days until the stage in which the first coleoptilar embryos were observed, at 21 days. The number of proembryos (still surrounded by the exine) and the number of developing embryos (embryos at different developmental stages, formed after the exine breakdown) found in control and AzaC-treated cultures were quantified at each time interval (Figures 9 and 10).

In control cultures, responsive microspores divided during the first days of culture and produced proembryos which reached a proportion of one third by 10 days (Figures 9A and 10A). Later, the number of proembryos slightly increased until day 12, remained relatively stable for several more days and progressively decreased until day 21 (Figures 9B and 10A). However, in AzaC-treated cultures, the proportion of proembryos at day 10 was significantly higher than in control cultures (Figures 9D and 10A). During the following days, the number of proembryos in AzaC-treated cultures progressively increased, until day 21 (Figures 9E and 10A). The proembryos formed during long AzaC treatments showed similar morphology and size to the proembryos formed in non-treated cultures at early stages (Figures 9A,D,E), and no aberrant embryo morphologies



were observed during long AzaC treatments. These observations suggested that, in long AzaC treatments, the proembryos that were formed in the presence of the drug during the first days of culture, later stopped developing.

In non-treated cultures, after the exine breakdown embryogenesis progressed and further cell proliferation and differentiation events, that occurred asynchronously, lead to the formation of embryos with various sizes and shapes, the so-called “developing embryos.” These developing embryos were found in significant proportions from day 17 and maintained high proportions on day 21 and later, until day 30 (Figures 9B,C and 10B). Developing embryos were not found at earlier stages, during the first time points studied, when proembryos were abundant in the cultures (10–12 days; Figure 10B). By contrast, in AzaC-treated cultures, the progression of embryogenesis was inhibited and developing embryos were found in extremely low proportions at all the time intervals analyzed (Figures 9E,F and 10B).

Discussion

DNA Hypomethylation by AzaC Induces Changes in the Chromatin Condensation Pattern and Promotes Microspore Reprogramming and Embryogenesis Initiation

In vivo exposure to 5-AzaC prevents the incorporation of methyl groups to DNA cytosines leading to DNA hypomethylation. Recently, we have shown that the microspore reprogramming to embryogenesis is accompanied by modifications in global DNA methylation which exhibits low levels after induction and early embryogenesis (Solís et al., 2012; El-Tantawy et al., 2014; Rodriguez-Sanz et al., 2014a). Therefore, with the aim of exploring whether epigenetic inhibitors could affect the DNA methylation dynamics during microspore embryogenesis, we studied the effects of the demethylating agent AzaC on the process and its potential application to improve microspore embryogenesis induction.

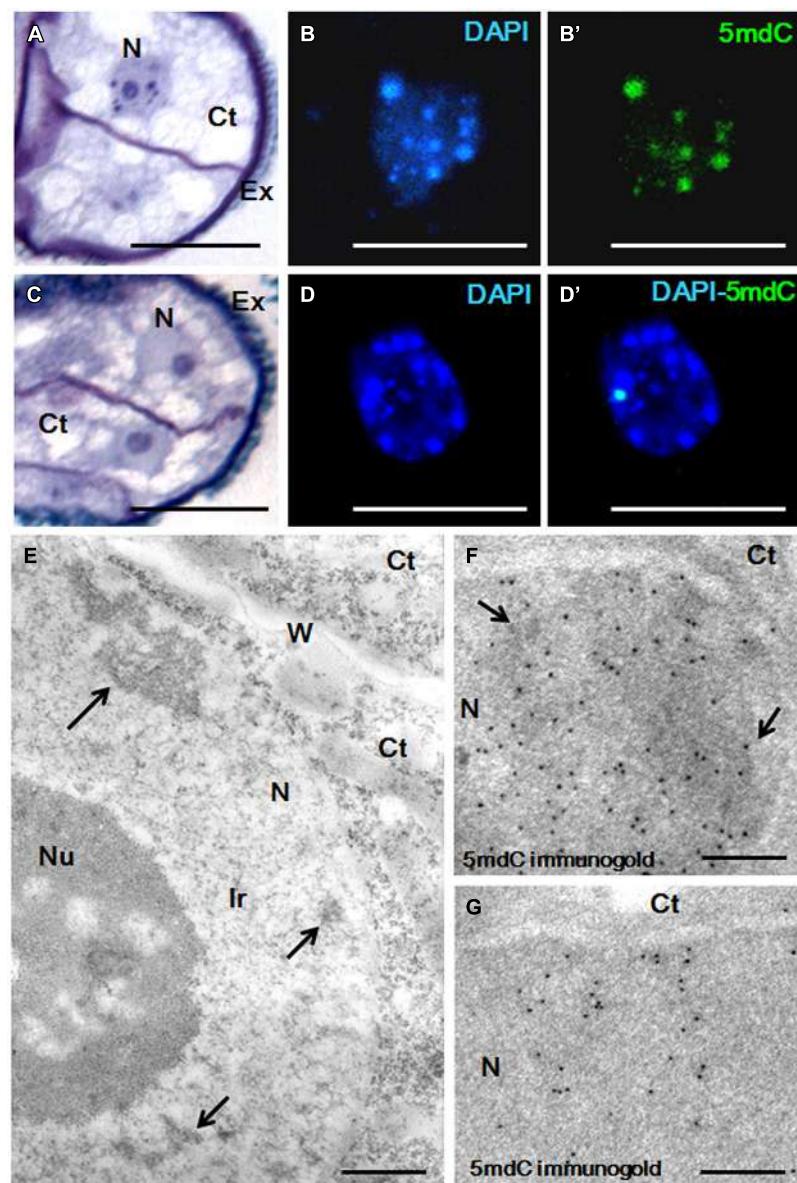


FIGURE 6 | Chromatin condensation patterns and methylated DNA distribution in microspore proembryos of *B. napus*. **(A–D)** High magnification light microscopy images of microspore proembryo nuclei in control **(A,B,B')** and 2.5 μ M AzaC-treated **(C,D,D')** cultures, observed after toluidine blue staining **(A,C)**, DAPI staining **(B,D)** and 5mdC immunofluorescence **(B',D')** by confocal laser scanning microscopy. The same nuclei are visualized under different microscopy modes in **(B,B')**, and in **(D,D')**. **(E–G)** Transmission electron microscopy (TEM) micrographs of nuclear regions of proembryos of control cultures. **(E)** Ultrastructural

organization of the nucleus that shows some condensed chromatin masses (arrows), an extensive interchromatin region (Ir) and a large nucleolus (Nu). **(F,G)** 5mdC immunogold labeling over nuclear regions of proembryo cells; large heterochromatin masses (arrows in **F**) are labeled by numerous gold particles, and nuclear regions with small condensed chromatin masses of different sizes show lower labeling (**G**). No gold particles are found on nucleolus and cytoplasms (Ct). Ex, exine; W, cell wall separating proembryo cells. Bars represent in **(A–D)** 10 μ m, in **(E)** 0.5 μ m, in **(F)**, **(G)** 0.2 μ m.

The present work was aimed to analyze the effects of the demethylating agent AzaC on microspore embryogenesis induction and progression, by comparing two different plant species, the monocot barley and the dicot rapeseed. These species are model systems for the process in which direct embryogenesis is induced, via different temperature stress treatments, in isolated microspores cultured in liquid media. The results of the short

AzaC treatments demonstrated a positive effect of the drug on microspore embryogenesis induction, at the low concentration of 2.5 μ M, increasing the percentage of microspore-derived proembryos formed, in the two systems.

AzaC has previously been tested as an additive in the culture medium of various *in vitro* systems of somatic embryogenesis and organogenesis, mainly through the culture of organs and tissue

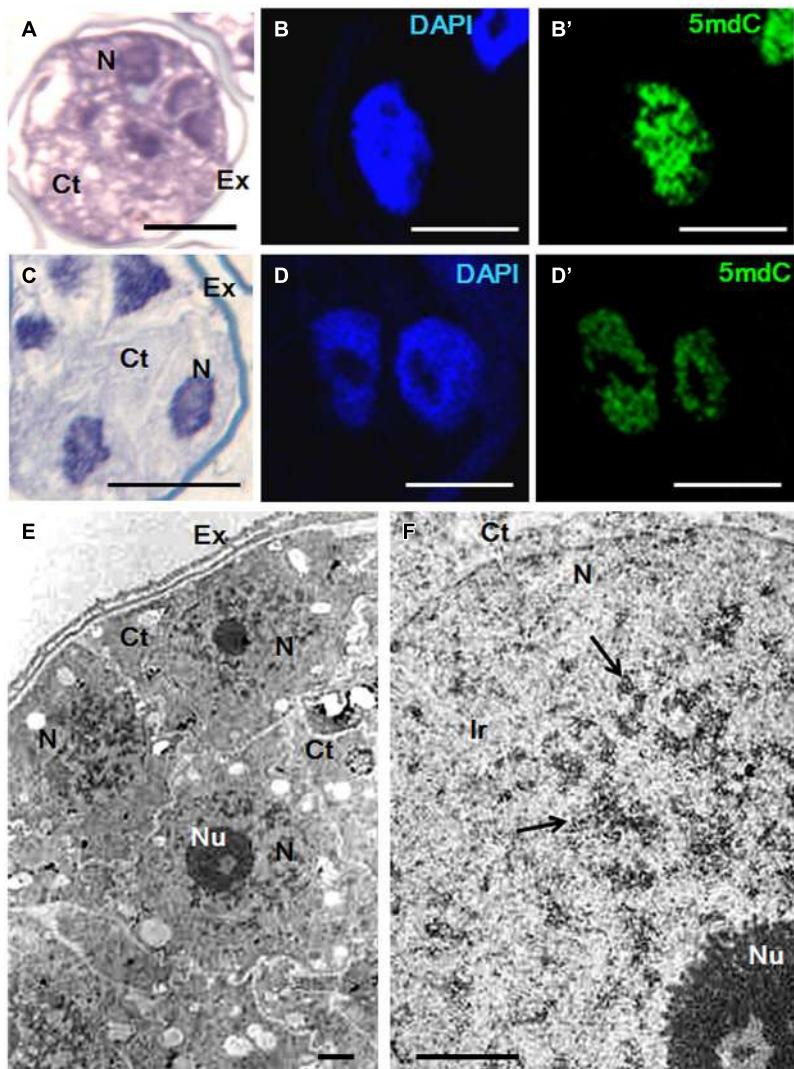


FIGURE 7 | Chromatin condensation patterns and methylated DNA distribution in microspore proembryos of *H. vulgare*. **(A–D)** High magnification light microscopy images of microspore proembryo nuclei in control (**A,B,B'**) and 2.5 μ M AzaC-treated (**C,D,D'**) cultures observed after toluidine blue staining (**A,C**), DAPI staining (**B,D**) and 5mdC immunofluorescence (**B',D'**) by confocal laser scanning microscopy. The same nuclei are visualized under different microscopy modes in (**B,B'**), and (**D,D'**).

(E,F) TEM micrographs of proembryos of control cultures. **(E)** Panoramic view of a proembryo surrounded by the microspore wall, the exine (Ex) showing several cells with one large nucleus (N) per cell and dense cytoplasms (Ct). **(F)** Detail of a nuclear region at high magnification; condensed chromatin masses (arrows) appear dense to electrons and forming numerous patches of different sizes, frequently connected by chromatin threads. Ir, interchromatin region; Nu, Nucleolus. Bars represent in **(A,C)** 20 μ m, in **(B,B',D,D')** 10 μ m, in **(E,F)** 1 μ m.

segments, with varying results. Most studies reported negative effects of the drug in the production of somatic embryos (Pedrali-Noy et al., 2001; Santos and Fevereiro, 2002; Yamamoto et al., 2005; Nic-Can et al., 2013; Teyssier et al., 2014); there are only a few examples in which AzaC promoted organogenesis or somatic embryogenesis (Li et al., 2001; Belchev et al., 2004; Tokaji et al., 2011; Fraga et al., 2012). In these previous studies, the range of concentration of AzaC has been very variable and high (from 10 to 200 μ M). Therefore, a dose response effect with possible secondary effects and cell toxicity could occur in these *in vitro* systems, as previously reported (Juttermann et al., 1994; Teyssier et al., 2014). In addition, data on AzaC effects on early events of

the process have not yet been analyzed. In the present work, lower concentrations of AzaC have been tested, 2.5, 5, and 10 μ M, and their effects on cell death have been evaluated; the results of these analyses reveal that cultures with the lowest AzaC dose (2.5 μ M) showed slightly lower proportions of dead cells than non-treated cultures, indicating that at this concentration the drug has no toxic effects on isolated microspore cultures. Therefore, 2.5 μ M was the concentration selected for the treatments. Moreover, the quantification of global DNA methylation indicates that 2.5 μ M AzaC significantly decreased the DNA methylation level of cells in microspore cultures of the two species studied, at precisely the same culture stage as when we detected significant increases in

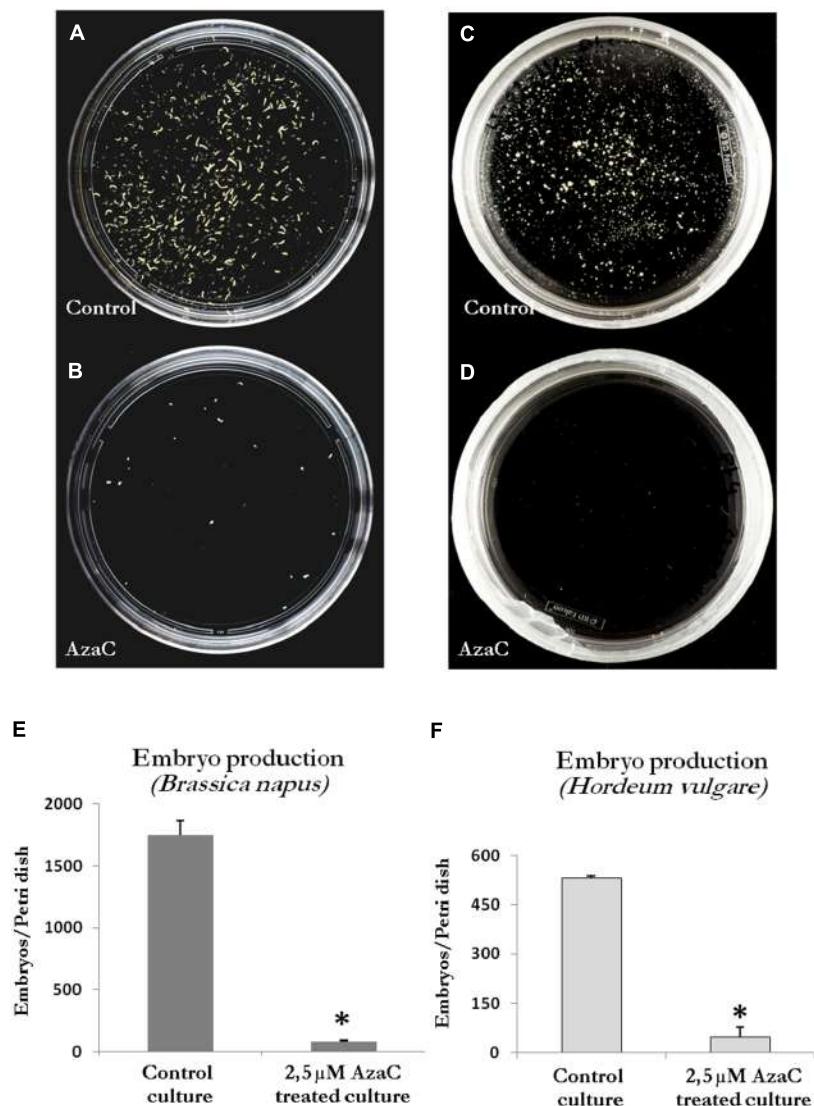


FIGURE 8 | Effects of long AzaC treatment on embryo production yield. (A–D) Plates showing the microspore-derived embryos produced in control **(A,C)** and 2.5 μM AzaC-treated **(B,D)** cultures of *B. napus* **(A,B)** and *H. vulgare* **(C,D)**, after 30 days. **(E,F)** Quantification of the embryo production in control

and 2.5 μM AzaC-treated cultures of *B. napus* **(E)** and *H. vulgare* **(F)**. In histograms **(E,F)**, columns represent mean values and bars represent SEM of the total number of embryos per Petri dish. Asterisks indicate significant differences with the non-treated/control culture sample (Student's *t*-test at $P \leq 0.05$).

proembryo formation. These results indicate that, in rapeseed and barley, while the stress treatment induces microspore reprogramming and proliferation, concomitantly, AzaC-induced DNA hypomethylation promotes microspore embryogenesis initiation and formation of proembryos a few days after culture initiation.

Reprogramming and acquisition of cellular totipotency involve activation of numerous genes associated with the new developmental program and/or repression of genes of the original cell program. The way in which differentiating plant cells remodel their gene expression program during the acquisition of cell totipotency is a central question which involves large-scale chromatin reorganization (Tessadori et al.,

2007). Changes in chromatin organization and variations in the level of global DNA methylation have been associated with several different *in vitro* plant regeneration processes (Loschiavo et al., 1989; Miguel and Marum, 2011). Also during microspore embryogenesis, remodeling of the chromatin organization patterns have been characterized in various species like pepper, tobacco, and rapeseed (Testillano et al., 2000, 2002, 2005; Bárány et al., 2005; Seguí-Simarro et al., 2011). In these previous studies, comparative analyses were performed between the gametophytic and the sporophytic pathways followed by the microspore, permitting the identification of defined nuclear changes that occurred when the microspore reprogrammed and switched to embryogenesis. These reports showed that

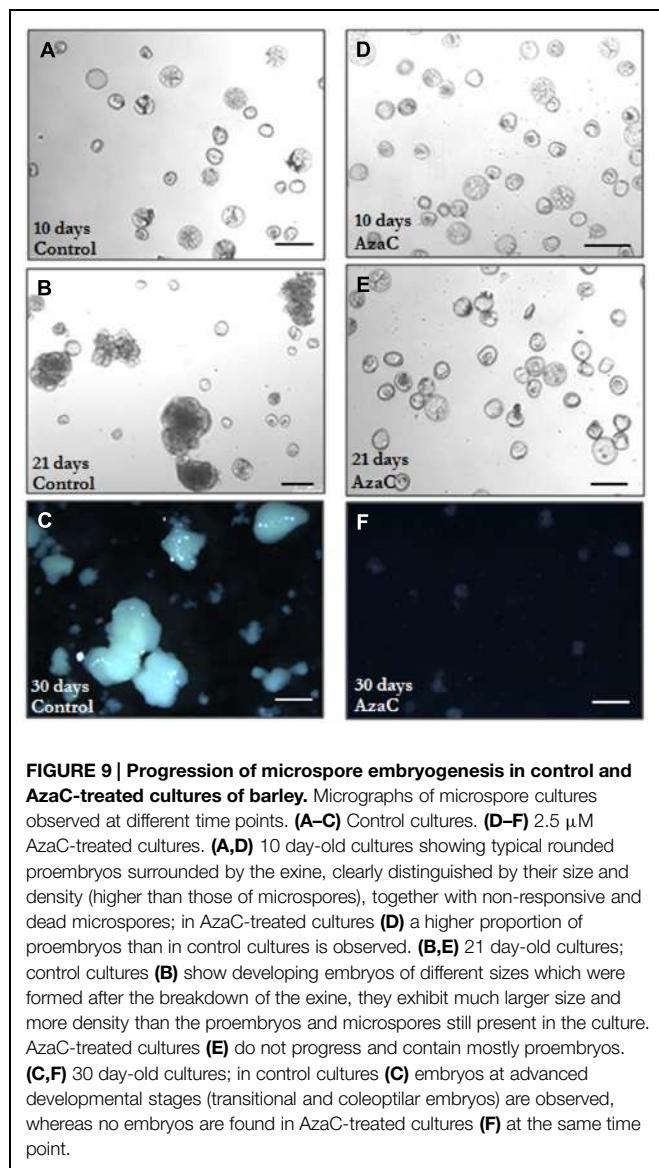


FIGURE 9 | Progression of microspore embryogenesis in control and AzaC-treated cultures of barley. Micrographs of microspore cultures observed at different time points. **(A–C)** Control cultures. **(D–F)** 2.5 μ M AzaC-treated cultures. **(A,D)** 10 day-old cultures showing typical rounded proembryos surrounded by the exine, clearly distinguished by their size and density (higher than those of microspores), together with non-responsive and dead microspores; in AzaC-treated cultures **(D)** a higher proportion of proembryos than in control cultures is observed. **(B,E)** 21 day-old cultures; control cultures **(B)** show developing embryos of different sizes which were formed after the breakdown of the exine, they exhibit much larger size and more density than the proembryos and microspores still present in the culture. AzaC-treated cultures **(E)** do not progress and contain mostly proembryos. **(C,F)** 30 day-old cultures; in control cultures **(C)** embryos at advanced developmental stages (transitional and coleoptilar embryos) are observed, whereas no embryos are found in AzaC-treated cultures **(F)** at the same time point.

the change of developmental program and the activation of proliferative activity (at the initiation of embryogenesis) affected the functional organization of the nuclear domains, which changed their architecture and functional state accordingly. Ultrastructural and *in situ* localization approaches revealed the pattern and functional states of chromatin and demonstrated the relation between the nuclear activity and the degree of chromatin condensation/decondensation. Regardless of the heterochromatin distribution pattern typical of each species, after microspore embryogenesis induction, the pattern of chromatin was less condensed in proembryos than in cells that follow the gametophytic development. Early microspore proembryos were characterized by a typical decondensed chromatin pattern, also found in proliferating cells of several plant species (Testillano et al., 2000, 2002, 2005; Bárány et al., 2005; Seguí-Simarro et al., 2011). *De novo* auxin biosynthesis and accumulation has been recently reported

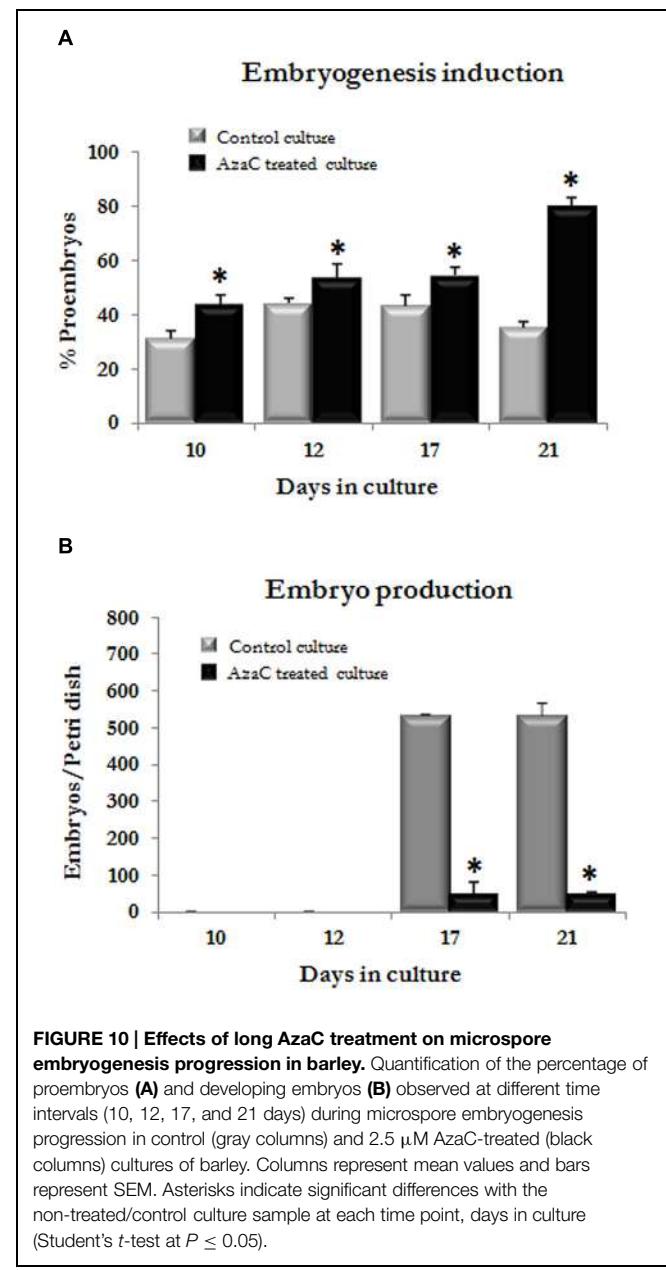


FIGURE 10 | Effects of long AzaC treatment on microspore embryogenesis progression in barley. Quantification of the percentage of proembryos **(A)** and developing embryos **(B)** observed at different time intervals (10, 12, 17, and 21 days) during microspore embryogenesis progression in control (gray columns) and 2.5 μ M AzaC-treated (black columns) cultures of barley. Columns represent mean values and bars represent SEM. Asterisks indicate significant differences with the non-treated/control culture sample at each time point, days in culture (Student's *t*-test at $P \leq 0.05$).

in early microspore embryogenesis, from the first divisions (Rodríguez-Sanz et al., 2015). This auxin accumulation has been related to the activation of proliferative activity in the reprogrammed microspore and early proembryo cells.

The results of the ultrastructural analysis of the chromatin condensation patterns together with the 5mdC immunofluorescence and immunogold assays presented here illustrate that AzaC-treatments not only decrease global DNA methylation levels but also modify the distribution pattern of the methylated DNA in the nucleus leading to more decondensed chromatin patterns in proembryo cells. In *B. napus*, the size and number of heterochromatin masses, enriched in 5mdC, diminished in proembryo cells treated with AzaC. Also in

barley, the hypomethylating drug affected methylated DNA distribution and chromatin condensation patterns, which changed into more decondensed chromatin threads. In animals, cell totipotency and pluripotency have been associated with a global chromatin reorganization and decondensation leading to the so-called “open chromatin state” in which specific histone modifications and DNA hypomethylation, among other factors, have been shown to be involved. This open chromatin structure is required for the cell to maintain its totipotent state, ready for transcriptional activation (Shi et al., 2008; Gaspar-Maia et al., 2011; Gonzalez-Muñoz et al., 2014). In animals, after fertilization and the formation of the zygote (totipotent) chromatin is decondensed and acquires specific epigenetic marks (Burton and Torres-Padilla, 2010). High mobility of core histones, remodeling of constitutive heterochromatin marks, and acquisition of specific permissive histone modifications have been suggested as required features for the chromatin state compatible with cellular reprogramming (Burton and Torres-Padilla, 2010; Boskovic et al., 2014; Lu and Zhang, 2015). In plants, cellular reprogramming has been associated with nuclear changes including chromatin decondensation, reduction in heterochromatin and changes in DNA methylation and histone modifications landscapes (Solís et al., 2012; She et al., 2013; El-Tantawy et al., 2014; Rodriguez-Sanz et al., 2014b). In *Arabidopsis*, after fertilization, distinct chromatin patterns have been reported in the zygote (totipotent) and endosperm (Pillot et al., 2010), patterns that have been associated with differential epigenetic and transcription patterns in the zygote/embryo and endosperm (Pillot et al., 2010) and could underlay the totipotency acquisition in the zygote. By contrast, DNA hypermethylation, and repressive histone modifications has been associated with heterochromatization and cell differentiation in animal and plant systems (Lippman et al., 2004; Solís et al., 2012; El-Tantawy et al., 2014; Rodriguez-Sanz et al., 2014b).

Recently, it has been shown that the change of developmental program of the microspore toward embryogenesis is accompanied by modifications in global DNA methylation (Solís et al., 2012; El-Tantawy et al., 2014; Rodriguez-Sanz et al., 2014a) and changes in histone epigenetic modifications (Rodriguez-Sanz et al., 2014b). These facts indicate that an epigenetic reprogramming occurs after the induction of the microspore to a totipotent state and embryogenesis initiation. Recent work by our group with *B. napus* (Rodriguez-Sanz et al., 2014b) suggested the participation of the dimethylated histone H3K9me2, a repressive mark, and histone methyl transferases (HKMTs) in microspore embryo cell differentiation and heterochromatinization events, whereas the acetylated histones H3Ac and H4Ac, permissive marks, and histone acetyl transferases (HATs) were involved in transcriptional activation and totipotency during microspore reprogramming. In addition, the reported changes of the DNA methylation (Solís et al., 2012) that occur after microspore embryogenesis induction lead to low methylation levels in early embryo stages. DNA hypomethylation is associated with the change of developmental program and with the activation of cell proliferation at the beginning of embryogenesis, and this DNA hypomethylation appears to be

related to a global change of gene expression (Solís et al., 2012). AzaC would facilitate/promote DNA hypomethylation and chromatin decondensation of cells stimulating reprogramming, totipotency acquisition, and early proembryo divisions and, therefore, increasing the efficiency of embryogenesis initiation. In mammalian cells, AzaC has been reported to induce expression of silenced genes, through demethylation of specific genome regions, and even to increase the expression of unmethylated genes by affecting histone methylation (Zheng et al., 2012). The DNA hypomethylation induced by AzaC could favor the deactivation of the gene expression program of the microspore to the pathway and the activation of a new gene expression program which promotes totipotency of a differentiating cell, the microspore, and the beginning of its active proliferation and cell cycle division.

In vivo exposure of *Allium cepa* root meristems to 5-AzaC (10^{-6} M) stimulated the rate of nucleologenesis and shortened its cycle time (De-La-Torre et al., 1991; Mergudich et al., 1992). In AzaC-treated proliferating root cells, nucleoli on the hypomethylated NORs were larger, a sign of high transcriptional activity, as demonstrated by the increase of the rate of [³H]uridine incorporation in AzaC-treated root cells (Mergudich et al., 1992). The vacuolated microspore, the most responsive stage for embryogenesis induction, has been characterized by a high transcriptional activity which is reflected by a large nucleolus and a decondensed chromatin pattern (Testillano et al., 2000, 2005; Seguí-Simarro et al., 2011). The positive effect of AzaC on microspore embryogenesis induction could also be due in part to the activation of nucleolar activity and nucleologenesis rate which would promote cell cycle divisions of the reprogrammed microspore.

Furthermore, the results presented here show that the same effects of AzaC (DNA hypomethylation, chromatin decondensation and an increase in microspore embryogenesis induction rates) are found in the two species studied, a monocot and a dicot plant, suggesting common epigenetic mechanisms during microspore embryogenesis induction in both phylogenetic groups.

DNA Methylation is Required for Microspore Embryo Differentiation and Long AzaC Treatment Prevents the Subsequent Embryo Development

In the present work, we have also analyzed the effects of the demethylating agent AzaC on the progression of microspore embryogenesis during subsequent developmental stages after the induction and the formation of proembryos. For this purpose, longer treatments of 2.5 μ M AzaC were applied to microspore cultures. The results revealed that, in contrast with short AzaC treatments which promoted embryogenesis initiation and proembryo formation, longer treatments prevented subsequent embryogenesis progression. The proembryos formed in AzaC-treated cultures during the first days of treatment were also observed during the following days and, although their development had stopped, they did not show any aberrant morphology.

During development, in relation to differentiation processes, the pattern of DNA methylation in the genome changes as a result of a dynamic process involving both *de novo* DNA methylation and demethylation. As a consequence, differentiated cells acquire a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription. The progress of the cellular differentiation has been related to a rapid increase in global DNA methylation levels in various plant developmental processes (Costa and Shaw, 2006, 2007; Malik et al., 2012). In mammals, heterochromatin increases dramatically during terminal cell differentiation and this has been linked to increased levels of DNA methylation (Politz et al., 2013). In *Arabidopsis*, embryos with loss-of-function mutations of the DNA methyltransferases MET1 and CMT3 (responsible of methylating DNA) develop improperly, indicating that DNA methylation is critical for plant embryogenesis (Xiao et al., 2006). Recent studies by our group have demonstrated the increase of global DNA methylation during microspore embryogenesis progression in rapeseed (Solís et al., 2012) and barley (El-Tantawy et al., 2014). This hypermethylation was associated with the heterochromatization that accompanies cell differentiation in advanced embryogenesis stages (Solís et al., 2012; El-Tantawy et al., 2014). In addition, the gene expression of the *MET1* DNA methyltransferase has been reported to increase during late stages of pollen maturation, tapetum developmental PCD, and differentiation of embryos originated from zygotes and microspores, in *B. napus* (Solís et al., 2012, 2014). This increase in *MET1* expression correlated with the increase in global DNA methylation and heterochromatization events (Solís et al., 2012, 2014). In the present work, the dynamics of DNA methylation has been altered by a demethylating agent, AzaC. The analysis of the effects of AzaC on the progression of microspore embryogenesis reported here showed that the drug clearly prevented embryo differentiation (hypermethylated stage), whereas AzaC promoted embryogenesis initiation (hypomethylated stage). The presence of the drug from the beginning until advanced stages blocked the process at the proembryo stage, which indicates that *de novo* DNA methylation

is required for subsequent microspore embryo differentiation processes.

Conclusion

Epigenetic inhibitors affecting DNA methylation, such as AzaC, provide a promising way for intervention through pharmacological assays to improve the efficiency of plant regeneration by stress-induced embryogenesis *in vitro* systems, as well as a convenient tool to investigate the role of DNA methylation dynamics in these processes. The results reported here demonstrated that AzaC increases microspore embryogenesis induction rates by inducing DNA hypomethylation and chromatin decondensation, at early stages. By contrast, subsequent embryo development is drastically affected by AzaC, suggesting that microspore-derived embryo differentiation requires *de novo* DNA methylation. The present study illustrates that low concentration and short duration of the AzaC treatment, at defined early stages, are critical points to achieve positive effects in terms of microspore embryogenesis efficiency, 2.5 μ M AzaC for 4 days from culture initiation is a suitable treatment for promoting the induction of the process in isolated microspore cultures of two different species, rapeseed and barley. The results suggest common epigenetic mechanisms in both monocot and dicot plant systems and open the way to design new biotechnological strategies for improving doubled-haploid production in crop breeding programs.

Acknowledgments

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Formation and excretion of autophagic plastids (plastolysomes) in *Brassica napus* embryogenic microspores

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The change in developmental fate of microspores reprogrammed toward embryogenesis is a complex but fascinating experimental system where microspores undergo dramatic changes derived from the developmental switch. After 40 years of study of the ultrastructural changes undergone by the induced microspores, many questions are still open. In this work, we analyzed the architecture of DNA-containing organelles such as plastids and mitochondria in samples of *B. napus* isolated microspore cultures covering the different stages before, during, and after the developmental switch. Mitochondria presented a conventional oval or sausage-like morphology for all cell types studied, similar to that found *in vivo* in other cell types from vegetative parts. Similarly, plastids of microspores before induction and of non-induced cells showed conventional architectures. However, approximately 40% of the plastids of embryogenic microspores presented atypical features such as curved profiles, protrusions, and internal compartments filled with cytoplasm. Three-dimensional reconstructions confirmed that these plastids actually engulf cytoplasm regions, isolating them from the rest of the cell. Acid phosphatase activity was found in them, confirming the lytic activity of these organelles. In addition, digested plastid-like structures were found excreted to the apoplast. All these phenomena seemed transient, since microspore-derived embryos (MDEs) showed conventional plastids. Together, these results strongly suggested that under special circumstances, such as those of the androgenic switch, plastids of embryogenic microspores behave as autophagic plastids (*plastolysomes*), engulfing cytoplasm for digestion, and then are excreted out of the cytoplasm as part of a cleaning program necessary for microspores to become embryos.

Keywords: androgenesis, cryomethods, electron microscopy, microspore embryogenesis, rapeseed

INTRODUCTION

Microspore embryogenesis is the most efficient biotechnological approach to obtain haploid individuals and doubled haploid (DH) lines. DHs are used as pure lines in breeding programs to produce hybrid seeds. They are also extremely valuable tools for plant genetic research (Forster et al., 2007; Seguí-Simarro, 2010). Microspore embryogenesis consists of the reprogramming of the microspores (the pollen precursors) toward embryogenesis. This developmental switch (also known as androgenesis) is generally induced through the application of stress. The microspores of some species can be induced by starvation, others by the application of cold temperatures to the inflorescences prior to *in vitro* culture, and others by the application of a heat shock to the *in vitro* cultured microspores, as is the case for *Brassica napus* microspores (reviewed in Shariatpanahi et al., 2006). Once microspores are reprogrammed, they undergo multiple changes to readapt themselves to the new developmental scenario. These changes include, among others, a profound remodeling of gene expression, the triggering of a (stress) response as a consequence of the inductive (stressing) treatment,

the suppression of the ongoing gametophytic program, and the initiation of embryogenesis (Maraschin et al., 2005; Seguí-Simarro and Nuez, 2008; Dunwell, 2010). At the subcellular level, there is also an extensive remodeling of cell ultrastructure, including a displacement of the nucleus to the center of the cell, a rearrangement of the cytokinetic machinery, a switch from an asymmetric to a symmetric division pattern, and a reduction in the number of plastids (Zaki and Dickinson, 1991; Hause et al., 1993; Telmer et al., 1995; Testillano et al., 2000; Shariatpanahi et al., 2006; Makowska and Oleszczuk, 2014).

The study of the ultrastructural changes associated to the androgenic switch started approximately 40 years ago with the pioneering works of Dunwell and Sunderland (1974a,b, 1975, 1976a,b,c). During these decades, these studies have been traditionally done by using transmission electron microscopy (TEM) in samples preserved with aldehyde-based chemical fixatives. The main disadvantage of these fixatives is the parallel generation of structural disorders in membranous elements of different subcellular compartments and organelles (McDonald and Auer,

2006). Among these artifacts, chemical fixatives may generate membrane retraction, fusion, and/or swelling, as well as vesiculation of large membranous elements (Gilkey and Staehelin, 1986). Such a change of the original cell ultrastructure frequently precludes the accurate identification and analysis of complex membranous structures (Gilkey and Staehelin, 1986; McDonald and Auer, 2006). Fortunately, there is an alternative to avoid the artifacts of chemical fixatives, which consists on the combined use of two cryotechniques for sample preservation: High Pressure Freezing and Freeze Substitution (HPF/FS). HPF consists on freezing the sample within milliseconds while subjected to high pressure (2100 bar). HPF/FS prevents the formation of ice crystals derived from freezing and provides an excellent ultrastructural preservation, much better than chemical fixation (Gilkey and Staehelin, 1986). These features make HPF/FS the method of choice for fine ultrastructural analysis. Using this method, Corral-Martínez et al. (2013) found evidence for the extensive formation of autophagosomes engulfing from small to large regions of cytoplasm, and the occurrence of massive autophagy prior to excretion of the partially digested cytoplasmic material to the apoplast. These were exclusive features of just induced microspores, not present in cells neither before nor long after the inductive stage. It seemed that in induced cells, the autophagosomes and the vacuolar system worked together as a cytoplasmic cleaning mechanism to adapt the cell to a new embryogenic scenario.

In this work, we also applied HPF/FS to study isolated microspore cultures of *B. napus*, in order to find out whether cytoplasmic organelles undergo similar autophagic processes. Indeed, the transformation of initially normal plastids into autophagic compartments has been previously described in plastids of other cell types, including senescing suspensor cells of *Phaseolus coccineus* (Nagl, 1977) and *Phaseolus vulgaris* (Gärtner and Nagl, 1980) and in petal cells of *Dendrobium* (van Doorn et al., 2011). For the present work, different stages of microspore embryogenesis (before and after the inductive treatment) were covered, including vacuolate microspores and pollen grains, both before induction, and induced and non-induced microspores, as well as microspore-derived embryos (MDEs), after the induction. We focused on the analysis of DNA-containing organelles such as plastids and mitochondria, and analyzed their ultrastructure and development during the process of embryogenesis induction and further MDE development. Our results demonstrate that many plastids (but not mitochondria) of embryogenic microspores undergo dramatic structural changes as a consequence of embryogenesis induction. We propose that these changes are related to autophagy and excretion of the engulfed material.

MATERIALS AND METHODS

PLANT MATERIAL

B. napus L. cv. Topas was used as the donor plants for isolated microspore culture. Donor plants were grown at 20°C under natural light in the greenhouses of the University of Colorado (Boulder, CO, USA) and the COMAV Institute (Universitat Politècnica de València, Valencia, Spain).

ISOLATED MICROSPORE CULTURE OF *BRASSICA NAPUS*

Flower buds from 3.3 to 3.4 mm were collected. The microspores were isolated in NLN-13 medium that consists on NLN medium (Nitsch and Nitsch, 1967) +13% sucrose. Isolated microspore cultures were performed as previously described (Corral-Martínez et al., 2013). Briefly, microspores were isolated and subjected to a heat stress treatment at 32°C for 24 h to induce embryogenesis, and then cultured in darkness at 25°C for progression of embryogenesis. Cultures were monitored on a daily basis under an inverted microscope. Dishes at different stages were collected and processed by HPF/FS as explained below.

PROCESSING OF SAMPLES FOR TEM

Three different types of *B. napus* samples were processed for TEM, including anthers containing microspores and pollen at different stages of microsporogenesis and microgametogenesis, 4-day-old cultured microspores, and MDEs at different developmental stages: globular, heart-shaped and torpedo embryos. All samples were randomly selected and three different sample batches were processed as previously described (Corral-Martínez et al., 2013; Seguí-Simarro, 2015a). Briefly, samples were fixed with a Baltec HPM 010 (Technotrade, Manchester, NH, USA) and a Leica EM HPM 100 (Leica Microsystems, Vienna, Austria) high pressure freezers. After HPF, samples were freeze substituted in a Leica AFS2 system. Substitution was performed with 2% OsO₄ in anhydrous acetone. Infiltration was carried out with increasing concentrations of Epon resin (Ted Pella, Redding, CA, USA) diluted in acetone, and polymerization was performed at 60°C for 2 days. A minimum of five resin blocks were randomly selected and sectioned for further analysis. A Leica UC6 ultramicrotome was used to obtain thin sections (~1 µm) and ultrathin (~80 nm) sections for observation at light microscopy and TEM, respectively. Ultrathin sections were mounted on formvar and carbon-coated, 200-mesh copper grids, stained with uranyl acetate and lead citrate, observed and photographed in a Philips CM10 TEM. A minimum of 100 electron micrographs were taken and analyzed at each of the stages studies. For the stage presenting atypical plastids, 242 electron micrographs were taken and studied. For the quantitative analysis of atypical plastids, 107 of these images, containing at least one plastid per image, were analyzed.

FESEM-FIB THREE-DIMENSIONAL RECONSTRUCTION OF SUBCELLULAR VOLUMES

The 3-D study of the plastids and mitochondria contained within the induced microspores was carried out with a FESEM-FIB (Auriga Compact, Zeiss) as described in Seguí-Simarro (2015b). This technique combines a Field Emission Scanning Electron Microscope (FESEM) and a Focused gallium Ion Beam (FIB) to sequentially mill the sample surface. Briefly, the sample-containing part of HPF/FS-processed resin blocks was smoothened with a diamond knife, separated, and placed into the specimen stage of the FESEM-FIB. The areas of interest (embryogenic microspores) were visualized using the secondary electron detector. Then, the sample was tilted to 52°. The ion beam was used to mill a window exposing the areas of interest. Then, the samples were milled with the FIB (operating at 1 nA)

removing 20 nm thick layers from the sample surface. Images were acquired every 40 nm. Two different stacks from two different areas of interest were acquired. The images were aligned with MIDAS and the reconstruction was made with 3 dmod, both included in the IMOD software package (Kremer et al., 1996).

IN SITU DETECTION OF ACID PHOSPHATASE ACTIVITY

The assay of acid phosphatase activity was performed over ultrathin sections according to Gärtner and Nagl (1980). The nature of this cytochemical reaction imposes the use of hydrated samples and temperatures above 0°C. For these reasons, this assay was performed on chemically fixed specimens, instead of using HPF for fixation. Three day-old *B. napus* microspore cultures were collected and fixed with 6.25% glutaraldehyde and 3% DMSO in 0.05 M cacodylate buffer (pH 7.4) at 4°C for 45 min. After three washes with 0.05 M cacodylate buffer, samples were incubated for 1 h at 37°C in a solution containing 10 ml 0.2 M Tris-maleate buffer (pH 5.2), 6 ml 0.02 M lead nitrate, 4 ml 0.1 M β-glycerophosphate disodium salt, and 30 ml distilled water. Control incubation was made replacing the substrate (β-glycerophosphate) by distilled water. After incubation, the samples were washed three times with 0.04 M Tris-maleate buffer (pH 5.2) at 4°C, 30 min each. Then, samples were postfixed with 1% OsO₄ in 0.05 M cacodylate buffer for 4 h, and washed three times with same buffer, 30 min each. Samples were dehydrated through increasing series of ethanol at 4°C, and then infiltrated with increasing concentrations of Spurr resin (Ted Pella, Redding, CA) in ethanol. Polymerization was carried out at 70°C for 24 h. Thin (~1 μm) and ultrathin (~80 nm) sections were obtained for light microscopy and TEM, respectively, using a Leica UC6 microtome. Ultrathin sections were mounted on formvar and carbon-coated, 200-mesh copper grids, stained with uranyl acetate and lead citrate, and finally observed and imaged in a Philips CM10 TEM. Thin (~1 μm) and ultrathin (~80 nm) sections were obtained for light microscopy and TEM, respectively, using a Leica UC6 microtome. Ultrathin sections were mounted on formvar and carbon-coated, 200-mesh copper grids, stained with uranyl acetate and lead citrate, and finally observed and imaged in a Philips CM10 TEM.

RESULTS

In this work, we performed *B. napus* isolated microspore cultures and analyzed the ultrastructure of DNA-containing organelles (plastids and mitochondria) during the different stages of the cultures, including before and after microspore isolation and induction. The first stage studied was the vacuolate microspore *in vivo*, still within the anther (Figure 1A). The identification and isolation of this particular developmental stage is essential for a successful induction, since it is known that this is the stage where the microspore is most sensitive to reprogramming treatments (Maraschin et al., 2005; Seguí-Simarro and Nuez, 2008; Dunwell, 2010). Just after the isolation, cultures exhibited mainly vacuolate microspores, as expected. Once induced, some microspores underwent multiple changes that transformed their morphology and architecture. As seen in Figure 1B, these microspores entered an embryogenic program defined by several cell divisions that generate embryo-like structures where the embryo proper and suspensor domains can be distinguished. In parallel, other microspores, not sensitive to induction, developed as pollen-like cells or just arrested in development (*mic* in Figure 1B). Dividing structures progressed as MDEs through the different

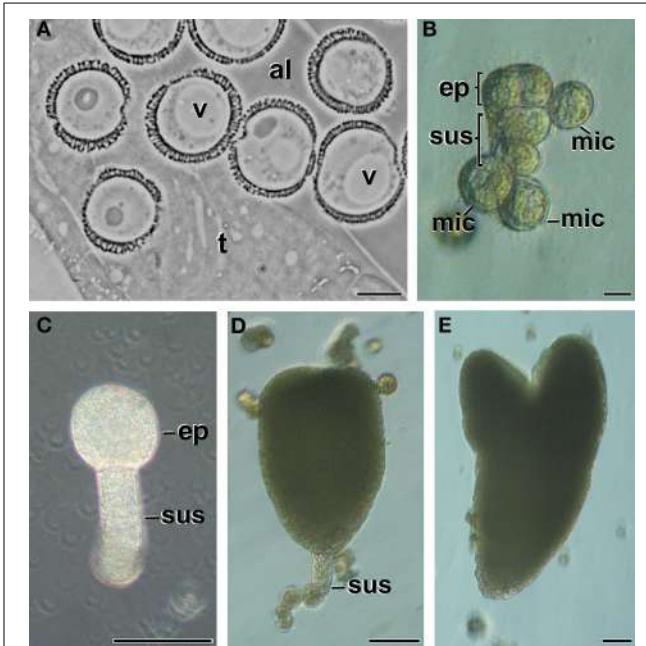


FIGURE 1 | Stages of *B. napus* microspore embryogenesis. (A) Vacuolate microspores during *in vivo* development within the anther. **(B)** Just induced, embryogenic microspore showing two clearly differentiated domains, the embryo proper domain (ep) and the suspensor (sus). Other microspores (mic) are not sensitive to induction and become arrested or enter a pollen-like development. **(C)** Globular MDE. **(D)** transitional MDE. **(E)** Torpedo MDE. al, anther locule; t, tapetum; v, vacuole. Bars: (A,B): 10 μm, (C–E): 50 μm.

stages of embryo development, including globular (Figure 1C), transitional (Figure 1D), heart-shaped and torpedo (Figure 1E) embryos, which showed an anatomy and external morphology remarkably similar to zygotic embryos (Seguí-Simarro and Nuez, 2008). Samples of all the stages shown in Figure 1 were collected at different culture stages, processed by HPF/FS, and observed under a TEM.

NON-INDUCED CELLS PRESENTED NORMAL DEVELOPMENT OF PLASTIDS

Vacuolate microspores, still within the anther or just after isolation (not yet induced; Figure 2A), presented only proplastids, still undifferentiated. Proplastids were typically round. Their stroma appeared less electron dense than in the rest of stages studied, and presented very few thylakoids. Pollen-like structures presented plastids clearly transformed into amyloplasts, as revealed by the large starch deposits present in most of the plastids (Figure 2B). Amyloplasts of pollen-like cells presented different sizes and shapes, rounded, and/or elongated, and accumulated one or more starch granules. These amyloplasts were remarkably similar to those found in *in vivo* pollen grains within the anther (Figure 2C). In summary, non-induced cells presented conventional proplastids and amyloplasts, similar to those previously described for equivalent stages in this and other species (Sangwan and Sangwan-Norreel, 1987; Zaki and Dickinson, 1990; Satpute et al., 2005).

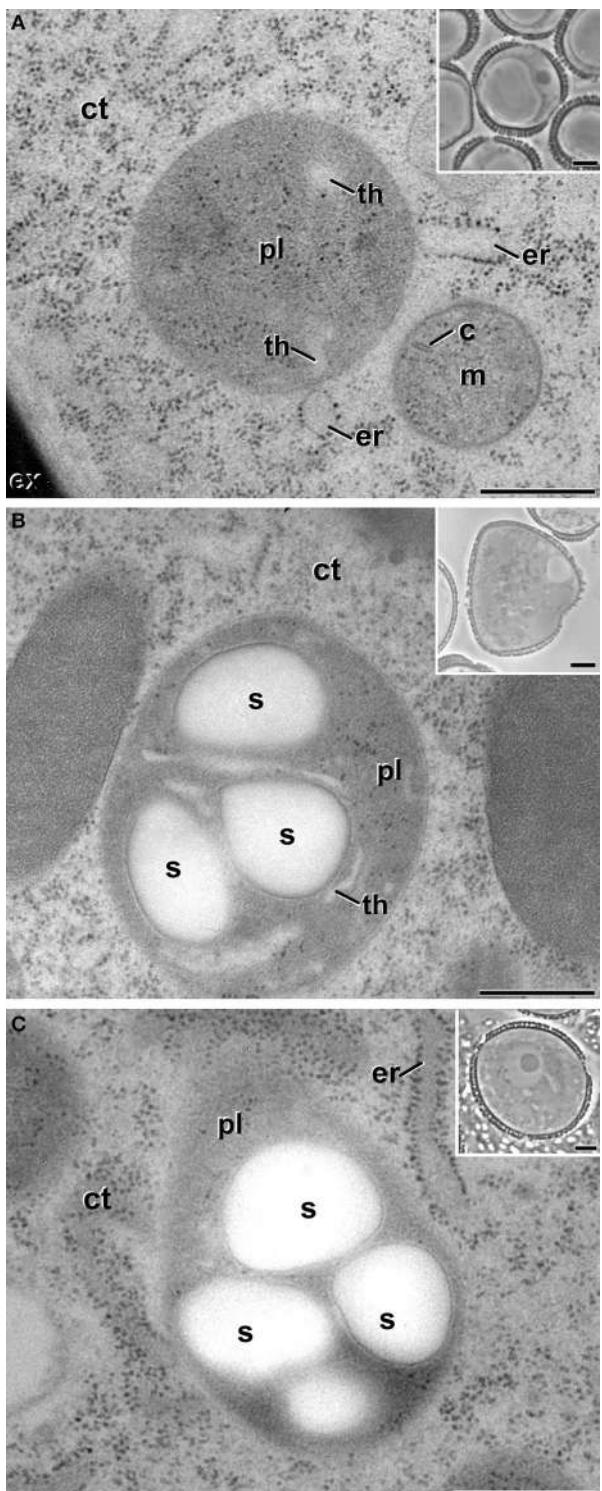


FIGURE 2 | Plastids (pl) of non-embryogenic *B. napus* cells. **(A)** Proplastid of a vacuolate microspore during *in vivo* development within the anther. **(B)** Amyloplast of an *in vitro* cultured, pollen-like structure. **(C)** Amyloplast of a pollen grain within the anther. Insets show light microscopy sections of the corresponding stages. **(C)**, mitochondrial crista; ct, cytoplasm; er, endoplasmic reticulum; ex, exine; m, mitochondria; s, starch; th, thylakoid. Bars: 500 nm, insets: 5 μ m.

EMBRYOGENIC MICROSPORES PRESENTED ATYPICAL PLASTIDS

After the inductive period, some microspores underwent multiple changes that transformed their morphology and ultrastructure. As seen in **Figure 1B**, these microspores entered an embryogenic program defined by several cell divisions that generated embryo-like structures. A qualitative (**Figure 3**) and quantitative (**Table 1**) study of plastids of these cells revealed that 60.7% of them presented conventional shapes, including round, elongated, bean-like, or sausage-like profiles (**Figure 3A**). In all of these plastids, the stroma appeared more electron dense than in vacuolate microspores. In parallel, electron light tubular and/or cisternal profiles appeared loosely arranged within the stromal matrix, indicating the onset of thylakoid formation. Only in very few examples, small starch granules could be observed in these plastids (s in **Figure 3A**).

However, many other plastids (39.3%) exhibited morphologies remarkably different from conventional (**Figures 3B–I**). The differences pertained principally to plastid shape and contents. As for plastid shape, the most striking difference was the presence of open plastid profiles surrounding cytoplasmic portions. Based on this observation, we identified two types of open plastid profiles. In the first type, we included elongated and curled dumbbell-shaped plastids (**Figures 3B,C**). Their extreme bending trapped portions of cytoplasm, as revealed by the presence of ribosomes and vesicles embedded in a matrix identical to that of the outer cytoplasm (**Figure 3B**). In the trapped cytoplasm, we never found cytoplasmic organelles such as plastids, mitochondria, Golgi stacks, or ER cisternae. In some cytoplasm-containing plastids, the connection between both cytoplasms was reduced to a thin channel or a pore of few nanometers (arrows in **Figures 3C,E**). In other plastids the channel was absent, suggesting that the membranes of opposite ends of the plastids were fused (**Figure 3F**), leaving a cytoplasmic portion isolated at the center of the closed plastid profile. The envelope of the internal cytoplasm-containing compartment was formed by a double membrane system identical to that found at the outer plastid envelope (insets in **Figures 3C,F,I**). Together, these observations suggested a dynamic process of plastid curling to engulf small regions of cytoplasm. The second type of profiles consisted of a relatively round plastid body engaged in the process of engulfing larger cytoplasmic areas (**Figures 3G,H**), or having them entirely engulfed (**Figure 3I**). In these cases, the large cytoplasmic area appeared in a lateral position within the deformed plastid, suggesting that the engulfment of large cytoplasmic areas imposes dramatic changes to the otherwise typical structure of the plastid. Overall, open plastid profiles suggesting engulfment of cytoplasm accounted for 15.2% of the atypical plastid profiles we identified.

Among all the atypical plastid profiles, the most frequent (68.5%) were those containing isolated cytoplasm portions. Most of these closed plastid profiles showed no structural abnormalities other than the engulfed cytoplasm. However, some of them presented more electron dense contents, indicating the onset of a change in the engulfed cytoplasm. Occasionally, plastids with an electron dense content (asterisk in **Figure 3G**) were engaged in a second round of cytoplasmic engulfment, suggesting that this might be a recurrent process. In addition, others showed one or more concentric membranous structures surrounding

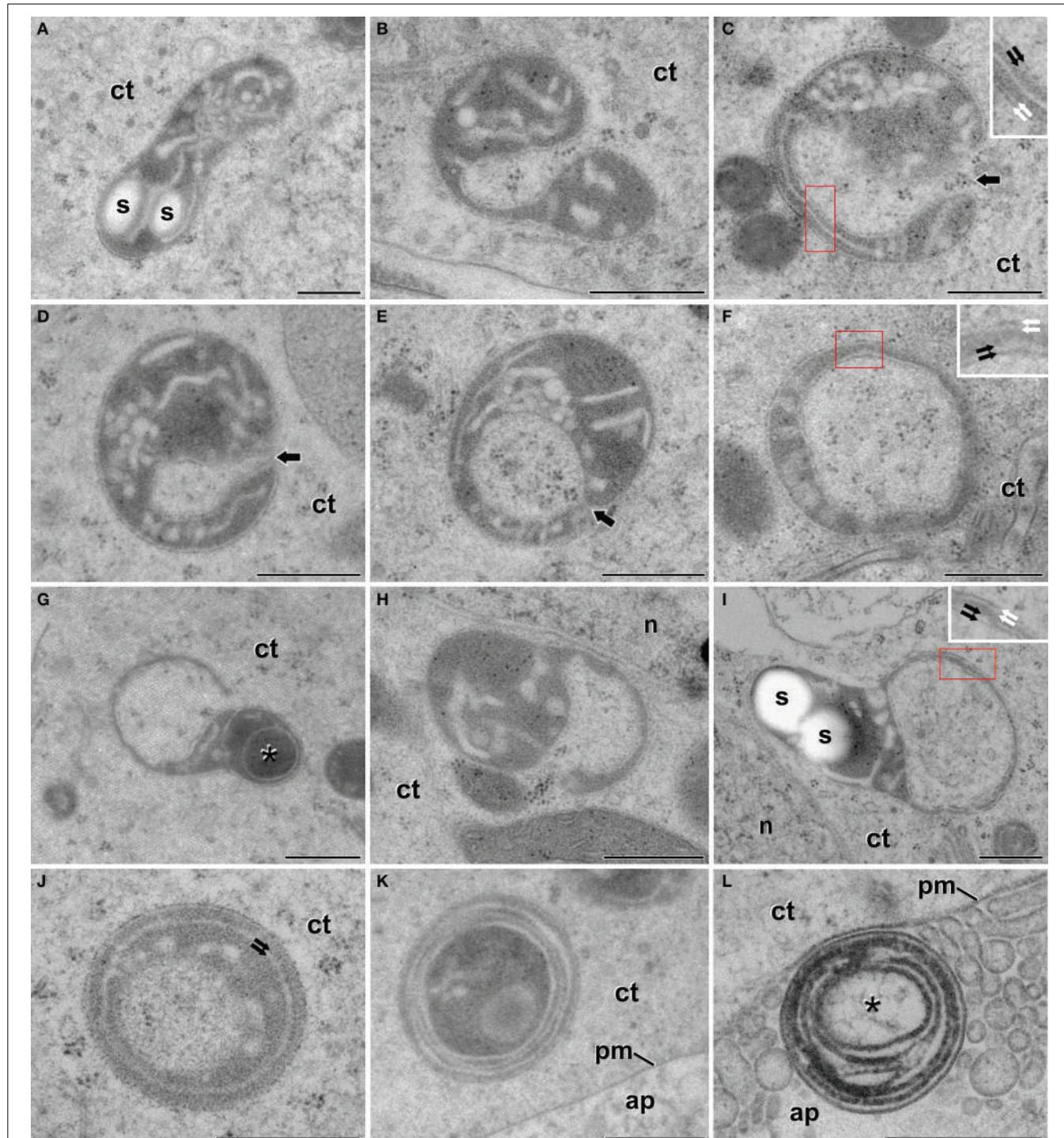


FIGURE 3 | Plastids of *B. napus* embryogenic microspores. (A) Shows a conventional, elongated plastid, with two small starch deposits (s). (B) Dumbbell-shaped plastid curled to engulf a small cytoplasmic region. (C,D) Plastids wrapped around a cytoplasmic region. Arrows indicate the thin cytoplasmic channel that still connects the engulfed cytoplasm. (E) Plastid engulfing a cytoplasmic region. Note that the connection between the trapped region and the cell cytoplasm is almost broken, but the point of closure is still evident (arrow). (F) Plastid showing an isolated cytoplasmic region. (G,H) Plastids engulfing large cytoplasmic regions. The asterisk in (G) indicates an electron dense cytoplasmic compartment. (I) Deformed plastid with a large cytoplasmic region entirely engulfed. (J) Plastid where the internal compartment contains

fibrillar material, different from the surrounding cytoplasm. Note the presence of two concentric membranes below the plastid envelope (arrows). (K) Plastid showing numerous concentric membranes and a dark and disorganized contents, both in and out of the internal compartment. Note the proximity to the apoplast (ap). (L) Multilamellar body at the apoplast (ap), surrounded by excreted cellular debris. The asterisk indicates an internal compartment with traces of fibrillar material. The red boxes in (C,F,I) correspond to the area enlarged in the corresponding inset, where a double membrane system can be seen at both the outer plastid envelope (white arrows) and the inner envelope of the engulfed cytoplasm (black arrows). ct, cytoplasm; n, nucleus; pm, plasma membrane. Bars: 500 nm.

the cytoplasmic compartment (**Figure 3J**). Most of the plastids with several concentric membranes presented dark and disorganized contents as well (**Figure 3K**). Interestingly, multilamellar bodies of a size similar to the plastids with concentric membranes were also found in the cytoplasm, close to the plasma membrane (**Figure 3K**), and in the apoplast (**Figure 3L**), together with cellular debris excreted as a consequence of embryogenesis

Table 1 | Quantitative analysis of plastids of embryogenic microspores.

	Number	Percentage (from total)	Percentage (from atypical)
Conventional	142	60.7%	
Atypical	92	39.3%	
Engulfing (open profiles)	14	6.0%	15.2%
Engulfed (closed profiles)	63	26.9%	68.5%
Concentric membranes/disorganized contents/multilamellar	15	6.4%	16.3%
Total	234	100%	100%

induction (Corral-Martínez et al., 2013). These multilamellar bodies presented an internal compartment with fibrillar material, similar to that present in lytic compartments. Closed plastid profiles with concentric membranes, dark, fibrillar, and disorganized contents, together with cytoplasmic and apoplastic multilamellar bodies, accounted for 16.3% of the atypical profiles observed. Altogether, these plastid profiles suggested the occurrence of plastid degradation and excretion out of the cell.

3-D RECONSTRUCTION OF SUBCELLULAR VOLUMES OF EMBRYOGENIC MICROSPORES

Theoretically, it might be possible that the atypical plastid profiles observed in TEM micrographs of embryogenic microspores correspond to polar sections of cup-shaped plastids. Alternatively, these plastid profiles might correspond to equatorial sections of ring-shaped plastids. In other words, the atypical plastid profiles we observed might be artifactual, and might not engulf cytoplasm actually. In order to rule out this possibility, and to figure out the actual 3-D structure of these plastids, we performed FESEM-FIB-based 3-D reconstructions and models of large cytoplasmic areas of embryogenic microspores (**Figure 4A**; **Supplementary Movie S1**). These models confirmed the presence of three morphologically different plastid types

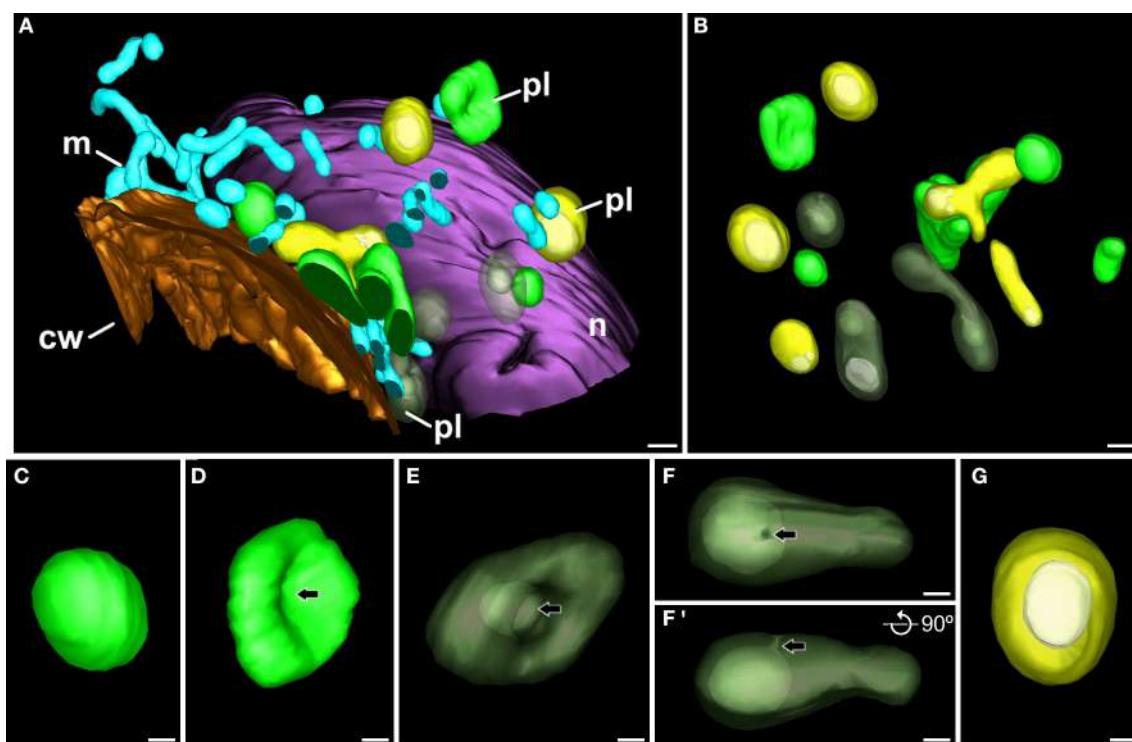


FIGURE 4 | 3-D model of a subcellular volume of a *B. napus* embryogenic microspore. (A) Modeled subcellular volume. **(B)** Model excluding all the cell structures but the plastids (pl). The different plastid types are modeled in different colors: conventional (light green), open profiles engulfing cytoplasm (dark green), and closed profiles (yellow) with the engulfed cytoplasm (white). **(C)** Conventional, round-shaped plastid. **(D)** Disc-shaped plastid with a slight central depression (arrow). **(E)** Plastid starting to engulf cytoplasm. The arrow points to a deep depression that

creates a cytoplasmic pocket within the plastid. **(F,F')** Cytoplasm-containing plastid where the internal cytoplasm is connected with the outer cytoplasm just by a narrow channel that ends in a small pore at the plastid surface. **(F')** is a 90° turn of this plastid, for a clear visualization of the narrow channel. Arrows point to the pore in **(F)** and to the narrow channel in **(F')**. **(G)** Round plastid (yellow) with the cytoplasmic contents (white) entirely isolated from the outer cytoplasm. cw, cell wall; m, mitochondrion; n, nucleus. Bars: **(A,B)**: 500 nm; **(C-G)**: 200 nm.

(Figure 4B), as previously observed in TEM micrographs. We modeled each plastid type in different colors. Plastids with conventional morphologies (oval, round, or elongated, not engulfing cytoplasm) were modeled in light green (Figures 4B–D). Some of them were round or oval (Figure 4C), and others exhibited a disc-like morphology with a slight central depression (arrow in Figure 4D; yellow arrow in Supplementary Movie S2), suggesting the onset of a process of membrane invagination. Plastids engulfing cytoplasm were modeled in dark green (Figures 4B,E–F'). These plastids presented different sizes and shapes. Some of them, similar in shape to the disc-shaped conventional plastid of Figure 4D, showed a profound invagination of their envelope, generating a cytoplasm-containing open pocket (arrow in Figure 4E, central plastid in Supplementary Movie S2). Other plastids were oval or elongated, and engulfed large portions of cytoplasm. In these plastids, the cytoplasm outside and inside the plastid was connected by a narrow channel of a few nanometers, leaving a pore at the surface of the plastid (arrows in Figures 4F,F'; Supplementary Movie S3 and red arrow in Supplementary Movie S2). The third plastid type, modeled in yellow (Figures 4B,G) showed an oval or elongated shape, and included one or more cytoplasm portions (modeled in white) fully isolated from the outer cytoplasm, as evidenced by the absence of connecting channels (Figure 4G, Supplementary Movies S4, S5). Together, the different plastids observed in 3-D models (Figures 4C–G) confirmed our observations from 2-D TEM micrographs, and indicated the occurrence of a mechanism whereby some plastids surround and engulf discrete cytoplasm regions, isolating them from the outer cytoplasm.

We also observed that cytoplasm engulfment was associated to a change in plastid morphology. In conventional plastids and in atypical plastids with open profiles (inner and outer cytoplasm still connected), elongated morphologies were more frequent than round morphologies, accounting for 57% of the conventional plastids and 71% of atypical plastids with open profiles. In the second case, the cytoplasmic volume was considerably smaller than the total plastid volume. In contrast, most (77%) of the atypical plastids with closed profiles (internal cytoplasm entirely isolated from the outer cytoplasm), showed round morphologies, and their cytoplasmic content appeared to occupy a larger fraction of the plastid volume (Figure 4G). These observations suggested that cytoplasm engulfment induced a change not only in plastid shape, but also in internal architecture, reducing the stromal volume compared to that of the engulfed cytoplasm.

In addition to conventional and atypical plastids, we also modeled the multilamellar bodies excreted to the apoplast (Figure 5). Figure 5A shows a model of an apoplast region and Figure 5B shows one of the several FESEM-FIB micrographs used to reconstruct it. This region corresponds to a swollen apoplast area with numerous membranous bodies embedded in a matrix of dense material (Corral-Martínez et al., 2013). Close to this area but separate from it, a multilamellar body (inset in Figure 5A, modeled in red; Supplementary Movie S6) similar to those observed in TEM images (Figures 3L, 5B) is observed. The multilamellar body is similar in size and shape to the atypical plastids and multilamellar bodies observed in the cytoplasm. The 3-D model showed that nearly half of the body is facing the apoplast,

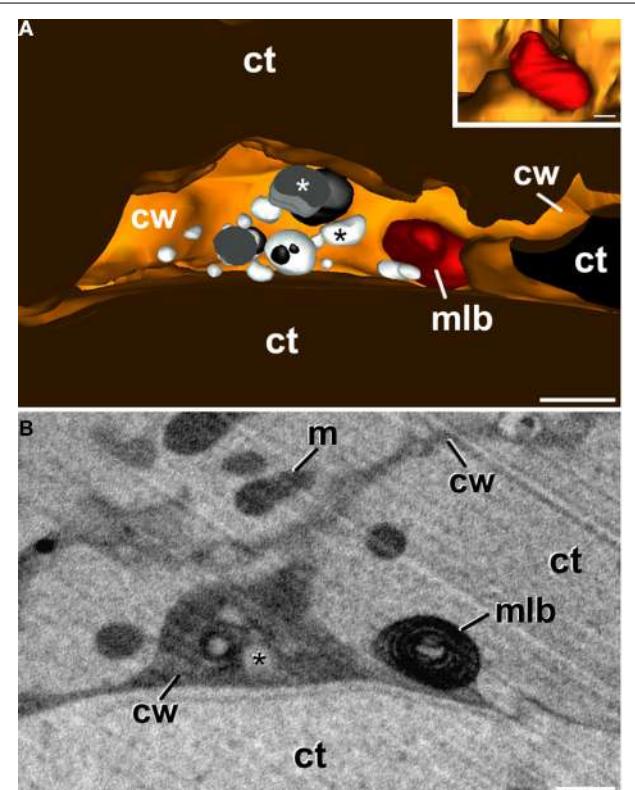


FIGURE 5 | 3-D reconstruction of a region from a newly-formed cell wall of a *B. napus* embryogenic microspore. (A) is a 3-D model showing the apoplast with several vesicular bodies of different electron density (white and black asterisks), and an excreted plastid, degraded to become a multilamellar body (mlb). The inset shows the plastid from a different perspective. Note that part of the plastid is tightly surrounded by the plasma membrane whereas the other part is facing the apoplast. **(B)** One of the FESEM-FIB micrographs used for the 3-D reconstruction showing the multilamellar body separated from the excreted cytoplasmic vesicular bodies (asterisk), embedded in a matrix of dense material. ct, cytoplasm; cw, cell wall; m, mitochondrion. Bars: 500 nm.

while the other half is still tightly wrapped by the plasma membrane, which clearly delineates the shape of the body (inset in Figure 5A). These observations are suggestive of a process of excretion of the multilamellar body, likely mediated by the fusion of its outer membrane with the plasma membrane, and independent of the excretion of the membranous and dense material.

CYTOPLASM-CONTAINING PLASTIDS SHOWED ACID PHOSPHATASE ACTIVITY

As seen in Figures 3G,J–L, some of the cytoplasm-containing plastids showed signs of degradation of their cytoplasmic contents and of the entire plastid. In order to elucidate a putative lytic activity in these organelles, similar to that found in autophagosomes of the cytoplasm of embryogenic microspores (Corral-Martínez et al., 2013), we performed an *in situ* acid phosphatase cytochemical assay (Figure 6). In embryogenic microspores we observed cytoplasm-containing plastids with different amounts of electron dense precipitates, indicative of different levels of acid phosphatase activity. Figure 6A shows a plastid containing

cytoplasm similar to that found out the plastid, together with few small precipitates distributed throughout the plastid, indicating a mild lytic activity. As a reference, this figure also includes a lytic cytoplasmic vacuole with an electron light lumen and numerous precipitates, indicating a more intense lytic activity. **Figure 6B** shows a plastid where most of the precipitates concentrate in the engulfed cytoplasm, suggesting that lytic activity is first initiated in the cytoplasmic cargo. **Figure 6C** shows a plastid where the cytoplasmic content seems already digested, as revealed by the electron translucent internal compartment, similar to that of lytic vacuoles (**Figure 6A**). This plastid exhibits electron dense precipitates dispersed throughout the stroma. Together, **Figures 6A–C** are suggestive of a process whereby cytoplasm-containing plastids first digest their cytoplasm, and then enter an auto-lytic process conduced to the entire degradation of the plastid. In contrast, pollen-like structures present in the same sections and therefore exposed to the same cytochemical assay did not show any precipitate, neither in their amyloplasts nor in their cytoplasmic

vacuoles (**Figure 6D**). Controls excluding β -glycerophosphate did not show any comparable precipitate in any of the studied cell types (**Figure 6E**).

MDEs PRESENTED CONVENTIONAL PLASTIDS

Embryogenic structures progressed as MDEs through the different stages of embryo development, including globular (**Figure 1C**), transitional (**Figure 1D**), heart-shaped, and torpedo (**Figure 1E**) MDEs. Plastids from cells of these MDE stages were also analyzed in order to check whether the unusual plastid profiles found in induced microspores persisted during further MDE development, or they were transient structures, exclusive of the first stages of MDE induction. As seen in **Figure 7**, the plastids found in the embryo proper domain (**Figure 7A**) and in the suspensor (**Figure 7B**) of globular MDEs were similar to those found in pollen grains (**Figure 2C**). All of them exhibited a round or oval shape, dense stroma, tubular, and/or lamellar thylakoids, and starch granules. No engulfed cytoplasmic regions were observed

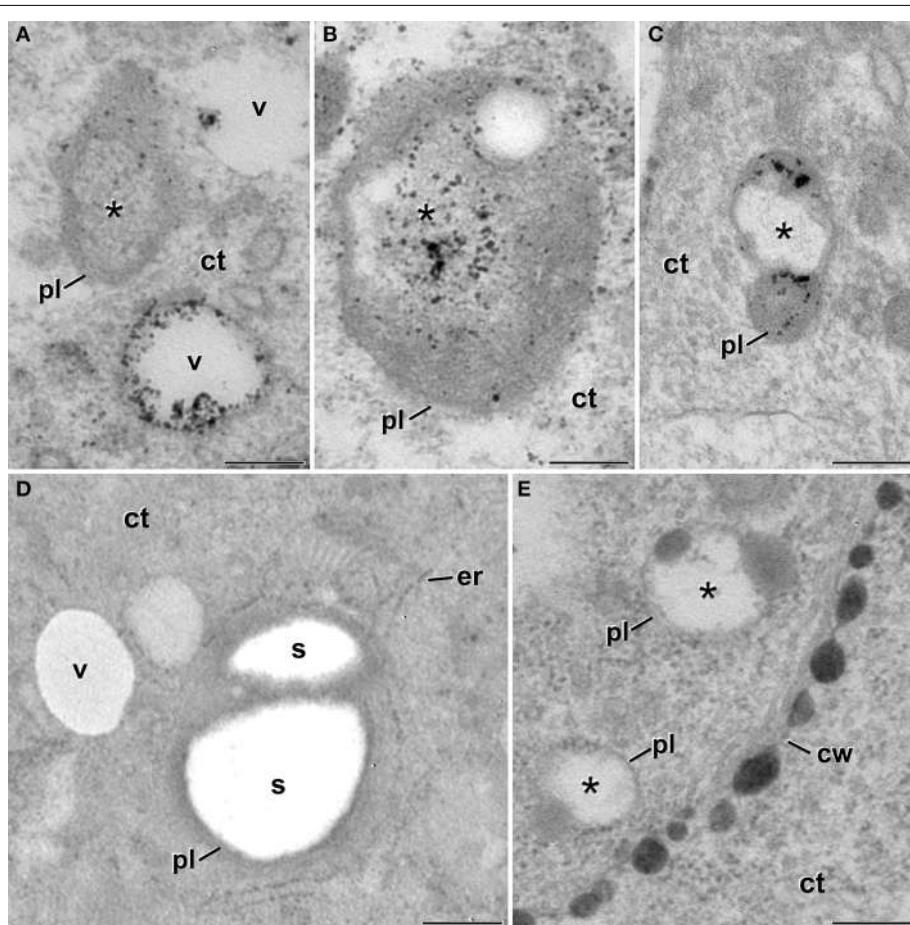


FIGURE 6 | Detection of acid phosphatase activity in cytoplasm-containing plastids of *B. napus* embryogenic microspores. **(A)** Cytoplasm-containing plastid (pl) showing a discrete acid phosphatase activity as revealed by the small and scarce dense precipitates. The cytoplasm compartment (asterisk) is devoid of precipitates. As a reference, two adjacent vacuoles (v) show different amounts of precipitates. **(B)** Cytoplasm-containing plastid where most of the dense precipitates

concentrate within the cytoplasm compartment (asterisk). **(C)** Plastid where all the cytoplasmic contents seems digested, and the dense precipitates (lytic activity) concentrate over the plastidial stroma. **(D)** Vacuoles and amyloplasts of a non-embryogenic, pollen-like structure. Note the total absence of dense precipitates. **(E)** Cytoplasm-containing plastids of a control sample without β -glycerophosphate. Note the total absence of dense precipitates. ct, cytoplasm; cw, cell wall; er, endoplasmic reticulum; s, starch. Bars: 250 nm.

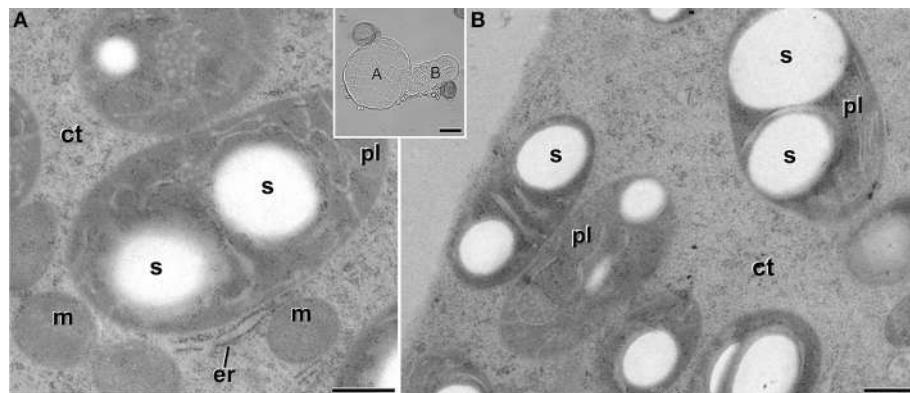


FIGURE 7 | Starch-containing plastids (pl) from *B. napus* globular MDEs.

The inset shows a light microscopy section of a globular MDE where the embryo proper (A) and suspensor (B) domains can be clearly

differentiated. Panels (A,B) show electron micrographs of plastids of embryo proper (A) and suspensor (B) cells. ct, cytoplasm; er, endoplasmic reticulum; m, mitochondria; s, starch. Bars: (A,B): 500 nm. Inset: 20 μm.

in any case. Cells of heart-shaped, transitional, and torpedo MDEs (data not shown) presented only conventional starch and thylakoid-containing plastids, structurally equivalent to those described for globular MDEs. Thus, it seemed that the unusual features observed in plastids of embryogenic microspores did not persist during MDE development. Instead, plastids adopted a conventional architecture, characterized by the presence of starch and thylakoids.

MITOCHONDRIA PRESENT A CONVENTIONAL ARCHITECTURE DURING ALL THE STAGES OF MICROSPORE EMBRYOGENESIS

In order to check whether the atypical features found in plastids of embryogenic microspores were extensive to other subcellular organelles, we also analyzed the ultrastructure of mitochondria in all the culture stages processed. Mitochondria of vacuolated microspores and pollen grains within the anther (Figures 8A,B) presented a conventional oval or sausage-like morphology, and a mitochondrial matrix where cristae can be easily identified as emerging from the inner mitochondrial membrane. This description also applied to microspores of pollen-like structures developing *in vitro* (data not shown), as well as of just induced, embryogenic microspores (Figure 8C) and MDEs (Figure 8D). Therefore, in contrast to the changes observed in plastids of embryogenic microspores, the mitochondria of embryogenic microspores presented a morphology and architecture equivalent to those of cells of any other culture stage, as well as of non-cultured *B. napus* cells.

DISCUSSION

THE ANDROGENIC SWITCH PRODUCES ATYPICAL PLASTIDS

We showed in this work that induction of embryogenesis produces dramatic changes in proplastids of embryogenic microspores, diverting them from their original fate (pollen amyloplasts), and transforming them into different, unique structures. It could be argued that this change in plastid architecture and function could be a side consequence of the stress treatment applied and the *in vitro* culture environment, since it is known that *in vitro* culture may alter the normal structure and function of plant cells. However, it must be noted that *in vitro*

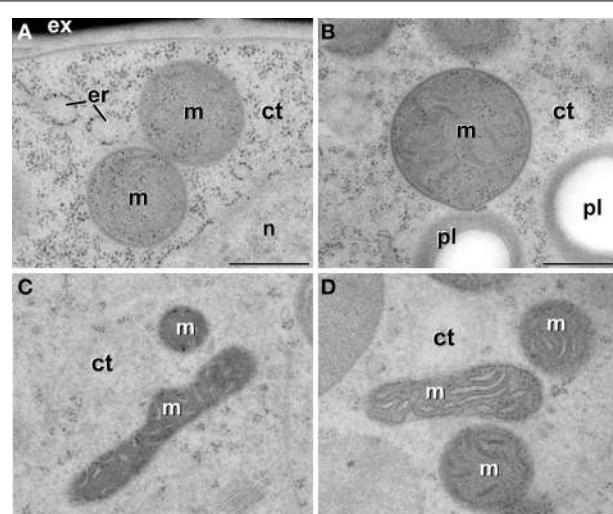


FIGURE 8 | Mitochondria (m) of *B. napus* vacuolate microspores during *in vivo* development within the anther (A), pollen grains within the anther (B), embryogenic microspores (C), and globular MDEs (D). ct, cytoplasm; er, endoplasmic reticulum; ex, exine; pl, plastid; n, nucleus. Bars: 500 nm.

microspore cultures produce not only embryogenic microspores, but also pollen-like structures, also submitted to same stressing conditions, but containing only amyloplasts, and not cytoplasm-containing plastids. In addition, *in vitro* developed MDEs showed only conventional plastids, indicating that the plastidial changes are a transient phenomenon. Furthermore, other DNA-content organelles such as mitochondria, present in the same cells that developed atypical plastids, did not undergo any change in response to the *in vitro* culture. Therefore, it seems clear that the occurrence of cytoplasm-containing plastids is not related to the *in vitro* culture conditions, including the heat shock treatment used to induce microspore embryogenesis. Instead, we postulate that the occurrence of this unusual plastid type is inherent to the androgenic switch. The biological significance and role in the context of microspore embryogenesis is discussed next.

CYTOPLASM-CONTAINING PLASTIDS OF EMBRYOGENIC MICROSPORES ARE ENGAGED IN AUTOPHAGY

A plastidial architecture similar to that described hereby for *B. napus* embryogenic microspores has been rarely reported in the literature. Similar observations have only been reported in plastids of suspensor cells of *P. coccineus* (Nagl, 1977) and *P. vulgaris* (Gärtner and Nagl, 1980), where plastids transformed into autophagic vacuoles during the senescence of the suspensor, and in petal cells of *Dendrobium*, where it was shown that plastids adopt autophagic functions, engulfing, and digesting portions of the cytoplasm (van Doorn et al., 2011). As seen, all these reports established a clear link between these plastid transformations and their engagement in autophagy. Our TEM images and 3-D reconstructions demonstrated that a significant percentage of the plastids of embryogenic microspores engulf and isolate entire organelle-free cytoplasmic portions, creating an independent intraplastidial compartment. Structural changes such as the reduction in the stromal volume and the number of thylakoids, suggests the onset of a new role for the cytoplasm-containing plastids. As seen in **Figure 3**, the compartmentalized cytoplasm is surrounded by a double membrane system structurally identical to the plastid envelope. This suggests that the intraplastidial compartment is originated from the plastidial double membrane. The 3-D models confirmed the physical continuity between the plastid envelope and the cytoplasmic pockets or internal cytoplasmic compartments. Such double membrane-bound compartments are remarkably similar to the widely described plant autophagosomes (Aubert et al., 1996; Otegui et al., 2005; Lundgren Rose et al., 2006; Reyes et al., 2011). The abundance of C-shaped and dumbbell-shaped plastid profiles found in embryogenic cells, together with the 3-D plastid models, suggests that the intraplastidial compartment is formed by curling and protrusion of a disc-shaped plastid, which wraps around a cytoplasm portion and eventually fuse their opposite ends to engulf it. Mechanistically, this process is also resembling the process of autophagosome formation in plant cells (Li and Vierstra, 2012), and specifically in *B. napus* embryogenic microspores, as a consequence of embryogenesis induction (Corral-Martínez et al., 2013). Furthermore, the acid phosphatase activity demonstrated not only in the internalized cytoplasm but also in the stroma, confirmed the lytic activity of these organelles. Based on all these evidences, it is reasonable to assume that we are observing plastids acting as autophagosomes and developing internal autophagic compartments that eventually lead to the digestion of the entire plastid. According to the term coined by Nagl (1977), they would be *plastolysomes*.

PLASTOLYSOMES OF EMBRYOGENIC MICROSPORES ARE EXCRETED TO THE APOPLAST

In *Dendrobium* and *Phaseolus*, the lytic plastidial compartments were filled with material of different levels of electron density, from dark to very light (Nagl, 1977; Gärtner and Nagl, 1980; van Doorn et al., 2011). This, together with the demonstration of acid phosphatase activity, led the authors to propose that the engulfed cytoplasm was digested and recycled. In embryogenic microspores, despite that many of the atypical plastids presented few or no structural evidences of digestion, evidences

of acid phosphatase activity was detected in many of them (**Figures 6A,B**). Furthermore, full plastid degradation, similar to those of *Dendrobium* and *Phaseolus* cells, was found in ~16% of the atypical plastids, and multilamellar bodies, similar in size to plastids, were found in the cytoplasm and also out of it, in the apoplast. **Figure 5** illustrates one of these bodies being excreted to the apoplast in a way that indicates that the body has been excreted independently, and not as part of a massive excretion of cytoplasmic material. All these observations made us think that the fate of cytoplasm-containing plastids would not only be their degradation, but also their excretion out of the cell. We propose that cytoplasm engulfment affects not only the structure of the plastid, but also its fate and subsequently, its function. The cytoplasm engulfment, the lytic activity, and the loss of stroma and grana may be related to a functional shift of the plastid, transforming itself into a vehicle for cytoplasm and plastid elimination through an orchestrated sequence of steps that involves cytoplasm engulfment, cytoplasm and plastid digestion, and finally excretion of the entire structure (**Figure 9**).

Recently, similar processes of autophagy associated to excretion of the digested material were demonstrated to occur in embryogenic microspores of *B. napus* (Corral-Martínez et al., 2013). It was proposed that these combined processes would be acting as a cleaning mechanism for massive removal of useless cytoplasmic material. However, this study showed few evidences of the usefulness of this process to eliminate plastids. In other words, autophagosome formation and excretion would not

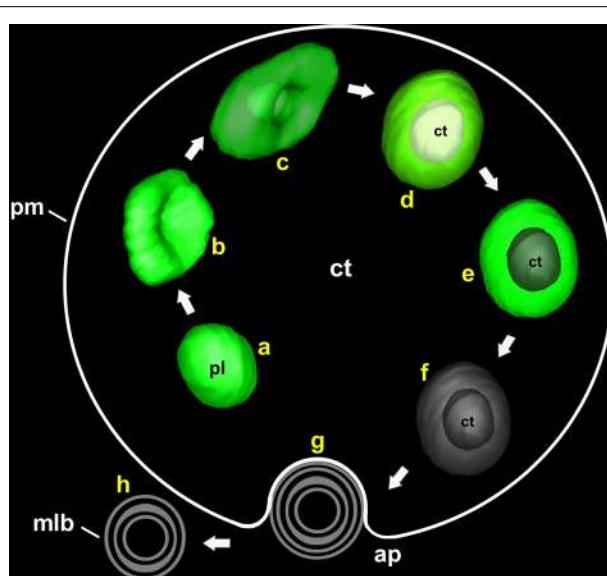


FIGURE 9 | Model of the different steps involved in the process of plastolysome formation and excretion to the apoplast. First, an initially conventional plastid (a) creates an invagination at its envelope (b). The invagination enters into the plastid until it wraps the cytoplasmic portion (c). Then, the plastid envelope closes around the cytoplasmic portion, isolating it from the outer cytoplasm (d). Once the cytoplasm is internalized, digestion of the cytoplasm (e) and then of the entire plastid (f) takes place. Finally, the remnants are excreted to the apoplast (g,h) in the form of a multilamellar body. ap, apoplast; ct, cytoplasm; mlb, multilamellar body; pl, plastid; pm, plasma membrane.

account for the reduction in the number of plastids previously described as associated to embryogenesis induction (reviewed in ShariatiPanahi et al., 2006; Makowska and Oleszczuk, 2014). Therefore, it seems reasonable to speculate that the processes described by Corral-Martínez et al. (2013) and hereby (formation and excretion of autophagosomes and plastolysomes) could be related as parallel parts of a *cleaning program* necessary to adapt the embryogenic microspore to its new developmental scenario (Seguí-Simarro and Nuez, 2008). In this context, plastolysome-mediated autophagy and excretion would be responsible for the elimination of small cytoplasm portions and, most importantly, ~40% of the plastids, according to our quantification of the plastids that enter this pathway. The cellular mechanisms, still unknown, that mediate the excretion of entire autophagosomes, might also govern the excretion of plastolysomes, even before their contents have been entirely digested and recycled. Further research should focus on elucidating such putative mechanisms and the molecular link between these two parallel ways of cytoplasmic cleaning.

IS THERE A POSSIBLE LINK BETWEEN PLASTOLYSOME FORMATION AND ANDROGENIC RECALCITRANCE?

In general, angiosperm microspores have only proplastids, which transform into amyloplasts in pollen grains (Clément and Pacini, 2001). This general rule, however, has some exceptions. In 1987, Sangwan and Sangwan-Norreel studied the relationship between the androgenic response of several species and the plastid types present in their microspores and pollen grains. They observed that responsive species present proplastids up to the bicellular pollen stage (the latter inducible stage). In contrast, in recalcitrant species proplastids differentiate to amyloplasts at the microspore stage or even before. Indeed, the differentiation of proplastids into amyloplasts has been related to the lack of embryogenic response of late pollen stages (Maraschin et al., 2005) and of recalcitrant, non-responsive species (Sangwan and Sangwan-Norreel, 1987). It is known that inhibition of starch biosynthesis blocks pollen development (Datta et al., 2002), whereas the presence of starch-free proplastids is needed for a microspore to be sensitive to induction (Nitsch and Nitsch, 1969; Sangwan and Sangwan-Norreel, 1987). Thus, it appears that starch biosynthesis in amyloplasts is a marker of irreversible microspore commitment toward gametogenesis (Clément and Pacini, 2001). In maize, a protein analysis during microspore development revealed that as soon as starch biosynthesis initiates in the plastids of microspores (not yet pollen), there is a drastic decrease in the synthesis of a number of polypeptides whereas other, new ones, are being synthesized *de novo* (Mandaron et al., 1990). These authors related this starch-mediated change in the pattern of protein synthesis with the androgenic competence.

Our results would add a new piece to this puzzle, explaining why amyloplasts-containing microspores cannot undergo embryogenesis: an amyloplast would not be able to transform into a plastolysome by the time when the stress treatment is applied. Therefore, amyloplast-containing cells would not be able to execute the cytoplasmic cleaning process as extensively as those having proplastids, not yet differentiated. Conversely, it could be speculated, according to the hypothesis of Mandaron et al. (1990),

that the activation of the genes for starch biosynthesis might eliminate proteins necessary for embryogenesis induction and/or produce inhibitors of embryogenesis that make microspores lose their embryogenic potential. In this context, the process we hereby describe would greatly contribute to reduce the impact of starch biosynthesis in the blockage of embryogenesis by targeting plastids to elimination through their engagement in autophagy. Obviously, these all are speculations that should be confirmed in further studies. We are currently examining HPF/FS-processed embryogenic microspores from other species, including recalcitrant crops, in order to validate the link found hereby between plastid elimination and embryogenic competence, and to verify to what extent the formation of plastolysomes is a common feature of microspore embryogenesis.

AUTHOR CONTRIBUTIONS

JMSS designed the research. VPV, ARS, and JMSS obtained and processed the samples. VPV and PCM performed the experiments. VPV and JMSS analyzed the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00094/abstract>

Supplementary Movie S1 | One of the models produced from 3-D reconstructions showing all of the subcellular structures present in the reconstructed volume. The nucleus is modeled in purple, the newly formed cell plate in dark yellow, the exine in red, the mitochondria in light blue, the vacuoles in white, and the different plastid types in different colors: conventional in light green, open profiles engulfing cytoplasm in dark green and closed profiles in yellow with the engulfed cytoplasm in white.

Supplementary Movie S2 | A series of consecutive FESEM-FIB slices covering three entire plastids at different stages in the process of cytoplasm engulfment. The disc-shaped plastid at the lower left quadrant is slightly bent, beginning to form a cytoplasmic pocket (yellow arrow) at one side. The central, large and elongated plastid is forming a deep cytoplasmic pocket around the cytoplasm being engulfed. The round plastid at the upper right quadrant engulfs a cytoplasm portion almost entirely, leaving a small pore (red arrow) that connects the inner and outer cytoplasm.

Supplementary Movie S3 | 3-D model of cytoplasm-containing plastids where the cytoplasm outside and inside the plastid is not totally isolated, but still connected by narrow channels, leaving a pore at the surface of the plastids.

Supplementary Movie S4 | 3-D model of cytoplasm-containing plastids where the engulfed cytoplasm is entirely isolated from the outer cytoplasm.

Supplementary Movie S5 | A series of consecutive FESEM-FIB slices covering an entire plastid containing a cytoplasmic portion totally isolated from the outer cytoplasm.

Supplementary Movie S6 | A series of consecutive FESEM-FIB slices covering a multilamellar body (encircled by a red line) being excreted to the apoplast. Note that approximately half of the body is still tightly wrapped by the plasma membrane, and the other half is exposed to the apoplast.

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Induction of Embryogenesis in *Brassica Napus* Microspores Produces a Callosic Subintinal Layer and Abnormal Cell Walls with Altered Levels of Callose and Cellulose

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The induction of microspore embryogenesis produces dramatic changes in different aspects of the cell physiology and structure. Changes at the cell wall level are among the most intriguing and poorly understood. In this work, we used high pressure freezing and freeze substitution, immunolocalization, confocal, and electron microscopy to analyze the structure and composition of the first cell walls formed during conventional *Brassica napus* microspore embryogenesis, and in cultures treated to alter the intracellular Ca^{2+} levels. Our results revealed that one of the first signs of embryogenic commitment is the formation of a callose-rich, cellulose-deficient layer beneath the intine (the subintinal layer), and of irregular, incomplete cell walls. In these events, Ca^{2+} may have a role. We propose that abnormal cell walls are due to a massive callose synthesis and deposition of excreted cytoplasmic material, and the parallel inhibition of cellulose synthesis. These features were absent in pollen-like structures and in microspore-derived embryos, few days after the end of the heat shock, where abnormal cell walls were no longer produced. Together, our results provide an explanation to a series of relevant aspects of microspore embryogenesis including the role of Ca^{2+} and the occurrence of abnormal cell walls. In addition, our discovery may be the explanation to why nuclear fusions take place during microspore embryogenesis.

Keywords: androgenesis, benzyl alcohol, caffeine, doubled haploids, microspore embryogenesis, rapeseed, cell wall carbohydrates

INTRODUCTION

Microspore embryogenesis is a fascinating experimental process whereby a haploid microspore is reprogrammed to become a haploid or doubled haploid (DH) embryo (Seguí-Simarro and Nuez, 2008a). This inducible pathway has a great biotechnological potential, since among many other advantages, it allows for faster and cheaper ways to obtain pure lines for hybrid seed production (Forster et al., 2007). In addition, it offers the possibility to be used as an *in vitro* model to study different basic and induced processes. Indeed, the androgenic switch is induced by the application of different types of abiotic stresses, including heat shock, cold, and starvation, among others (Shariatpanahi et al., 2006). Once induced, the cellular responses to abiotic stresses coexist with

a developmental switch toward embryogenesis, and with the cessation of the old gametophytic program (Malik et al., 2007; Seguí-Simarro and Nuez, 2008a). Conceivably, all these changes must imply a profound remodeling at the genetic and molecular levels, and also in cell architecture. Among all the changes undergone by the embryogenic microspore, one of the aspects that attracted the attention of the first cell biologists that studied this process was how induced cells are divided (Zaki and Dickinson, 1991; Simmonds and Keller, 1999).

In somatic-type plant cells, the first structural marker of cell division is the microtubular pre-prophase band (PPB), which defines the future division plane (Pickett-Heaps and Northcote, 1966). By late anaphase phragmoplast initials are formed, and at early telophase, a tubulo-vesicular network (TVN) cell plate is assembled in the middle of a solid phragmoplast (Seguí-Simarro et al., 2004; Austin et al., 2005). At mid telophase, a ring-shaped transitional phragmoplast marks the transformation of the central region of the cell plate into a wide tubular network and then into a maturing, planar fenestrated sheet, while the actively growing peripheral zone expands centrifugally and eventually fuses with the mother cell wall. Finally, at late telophase the peripheral zone matures too, and the cell plate is transformed into a new cell wall (reviewed in Seguí-Simarro et al., 2008). These orchestrated changes in cell plate structure are accompanied by the deposition of different polysaccharides in a timely manner (reviewed in Worden et al., 2012; Drakakaki, 2015). The first polysaccharides present in the nascent cell plate would be pectins and hemicelluloses. Then, the synthesis of copious amounts of callose in the cell plate lumen is responsible for the transformation of the TVN cell plate into a maturing tubular network, and for the widening of these tubules into fenestrated sheets (Samuels et al., 1995). The final transformation of the planar fenestrated sheet-type cell plate into a new primary cell wall involves the progressive replacement of callose deposits by cellulose fibrils (Kakimoto and Shibaoka, 1992; Samuels et al., 1995; Otegui and Staehelin, 2000). Proper cellulose deposition appears essential for cell plate stabilization, as revealed by the aborted cell plates present in cellulose-deficient mutants (Zuo et al., 2000; Beeckman et al., 2002). Finally, cellulose combines with the already secreted hemicellulose molecules into a cellulose-hemicellulose network, while pectic polysaccharides reorganize to form the pectin-rich middle lamella (Carpita and McCann, 2000). In the final, somatic-type primary cell wall, callose is absent with the exception of the region around plasmodesmata, where it is supposed to play a regulatory role in cell-to-cell movement of molecules (Levy et al., 2007).

Based on this canonical pattern, some specialized cell types have developed alternative division mechanisms adapted to their function. This is the case, for example, of microspores. The first pollen mitosis (PMI) that transforms a microspore into a young pollen grain is characterized by the absence of a previous PPB (Van Lammeren et al., 1985), and by the building of an asymmetric phragmoplast (Brown and Lemmon, 1991), giving rise to the large, vegetative cell and the small, generative cell of the pollen grain. The cell wall formed around the generative cell is also special, since it is

hemispherical and transiently rich in callose (Park and Twell, 2001). However, soon it was found that microspores committed to *in vitro* embryogenesis significantly diverge from these cell division patterns. First, embryogenic microspores usually divide symmetrically, as opposed to *in vivo* microspores, in a mechanism more similar to somatic-type cytokinesis than to PMI cytokinesis (Zaki and Dickinson, 1991). Accordingly, it was found that embryogenic microspores show the typical PPB of somatic-type dividing cells (Simmonds and Keller, 1999). The cell walls of induced microspores and microspore-derived embryos (MDEs) present other unique features. For example, it was recently shown that embryogenic microspores exhibit abundant deposits of excreted cytoplasmic material (Corral-Martínez et al., 2013). These walls are also characterized by altered levels of certain cell wall components, such as xyloglucans, pectins (Barany et al., 2010), and arabinogalactan proteins (El-Tantawy et al., 2013). The major cell wall polymer, cellulose, has also been investigated, but unfortunately, the results are somehow confusing. For example, Schulze and Pauls (2002) assumed that the first cell walls produced in *Brassica napus* embryogenic microspores are somatic-type and therefore, cellulose-rich. In olive, Solis et al. (2008) described the presence of cellulose in some embryogenic microspores, at both the inner cell walls and the “thick peripheral wall localized below the exine.” However, Dubas et al. (2013) recently demonstrated by calcofluor white staining that cellulose was absent from the first cell walls formed in few-celled *B. napus* embryogenic microspores, whereas older MDEs presented abundant cellulose signal in their walls.

The other major cell plate-forming polymer, callose, is a β -1,3-glucan essential not only for somatic-type cell plate formation, but also for the cellular response to biotic and abiotic stresses (reviewed in Stone and Clarke, 1992; Chen and Kim, 2009). During microspore development, the presence of callose as the main component of the tetrad cell walls appears critical for a proper development of the microspore, since rice *GSL5* knockout mutants, defective in callose deposition during post-meiotic cytokinesis, exhibit a severe male-sterile phenotype (Shi et al., 2014). Later on, the deposition of a thin callose layer below the exine was proposed to have a role in exine patterning (Dong et al., 2005). In immature pollen grains, callose is found in the cell walls of the generative cell (Gorska-Brylass, 1967; Heslop-Harrison, 1968). Upon germination, callose progressively accumulates beneath the intine and is a major component of the pollen tube (Meikle et al., 1991; Ferguson et al., 1998). In the particular case of the first embryogenic divisions of the microspore, Telmer et al. (1995) found callose at the connections of the new cell wall with the mother cell wall, attributing it to alterations in the plasma membrane or the cell wall physiology. Other than that, little is known about the role of callose in the development of the MDE.

In this work we present a detailed study of the cell walls present in *B. napus* microspores during the initial stages of their induction toward embryogenesis. We analyzed a number of confocal three-dimensional series obtained from cultured microspores subjected to different experimental treatments, and a collection of transmission electron microscopy (TEM) images

obtained from samples processed by high pressure freezing and freeze substitution (HPF-FS). HPF-FS is known as the best procedure to preserve even the most labile and/or transient ultrastructural elements of *in vivo* physiological processes (Gilkey and Staehelin, 1986), as well as during the androgenic switch (Corral-Martínez et al., 2013). Our samples covered all the stages of *B. napus* microspore embryogenesis, the different cell types formed, and the subcellular changes undergone as a consequence of embryogenesis induction. Together, our results shed light on the dynamics of callose and cellulose deposition during the initials of the androgenic switch, and its significance for further MDE development.

MATERIALS AND METHODS

Plant Materials

Brassica napus L. donor plants of the highly embryogenic cv. Topas were grown as previously described (Seguí-Simarro et al., 2003). Plants were grown in the greenhouses of the COMAV Institute (Universitat Politècnica de València, Spain), the University of Colorado (Boulder, CO, USA), and the Plant Research International (Wageningen, The Netherlands).

Brassica napus Microspore Cultures

Flower buds containing mostly vacuolated microspores were selected and processed as previously described (Custers, 2003). Briefly, buds were surface sterilized with 2% sodium hypochlorite for 10 min, and washed three times in sterile distilled water. To release the microspores, buds were gently crushed in filter sterilized NLN-13 medium with the back of the plunger of a disposable 50 ml syringe. NLN-13 medium consists of NLN medium (Lichter, 1982) + 13% sucrose. Then, the slurry was filtered through 30 µm nylon cloths. The filtrate was transferred to 15 ml conical tubes and centrifuged at 800 rpm for 4 min. After discarding the supernatant, the pellet of microspores was resuspended in 10 ml of fresh NLN-13 medium. This procedure was additionally repeated twice for a total of three centrifugations and resuspensions. Before the last centrifugation step, microspore concentration was calculated using a hemacytometer. The required volume of NLN-13 medium was added to adjust suspension to a concentration of 4×10^4 microspores per ml. For caffeine and benzyl alcohol (BA) experiments, NLN-13 medium supplemented with 1 mM caffeine and 100 µM BA were used, respectively. Adjusted microspore suspension was distributed in sterile culture dishes. Dishes were incubated in darkness for 24 h at 32°C to induce embryogenesis, and then continuously at 25°C for MDE progression up to the time of sample collection and processing.

Callose and Cellulose Staining for Confocal Laser Scanning Microscopy

Samples of *B. napus* microspore cultures were collected at day 1, 2, 3, 4, and 6 after isolation, and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) pH 7.4. For callose staining, samples were placed in glass slides with 0.9% agarose, and

stained with 10 µg/ml propidium iodide (PI) in PBS for 10 min. PI was used in order to have a reference of the subcellular staining pattern of aniline blue. PI is a general stain for nucleic acids, binding both DNA and RNA. Thus, when a previous RNase treatment is omitted, the cytoplasm is also stained with PI (Suzuki et al., 1997). After three washes with PBS, samples were stained with 0.1% aniline blue (Evans et al., 1984) dissolved in PBS for 20 min, and then washed with the same buffer.

For cellulose staining, we used two different fluorescent stains, Calcofluor White ST and Pontamine Fast Scarlet (S4B). Calcofluor White was used diluted to 0.05% in water (Dubas et al., 2013). PI was also used better identification of the developmental stage of the microspore as described above. Both stains were added directly to the fixed microspores and slides were mounted with Vectashield and kept in darkness for at least 15 min before microscopic analysis. For S4B staining, samples were stained with 0.01% S4B in PBS for 30 min (Anderson et al., 2010), then washed thrice with PBS, and finally mounted with a mix of Vectashield and DAPI (1.25 µg/ml according to Custers et al., 1994) in a 1:4 proportion. Double staining with aniline blue and S4B was also performed as described above but combining both stains at 0.05 and 0.01%, respectively. Finally, preparations were observed with a Leica CTR 5500 and a Zeiss LSM 780 confocal laser scanning microscopes. Digital 3-D series of images were processed with Leica Application Suite Advanced Fluorescence (LAS AF) and FIJI software.

Processing of *B. napus* Anthers and Microspore Cultures for TEM

Anthers carrying microspores and pollen grains at different stages of microsporogenesis and microgametogenesis were excised, transferred to aluminum sample holders, cryoprotected with 150 mM sucrose, frozen in a Baltec HPM 010 high-pressure freezer (Technotrade, Manchester, NH, USA) and a Leica EM HPM-100 high-pressure freezer (Leica Microsystems, Vienna), and then transferred to LN₂. Cultured microspores and small MDEs were recovered from culture dishes by gently spinning culture media. Larger MDEs were manually picked up from cultures. These samples were transferred to aluminum sample holders, cryoprotected with their same glucose-rich culture medium and high-pressure frozen. All samples were freeze substituted in a Leica AFS2 system (Leica Microsystems, Vienna) with 2% OsO₄ in anhydrous acetone at -80°C for 7 days, followed by slow warming to room temperature over a period of 2 days. After rinsing in several acetone washes, they were removed from the holders, incubated in propylene oxide for 30 min, rinsed again in acetone, and infiltrated with increasing concentrations of Epon resin (Ted Pella, Redding, CA, USA) in acetone according to the following schedule: 4 h in 5% resin, 4 h in 10% resin, 12 h in 25% resin, and 24 h in 50, 75, and 100% resin, respectively. Polymerization was performed at 60°C for 2 days. Using a Leica UC6 ultramicrotome, thin sections (1 µm) were obtained for light microscopy observation, and ~80 nm sections were obtained for electron microscopy. Sections were mounted on formvar-coated, 200 mesh copper grids, stained with uranyl acetate

and lead citrate, and observed in a Philips CM10 electron microscope.

Immunogold Labeling

For the immunodetection of callose we used an anti-callose, mouse IgG monoclonal antibody (Biosupplies, Australia) crossreacting with linear β -1,3-oligosaccharide segments in β -1,3-glucans (Meikle et al., 1991). Immunogold labeling was performed in HPF-fixed, OsO₄-treated, epoxy-embedded samples. This type of processing is not usually employed for immunogold labeling because it may preclude the immunolocalization of protein epitopes. Notwithstanding this, we used these samples for immunolocalization because the epitope studied (callose) is a carbohydrate, insensitive to the effects of OsO₄ and the heat used to polymerize the resin. This way, we were able to combine specific immunolabeling with the excellent ultrastructural preservation provided by the use of these methods. 80–100 nm sections were deposited on Formvar and carbon-coated, 200-mesh nickel grids. Sections were hydrated with distilled water for 1 min, 1x PBS for 1 min, and blocked with 5%BSA in PBS for 5 min. Then, sections were incubated for 1 h at 25°C with anti-callose antibody, diluted 1:5,000 in 1% BSA. Next, sections were subjected to three 4-min washes with PBS and incubated for 45 min at 25°C with a goat anti-mouse secondary antibody conjugated to 10 nm colloidal gold (BBI Solutions, UK), diluted 1:25 in 1% BSA. Then, sections were subjected to three 4-min washes with PBS and one with distilled water. Finally, sections were counterstained with 0.5% uranyl acetate in 70% methanol and lead citrate, 10 min each.

RESULTS

In *B. napus* vacuolate microspores (**Figure 1A**), embryogenesis starts with a set of cell divisions still within the microspore exine (**Figure 1B**, left). In parallel, other microspores insensitive to induction may develop as pollen-like structures (**Figure 1B**, right) or just arrest and die. Successive divisions in embryogenic microspores give rise to exine rupture and to the emergence of young MDEs where the embryo proper and suspensor domains can soon be distinguished (**Figure 1C**). MDEs then follow a typical embryogenic pattern proceeding through the globular (**Figure 1D**), heart-shaped (**Figure 1E**), torpedo, and cotyledonar stages (**Figure 1F**). Samples of all the stages shown in **Figure 1** were collected at different culture stages and studied as follows.

Embryogenic Microspores Occasionally Present Discontinuous, Incomplete Cell Plates, and Nuclear Fusion Profiles

Some embryogenic microspores presented continuous cell walls morphologically similar to conventional walls with the only exception of the irregularities and deposits of excreted material (arrowheads in **Figure 2A**) previously described (Corral-Martínez et al., 2013). However, we also observed often dividing cells with abnormal cell plates and cell walls. Abnormal cell plates were characterized by an irregular architecture, with numerous tubular profiles and abundant openings (fenestrae)

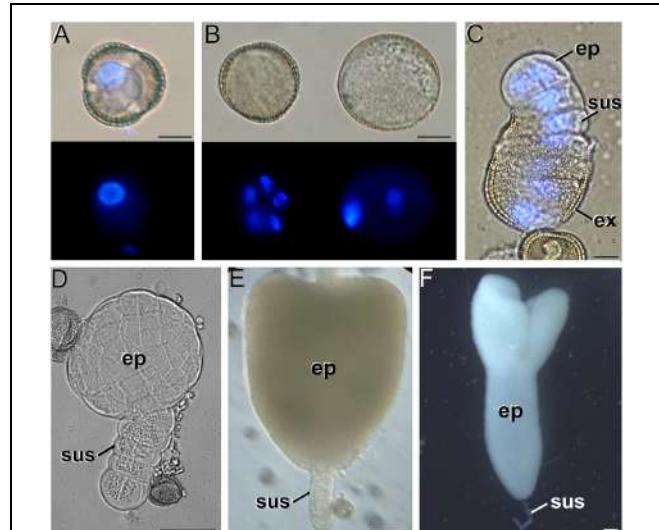


FIGURE 1 | The different stages of *Brassica napus* microspore cultures. **(A)** Isolated microspore, prior to induction. **(B)** Induced, embryogenic microspore (left) and pollen-like structure (right). Top and bottom images in **(A,B)** were taken under phase contrast and fluorescence with DAPI-stained nuclei, respectively. Note the presence of up to five nuclei in the embryogenic microspore, and the presence of a brighter (generative) and a faintly fluorescing (vegetative) nucleus in the pollen-like structure. **(C)** Quadrant-like embryo emerging from the exine (ex), where the suspensor (sus) and the embryo proper (ep) domains can be clearly identified. Superimposed phase contrast + fluorescence image where the DAPI-stained nuclei are shown in blue. **(D)** Globular embryo. **(E)** Heart-shaped embryo. **(F)** Cotyledonar embryo. Bars in A–C: 10 μ m; D: 50 μ m; E, F: 100 μ m.

that permitted the contact between the cytoplasm of daughter cells (white arrows in **Figures 2A,B**). In conventional cell plates, planar fenestrated profiles typically arise at the mid stage of cytokinesis. This stage is defined by the presence of a transitional, ring-shaped phragmoplast (Austin et al., 2005) and the targeted delivery of Golgi-derived vesicles to the growing edges of the cell plate, but not to the maturing central part (Seguí-Simarro et al., 2004). However, in the unusual cell plates of embryogenic microspores such a ring phragmoplast was absent, as revealed by the close proximity of cell organelles and dense vacuoles (Corral-Martínez et al., 2013) to both central and peripheral domains of the cell plate (asterisks in **Figures 2A,B**). In addition, the few Golgi-derived vesicles identified were observed randomly dispersed throughout the tubular cell plate (**Figure 2B**). These data would be indicating that in these cells, the final stage of cytokinesis (the formation of a planar fenestrated sheet) is disturbed. In parallel, we identified embryogenic microspores with binucleated cells (**Figure 2C**) and cells containing nuclei larger than usual, occasionally with a peanut-like morphology (**Figure 2A**), indicative of the recent occurrence of nuclear fusion events (Seguí-Simarro and Nuez, 2007, 2008b; Corral-Martínez et al., 2011).

Thus, we analyzed embryogenic microspores at previous stages of cell division in order to identify the initials of the cytokinesis defects. A detailed observation of mitotic cells did not reveal any structural abnormality with respect to the

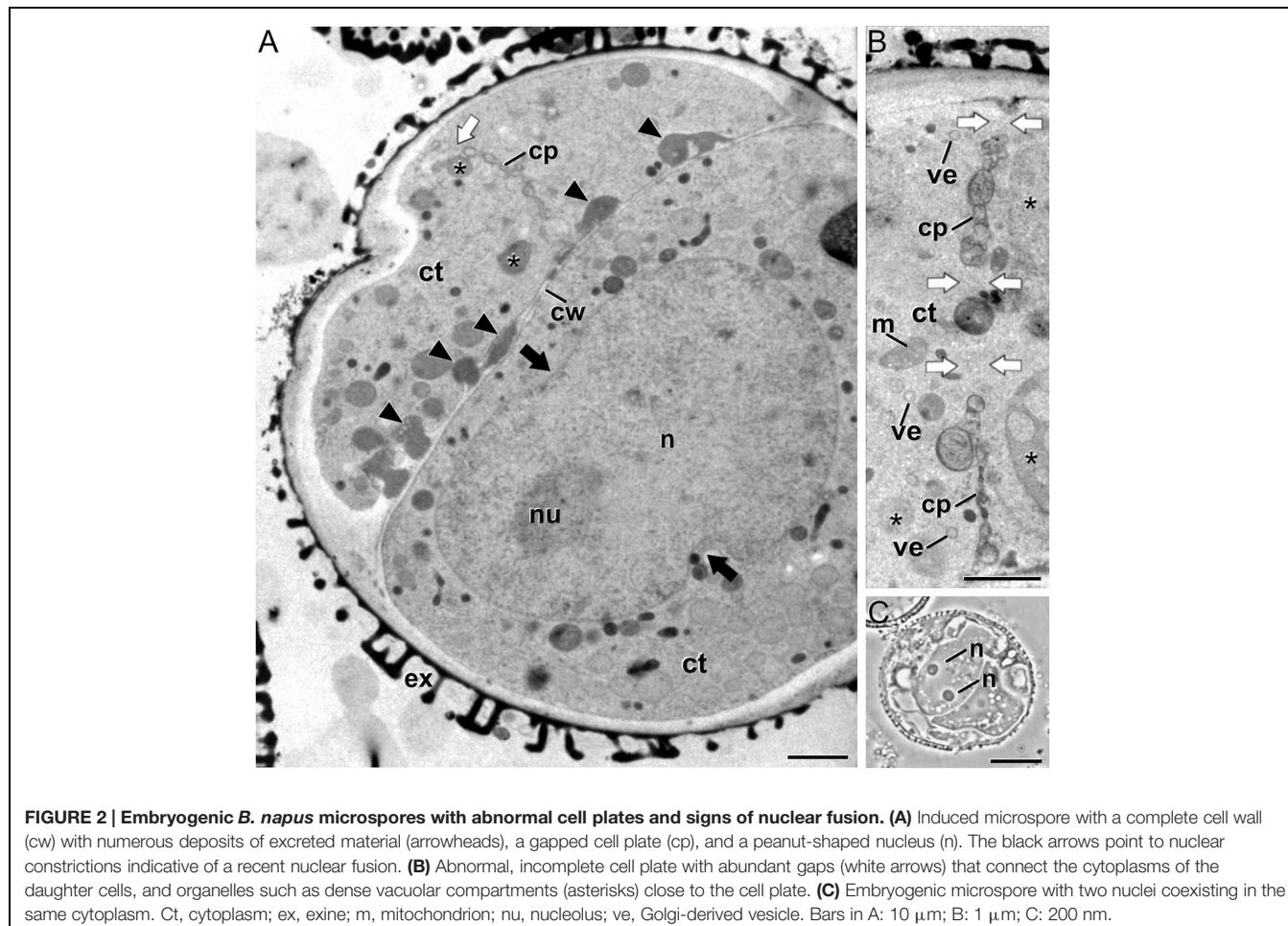


FIGURE 2 | Embryogenic *B. napus* microspores with abnormal cell plates and signs of nuclear fusion. (A) Induced microspore with a complete cell wall (cw) with numerous deposits of excreted material (arrowheads), a gapped cell plate (cp), and a peanut-shaped nucleus (n). The black arrows point to nuclear constrictions indicative of a recent nuclear fusion. **(B)** Abnormal, incomplete cell plate with abundant gaps (white arrows) that connect the cytoplasms of the daughter cells, and organelles such as dense vacuolar compartments (asterisks) close to the cell plate. **(C)** Embryogenic microspore with two nuclei coexisting in the same cytoplasm. Ct, cytoplasm; ex, exine; m, mitochondrion; nu, nucleolus; ve, Golgi-derived vesicle. Bars in A: 10 μ m; B: 1 μ m; C: 200 nm.

normal architecture of the mitotic machinery. As seen in **Figure 3A**, metaphasic cells presented the typical condensed chromosomes and an organelle-free region where the abundant parallel microtubules of the mitotic spindle could be identified. Images of cells showing the microtubular scaffold of the mitotic spindle and the solid phragmoplast appeared normal, as well as the cell plate initials that appeared correctly assembled (data not shown). Later on, at early telophase, the TVN cell plate typical from the solid phragmoplast stage was clearly identified together with abundant vesicles and microtubules near the cell plate (**Figure 3B**). Together, these observations pointed to the existence of problems during late stages of cytokinesis, but not before. It seemed that at the ring phragmoplast stage, where cell plate flattening and maturation starts, cytokinesis was somehow blocked and abnormal cell walls were finally formed.

The Presence of a Thick, Distinct Layer Beneath the Intine is a Differential Feature of Embryogenic Microspores

Soon after induction, we observed that cultured microspores presented an extra layer deposited just between the intine and the plasma membrane (**Figure 4A**). From now on, we will refer to this layer as the *subintinal layer*. The subintinal layer is defined

as a continuous layer of irregular thickness, alternating thick and thin domains, with an electron light appearance in osmium-treated, epoxy-embedded samples. Such a layer, including all the mentioned features, was exclusively observed in embryogenic microspores (**Figure 4B**). Pollen-like structures also presented a thickened inner layer (**Figure 4C**). However, this layer was homogeneous in thickness and very similar to the intine in terms of electron density. Indeed, at low magnification the coat of pollen-like structures appeared to be composed by the exine and a thickened intine, in contrast to the three layers of embryogenic microspores (compare **Figures 4B,C**). This suggested an independent nature for the subintinal layer. As expected, this layer was absent from young microspores and pollen grains not subjected to isolation and induction treatments (**Figures 6A,B**, respectively). Therefore, it seemed that the subintinal layer is a structure assembled exclusively in embryogenic microspores.

Embryogenic Microspores Present Callose-rich Subintinal Layers and Cell Walls

The subintinal layer of embryogenic microspores had in general a texture resembling the callose-rich wall of meiocytes

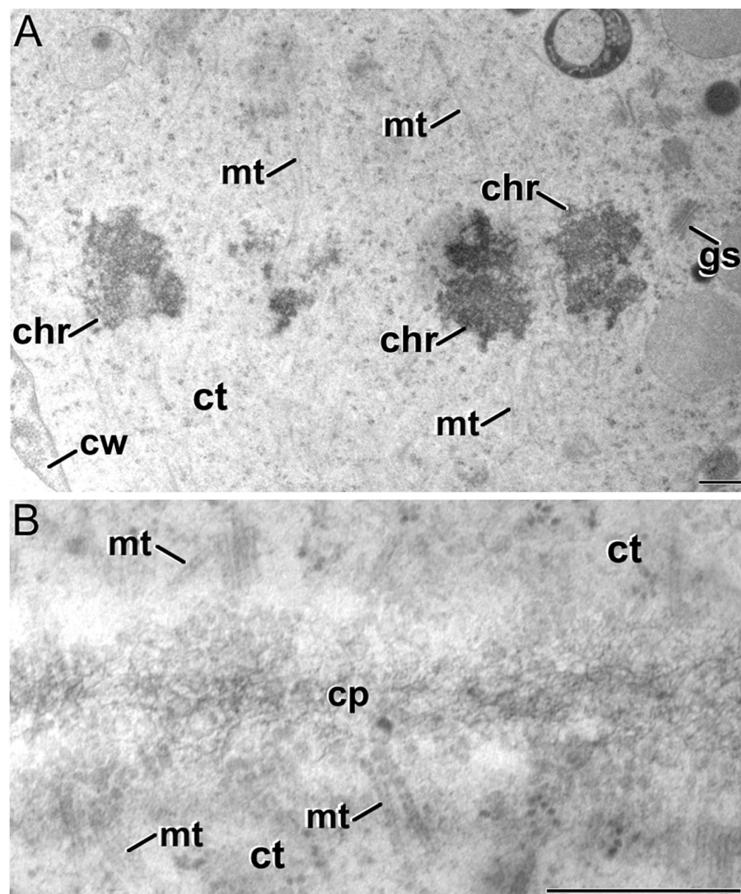


FIGURE 3 | Dividing cells of *B. napus* embryogenic microspores. **(A)** Metaphasic chromosomes (chr) at the cell equator of a dividing cell. **(B)** Tubulo-vesicular network (TVN) cell plate (cp) of a dividing cell at early telophase. ct, cytoplasm; cw, cell wall; gs, Golgi stack; mt, microtubules. Bars: 500 nm.

(Supplementary Figures S1A,B), as it can be deduced by comparing **Figure 4B** and Supplementary Figure S1C. For this reason, we investigated the putative callosic nature of this layer by two parallel approaches: staining with aniline blue, a callose-binding fluorescent dye, and immunogold labeling with anti-callose antibodies. Just after the induction treatment, abundant aniline blue fluorescence was found at specific regions throughout the embryogenic microspore. **Figure 5** shows a series of confocal slices from a representative embryogenic microspore, covering different planes from pole to pole. In this series, it is observed that callose accumulated below the exine in a non-uniform manner, alternating wide regions with bright fluorescence and thin regions with faint fluorescence (**Figure 5**). Interestingly, we also found aniline blue staining in the newly formed cell walls separating the daughter cells. Fluorescence was not uniform, indicating a differential callose accumulation at different cell wall domains. In some cell walls, cytoplasmic bridges were found connecting daughter cells through the regions devoid of aniline blue staining (Supplementary Figure S2). This finding, consistent with our observations in TEM micrographs (**Figure 2B**), confirmed the existence of incomplete, callose-rich cell walls. On the contrary, no evident aniline blue fluorescent

signal was observed at the thickened coat of pollen-like structures or in the cell walls of small globular MDEs (Supplementary Figures S3A,B). Therefore, we confirmed the unusual and transient accumulation of callose in embryogenic microspores.

Then, we performed a more comprehensive study at different stages before induction (during *in vivo* microspore development), just after induction, and 10 days after induction (in MDEs) by means of immunogold labeling with anti-callose antibodies. Positive controls with tomato meiocytes (Supplementary Figure S1D) showed an intense labeling of the callosic walls. Negative controls excluding the secondary antibody provided no labeling at all (Supplementary Figures S1E,F). Vacuolate microspores, during their *in vivo* development within the anther, showed almost no callose at their coat (**Figure 6A**). The exine was completely devoid of labeling and only clusters of 2–3 gold particles could be rarely observed at the intine, usually close to the plasma membrane. This was consistent with the thin callose layer described as necessary for exine formation and pollen viability (Dong et al., 2005). Pollen grains maturing within the anther showed no labeling at any layer of the pollen coat (**Figure 6B**) except for mature pollen, where anti-callose labeling was found below the pollen apertures (data

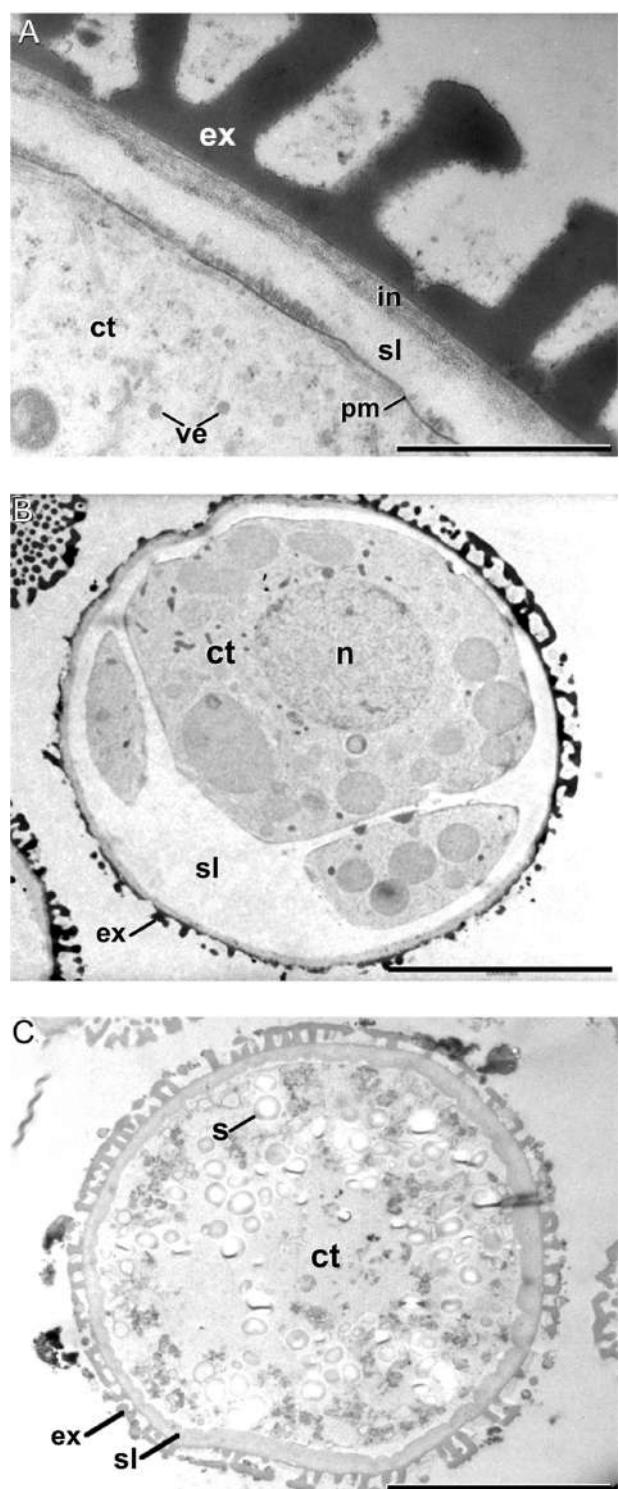


FIGURE 4 | The subintinal layer of *B. napus* embryogenic microspores.

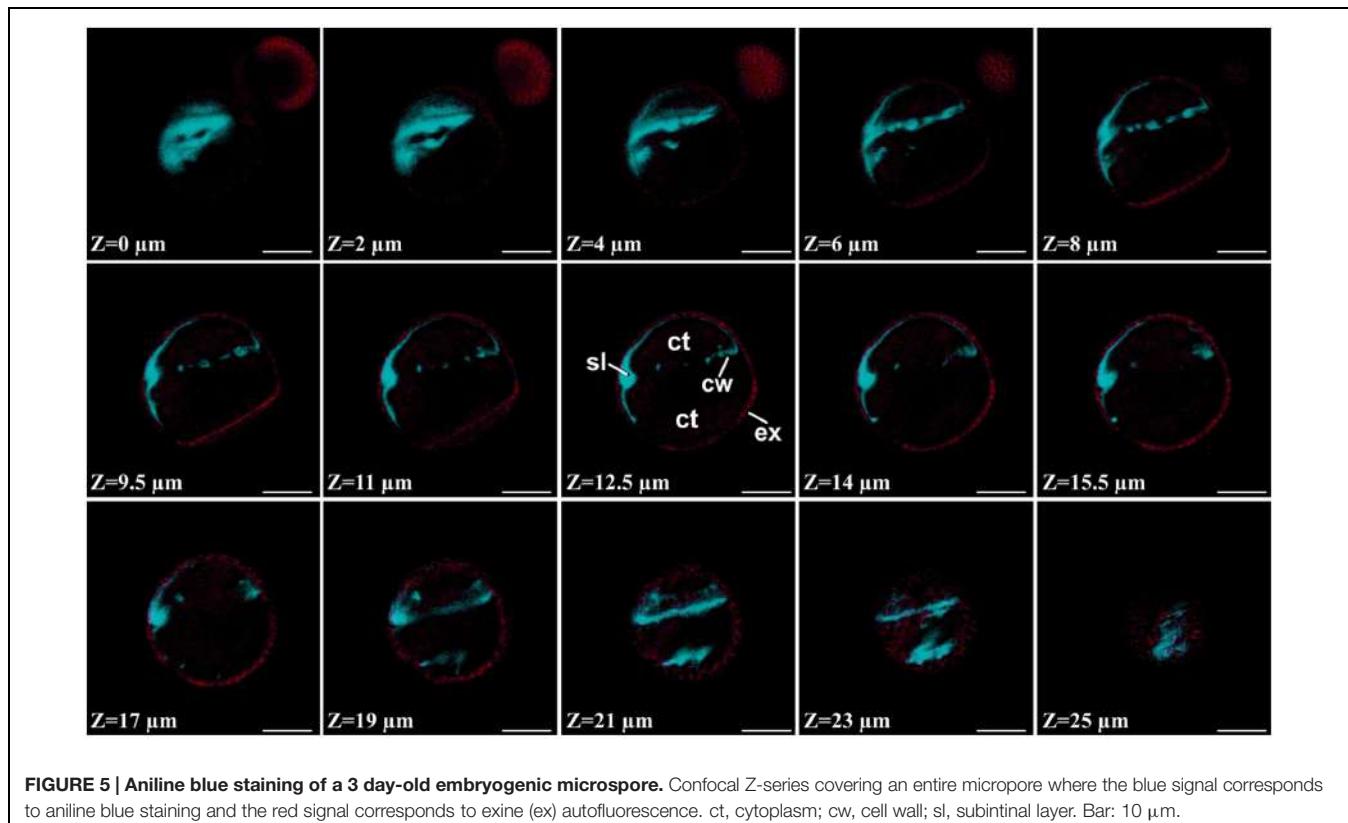
(A) Electron light subintinal layer (sl) deposited beneath the intine (in) of a embryogenic microspore. (B) Overview of an embryogenic microspore showing a continuous subintinal layer. (C) Pollen-like structure showing a thickened, uniform and electron dense intine. Ct, cytoplasm; ex, exine; n, nucleus; pm, plasma membrane; s, starch granule; ve, vesicle. Bars in A: 200 nm, B, C: 10 μ m.

not shown). After induction, the few cells enclosed within the embryogenic microspores presented a specific anti-callose labeling restricted to the subintinal layer (**Figure 6C**). In contrast, the thickened inner layer of pollen-like structures showed no labeling (**Figure 6D**). This different labeling pattern between embryogenic and pollen-like structures was consistent with their differences at the ultrastructural level. In addition to their different thickness and electron density, these layers also differed in callose contents.

Anti-callose antibodies also decorated the new cell walls created after the first embryogenic divisions. Young, developing cell plates (**Figure 6E**) but also older, mature cell walls (**Figure 6F**) showed immunogold labeling dispersed throughout the wall. Callose is a common component of developing somatic-type cell plates, but is progressively replaced by cellulose during the maturation stages (Samuels et al., 1995). With the exception of plasmodesmata, callose is absent from mature cell walls. Therefore, its persistence should be considered an abnormal feature of embryogenic microspores. In walls with deposits of excreted material (Corral-Martínez et al., 2013), callose was always absent from these deposits (**Figure 6G**). This suggested that callose deposition and secretion of cytoplasmic material were independent processes. In older stages of microspore embryogenesis, the cell walls of suspensor-bearing MDEs did not show decoration with anti-callose antibodies (**Figure 6H**), as expected for mature cell walls derived from somatic-type cytokinesis. In other words, the unusual presence of callose in mature cell walls of few-celled embryogenic microspores appeared to be a transient phenomenon, associated to the first stages of this developmental switch, and absent from MDEs with a clearly established embryogenic pattern.

Cellulose is Absent in the Callose-Rich Cell Walls of Just Induced Embryogenic Microspores

Due to the abnormal pattern of callose deposition observed in embryogenic cells, we next studied whether this might have an influence on its subsequent replacement by cellulose. For this, we stained microspore cultures with two cellulose-specific stains: calcofluor white, conventionally used to stain cellulose, and Pontamine Fast Scarlet 4B (S4B), described as more specific for cellulose than calcofluor white (Thomas et al., 2013). Calcofluor white staining revealed a noticeable and continuous cellulose signal at the intine of pollen-like structures, being more intense at the regions just below the apertures (Supplementary Figure S4A). In contrast, the induced microspores and MDEs exhibited variable patterns of cellulose deposition (Supplementary Figures S4B,C). To further investigate this unusual observation and the possible interaction between cellulose and callose, we performed a double staining of just induced microspore culture samples with aniline blue and S4B. We analyzed 289 cultured microspores from different randomly chosen microscope fields. From this microspore population, 217 (75.1%) showed no staining, only exine autofluorescence. Most likely, these microspores would be either dead or arrested in development, as usual in microspore



cultures of all inducible species (Seguí-Simarro and Nuez, 2008a). The remaining 72 microspores (24.9%) showed aniline blue staining, S4B staining, or both. We carefully analyzed these 72 microspores and categorized them into four groups according to their morphology, internal architecture and double staining pattern.

- **Group 1:** 12.5% of the microspores presented a slightly lobulated shape and a size similar to vacuolate microspores (**Figures 7A–D**). No embryogenic divisions were identified in their cytoplasm. No S4B staining was observed in any part of any of these cells. However, intense aniline blue fluorescence was consistently found at the region below the apertures (**Figure 7A**), in some cases extended beyond these regions (**Figure 7B**), and even along the entire subintinal layer (**Figure 7C**). Some of them also presented small stubs penetrating into the cytoplasm (arrows in **Figure 7D**), although a clear cell wall could not be observed in phase contrast images. Together, these microspores with increasing levels of aniline blue staining at their periphery were suggestive of different stages in the formation of the subintinal layer, prior to the first embryogenic division. Such formation would start at the region of the apertures, and would extend centrifugally to eventually cover the whole microspore.

- **Group 2:** 40.3% of the microspores presented the typical features of embryogenic microspores. In addition to the morphological features mentioned above for the first group, we clearly observed in all of them continuous (but variable in thickness) subintinal layers and cell walls, positive for aniline blue staining but negative for S4B staining (**Figure 7E**).

- **Group 3:** 43.1% of the microspores clearly showed pollen-like features, such as enlarged size, oval shape, dense cytoplasm, absence of symmetric divisions, and abundance of starch granules. When observing the S4B staining, these cells revealed another common feature: all of them but one presented a bright and continuous staining at the thickened intine layer (**Figure 7F**). This trait was remarkably different from the embryogenic microspores, devoid of cellulose at any cell wall region. Some of the cellulose-containing, pollen-like structures (15.3% of the total) were also positive for aniline blue staining at the regions below the apertures (data not shown), and only one (1.4% of the total) was positive only for aniline blue. In some double stained pollen-like structures, starch granules seemed clearly delineated by aniline blue staining (**Figure 7F**). To verify this, we isolated starch rich, mature pollen grains from anthers and stained them with aniline blue, confirming that pollen starch granules were also stained by aniline blue (Supplementary Figure S5).

- **Group 4:** 4.2% of the microspores presented abnormal patterns of development, combining features of pollen-like structures such as starch grains and large sizes with features of embryogenic microspores, such as cell divisions and new cell walls (**Figure 7G**). In addition, these cells presented high levels of cytoplasmic vacuolation, exine rupture at different regions of their surface, and asymmetric division patterns. These structures have been previously described as non-embryogenic, arresting, and dying after one or few division rounds (Corral-Martínez et al., 2013). Two microspores of this group (2.8% of the total) showed only cellulose staining, which delineated both the inner

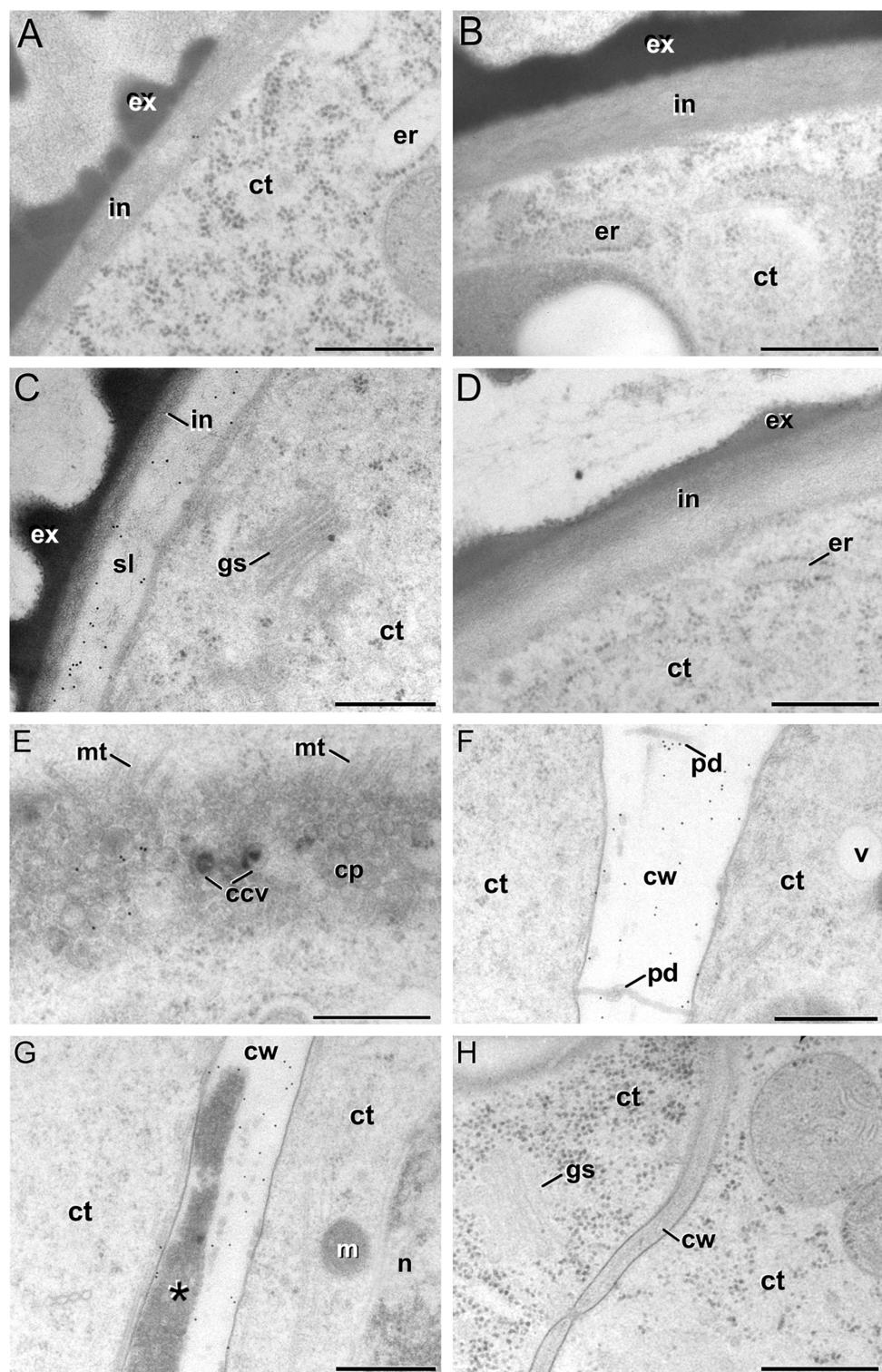


FIGURE 6 | Anti-callose immunogold labeling. **(A,B)** *In vivo* vacuolate microspore **(A)** and pollen grain **(B)**. Note the absence of a subintinal layer in these cell types. **(C)** Embryogenic microspore with abundant gold particles decorating the subintinal layer (sl). **(D)** Pollen-like structure with a thickened intine without anti-callose gold particles. **(E–G)** Developing cell plate **(E)** and mature cell wall **(F,G)** of embryogenic microspore cells, decorated with anti-callose gold particles. Note in **(G)** that callose labeling is absent from the deposits of excreted material (asterisk). **(H)** MDE cell without anti-callose gold particles in the cell wall. Ccv, clathrin-coated vesicle; ct, cytoplasm; cp, cell plate; cw, cell wall; er, endoplasmic reticulum; ex, exine; gs, Golgi stack; in, intine; m, mitochondria; mt, microtubules; n, nucleus; pd, plasmodesma; v, vacuole. Bars: 500 nm.

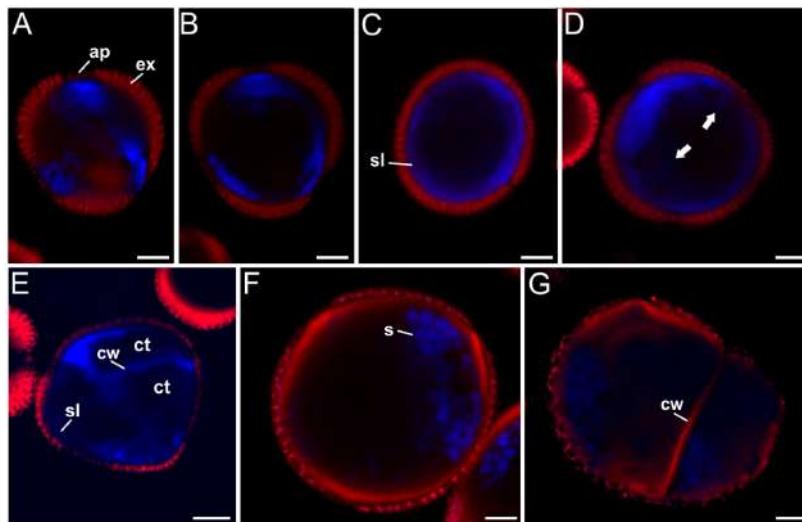


FIGURE 7 | Callose (blue) and cellulose (red) double staining with aniline blue and S4B. **(A–D)** Unicellular (not yet divided) microspores developing a callose-rich subintinal layer (group 1). **(E)** Three-celled microspore (group 2) with callose-rich subintinal layer (sl) and inner cell walls (cw). Note the absence of red cellulose signal. **(F)** Pollen-like structure (group 3) showing cellulose signal (red) below the exine, but not blue callose signal. **(G)** Microspore with unusual patterns of development (group 4) showing cellulose signal (red) below the exine and in the inner cell wall. The blue spots in **(F–G)** correspond to unspecific staining of starch granules by aniline blue. The dark red signal corresponds to exine (ex) autofluorescence. Bars: 5 μ m.

cell walls and the thickened intine (Figure 7G), and only one (1.4% of the total) presented both, cellulose and callose signal, mostly at the regions below the apertures.

As for MDEs, in young 4–8 MDEs, cellulose deposition was very scarcely found in the outer walls, being absent from the inner walls (data not shown). Equivalent (quadrant and octant) suspensor-bearing MDEs presented a similar cellulose pattern at the embryo proper domain, whereas suspensor cell walls were clearly delineated by cellulose staining (Supplementary Figure S4B). This pattern, however, changed from globular MDEs onward (Supplementary Figure S4C), where staining consistently decorated all the cell walls of the embryo proper in a pattern similar to a conventional mature cell wall. Together, these results indicated that the first embryogenic divisions of the induced microspore are defined by abnormal patterns of callose and cellulose deposition, establishing a clear correlation between the morphological features that define a microspore as embryogenic, the presence of callose at both the subintinal layer and the cell walls, and the total absence of cellulose. This pattern, however, changes in MDEs, where cellulose is progressively more present in the cell walls of developing MDEs, being abundant at the stages of globular and beyond, as expected for normal somatic-type cell walls.

Altered Ca^{2+} Levels Modify the Patterns of Callose and Cellulose Deposition in the New Cell Walls of Embryogenic Microspores

Once demonstrated that the abundance of callose in the newly formed cell walls is a feature inherent to the developmental switch toward embryogenesis, we wanted to find the cause of such

abundance. Due to the role assigned to Ca^{2+} in the regulation of callose deposition and in the inhibition of cellulose synthesis at the cell plate (Hayashi et al., 1987; Verma and Hong, 2001), we applied caffeine and BA to microspore cultures, and observed their effects in embryogenic microspores. Caffeine is known to redistribute the intracellular Ca^{2+} by altering transmembrane calcium gradients, thereby reducing the levels of Ca^{2+} available for callose synthases at the cell plate (Samuels and Staehelin, 1996). As a consequence, Ca^{2+} -deprived callose synthases cannot synthesize callose at normal levels (Kakimoto and Shibaoka, 1992). In turn, BA is a membrane fluidizer that induces major but transient elevations of cytosolic Ca^{2+} (Saidi et al., 2009).

When 1 mM caffeine was added to microspore cultures, callose-containing cell walls were observed between daughter cells, but they were thinner and aniline blue staining was less intense and extensive (Figure 8A) than in control cells. The subintinal layer was also thinner, discontinuous and irregularly stained by aniline blue. Interestingly, S4B staining revealed the continuous presence of cellulose along the cell walls and subintinal layer of ~50% of the cells (Figure 8A). As opposed to control cultures, 21 day-old caffeine-treated cultures did not produce MDEs. In contrast, 100 μ M BA-mediated increase of intracellular Ca^{2+} levels produced an increase in callose deposition, principally in the subintinal layer as revealed by the abundant aniline blue staining (Figure 8B). S4B staining, however, evidenced the absence of cellulose in these cells (Figure 8B'). Interestingly, 21 days after induction, BA-treated cultures produced ~600 MDEs/ml (Supplementary Figure S6), which represented 240% of the yield of control cultures (~250 MDEs/ml). Together, these results indicated that alteration of Ca^{2+} levels by caffeine and BA affected the amount of callose deposited in the cell walls of embryogenic

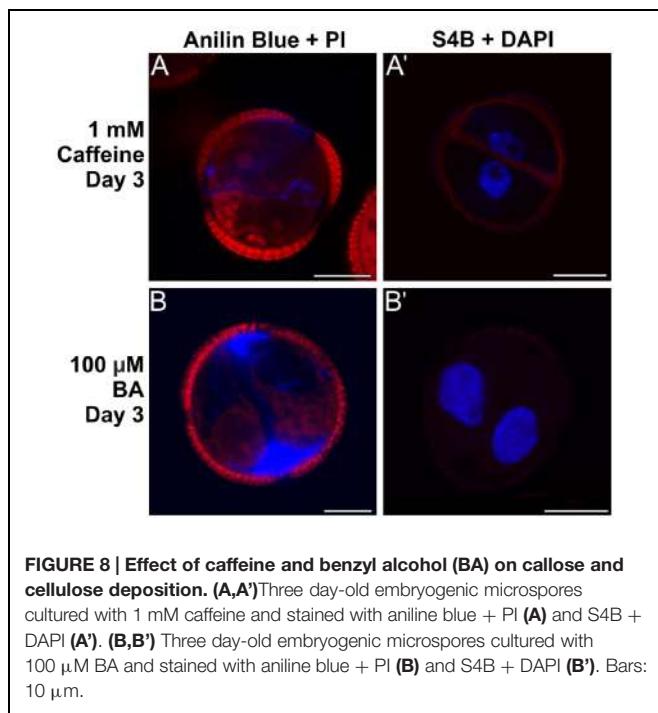


FIGURE 8 | Effect of caffeine and benzyl alcohol (BA) on callose and cellulose deposition. **(A,A')** Three day-old embryogenic microspores cultured with 1 mM caffeine and stained with aniline blue + PI **(A)** and S4B + DAPI **(A')**. **(B,B')** Three day-old embryogenic microspores cultured with 100 μ M BA and stained with aniline blue + PI **(B)** and S4B + DAPI **(B')**. Bars: 10 μ m.

microspores. Caffeine-mediated Ca^{2+} redistribution reduced callose deposition and allowed for the synthesis of cellulosic (conventional) walls in some microspores, which suggested that the lack of cellulose was due to a Ca^{2+} -mediated massive deposition of callose. On the other hand, BA-mediated increase of intracellular Ca^{2+} produced more callose in the subintinal layer and, as expected, cellulose-free cell walls. In addition to this, it seemed that the addition of BA was markedly beneficial for microspore embryogenesis, since 2.4x more MDEs were produced.

DISCUSSION

It is clear from this and other works (Solis et al., 2008; Barany et al., 2010; Dubas et al., 2013) that the cell wall is a highly dynamic structure whose architecture, arrangement, and composition changes dramatically as the microspore enters embryogenesis and then transforms into a MDE. We have demonstrated that a direct consequence of the exposure of microspores to the inductive treatment is the production of unusual walls with a unique composition. The microspore intine, described to have a pectocellulosic nature (Sitte, 1953), retained a cellulose-rich composition in pollen-like structures, as previously described for pollen (Heslop-Harrison, 1979). In contrast, the subintinal layer presented a dramatically different composition, indicating that it is not an extension of the intine, but a different structure originated *de novo* upon induction. Interestingly, the two cell walls created *de novo* in embryogenic microspores (inner cell walls and the subintinal layer) had the same appearance and composition, which was different from previously existing walls (the intine), but also from the walls of MDEs. It seems that during

the first stages of the switch, the mechanisms of synthesis and/or deposition of cell wall components are altered, giving rise to the transient formation of atypical walls. The origins, implications and consequences of the presence of these walls are discussed next.

The Subintinal Layer is an Early Marker of the Androgenic Switch

The presence of additional layers beneath the coat of embryogenic microspores in different species has been previously reported in the literature, but only occasionally (Rashid et al., 1982; Zaki and Dickinson, 1990; González-Melendi et al., 1995; Solis et al., 2008; Dubas et al., 2013). Perhaps, the reason why this layer has not been studied more in detail relies on the fact that TEM images of chemically fixed embryogenic microspores frequently showed cell retraction and waviness of plasma membrane (Telmer et al., 1993; González-Melendi et al., 1995; Maraschin et al., 2005; Seguí-Simarro et al., 2006), making it difficult to figure out whether the electron light spaces observed are real structures or just retraction artifacts. In addition, it is known that aldehyde fixation may induce callose deposition (Hughes and Gunning, 1980), which may raise doubts about the presence of callose in chemically fixed embryogenic microspores. We demonstrated that the callose-rich subintinal layer of embryogenic microspores is a real entity, not induced by chemical fixation. Thus, it is pertinent to ask whether this layer is a cause or a consequence of the developmental switch.

In plant development, the synthesis of a callose-rich layer is generally related to the developmental fate of the cells that synthesize it. The self-aggregating properties of the helical callose molecules, and the dense, insoluble nature of the highly hydrated but semipermeable callose gels make callose deposits ideal for creating physical and chemical barriers (Albersheim et al., 2011). Indeed, during meiosis callosic walls isolate the developing microspore and megasporocyte mother cells, acting as a physical barrier against premature swelling and bursting (Zhang et al., 2002), and also as a ‘molecular filter’ to allow for the expression of their specific developmental programs without the interference of the surrounding diploid tissues (Heslop-Harrison and Mackenzie, 1967; Abramova et al., 2003). Aside of natural processes, callose has been observed in internodal cells of *Humulus lupulus* committed to organogenesis (Fortes et al., 2002), and in cells of *Trifolium* (Maheswaran and Williams, 1985) and *Cichorium* (Dubois et al., 1991) reprogrammed to somatic embryogenesis. The deposition of a callose layer covering *in vitro*-induced young globular embryos was reported during somatic embryogenesis in *Camellia japonica* leaves (Pedroso and Pais, 1992). Most importantly, it was found that when zygotic embryos of *Eleutherococcus senticosus* are exposed to osmotic stressors such as mannitol or sucrose, single epidermal cells develop a surrounding callose layer between the plasma membrane and the cell walls, similar to the subintinal layer we hereby describe, and then enter somatic embryogenesis (You et al., 2006). These authors stated that plasmolysis-induced callose would interrupt cell-to-cell communication, which in turn would stimulate the embryogenic reprogramming. In line with these findings, we

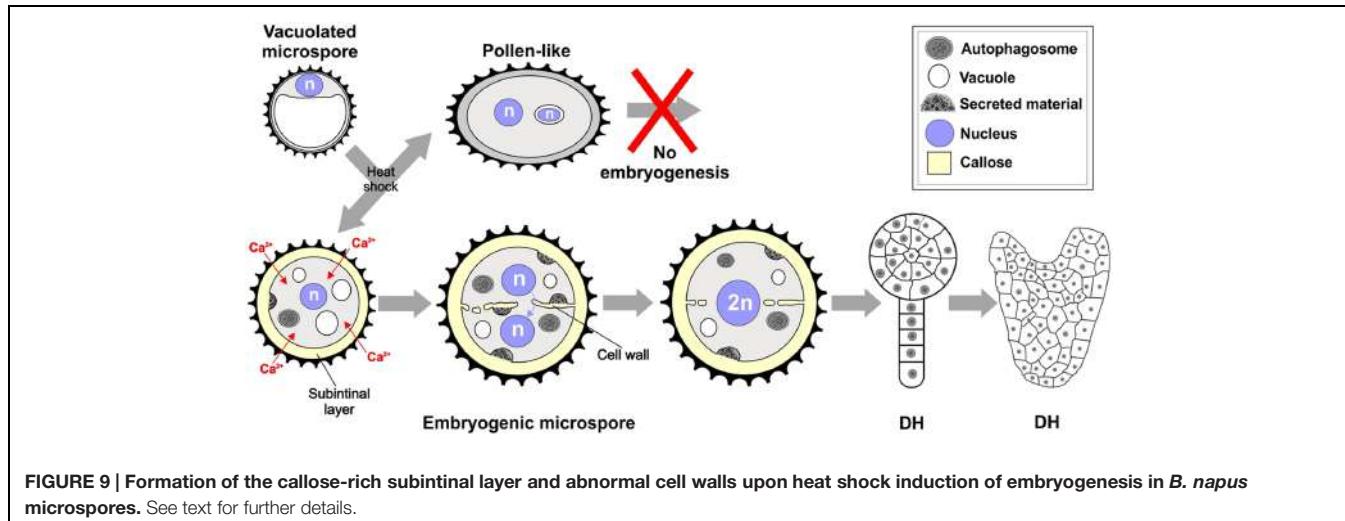


FIGURE 9 | Formation of the callose-rich subintinal layer and abnormal cell walls upon heat shock induction of embryogenesis in *B. napus* microspores. See text for further details.

observed that all the embryogenic microspores developed a callosic subintinal layer, whereas those non-embryogenic, did not develop it. Since both structures were exposed to the inductive heat shock and only the embryogenic developed an additional subintinal layer, we deduce that this layer would not be a direct consequence of the heat shock. It could also be argued that this layer could be related to the callose layer that appears beneath the intine in germinating pollen grains (Ferguson et al., 1998). However, we never found such a layer in the non-embryogenic, pollen-like structures, which accounts against this possibility. Instead, we propose that the subintinal layer is related to the change in developmental fate toward embryogenesis. We even found some microspores that developed the callosic layer even before the first clear evidence of inner cell walls was found in them (Figures 7A–D). Thus, we propose that the callosic subintinal layer would be formed in microspores prior to the first embryogenic division, and this would isolate them from the outer environment (the liquid culture media or the cultured anther tissue), creating a suitable environment to start a different developmental program, the embryogenic program. It can also be concluded that the deposition of a callose-rich layer, as a step previous to *in vitro* embryo development, would be a common marker associated to reprogrammed cells during different types of *in vitro* morphogenesis, including somatic and microspore embryogenesis.

The Abnormal Inner Walls of Embryogenic Microspores are Due to Disrupted Cytokinesis

We showed that the cell walls of embryogenic microspores are abnormal in terms of both structure and composition. For us, the most reasonable cause for this is that cytokinesis proceeds normally up to the fenestrated sheet-transitional phragmoplast stage of cytokinesis, and then it is disrupted. Our results are consistent with this hypothesis, since we did not observe any structural alteration up to this stage. Once the cell plate is established at the cell equator, callose synthesis is the driving

force that transforms the architecture of the developing cell plate. Callose is an amorphous polymer proposed to provide the fluidity needed to rapidly develop an initial membranous scaffold (Samuels et al., 1995) and to respond to the guidance mechanisms that insert the cell plate at specific sites of the parental wall (Xie et al., 2012). Newly synthesized callose widens the cell plate membranous tubules, as it spreads over the membrane inner surface (Samuels et al., 1995). At the end of this spreading phase, somatic-type cell plates resemble irregular sheets with numerous openings (Samuels et al., 1995; Seguí-Simarro et al., 2004, 2008). This is how the cell walls look like in our micrographs of abnormally walled *B. napus* embryogenic microspores (Figures 2A,B). Once the spreading phase is accomplished in somatic-type cytokinesis, the transition from a fluid and wrinkled immature cell plate to a stiff and straight cell wall is associated to deposition of cellulose and pectins and the parallel removal of callose (Kakimoto and Shibaoka, 1992; Samuels et al., 1995; Drakakaki, 2015). However, we showed that callose is not replaced by cellulose in the gapped and incomplete walls of embryogenic microspores. Callose does not form crystalline associations with adjacent strands, as cellulose does (Samuels et al., 1995). Thus, a callose-rich cell wall would in principle be more fluid than a conventional, cellulose-rich cell wall with no callose. In addition, β -1,3-glucans such as callose tend to gelate. It was suggested that the formation of hydrogels by callose would allow insertion or co-gelation with other polymers in the cell plate (Brown and Lemmon, 2009). It is also known that callose gelation can be increased upon heating (Stone and Clarke, 1992). Conceivably, the combination of a mild heat stress, callose accumulation and reduced cellulose contents would confer the newly formed cell walls of embryogenic microspores new properties, different from those of conventional walls. These walls would be more difficult to flatten and close through the conventional mechanisms of cell plate maturation, giving rise to irregular final walls, wrinkled and incomplete, as those shown in Figures 2A,B.

All this considered, we propose that the very first division rounds in embryogenic microspores are impaired at the stage

of callose replacement by cellulose and as a consequence, the cell walls produced are defective. In support of this, similar phenotypes of abnormal cell plates linked to callose abundance and/or scarce or absent cellulose have also been reported in other experimental systems. For example, in the *Arabidopsis* cytokinesis-defective *cyt1* mutant, the persistence of callose affected cell plate maturation, preventing cellulose synthesis and producing incomplete cell walls (Nickle and Meinke, 1998). Onion root cells treated with the herbicide dichlobenil, that prevents cellulose synthesis, showed reticulate and wavy cell plates with dramatically increased levels of callose (Vaughn et al., 1996). Furthermore, the *Arabidopsis rsw1* null mutant, where the activity of the RSW1/CeSA1 cellulose synthase gene is knocked down, produced thin, highly undulated, and frequently interrupted primary cell walls (Beeckman et al., 2002).

Increased Ca^{2+} Levels may be Responsible for Prolonged Callose Synthase Activity and Cellulose Inhibition

According to our results, it is pertinent to ask why callose persists and cellulose is not deposited in the first cell walls formed in embryogenic microspores. One candidate to mediate in this process is Ca^{2+} signaling, a highly conserved mechanism for temperature sensing among land plants (Horvath et al., 2012). Heat stress, among other treatments, is known to alter the properties of plant cell plasma membranes (reviewed in Horvath et al., 2012). Heat shock tends to fluidize plasma membranes, becoming leaky to ions and thus favoring uncontrolled influxes of Ca^{2+} and rapid, transient and proportional elevations of cytosolic Ca^{2+} in moss cells (Saidi et al., 2009), higher plants (Gong et al., 1998), and in *B. napus* embryogenic microspores (Pauls et al., 2006). In addition, high levels of intracellular Ca^{2+} are required for the activity of cell plate-specific, Ca^{2+} -dependent callose synthases (reviewed in Verma, 2001; Drakakaki, 2015). Related to this, our experiments with caffeine and BA suggested a possible relationship between the intracellular Ca^{2+} levels and the amount of callose deposited, which shows that the synthases involved in the development of the new cell wall and the subintinal layer are Ca^{2+} -sensitive. In somatic-type cell walls, high Ca^{2+} levels are known to cause the shift toward callose production, whereas Ca^{2+} levels must be reduced for cellulose synthesis to initiate during cell plate maturation. Indeed, prolonged high Ca^{2+} levels may extend callose synthesis in time and inhibit the shift from callose to cellulose synthesis (Verma, 2001). Our hypothesis is that a similar scenario might be occurring during the formation of the first cell walls in *B. napus* embryogenic microspores. Our experiments showed that caffeine decreased the presence of callose in both the subintinal layer and the inner cell walls, probably due to a reduction of the Ca^{2+} available for callose synthases. As a consequence, cellulose could be produced. In turn, the use of BA produced even more callose in the subintinal layer, probably due to an increase in the Ca^{2+} levels. In summary, we postulate that heat shock would alter the plasma membrane properties to allow for a Ca^{2+} influx that, among other effects (alteration of phragmoplast integrity, for example), would keep callose synthases active and, at the

same time, would inhibit cellulose synthases. In this context, it is interesting to note that the effect of BA added to the effect of heat shock not only in terms of Ca^{2+} influx and callose synthesis, but also of MDE yield. Indeed, several lines of evidence point to the redundancies of the cellular responses triggered by temperature-induced Ca^{2+} signaling and BA-mediated Ca^{2+} influx (Sangwan et al., 2001; Saidi et al., 2009; Horvath et al., 2012). Thus, it is tempting to speculate that BA may enhance or replace the effect of heat shock in cell wall remodeling and perhaps, in the activation of the whole embryogenic program in *B. napus* microspores.

Callose-rich and Cellulose-deficient Walls may Promote Genome Doubling

Cytokinetic defects typically induce the eventual formation of polyploid cells. Incomplete cell walls with large holes allow for the contact between the nuclei of daughter cells. Even those with smaller gaps may end up producing larger holes, since they are prone to break and collapse into pieces. These abnormal walls are known to facilitate the coexistence of nuclei of different cells in the same cytoplasm (Sunderland and Dunwell, 1974) and their eventual fusion, as demonstrated to occur in many other *in vitro* culture systems and cytokinesis-defective mutants (Mayer et al., 1999; Kasha et al., 2001; Muller et al., 2002; Strompen et al., 2002; Testillano et al., 2004; González-Melendi et al., 2005; Seguí-Simarro and Nuez, 2007; Corral-Martínez et al., 2011; De Storme et al., 2013). Our results showed that the instability and fluidity of callose-rich and cellulose-deficient cell walls, together with the severe cell wall deformations produced by the accumulation of excreted cytoplasmic material (Corral-Martínez et al., 2013), are behind the occurrence of incomplete, broken, or fenestrated cell walls. Hence in some embryogenic microspores, impairment of cytokinesis mediated by excessive callose deposition would generate the structural context necessary to facilitate nuclear fusion and therefore genome duplication, a necessary step widely accepted to produce DH cells, embryos and plants. On the other hand, cells where callose deposition is not so persistent would develop normal walls, which, according to this hypothesis, would preclude nuclear fusion during these initial stages, giving rise to haploid embryos. In turn, it is reasonable to assume that there will also be few embryogenic microspores whose initial phase of defective cytokinesis would last longer, being their cells more prone to undergo more than one round of nuclear fusions. It may also be possible that in a single embryogenic microspore, normally dividing cells coexist with others undergoing defective cytokinesis, giving rise to mixoploid and/or polyploid MDEs, known to occur in *B. napus* (Abdollahi et al., 2012) as well as in other species (Sato et al., 2005).

CONCLUSION

We showed in this work that one of the first signs of embryogenic commitment is the formation of callose-rich, cellulose-deficient subintinal layers and irregular, gapped, and incomplete cell walls (**Figure 9**), both of them absent in pollen-like structures. This is due to the combination of three factors: the formation of large

deposits of excreted cytoplasmic material described in Corral-Martínez et al. (2013), the massive deposition of callose, and the inhibition of cellulose synthesis. Callose would be produced by Ca^{2+} -dependent callose synthases activated by a raise in intracellular Ca^{2+} levels, which is a direct consequence of the increased permeability of the plasma membrane as a result of the inductive heat shock. Few days after the heat shock, abnormal cell walls are no longer produced, and MDEs present conventional, somatic-type cell walls.

These findings provide new insights that contribute to understand this intriguing, yet largely unknown developmental switch. In turn, our results pose new, open questions. One of them is why pollen-like structures, exposed to the same physico-chemical environment, do not produce callose-rich cell walls. A possible explanation is that these structures may have irreversibly entered the gametophytic-like pathway before the application of the inductive treatment, and this may somehow make them insensitive to the effects of heat shock exposure in terms of induction of embryogenesis. Another question is how and when the cell wall-associated phenomena we described cease, and conventional cell walls are formed in MDEs. It is possible that once the cells return to normal conditions, newly formed conventional walls coexist with the few preexisting callose-rich walls, as we have observed in some globular MDEs. After 1000s of conventional cytokinesis, the relevance of callosic walls would become negligible. However, we cannot rule out the possibility of callose being eliminated after the heat shock by specific 1,3- β glucanases. In favor of this hypothesis, Borderies et al. (2004) correlated the development of maize MDEs with the secretion of several proteins to the culture medium, one of them being a 1,3- β glucanase. It would be interesting to know whether *B. napus* MDEs produce

1,3- β glucanases as well. Finally, it would also be interesting to know whether the process we hereby describe or similar phenomena are triggered in other species where microspore embryogenesis is induced by other stresses different from temperature changes.

AUTHOR CONTRIBUTIONS

JMSS designed the research; VPV, PCM, ARS, and JMSS obtained and processed the samples. VPV, ARS, and PCM performed the experiments. JMSS wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01018>

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Effect of ovary induction on bread wheat anther culture: ovary genotype and developmental stage, and candidate gene association

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Ovary pre-conditioned medium and ovary co-culture increased the efficiency of green doubled haploid plant production in bread wheat anther culture. The positive effect of this medium led to a 6- and 11-fold increase in the numbers of embryos and green plants, respectively, having a greater effect on a medium-low responding cultivar. Ovary genotype and developmental stage significantly affected microspore embryogenesis. By the use of Caramba ovaries it was possible to reach a 2-fold increase in the number of embryos and green plants, and to decrease the rate of albinism. Mature ovaries from flowers containing microspores at a late binucleate stage raised the number of embryos and green plants by 25–46% as compared to immature ovaries (excised from flowers with microspores at a mid-late uninucleate stage). The highest numbers of embryos and green plants were produced when using mature Caramba ovaries. Ovaries from Galeón, Tigre, and Kilopondio cultivars successfully induced microspore embryogenesis at the same rate as Caramba ovaries. Moreover, Tigre ovaries raised the percentage of spontaneous chromosome doubling up to 71%. Attempts were made to identify molecular mechanisms associated to the inductive effect of the ovaries on microspore embryogenesis. The genes *TAA1b*, *FLA26*, and *WAL16* associated to wheat microspore embryogenesis, the *CGL1* gene involved in glycan biosynthesis or degradation, and the *FER* gene involved in the ovary signaling process were expressed and/or induced at different rates during ovary culture. The expression pattern of *FLA26* and *FER* could be related to the differences between genotypes and developmental stages in the inductive effect of the ovary. Our results open opportunities for new approaches to increase bread wheat doubled haploid production by anther culture, and to identify the functional components of the ovary inductive effect on microspore embryogenesis.

Keywords: wheat, anther culture, ovary co-culture, ovary developmental stage, ovary genotype, fasciclin-like arabinogalactan, *FERONIA*

Introduction

The production of doubled haploid (DH) plants is a valuable tool for plant breeding, genetics and functional genomics (Forster et al., 2007; Ferrie et al., 2011; Germanà, 2011), as it is a rapid, direct method to obtain homozygous plants. Of all the different methods for bread wheat (*Triticum aestivum* L.) DH production, microspore embryogenesis, through anther or microspore culture, has the greatest potential. However, this technique has major limitations due to the low percentage of microspores that enter the sporophytic pathway, the high percentage of albinism of many genotypes and the low percentage of spontaneous chromosome doubling (for review see Jauhar et al., 2009; Lantos et al., 2013). Therefore, the lack of protocols that can be successfully applied to a broad range of genotypes is still a bottleneck for bread wheat DH production.

Both anther and isolated microspore cultures are based on the switch of microspores from their normal pollen development toward an embryogenic pathway. However, each method had specific requirements (Soriano et al., 2007). In isolated bread wheat microspore culture, the presence of “nurse factors” in the culture medium can be critical in order to initiate and sustain microspore embryogenesis efficiently (Zheng et al., 2002). Although different flower tissues have been assayed as “nurse factor” donors, namely anthers, ovaries, glumes, and whole florets, ovaries are used most frequently. Ovary co-culture and/or an ovary pre-conditioned medium (in which ovaries have been grown for different time periods, OVCM), had been reported to increase embryogenesis and green plant regeneration in isolated wheat microspore cultures (Mejia et al., 1993; Puolimatka et al., 1996; Hu and Kasha, 1997; Puolimatka and Pauk, 1999; Zheng et al., 2002). Furthermore, the use of OVCM can be essential in order to produce embryos in recalcitrant genotypes (Zheng et al., 2002).

In bread wheat anther culture, early studies suggested that ovary co-culture stimulated regeneration, but it was not a prerequisite for high efficiency (Datta and Wenzel, 1987), as the anther wall could provide the necessary “nurse factors” (Bruins et al., 1996). Therefore, ovary co-culture and/or ovary pre-conditioned media have not traditionally been used. However, Broughton (2008) demonstrated the beneficial effect of ovary co-culture on embryo production in anther cultures of different Australian bread wheat cultivars. Ovary co-culture has occasionally been used in anther cultures by Soriano et al. (2007); Chauhan and Khurana (2011) and Tadesse et al. (2013). In durum wheat, ovary co-culture also enhanced the number of embryo and calli in anther culture, but had no effect on green plant regeneration (P'aitil et al., 1999).

In isolated microspore cultures, it has been shown that ovaries at different developmental stages have a promoting effect on embryogenesis (Zheng et al., 2002). However, in most studies immature ovaries excised from the flowers used for microspore culture (with microspores at a mid-late uninucleate stage) are used for co-culture or OVCM. In anther cultures, immature and mature ovaries (from flowers containing microspores at a late binucleate stage) have been used by Broughton (2008) and Soriano et al. (2007), respectively. In both anther and isolated

microspore culture, ovaries of the same genotype as the anthers are normally used (Hu and Kasha, 1997; Kunz et al., 2000; Broughton, 2008). Furthermore, Zheng et al. (2002) reported that a wide range of genotypes promoted microspore embryogenesis in isolated microspore culture. However, there are no reports of assays to determine the effect of ovary genotypes and their developmental stage for ovary co-culture or OVCM on wheat anther culture.

Although different authors have addressed the identification of the functional compounds released by the ovaries, their nature and the basis of the inductive effect on microspore embryogenesis, still remain unknown (El-Tantawy et al., 2013). Different promoting compounds have been proposed as candidate “nurse factors,” including the auxin PAA or its analogs, organic substances that buffer the medium, or compounds that metabolize the excess of nitrogen or detoxify the culture medium (Ziauddin et al., 1992; Hu and Kasha, 1997; Puolimatka and Pauk, 1999). Great attention has been paid to the arabinogalactan proteins (AGPs), as they were identified in microspore culture medium of barley and maize and promoted zygotic and microspore embryogenesis in maize (Paire et al., 2003; Borderies et al., 2004). Correspondingly, specific AGP genes were expressed in anther and isolated microspore during culture, such as *ECA1* in barley (Vrinten et al., 1999), *TaAGP31-like* in wheat (Sánchez-Díaz et al., 2013), *AGP15*, *AGP23*, *AGP2*, and *BnAGP Sta 39-4* in rapeseed (Joosen et al., 2007; Malik et al., 2007; Tsuwamoto et al., 2007; El-Tantawy et al., 2013). Moreover, the incorporation of AGP sources, Larcoll and arabic gum in the culture medium with or without ovary co-culture enhanced embryo production in wheat (Letarte et al., 2006). However, other proteins were also identified in the maize microspore culture medium, including one cell-wall invertase, two thaumatin, one 1-3 beta-glucanase, two chitinases and some oligosaccharides (Borderies et al., 2004). Furthermore, it is known that more than 25,000 genes were expressed in ovaries during at least one stage of development and 61.5% occurred in all stages, including genes associated to zygotic and microspore embryo development such as BABY BOOM (BBM) (Boutilier et al., 2002), and genes associated with ovary-pollen interactions, such as the defensin-like LURE or cysteine-rich proteins (Tran et al., 2013).

The objective of this work was to evaluate the effect of ovary genotype and ovary developmental stage on the efficiency of bread wheat anther culture when an ovary pre-conditioned medium and ovary co-culture were used. Furthermore, attempts were made to identify candidate genes associated with the ovary induction effect.

Materials and Methods

Material and Growing Conditions of the Donor Plants

The spring cultivars of bread wheat “Pavon” and “Caramba” were used as donor plants for anther culture and ovary co-culture. “Pavon” has a high microspore embryogenesis response, whereas “Caramba” has a medium-low response. The agronomically important cultivars Galeón (spring), Tigre (alternative), and

Kilopondio (alternative) were also used for ovary co-culture. Donor plants were grown as described by Soriano et al. (2007).

Preparation of Ovary Pre-conditioned Medium and Ovary Co-culture (OVPCM)

Ovaries were excised from flowers that contained microspores at a late binucleate stage of development, unless stated otherwise. Ovaries from the same spike were cultured in MS3M medium [MS medium modified by Hu and Kasha (1997), containing 62 g/l maltose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 1 mg/l benzyladenine (BA)], supplemented with 200 g/l Ficoll type 400 (MS3MF200) (Soriano et al., 2008). Cultures were kept in the dark at 25°C for 5 days before anther culture. The ovaries (eight ovaries per 1.5 ml) were kept in MS3MF200 medium during anther culture (co-culture, OVPCM).

Anther Culture

The microspore developmental stage was determined by DAPI (4', 6'-diamidino-2-phenylindole) staining (Vergne et al., 1987). Anthers containing the majority of microspores at the mid- to late-uninucleate stage were pre-treated for 5 days in 127.5 g l⁻¹ mannitol, 5.9 g l⁻¹ CaCl₂ plus macronutrients from FHG medium (Hunter, 1987) solidified with 8 g l⁻¹ Sea Plaque agarose (Soriano et al., 2008).

After pre-treatment, 30 anthers were inoculated in 1.5 ml of OVPCM medium, unless stated otherwise.

Experiment 1. Effect of OVPCM Medium on Anther Culture Response

After mannitol stress treatment, anthers from the same spike were randomly distributed on 3 cm Petri dishes containing 1.5 ml of control medium (MS3MF200) or 1.5 ml of OVPCM. Pavon and Caramba ovaries were used in this experiment for OVPCM preparation.

Experiment 2. Effect of the Ovary Genotype and Ovary Developmental Stage used in OVPCM on Anther Culture Response

Pavon and Caramba ovaries from spikes containing microspores at a late binucleate stage (mature ovaries) and microspores at a mid-late uninucleate stage (immature ovaries) were cultured in MS3MF200 for OVPCM preparation. Mannitol pre-treated anthers from Pavon and Caramba were randomly distributed in OVPCM medium with mature or young ovaries from Pavon and Caramba.

Experiment 3. Candidate Ovary Genotype for OVPCM

OVPCM was prepared with mature ovaries from the Caramba, Galeón, Tigre, and Kilopondio cultivars. Anthers from Pavon and Caramba were used in this experiment.

Cultures from all experiments were kept in the dark at 25°C. After 10–12 days, plates were replenished with 1.5 ml of the MS3MF400 (MS3M supplemented with 400 g/l Ficoll 400). After 30 days, embryos were transferred to J25-8 medium (Jensen, 1977) for regeneration. Embryos were kept in the dark at 25°C for 2 days and then transferred to the light. Ploidy analysis was performed with a PAS cytometer (Partec) as described by Soriano et al. (2007).

Statistical Analysis

All the experiments were performed with at least two different batches of plants. Each experiment consisted of 10–12 replicates of 30–35 anthers per treatment and genotype. Within replicates, pre-treated anthers from the same spike were randomly distributed between treatments. The following variables were recorded: number of embryos, number of green and albino plants and number of DH plants, all per 100 anthers. Percentage of regeneration (number of regenerated plants per 100 embryos), percentage of green plants (number of green plants per 100 total plants) and percentage of spontaneous chromosome doubling (number of doubled haploids per 100 analyzed plants) were calculated. All experiments were established in a completely randomized design. Statistical analysis was performed using SAS software (SAS Institute Inc., Cary, NC, and Version 9.1). Normality and homogeneity of variance were tested using Kolmogorov-Smirnov and Levene's tests, respectively. Data were transformed using the square root ($x + 0.5$) to meet parametric assumptions, except percentage of regeneration and percentage of green plants that did not need transformation. The GLM (Generalized Linear Model) procedure was used to conduct the ANOVA for all variables except for chromosome doubling percentage, which was analyzed using the FREQ procedure to do the Chi Square test. The Duncan method ($\alpha \leq 0.05$) was used for the Mean separation.

Selection of Candidate Genes, RNA Isolation, and Quantitative RT-PCR

Candidate genes were selected from the genes associated to the initial phases of microspore embryogenesis in wheat (Sánchez-Díaz et al., 2013) that were expressed in ovaries cultured in OVPCM by semi-quantitative RT-PCR analysis (data not shown): Ta.5024.1 (*WHEAT ALUMINIUM INDUCED-6, WALI6*), Ta.9528.1 (a fatty acyl CoA-reductase, *Ta.ANATHER-SPECIFIC1, TAA1b*), and Ta.1839.1 (*FASCICLIN-26, FLA26*). Two genes also expressed in ovaries cultured in OVPCM were included in the analysis: Ta.13696.1 (*COMPLEX GLYCAN LESS 1, CGL1*), a gene previously reported to be associated with AGPs or their derivate, and Ta.14561.2 (*FERONIA, FER*), a receptor-like kinase gene associated to ovary signaling processes. The consensus sequence of wheat genes based on HarvEST:Wheat v. 1.59 was used for designing specific primer pairs by PRIMEREXPRESS software (Applied Biosystems) (Table S1).

For RNA isolation, mature and immature ovaries from Pavon and Caramba at 0, 5, 10, and 15 days of culture in MS3MF200 medium (0dC, 5dC, 10dC, and 15dC) were frozen in liquid nitrogen. Mature ovaries from Galeón, Tigre and Kilopondio were frozen at 0dC, 5dC, 10dC, 15dC, and 20dC. Total RNA was isolated from frozen samples using TRIzol Reagent (Gibco BRL), and passed through RNeasy columns (Qiagen) for further clean up, following the manufacturer's instructions in both cases. Double-stranded cDNA was synthesized from the poly(A)+ mRNA present in the isolated total RNA (10.0 µg total RNA) using the M-MLV Reverse Transcriptase kit from Promega. Real-time qPCR reactions were performed with the Fast Maxima SYBR Green Master Mix (Applied Biosystems) and Rox (Rox Reference Dye, Invitrogen). qPCRs reactions for primer-dimer formation and primer efficiency were also performed (data not shown). The

reaction conditions were optimized to 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and a dissociation curve from 65 to 95°C was plotted after each run in the PCR7500 Fast Real-Time PCR System (Applied Biosystems), using gene Ta.27771 as reference (Paolacci et al., 2009). Data from three biological and two technical replicates were analyzed, using the Livak calculation method (Livak and Schmittgen, 2001).

Results

Anther Culture Efficiency

Experiment 1. Effect of OVPCM Medium on Anther Culture Response

Anther culture response was studied in control (MS3MF200 medium) and OVPCM (MS3MF200 ovary pre-conditioned medium and ovary co-culture) cultures, using anthers of the high-responding cultivar Pavon and the medium-low responding cultivar Caramba (Table 1). As expected, Pavon rendered a significantly higher number of embryos, green and albino plants than Caramba (3–5 times higher). Regeneration percentage from Pavon was also higher than from Caramba but both cultivars produced a similar percentage of green plants. Significant differences between control and OVPCM media were observed for all the variables studied. OVPCM gave rise to a 6-fold and 11-fold increase in the number of embryos and green plants, respectively. However, the percentages of plant regeneration and green plants were reduced by 40 and 30%, respectively in OVPCM medium.

A significant anther genotype x culture medium interaction was observed for the number of green plants and the regeneration percentage, OVPCM showing a larger positive effect in the lower-responding cultivar Caramba (Table 1 and Figure 1). In Pavon, 262 green plants were obtained with OVPCM and 27.2 in the control medium, representing a 9.6-fold increase. However, a 16.7-fold increase was observed with OVPCM in Caramba (from 3.8 to 63.8). The regeneration percentage was also enhanced, a 1.3 and a 4-fold increase being observed in Pavon and Caramba with OVPCM, respectively.

Experiment 2. Effect of the Ovary Genotype and Ovary Developmental Stage used for OVPCM on Anther Culture Response

Immature and mature ovaries corresponding to mid-late uninucleate and late binucleate stages from Pavon and Caramba were used for OVPCM preparation. ANOVA analysis showed that the numbers of embryos and green plants were affected not only by the anther genotype, but also by the ovary genotype and ovary developmental stage (Table 2). Once more, Pavon anther cultures produced a 6-times higher number of green plants than Caramba. When the ovary genotype effect was studied, Caramba ovaries rendered twice as many embryos and green plants as Pavon (Figure 2). A greater percentage of green plants was also obtained with Caramba ovaries. Finally, significant differences were also found between ovary developmental stages (Table 2 and Figure 2). Mature ovaries significantly raised the number of embryos (25%), the number of green plants (46%), and percentage of green plants (17%), in comparison to immature ovaries.

Caramba ovaries enhanced the anther culture response of both cultivars, but a significant anther genotype x ovary genotype interaction was detected for all the variables studied (Table 2).

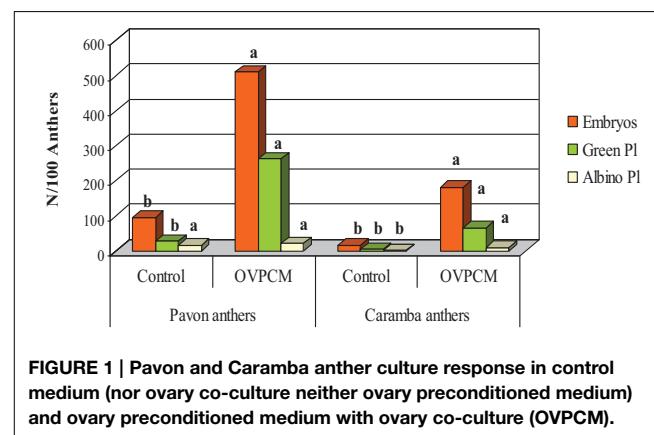


FIGURE 1 | Pavon and Caramba anther culture response in control medium (nor ovary co-culture neither ovary pre-conditioned medium) and ovary preconditioned medium with ovary co-culture (OVPCM).

TABLE 1 | Effect of ovary pre-conditioned medium and ovary co-culture (OVPCM) on anther culture of bread wheat cultivars Pavon and Caramba.

Source of variation <i>p</i> -value ²	Embryos	Green PI	Albino PI	Reg (%)	Green PI (%)
Genotype (G)	< 0.001	< 0.001	< 0.001	< 0.001	0.103
Culture medium (T)	< 0.001	< 0.001	0.045	< 0.001	< 0.001
G x T	0.097	0.001	0.176	0.021	0.065
Batch	0.014	0.017	0.024	0.124	0.051
R ²	0.80	0.84	0.34	0.63	0.50
ANTHER GENOTYPE					
Pavon	309.97a*	148.53a	18.67a	48.79a	77.65a
Caramba	92.18b	31.54b	5.00b	29.62b	85.94a
OVPCM					
Control	57.64b*	15.94b	8.42b	48.82a	89.77a
OVPCM	369.08a	177.21a	16.60a	31.42b	64.22b

p-values of the analysis of variance for the numbers of embryos, green plants, albino plants, all of them referred to 100 anthers, and percentages of regeneration and green plants.

*Values followed by the same letter within cultivar and treatment are not significantly different (*P* < 0.05).

TABLE 2 | Effect of ovary genotype and ovary developmental stage used for OVPCM on anther culture of Pavon and Caramba cultivars of bread wheat.

Source of variation p-value ²	Embryos	Green PI	Albino PI	Reg (%)	Green PI (%)
An Gen(1)	< 0.001	< 0.001	0.055	< 0.004	< 0.001
Ov Gen(2)	< 0.001	< 0.001	0.008	0.103	< 0.001
An Gen x Ov Gen	0.042	0.001	0.018	0.030	0.017
Ov Stage (3)	0.048	0.011	0.513	0.334	0.041
An Gen x Ov Stage	0.476	0.118	0.887	0.530	0.187
Ov Gen x Ov Stage	0.069	0.056	0.272	0.225	0.242
1 × 2 × 3	0.227	0.210	0.781	0.314	0.284
Batch	< 0.001	< 0.001	< 0.001	0.129	< 0.001
R ²	0.60	0.68	0.26	0.24	0.48
ANTHER GENOTYPE					
Pavon	242.32a*	143.02a	52.84a	79.78a	63.39a
Caramba	75.72b	24.03b	27.63a	69.82b	37.15b
OVARY GENOTYPE					
Pavon	112.87b*	58.78b	40.01b	78.70a	47.58b
Caramba	219.05a	125.14a	43.35a	71.75a	55.94a
OVARY STAGE					
Immature	148.63b*	72.42b	40.82a	73.77a	47.54b
Mature	186.08a	106.21a	42.26a	76.76a	55.52a

p-values of the analysis of variance for the numbers of embryos, green plants, albino plants, all of them referred to 100 anthers, and percentages of regeneration and green plants.

*Values followed by the same letter within anther genotype, ovary genotype, and ovary stage are not significantly different ($P < 0.05$).

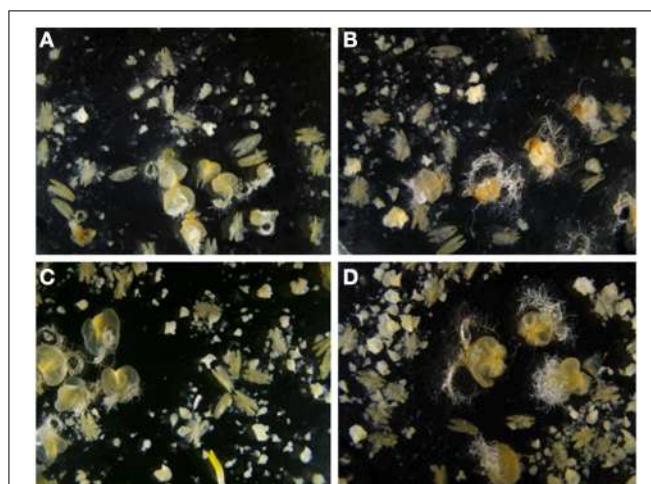


FIGURE 2 | Effect of the ovary developmental stage and ovary genotype used for OVPCM on Pavon anther cultures. (A) OVPCM with Pavon immature ovaries. **(B)** OVPCM with Pavon mature ovaries. **(C)** OVPCM with Caramba immature ovaries. **(D)** OVPCM with Caramba mature ovaries.

In the low-responding cultivar Caramba, the number of embryos and green plants increased 5 and 10 times, with Caramba ovaries (Figures 3A,B). However, in the high-responding cultivar Pavon, Caramba ovaries produced only a 2 and 2.5 times higher percentage of embryos and green plants.

No significant ovary genotype x ovary developmental stage interaction was observed for any of the variables; however this interaction was close to the significance level for number of embryos and green plants with p-values of 0.07 and 0.06,

respectively (Table 2). The positive effect of OVPCM with mature ovaries from Pavon as compared to immature ovaries was higher than that observed between Caramba ovaries. Thus, OVPCM with mature ovaries from Pavon produced a 50% higher number of embryos than immature ovaries (Figures 2A,B), whereas a 15% higher number of embryos was produced with mature ovaries from Caramba (Figures 2C,D). A similar trend was observed for the number of green plants (data not shown). However, we should point out that the highest number of embryos and green plants was obtained with mature ovaries from Caramba and the lowest with immature ovaries from Pavon (Figures 2A,D).

Experiment 3. Candidate Ovary Genotype for OVPCM

Our aim was to find out whether ovaries from other wheat cultivars could further improve the number of green plants in anther culture. Thus, the agronomically important cultivars Galeón, Tigre, and Kilopondio were assayed as donors of mature ovaries for OVPCM and were compared with mature ovaries from Caramba, which had shown the highest inductive effect. Nor were statistically significant differences observed between cultivars for number of embryos and green plants, or for the regeneration and green plant percentages (Table 3). However, differences in the final number of green DH plants were observed between ovary genotypes since they induced distinct rates of spontaneous chromosome doubling (Figure 4). The highest percentage of DH plants was obtained with ovaries from Tigre (71%), followed by Galeón with 58%, whereas Caramba and Kilopondio produced similar percentages of chromosome doubling (45–50%). Accordingly, Tigre ovaries produced the highest number of DH plants, with 111 DH plants/100 anthers.

Although Galeón ovaries rendered the highest number of embryos and a high percentage of spontaneous chromosome doubling, this cultivar produced the lowest number of DH plants

(58 DH plant/100 anthers), due to its low regeneration and green plant percentage.

Gene Expression Analysis

In order to identify the molecular mechanisms associated to the ovaries' inductive effect on microspore embryogenesis, genes associated to wheat microspore embryogenesis, glycan biosynthesis or degradation, as well as to the ovary signaling process, were considered. Candidate genes were selected based on their expression in Caramba mature ovaries cultured in OVPCM by semi-quantitative RT-PCR analysis (data not shown). The expression pattern of the candidate genes (*WALI6*, *FLA26*, *TAA1b*, *CGL1*, and *FER*) was characterized by quantitative RT-PCR in immature and mature ovaries from Pavon and Caramba at 0, 5, 10, and 15 days of culture (0dC, 5dC, 10dC, and 15dC). At 0dC, mature ovaries were bigger with a more developed stigma than immature ovaries (Figures 5A,E). Both mature and immature ovaries from Caramba enlarged during culture and their stigmatic branches

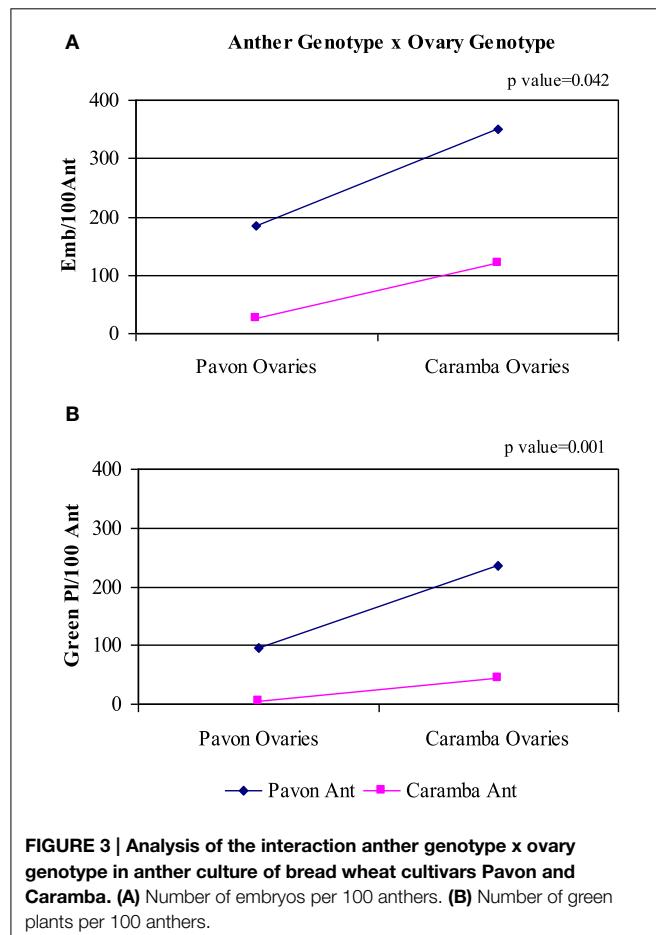


FIGURE 3 | Analysis of the interaction anther genotype x ovary genotype in anther culture of bread wheat cultivars Pavon and Caramba. (A) Number of embryos per 100 anthers. (B) Number of green plants per 100 anthers.

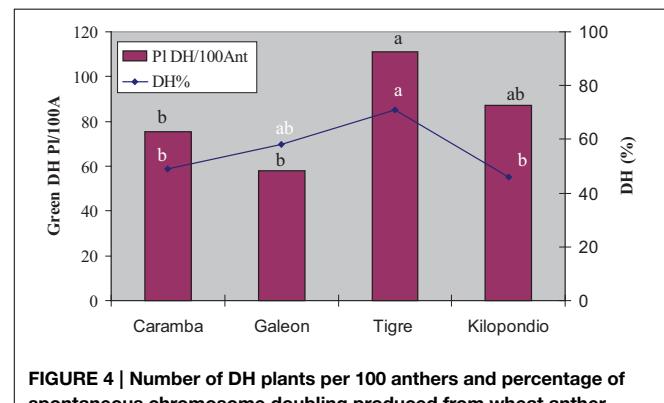


FIGURE 4 | Number of DH plants per 100 anthers and percentage of spontaneous chromosome doubling produced from wheat anther culture with different ovaries genotypes used for OVPCM.

TABLE 3 | Effect of ovary genotype used for OVPCM on anther culture of Pavon and Caramba cultivars of bread wheat.

Source of variation p-value ²	Embryos	Green PI	Albino PI	Reg (%)	Green PI (%)
An Gen	< 0.001	< 0.001	0.661	< 0.001	< 0.001
Ov Gen	0.776	0.943	0.277	0.715	0.378
An Gen x Ov Gen	0.714	0.990	0.439	0.296	0.613
Batch	0.701	0.018	0.545	< 0.001	0.061
R ²	0.29	0.45	0.09	0.43	0.45
ANTHER GENOTYPE					
Pavon	300.16a*	166.79a	71.91a	80.99a	65.86a
Caramba	148.39b	38.23b	67.17a	71.07b	36.88b
OVARY GENOTYPE					
Caramba	240.34a*	130.32a	69.78a	79.74a	54.90a
Galeon	298.06a	135.12a	84.86a	77.80a	54.92a
Tigre	242.99a	123.53a	64.04a	76.06a	60.11a
Kilopondio	235.22a	121.54a	63.20a	78.35a	58.31a

²p-values of the analysis of variance for the numbers of embryos, green plants, albino plants, all of them referred to 100 anthers, and percentages of regeneration and green plants.

*Values followed by the same letter within anther genotype and ovary genotype are not significantly different ($P < 0.05$).

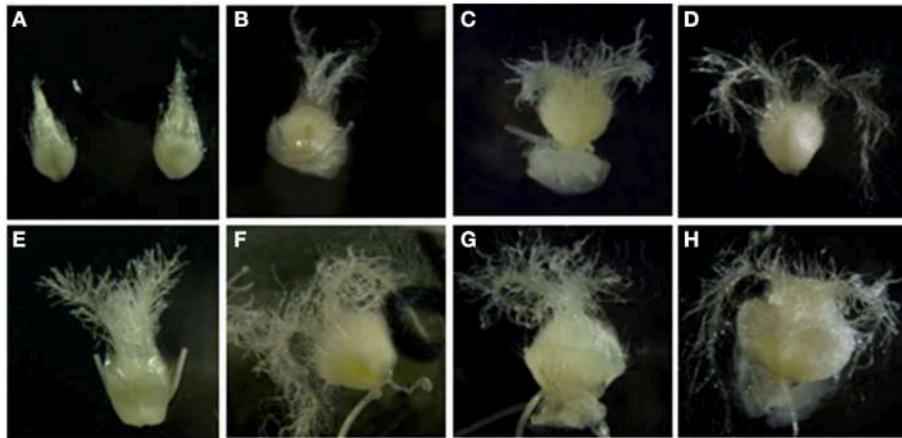


FIGURE 5 | Immature (A–D) and mature (E–H) ovaries from Caramba in OVPCM at 0 (A,E), 5 (B,F), 10 (C,G) and 15 days of culture (D,H).

spread, showing a similar morphology to the pollen reception stage at 10dC and 5dC in immature and mature ovaries, respectively (**Figures 5B–D,F–H**).

Differences in gene expression patterns were observed between ovary genotypes and ovary developmental stages (**Figure 6**). The gene *TAA1b* that encoded for a fatty acyl CoA-reductase, showed the greatest difference between genotypes, as the gene was only strongly induced in Pavon immature and mature ovaries at 10dC and 5dC, respectively. *WALI6*, a cysteine-rich serine protease inhibitor, showed the highest expression level during culture and the greatest differences between ovary developmental stages. This gene was induced after 5dC in Pavon and Caramba mature ovaries, and reached the highest level of expression at 5dC in Caramba and at 10dC in Pavon. *CGL1*, that encodes the first enzyme in the complex glycan biosynthesis pathway, showed a different expression pattern depending on ovary genotype and developmental stage. In immature ovaries, expression increased during culture in both genotypes, reaching higher levels in Pavon. In mature ovaries, the highest level was observed at 10dC in Caramba.

The two following genes, the fasciclin-like arabinogalactan gene *FLA26* and the receptor-like kinase *FER*, increased their expression during culture, in both genotypes and in both ovary developmental stages (**Figure 6**). However, it is noteworthy that mature Caramba ovaries, which produced the highest inductive effect on microspore embryogenesis, showed the highest level of expression at the beginning of culture (0dC) and at the latest stage (15dC).

The expression patterns of *FLA26* and *FER* were also characterized by quantitative RT-PCR in mature ovaries from Galeón, Tigre, and Kilopondio cultivars that showed a similar inductive effect to Caramba. Analysis was performed at 0, 5, 10, 15, and 20 days of culture (0dC, 5dC, 10dC, 15dC, and 20dC) (**Figure 7**). The *FLA26* pattern of expression in the three genotypes was comparable to that observed in mature Caramba ovaries (**Figure 6**), as its expression increased during culture, reaching the highest level at 15dC in Galeón, and at 15dC–20dC in Kilopondio and Tigre. Expression levels of *FLA26*

in Kilopondio and Tigre ovaries were the closest to those of Caramba, but Galeón showed the highest expression level at 15dC. In the analysis of *FER*, some similarities in the expression pattern of the three genotypes and Caramba were found as the level of expression at the beginning of the culture (0dC) was high. However, slight differences were also observed, since the level of expression remained close to the 0dC level, except at 15dC in Galeón and at 10–15dC in Kilopondio, with a decrease in their expression level, and a high increase at 10dC in Tigre.

Discussion

Ovary Pre-conditioned and Co-culture Medium (OVPCM) Increases Wheat Anther Culture Response

Our study shows that the utilization of an ovary pre-conditioned medium and ovary co-culture (OVPCM) has a positive effect on anther culture of high and medium-low responding cultivars of bread wheat, leading to a 6- and 11-fold increase in the numbers of embryos and green plants, respectively. Although it is well-known that ovary co-culture and/or an ovary pre-conditioned medium can be essential in wheat isolated microspore culture (for review see Zheng, 2003), few studies have addressed their effect on wheat anther culture response, even though this system is widely used for the routine production of doubled haploids (Jauhar et al., 2009; Lantos et al., 2013; Broughton, personal communication). Only Broughton (2008) conducted a comparative study of the use of ovary co-culture or ovary pre-conditioned medium, concluding that co-culture without pre-conditioning produced the highest number of green plants. Despite these data, we adopted the use of OVPCM as “nurse factors” that are immediately available at the time of anther culture, which is a great advantage. Furthermore, 5 days of ovary co-culture, which is the time used for ovary pre-conditioned medium in this study, was sufficient to produce enough “nurse factors” to induce microspore embryogenesis in isolated wheat microspore culture (Puolimatka and Pauk, 1999). Moreover, when isolated microspore culture was assayed in recalcitrant

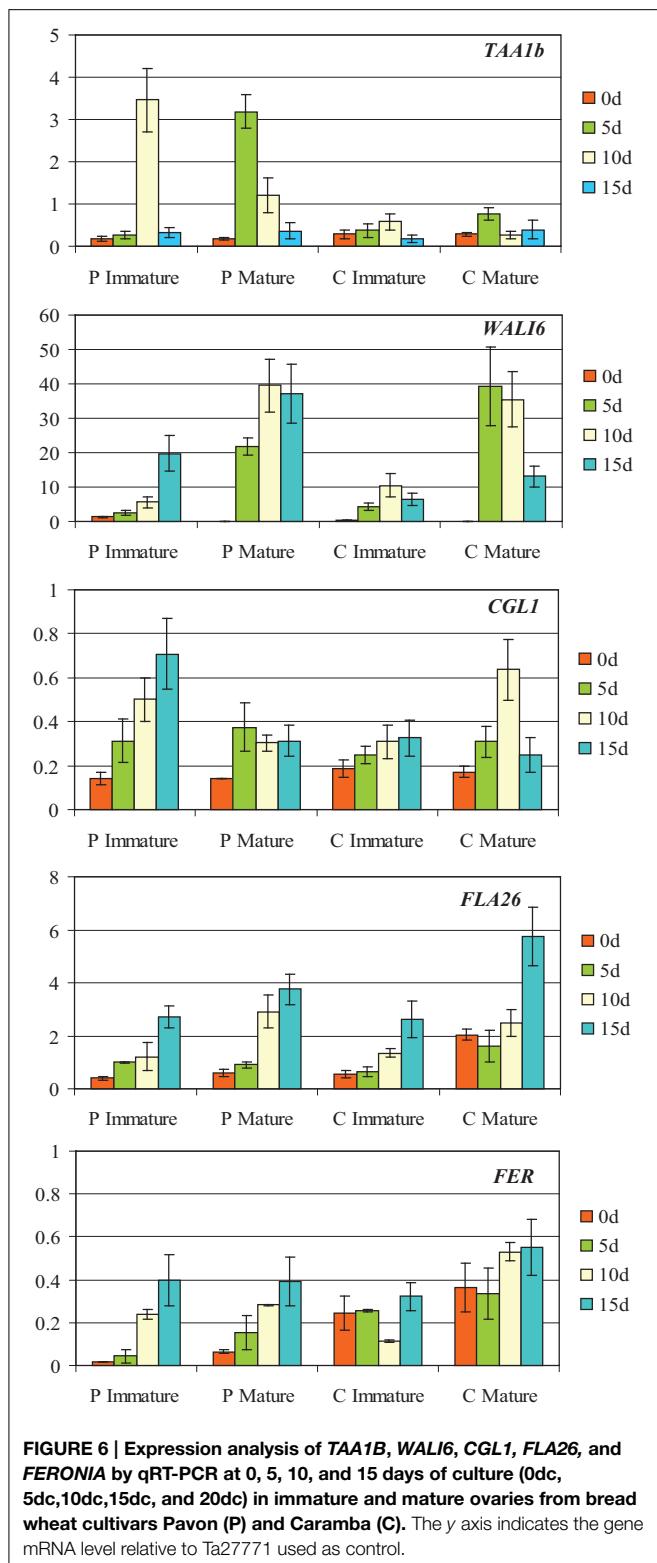


FIGURE 6 | Expression analysis of *TAA1B*, *WAL16*, *CGL1*, *FLA26*, and *FERONIA* by qRT-PCR at 0, 5, 10, and 15 days of culture (0dc, 5dc, 10dc, 15dc, and 20dc) in immature and mature ovaries from bread wheat cultivars Pavon (P) and Caramba (C). The y axis indicates the gene mRNA level relative to Ta27771 used as control.

cultivars, ovary co-culture was not effective but an ovary pre-conditioned medium was essential (Zheng et al., 2002).

In this study, the effect of OVPCM depended on the genotype, having a greater effect on the medium-low responding cultivar

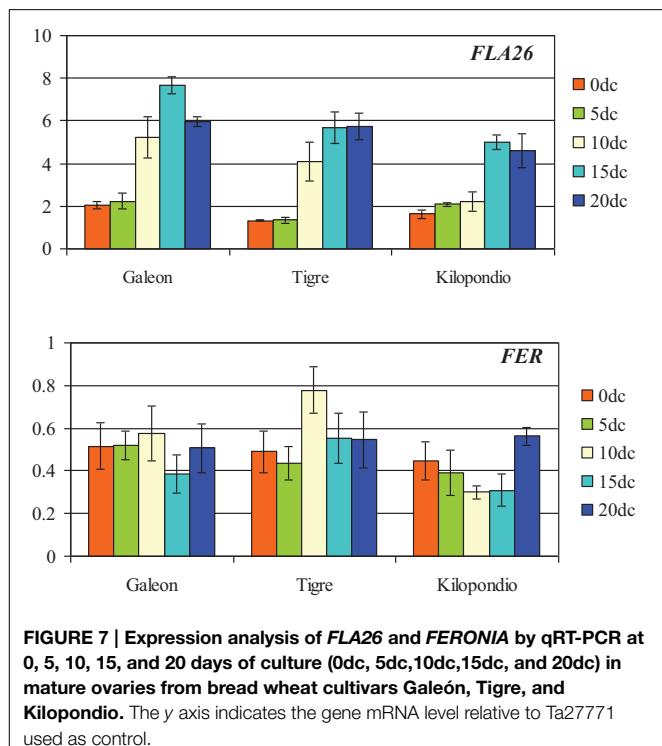


FIGURE 7 | Expression analysis of *FLA26* and *FERONIA* by qRT-PCR at 0, 5, 10, 15, and 20 days of culture (0dc, 5dc, 10dc, 15dc, and 20dc) in mature ovaries from bread wheat cultivars Galeón, Tigre, and Kilopondio. The y axis indicates the gene mRNA level relative to Ta27771 used as control.

Caramba than in the high responding cultivar Pavon. Thus, OVPCM raised the number of green plants up to 16 times and the percentage of regeneration 4 times in Caramba. These data are in accordance with those reported in microspores culture, showing a greater improvement in recalcitrant genotypes (Hu and Kasha, 1997; Zheng et al., 2002). Conversely, the study by Broughton (2008) in anther culture showed no interaction between the effect of ovary co-culture and anther genotype.

Previously, not much attention has been paid to the effect of the ovary genotype on isolated microspore culture in wheat, as the initial studies concluded that a wide range of genotypes were effective, and no clear differences between genotypes were shown (Bruins et al., 1996; Liu et al., 2002; Zheng et al., 2002). Thus, the ovaries from the same spikes from which the anthers are excised have traditionally been used for co-culture or OVCM. Surprisingly, in this study, a significant effect of the ovary genotype on microspore embryogenesis efficiency was observed. Caramba ovaries greatly increased the number of embryos and green plants and the percentage of green plants as compared to Pavon ovaries. As far as we know, only two references have previously suggested ovary genotype differences in the inductive effect on isolated microspore culture. Firstly, Mezja et al. (1993) mentioned the low capacity of Pavon ovaries in microspore induction, and secondly, Bruins et al. (1996) indicated that ovaries of one cultivar produced higher rates of microspore viability as compared to a mixture of ovaries from four cultivars.

It is well-known that microspores should be at a mid-late uninucleate stage to induce microspore embryogenesis efficiently (Datta, 2005). For ovary co-culture and pre-conditioned medium, immature ovaries from the same flowers that are used for anther culture have traditionally been used (Mezja et al., 1993;

Hu and Kasha, 1997; Puolimatka and Pauk, 1999; Broughton, 2008). However, Zheng et al. (2002) indicated that ovaries from flowers containing early uninucleate to binucleate microspores were all effective in promoting microspore embryogenesis. In this study we have demonstrated that the ovaries' inductive effect depends on the developmental stage. Co-culture with mature ovaries enhanced the number of embryos and green plants by 25–46% respectively as compared to immature ovaries in both cultivars of wheat. The percentage of green plants was also significantly higher with mature ovaries. These results differ from those reported by Lu et al. (2008) showing that barley microspores co-cultured with florets containing microspores at uninucleate stage produced a higher number of embryos than those co-cultured with florets at binucleate stage. In our study, the effect of the ovary developmental stage depended on the ovary genotype. Immature ovaries from Pavon produced half as many green plants as mature Pavon ovaries, whereas mature ovaries from Caramba rendered only a slightly higher number of green plants than immature ovaries. Overall, the highest number of green plants was obtained with mature Caramba ovaries.

Taking into account that anther culture efficiency depends on the ovary genotype, it would be desirable to identify a donor ovary genotype that could produce the maximum induction rate and that could be used universally for co-culture in wheat anther culture. Therefore, ovaries from three agronomically important cultivars in Spain were compared with Caramba ovaries for OVPCM, obtaining a similar number of embryo and green plants with all. Unexpectedly, Tigre ovaries induced the highest number of DH plants, due to a high percentage of spontaneous chromosome doubling. It has been suggested that spontaneous doubling takes place by a nuclear fusion mechanism during stress treatment and culture in barley (Kasha et al., 2001; González-Melendi et al., 2005). Thus, “nurse factors” from Tigre ovaries seem to promote nuclear fusion during culture. The rates of spontaneous doubling have been described to be affected by the genotype, the type of stress treatment and the stage of the microspores at the time of culture (for review see Castillo et al., 2009). To our knowledge, this is the first time that the ovary genotype used for OVPCM has been reported to greatly affect chromosome doubling, however more studies are needed to clarify the mechanism through which Tigre ovaries lead to an increased nuclear fusion.

Identification of Candidate Genes Associated to the Ovary Inductive Effect in OVPCM on Microspore Embryogenesis

Since early studies showed that the use of “nurse cultures” enhanced differentiation and proliferation in several systems (see review Boutilier et al., 2005), attempts have been made to identify these “nurse factors.” The nature of these compounds, when using ovary co-culture and/or ovary preconditioned medium systems, has been discussed extensively although few candidates have been recognized until now (Borderies et al., 2004; Asif et al., 2014). The differences in microspore embryogenesis efficiency due to ovary genotype and developmental stage found in this study were decisive in identifying the molecular mechanisms associated with the inductive effect of the ovaries. Five genes were

selected as candidate genes among those connected with ovary biological functions, ovary metabolism during the co-culture, microspore embryogenesis or cell wall modification.

Three of the candidate genes, *TAA1b*, *FLA26*, and *WALI6* have been associated with wheat microspore embryogenesis response (Sánchez-Díaz et al., 2013). *TAA1b* was induced in the first stages of microspore embryogenesis when most of the microspores presented a star-like morphology, whereas *FLA26* and *WALI6* were induced at later stages, in multicellular structures confined inside the exine wall. However, *TAA1b*, that encoded a fatty acyl-CoA reductase involved in signaling (Wang et al., 2002), and *WALI6*, a cysteine-rich serine protease inhibitor induced by wounding or metal stress (Richards et al., 1994), showed expression patterns that could not be associated to the inductive effect of the ovaries. In this sense, *TAA1b* was only strongly induced in Pavon and *WALI6* in mature ovaries of both genotypes. Another gene that exhibits an expression pattern that was not associated with the inductive effect was *CGL1*. This gene is involved in the biosynthesis of complex glycans that are part of membrane glycoproteins and secretory proteins (von Schaewen et al., 2008) and showed the highest level of expression in immature Pavon ovaries, which had the lowest inductive effect. Finally, the expression of *FLA26* and *FER* showed a pattern characterized by having a higher level in Caramba than in Pavon ovaries at the onset of culture. In addition, the expression levels of both genes increased during culture, reaching a maximum at 15 days and being higher in Caramba mature ovaries, which showed the highest induction rate. Therefore, this pattern could be associated to the inductive effect of the ovaries. Nevertheless, some differences should be highlighted as *FER* expression levels were almost 10 times lower than *FLA26*, and *FER* expression was maintained or increased slightly during culture whereas *FLA26* showed a higher level at 15–20dC.

The period at which the “nurse factors” should be present in the medium has been widely studied (Hu and Kasha, 1997; Puolimatka and Pauk, 1999; Zheng et al., 2002). It has been established that the effect of the ovaries begins between 5 and 10 days of co-culture and that this initial effect is related to the rates of dividing microspores or maintenance of embryogenic development. However, the number, size and embryos frequency increase after 21 days of co-culture (Hu and Kasha, 1997). Thus, the beneficial effect of ovary factors extends throughout the induction culture period, probably due to the presence of different “nurse factors” with different functions (Hu and Kasha, 1997) that meet the requirements of the induced microspores at each stage of the embryonic development. In this sense, the induction of a different set of functional genes throughout wheat microspore embryogenesis has been described recently (Sánchez-Díaz et al., 2013).

The receptor-like kinase FERONIA (*FER*), which belongs to the CrRLK1L-1 subfamily, was originally identified for its role as a communication factor during fertilization acting in termination of pollen tube growth (Huck et al., 2003; Rotman et al., 2003; Escobar-Restrepo et al., 2007). Therefore, its function would fit with the idea that ovary factors are related to ovary biological functions as suggested by Mezja et al. (1993). However, other receptor-like kinase, ERECTA, associated with cell-cell signaling

related to cell proliferation and growth in ovaries, anthers and embryos (Shpak, 2013) was only expressed in fresh ovaries at 0 days of culture (data not shown).

In recent years, new data suggest that FER plays a more general role in complex processes, intervening as a master regulator of cell growth (Yu et al., 2012, 2014; Duan et al., 2014). These data are consistent with the fact that the “nurse factors” were not ovary-specific (Puolimatka and Pauk, 1999). FER could act in signal transduction as a co-receptor to enhance the activity of other receptor kinase, or as receptor of the peptide secreted RALF (rapid alkalinization factor) (Haruta et al., 2014; Kessler et al., 2015). It has been reported that FER participates in a feedback signaling network in growing cells but also in stress conditions, through an interplay with the brassinosteroid signaling (Guo et al., 2009; Hofte, 2015). In this network, the cell wall secretions promote cell expansion, leading to the activation of mechanoreceptors that trigger an increase in cytosolic Ca^{2+} , alkalinization of the apoplast, ROS production and growth inhibition (Rojas et al., 2011). All components of this signaling network are important to microspore embryogenesis induction. Increased Ca^{2+} concentration resulted in a higher osmolality and a larger number of embryo-like structures and plant production (Hoekstra et al., 1997). A shift toward alkalinization upon embryogenesis induction could be behind the cytoskeletal rearrangements (Pauls et al., 2006). Žur et al. (2014) suggested that a certain H_2O_2 threshold was important for successful microspore embryogenesis induction, and ROS-induced signal transduction has been associated with the stress treatment (Jacquard et al., 2006; Žur et al., 2009). Finally, the incorporation of brassinosteroids in the culture medium of *Brassica* microspores significantly increased the percentage of embryogenesis (Ferrie et al., 2005).

Compounds secreted by the cell wall are also involved in microspore embryogenesis. In fact, these compounds have been among the first to be associated with the induction effect of ovary co-culture and/or ovary preconditioned medium (Borderies et al., 2004; Matthys-Rochon, 2005). Among them, AGPs, a large family of cell surface hydroxyproline-rich glycoproteins (HRGPs), were found to induce wheat microspore embryogenesis (Letarte et al., 2006). *FLA26* encoded for a fasciclin-like arabinogalactan protein (FLAs), a subclass of AGPs that contains an AGP-like glycosylated domain and two fasciclin-like domains (Faik et al., 2006). The FLAs are widely distributed in various plants but little information is available as compared to

other AGP families. Nevertheless, FLAs are known to be involved in cell-to-cell communication associated with plant growth and development (Johnson et al., 2003; Shi et al., 2003; Faik et al., 2006; Zang et al., 2015). The *FLA26* gene was highly expressed in microspore embryogenesis, fresh anthers, excised embryo and roots, indicating a general function in wheat (Sánchez-Díaz et al., 2013). However, other *FLAs* genes (*FLA25* and *FLA14*) that were also expressed in microspore embryogenesis induction, were not expressed in ovaries in culture (data not shown). Also, it is known that different AGPs are expressed in reproductive tissues (Cheung et al., 1995; Coimbra et al., 2008). Accordingly, in this study the two *AGPs* analyzed (*AGP5* and *AGP9*) were expressed in fresh ovaries but not during culture (data not shown).

In this study we have demonstrated that the use of ovary pre-conditioned medium and ovary co-culture increased the efficiency of green doubled haploid plant production in bread wheat anther culture. In addition, ovary genotype and stage of development are critical factors, increasing embryogenesis induction and chromosome doubling percentages at early stages of microspore embryogenesis, and enhancing green plant regeneration in later stages. A fasciclin-like arabinogalactan protein, *FLA26*, and the ovary signaling gene *FERONIA* have been associated with the inductive effect of the ovaries. Our results represent a breakthrough in identifying the molecular mechanisms associated with microspore embryogenesis induction, and suggest new approaches to increase the efficiency of anther culture that could have special relevance in bread wheat plant breeding.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00402/abstract>

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Current insights into hormonal regulation of microspore embryogenesis

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Plant growth regulator (PGR) crosstalk and interaction with the plant's genotype and environmental factors play a crucial role in microspore embryogenesis (ME), controlling microspore-derived embryo differentiation and development as well as haploid/doubled haploid plant regeneration. The complexity of the PGR network which could exist at the level of biosynthesis, distribution, gene expression or signaling pathways, renders the creation of an integrated model of ME-control crosstalk impossible at present. However, the analysis of the published data together with the results received recently with the use of modern analytical techniques brings new insights into hormonal regulation of this process. This review presents a short historical overview of the most important milestones in the recognition of hormonal requirements for effective ME in the most important crop plant species and complements it with new concepts that evolved over the last decade of ME studies.

Keywords: crop species, hormonal regulation, microspore embryogenesis, plant growth regulators, phytohormone crosstalk

Introduction

Plant growth regulators are known as key signaling molecules controlling plant growth and development, and initiating signal transduction pathways in response to environmental stimuli (Kohli et al., 2013). The role played by PGRs in ME has been examined widely but usually using the traditional 'one-factor-at-a-time' and 'trial-and-error' techniques. Hormonal requirements determined through such empirical methods were usually optimized for particular cultivars or genotypes. Once identified, positively acting combinations of PGRs were usually used standardly for years with small modifications introduced in the case of less responsive genotypes. Only in a few cases endogenous levels of PGRs were analyzed and taken into consideration in studies examining their influence on ME effectiveness (Dollmantel and Reinert, 1980; Delalonde and Coumans, 1998; Gorbunova et al., 2001; Lulsdorf et al., 2012). Moreover, usually only one or two

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; AZI, 7-azaindole; BAP, 6-benzylaminopurine; BL, brassinolide; BR, brassinosteroid; CPIBA, chlorophenoxyisobutyric acid; DIC, 3,6-dichloro-2-methoxybenzoic acid (dicamba); 2,4-D, 2,4-dichlorophenoxyacetic acid; EBr, 4-epibrassinolide; ETP, Ethephon; GAs, gibberellins; GA₃, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; 2iP, N6-(2-isopentenyl)-adenine; JA, jasmonic acid; ME, microspore embryogenesis; NAA, naphthalene-1-acetic acid; 1-NOA, 1-naphthoxyacetic acid; NPA, N-1-naphthylphthalamic acid; OCPIB, o-chlorophenoxy-isobutyric acid; PAA, phenylacetic acid; PCIB, p-chlorophenoxyisobutyric acid; PGR(s), plant growth regulator(s); PIC, 4-amino-3,5,6-trichloropicolinic acid (picloram); TDZ, N-phenyl-N'-1,2,3-thiadiazol-5-ylurea (thidiazuron); mT, 6,3-hydroxybenzylaminopurine (metatopoline); KN, N6-furfuryladenine (kinetin); SA, salicylic acid; TIBA, 2,3,5-triiodobenzoic acid; Z, zeatin; ZR, zeatin riboside.

groups of phytohormones were analyzed whereas there is growing evidence indicating that – as could be expected – it is the complex PGR crosstalk and its interaction with the plant's genotype and environmental factors which controls the initiation and the course of the process. The complexity of the PGR network which could exist at the level of biosynthesis, distribution, gene expression, or signaling pathways, renders the creation of an integrated model of ME-control crosstalk impossible at present. However, the analysis of the published data together with the results received recently with the use of modern analytical techniques bring new insights into hormonal regulation of this process. New concepts that evolved over the last decade of ME studies together with a short historical review showing the most important milestones in the recognition of hormonal requirements for effective ME are presented below. The review concerns the most important crop plants, both model species, and species well-known for their recalcitrance to most *in vitro* approaches like oat, rye, grain legumes, and cassava (Supplementary Table S1).

Auxins and Cytokinins

Particularly important for *in vitro* cultures is the concerted action of auxins and cytokinins which control cell division and morphogenesis. These two hormone groups usually act antagonistically but their effects are modulated by plant genome and tissue specificity (Moubayidin et al., 2009). Various combinations of auxins and cytokinins have been used in media designated for *in vitro* anther culture, whereas in the majority of isolated microspore cultures exogenous PGRs were not required for ME initiation (for details see Supplementary Table S1). For several plant species instead of exogenous PGRs, co-culture with so-called immature 'ovaries' (accurately, pistils) is critical to sustain microspore-derived embryo development (Hul and Kasha, 1997; Li and Devaux, 2001; Zheng et al., 2002; Lantos et al., 2009). Similar or even better results could be received through the use of conditioned medium, prepared by culturing isolated 'ovaries' or microspores of responsive plant genotypes. In isolated microspore culture of recalcitrant wheat cultivars (Zheng et al., 2002) live 'ovary' co-culture alone was not effective, while the addition of medium preconditioned by 'ovaries' increased the yield of microspore-derived embryos more than 100-fold. Similarly, conditioned medium extracted from actively growing microspores of barley broke the recalcitrancy of isolated oat microspores and resulted in regeneration of fertile green plants (Sidhu and Davies, 2009). Despite many attempts, the effect of 'ovary' co-culture could not be successfully substituted by any treatment or any exogenously applied substance. It is supposed that the 'ovaries' are a sources of active signaling molecules that increase microspore-derived embryo yield and improve green plant regeneration. The involvement of auxin-like substances and/or arabinogalactans (Baldwin et al., 1993; Borderies et al., 2004; Letarte et al., 2006) has been postulated, but the mechanism of their influence remains unexplained.

Among auxins, the first and most widely used for ME initiation was IAA (Guha and Maheshwari, 1964). It is

an essential phytohormone ubiquitous throughout the plant kingdom and involved in the regulation of a wide spectrum of physiological processes (Davies, 2010). Later on it was frequently replaced by more stable synthetic auxin analogs: 2,4-D, NAA, DIC or PIC and its combinations (for details see Supplementary Table S1; Table 1). 2,4-D is one of the most often used culture media supplements, applicable for both dicotyledonous and monocotyledonous plants (Raghavan, 2004). Its high effectiveness in the induction and maintenance of callus and suspension cultures from somatic tissues resulted in its application as ME stimulus. Other synthetic auxin analogs were also first tested in somatic tissue cultures as inducers of embryogenesis (PIC, DIC) or organogenesis (NAA; Gaspar et al., 1996). Natural auxins: IBA and PAA are used less frequently (for details see Supplementary Table S1). For decades, IBA has been used commercially for plant propagation, being more effective than IAA in stimulation of adventitious root formation. Its effectiveness can be at least partially explained by its higher stability and lower predisposition to the formation of inactive conjugates. Its possible direct involvement in ME initiation has been postulated recently (Dubas et al., 2013a). Similarly, PAA was applied mainly for stimulation of plant regeneration, its beneficial effects on androgenic plant production having been reported for wheat and barley (Ziauddin et al., 1992). In addition to acting as an active auxin, PAA may inhibit polar auxin transport (Morris and Johnson, 1987), regulating the level of free IAA. In plants, it is present at levels 10- to 100-fold lower in comparison with IAA. Due to its low activity (Normanly et al., 2010), it is usually supplemented in much higher concentrations to media used for ME (Table 1).

TABLE 1 | The most popular PGRs and their concentration ranges [mg l⁻¹] used standardly in media dedicated for ME induction and microspore-derived embryo regeneration.

PGRs	Induction media	Regeneration media
Auxins and synthetic auxin analogs		
IAA	1–4	0.01–3.5
IBA	0.5–1	1–2
PAA	1–100	1–4
2,4-D	0.1–8	0.5–3
NAA	0.5–2.5	0.05–5
Dicamba	0.1–2.5	-
Picloram	0.07–4	-
Anti-auxin and auxin transport inhibitors		
PCIB	1–5	-
TIBA	0.05–2	0.1–1
Cytokinins		
BAP	0.05–3	0.1–5
Kinetin	1	0.1–5
Z/ZR	0.1–1	0.5–2.2
TDZ	0.1–1	-
2IP	0.0001–0.4	0.1
Other PGRs		
GA ₃	0.001–5	0.01–0.1
ABA	0.001–10	0.05–3

For more details see review in Supplementary Table S1.

Interestingly, besides auxins, several inhibitors of auxin biosynthesis (AZI) or auxin polar transport TIBA, NPA, or 1-NOA as well as anti-auxins OCPIB, PCIB have been used quite frequently (for details see Supplementary Table S1; **Table 1**). All these chemicals influence embryo development through disruption of auxin homeostasis (Lankova et al., 2010). However, depending on the plant species, type of substance and procedures used, the results were ambiguous or even contradictory. For example, preculture of *Nicotiana tabacum* anthers in the presence of the inhibitor of AZI and anti-auxin (OCPIB) resulted in enhanced plantlet formation (Dollmantel and Reinert, 1980). In contrast, IAA-oxidase activator, CPIBA, IAA transport inhibitor (quercetin), and IAA-oxidase inhibitor (dopamine) did not give positive results in maize anther cultures (Delalonde and Coumans, 1998). Due to structural similarity, anti-auxins (Jönsson, 1961) can compete with IAA at the binding site of its receptors (McRae and Bonner, 1953) and exhibit some antagonistic effects. Lower concentration of PCIB (20 μ M) enhanced the development of microspore-derived embryos of *Brassica juncea* and *B. napus*, while higher doses were detrimental and resulted in a high frequency of morphologically abnormal embryo formation (Agarwal et al., 2006; Ahmadi et al., 2012). In *B. rapa*, critical ME-stimulating concentration of PCIB was twofold higher (40 μ M), but similarly its overdose decreased microspore-derived embryo yield and increased the frequency of morphological abnormalities (Zhang et al., 2011). The effect of TIBA (1 μ M), which conjugates specifically to the ingestion site and inhibits polar transport of IAA, on barley ME was highly genotype-dependent (Cistué et al., 1999). Although it decreased the number of dividing microspores in some cultivars, a tendency to produce a higher percentage of embryos and to improve embryo quality was also observed. Higher doses (2–4 μ M) of TIBA were beneficial for low responsive cultivar, increasing well developed embryo production and reducing albinism. TIBA could also affect later phases of microspore-derived embryo development. The treatment applied to *B. napus* cv. Topas at the preglobular/globular stages of embryo development resulted in altered shoot apical meristem development and in production of one fused cotyledon, which indicates a continuation of radial symmetry (Ramesar-Fortner and Yeung, 2006). However, tillers pre-treatment with 5 μ M PCIB or 10 μ M TIBA had no effect on ME induction in triticale anther culture (Žur et al., 2015). Similar to TIBA in the report of Cistué et al. (1999), PCIB stimulated plant regeneration but only in the highly recalcitrant triticale genotype. The supplementation of ME-induction medium with the same concentrations of TIBA or PCIB did not improve the efficiency of the process in the case of the recalcitrant genotype and significantly decreased the number of microspore-derived embryos produced by the responsive one (Žur et al., 2015).

The negative effect of NPA on embryogenesis was observed in microspore suspension of oak (Rodriguez-Sanz et al., 2014). This compound together with 1-NOA are potent synthetic auxin inhibitors (Lankova et al., 2010). It was proved that NPA strongly inhibits auxin efflux (Petrásek et al., 2003), whereas 1-NOA blocks both auxin influx and efflux. NPA interferes with actin dynamics being under the control of auxin itself, while 1-NOA action has been suggested to be related to the dynamics of

membrane vesicle transporting auxin carriers (Titapiwatanakun and Murphy, 2009; Lankova et al., 2010).

Two major properties of cytokinins that predispose these adenine derivatives for *in vitro* cultures are their abilities to induce cell division and differentiation. Their effects result from co-action with auxins, but each of these PGR groups seems to control different phases of the cell cycle: auxins – DNA replication, whereas cytokinins – mitosis and cytokinesis (Gaspar et al., 1996). Among cytokinins, kinetin (N6-furfuryladenine; KN), 6-benzylaminopurine (BAP), and zeatin (Z) have been frequently tested both in ME induction and regeneration media (for details see Supplementary Table S1; **Table 1**). Other kinds of cytokinins, like thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea; TDZ), N6-(2-isopentenyl)-adenine (2iP) or metatopoline (6-3-hydroxybenzylaminopurine, mT) are less popular ingredients of culture media (Kumar et al., 2003; Grewal et al., 2009; Esteves et al., 2014). TDZ has been used successfully *in vitro* to induce adventitious shoot formation and to promote axillary shoot proliferation. It is particularly effective with recalcitrant woody species. However, prolonged exposure to this cytokinin may cause problems such as hyperhydricity and abnormal shoot or root development (Lu, 1993). 2iP was used for ME initiation in anther culture of *Cicer arietinum* (Grewal et al., 2009), whereas regeneration medium supplemented with mT proved beneficial for green plants production in microspore culture of barley (Esteves et al., 2014).

Abscisic Acid

Besides auxins and cytokinins, ABA, known as a ubiquitous plant stress hormone, has been claimed to play a role in ME-inducing signal transduction system (Maraschin et al., 2005; Žur et al., 2012, 2015; Dubas et al., 2013b; Ahmadi et al., 2014). It is well documented that plant cells and tissues usually increase their ABA level in response to different biotic and abiotic stresses (Zeevaart and Creelman, 1988; Christmann et al., 2004; Cutler et al., 2010). During ME induction various stress conditions (e.g., starvation, cold, osmotic stresses) have been commonly used as a trigger of microspore switch toward sporophytic development pathway (Touraev et al., 1997; Zoriniants et al., 2005). ABA level increases in tissues and microspores exposed to these stresses and many reports have suggested a causal involvement of ABA in ME induction, describing the positive influence of ABA accumulation on the effectiveness of this process (Reynolds and Crawford, 1996; van Bergen et al., 1999; Wang et al., 1999; Žur et al., 2008, 2012). Furthermore, a positive relationship has been shown to exist between higher regeneration efficiency and increased endogenous ABA level during ME induction by osmotic stress in barley (Hoekstra et al., 1997; van Bergen et al., 1999). These positive ABA effects on ME have been confirmed in several manipulative experiments with a treatment with exogenous ABA or its inhibitor fluridone (Imamura and Harada, 1980; Reynolds and Crawford, 1996; Wang et al., 1999; Guzman and Arias, 2000). In *Hordeum* species the addition of 10⁻⁷ M ABA enhanced the regeneration of plants at sub-optimal anther pre-treatment conditions, while ABA-biosynthesis inhibitor fluridone

strongly reduced regeneration efficiency, particularly green plant production (Hoekstra et al., 1997; van Bergen et al., 1999). In another study of rapeseed, ME efficiency was improved by exogenous ABA treatment – 0.5 mg ABA l⁻¹ for 12 h enhanced ME threefold compared with untreated cultures and increased normal plantlet regeneration by 68% (Ahmadi et al., 2014). In turn Žur et al. (2008, 2012) observed a significant endogenous ABA increase during ME induction by low temperature stress in triticale. However, there was no linear relationship between the extent of ABA accumulation and ME efficiency in the population of 72 triticale DH lines (Žur et al., 2012). On the contrary, higher level of endogenous ABA significantly diminished green plant regeneration efficiency. Therefore, it seems that the induction of ME requires a certain genotype-specific threshold level of ABA, which initiates a signaling cascade switching the program of embryogenic development. Moreover, it seems that a specific PGRs homeostasis and auxins/cytokinins/ABA crosstalk is a more important prerequisite for effective ME than the level of individual PGRs (Žur et al., 2015). It has also been discovered that microspores' membrane fluidity may indirectly affect the level of ABA accumulation within the cell (Dubas et al., 2013b). Those findings verified the hypothesis about the influence of ABA on ME induction in rapeseed and pointed out that increased ABA concentration (to about 2.1 μM) in heat-treated microspores enhanced ME. Altogether, the role of ABA in microspore reprogramming is complex – it acts as a common anti-stress factor increasing microspores' viability during ME induction, and on the other hand, ABA-induced signaling cascade plays a vital role in the activation of many genes (mainly controlling the synthesis of LEA proteins), in the activity of enzymes and in the ox-redox status as well as interacts with other PGRs (Maraschin et al., 2005; Žur et al., 2012, 2014, 2015; Ahmadi et al., 2014).

Other Plant Growth Regulators

Despite many studies on microspore and anther culture in crop species, the effects of phytohormones such as gibberellins (GAs), brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), or ethylene on ME are not fully recognized.

GAs are involved in a wide range of developmental responses (Moshkov et al., 2008). They are required for normal pollen, anther and seed development, and are probably involved in a broad spectrum of responses to abiotic stress (Colebrook et al., 2014), but a complete understanding of their specific function remains elusive (Swain and Singh, 2005). Only scarce information is available on GAs in cultured cells. Plant tissue cultures can generally be induced to grow and differentiate without GAs. One of the most bioactive forms, GA₃ is generally used in plant tissue to stimulate stem elongation. It was also supposed to be an essential ingredient of media for culturing cells at low densities (Stuart and Street, 1971). In microspore cultures of *B. napus* and *Solanum tuberosum*, GA₃ improved plantlet regeneration (Supplementary Table S1), mainly via elongation of the embryo axis and acceleration of its maturation (Haddadi et al., 2008). Similarly Ahmadi et al. (2012) reported that the highest percentage of normal *B. napus* plantlet regeneration

(40%) was received as a result of 0.05–0.1 mg l⁻¹ GA₃ treatment. More attention has been attracted by a wide range of synthetic substances, called 'anti-gibberellins,' which block GAs biosynthetic pathways. In the studies of Biddington et al. (1992), the addition of paclobutrazol into induction media inhibited embryo production in anther cultures of Brussels sprout. However, the authors suggested that this effect could be caused not only by inhibition of GA-biosynthesis but also by inhibition of sterol biosynthesis. Another inhibitor of GA-biosynthesis, uniconazole, applied to *B. napus* embryo at the globular stage of development significantly reduced axis elongation (Hays et al., 2002).

Brassinosteroids (BRs) are a class of plant steroidal hormones that regulate multiple developmental and physiological processes essential for plant growth and development. Their involvement in cell elongation and division, vascular differentiation, senescence, flowering time, male fertility, pollen development, seed size, photomorphogenesis, and resistance to biotic and abiotic stresses has been reported (Clouse et al., 1996; Li and Chory, 1999; Ye et al., 2010; Clouse, 2011). BRs, in particular 24-epibrassinolide (EBr), increased frequency of induction of both somatic (Azpeitia et al., 2003; Pullman et al., 2003) and ME (Ferrie et al., 2005; Malik et al., 2008). The role of EBr may be related to protection against abiotic stresses as its positive impact on the acquisition of thermotolerance was reported (Divi et al., 2010). Other BR, brassinolide (BL) also enhanced embryogenesis and the quality of microspore-derived embryos in *B. napus* and *B. juncea* (Ferrie et al., 2005; Belmonte et al., 2010). The addition of BRs did not affect plant regeneration but seems to influence chromosome doubling. Moreover, depletion of cellular BL decreases microspore-derived embryo production and disrupts the architecture of the apical meristems of *B. napus* (Belmonte et al., 2010).

As ME is induced by stress treatment it could be supposed that not only ABA, but also other stress hormones like jasmonic acid (JA), salicylic acid (SA), or ethylene can be involved in this process.

JA is widely distributed in the plant kingdom and regulates a wide range of processes from growth and photosynthesis to reproductive development. The most important is the role connected with plant defense reactions against biotic and abiotic stresses (Santino et al., 2013). In anther cultures of barley, ME-induction treatment resulted in higher expression of three genes encoding enzymes involved in JA biosynthesis (Jacquard et al., 2009). Ahmadi et al. (2014) claimed that the supplementation of induction medium with 1.0 mg l⁻¹ JA for 24 h improved embryo yield in microspore cultures of *B. napus*. Moreover, the addition of 0.5 mg l⁻¹ JA for 12 h resulted in better plantlet regeneration.

SA, a plant phenolic derivative, is now considered to be a hormone-like endogenous regulator and its role in the defense mechanisms against biotic and abiotic stress is well documented (Catinot et al., 2008). Being a mobile molecule, SA is capable of acting as a cell signal that senses, amplifies, and transmits information initiating the embryogenic program (Mulgund et al., 2012). There are several papers that describe the application of SA to culture media in order to improve ME efficiency. In the above-mentioned work, Ahmadi et al. (2014) reported

a positive effect of short-term application (6 h) of 0.2 and 0.5 mg l⁻¹ of SA on *B. napus* microspore-derived embryo yield. The mechanism of SA action could be connected with its ability to increase the activity of superoxide dismutase (H₂O₂-producing enzyme), and to inhibit ascorbate peroxidase and catalase activities (H₂O₂-decomposing enzymes), thus leading to endogenous H₂O₂ accumulation, which is supposed to initiate ME (Larqué-Saavedra, 1978, 1979; Leslie and Romani, 1988; Luo et al., 2001; Žur et al., 2014).

Ethylene is a gaseous plant hormone involved in many developmental processes – seed germination, root development, flower senescence, abscission, and fruit ripening (Kumar et al., 2009). Its biosynthesis is tightly regulated by internal signals and environmental stresses, like wounding, low temperature, hypoxia, or pathogen attack (Wang et al., 2002). Its role in *in vitro* callus growth, organo- and embryogenesis has been suggested (Kumar et al., 2009). It was reported that embryogenesis in barley can be stimulated by both promoters and antagonists of ethylene, depending on the genotype (Cho and Kasha, 1989). It suggests that the response depends upon how much ethylene is being produced and that an optimum level of ethylene is required for ME initiation. More often, positive effects induced by substances known as inhibitors of ethylene action – silver nitrate (Prem et al., 2005), activated charcoal (Prem et al., 2008), AVG or cobalt chloride (Leroux et al., 2009) were observed. On the other hand, there is also evidence reporting benefits from ethylene precursor ACC or promoter ETP. Their application increased ME initiation in anther culture of barley (Evans and Batty, 1994) and oat (Kiviharju et al., 2005).

New Concepts Describing PGRs Involvement in ME Regulation

New Kinds of PGRs Possibly Involved in ME Regulation

Although IBA is commonly considered to be only an IAA precursor and storage form (Woodward and Bartel, 2005; Korasick et al., 2013), some evidence suggests that it could act directly as an active auxin (Ludwig-Muller, 2000; Poupart and Waddell, 2000; Zolman et al., 2000). Recent results shown by Dubas et al. (2013a) suggest that the increased level of IBA in *B. napus* microspores under heat shock treatment might be used as a marker of cell embryogenic competence. However, IBA accumulation was not sufficient for ME initiation in the case of recalcitrant genotypes. Similar results were received in anther cultures of triticale (Žur et al., 2015), where higher concentration of IBA seems to be advantageous for effective ME induction. However, because IBA pool in triticale anthers comprises only about 1% of the total auxins content, it is questionable whether differences in its concentration are of any significance. In the same report, *trans* and *cis* isomers of *tZ*, *cZ* and *tZR*, *cZR* were detected in anthers of eight DH lines of triticale. Interestingly, *cZ* commonly regarded as cytokinin derivative without any or with low biological activity, prevailed significantly and positively correlated with ME induction. Similarly, in reports of Emery et al. (1998), Vyrubalová et al. (2009), and Kudo et al. (2012) *cZ*

appeared to be the dominant form of cytokinins in specific plant organs and/or stages of development. In a recently published report, Gajdošová et al. (2011) concluded that *cZ* can be qualified as a regulator of cytokinin responses in plants under growth-limiting conditions. Another finding of Žur et al. (2015) was a relatively high concentration of KN-like compound and its negative correlation with ME efficiency. KN was the first compound identified as cytokinin, but for many years it was classified as a product of DNA rearrangement not produced by plant cells. This opinion started to change in the last decade, as sources of KN in biological samples were found in cellular DNA, plant tissues and extracts (Barciszewski et al., 1996, 1999, 2007; Ge et al., 2005). The role of endogenous KN and the molecular mechanisms of its action are not well understood, although some data indicate its strong antioxidant properties and some ABA- and JA-antagonistic effects (Barciszewski et al., 2000).

High Concentrations of PGRs as a Stress Factor

Higher concentration (5–10 mg l⁻¹) of 2,4-D stimulated ME initiation in some recalcitrant plant species, namely oat (Kiviharju and Tauriainen, 1999), *Triticum turgidum* (Jauhar, 2003), and cassava (Perera et al., 2014). It has been suggested that 2,4-D is not only an auxin analog but at higher concentrations acts as a stress factor effectively triggering embryogenic pathway of cell development (Gaj, 2004). The observed effect is probably the result of concerted PGRs action as evidence that 2,4-D regulates the activity of genes associated with auxin, ABA and ethylene biosynthesis has been reported (Raghavan et al., 2006). Short-term treatment with extremely high concentration of this substance (15–45 mg l⁻¹ 2,4-D for 15–45 min) has been recently used as an effective substitute of classical heat shock treatment (Ardebili et al., 2011) for *B. napus* ME initiation. It has been proposed as an alternative for plant species whose microspores are extremely sensitive to classical stresses.

Endogenous Level of PGRs and their Interaction with their Exogenously Applied Analogs

Inconsistent or even contradictory effects of various PGRs and their inhibitors suggest that the endogenous level of natural phytohormones and its balance with exogenously applied ones can be crucial both for yield and quality of microspore-derived embryos.

The first report pointing out that endogenous auxins level can determine anther culture responsiveness was published as early as by Dollmantel and Reinert (1980). Next, the results published by Gorbunova et al. (2001) indicated that wheat genotypes with high endogenous IAA content required lower concentration of 2,4-D in induction medium. Other data showing that anti-auxin (PCIB) and auxin transport inhibitor (TIBA) can stimulate microspore-derived embryo formation, probably due to overcoming the inhibitory effect of high auxin concentration, were published by Cistué et al. (1999) and Agarwal et al. (2006). Also in the case of triticale (Žur et al., 2015), for which ME-induction medium was supplemented

with 1 mg l^{-1} DIC, 1 mg l^{-1} PIC, and 0.5 mg l^{-1} KN, anther cultures of responsive DH lines were characterized by significantly lower endogenous/exogenous auxins ratio in comparison to recalcitrant genotypes. In the same cultures, higher embryogenic potential was associated with significantly higher endogenous/exogenous cytokinins ratio.

Generally, exogenous PGRs are not required for ME in *Brassica* species. However, too low levels of endogenous auxins and/or cytokinins could disturb the proper course of the process, especially the transition from the radial to the bilateral microspore-derived embryo symmetry, as it was observed in *B. napus* (Ramesar-Fortner and Yeung, 2006). Similarly, the addition of $0.1\text{--}0.3 \text{ mg l}^{-1}$ BAP significantly improved microspore-derived embryo yield in several *B. rapa* subspecies (Takahashi et al., 2012). Recently, Prem et al. (2012) and Dubas et al. (2014) showed how endogenous auxin distribution influenced embryo development in microspore suspension of *B. napus*. Precise endogenous auxin estimation in transgenic DR5rev::GFP or DR5::GUS microspores of highly embryogenic spring rape line (Dubas et al., 2014) revealed IAA concentration at $1.01 \mu\text{M}$ in microspores under ME-initiating heat treatment (1 day at 32°C). It could be supposed that such IAA concentration is optimal for further embryo development.

Crosstalk of Various PGRs and their Interaction with Stress Treatment and Plant Genotype in ME Initiation

A number of reviews highlighting phytohormone crosstalk in plant growth, development and response to abiotic and biotic stresses have been published recently (Depuydt and Hardtke, 2011; Hou et al., 2013; Kohli et al., 2013; Lyons et al., 2013; Wang et al., 2013). It is also well known that altered homeostasis of PGRs is one of the most dynamic changes in response to stress conditions (Kohli et al., 2013). As tissue/cell sensitivity to PGRs also changes during plant development in response to environmental or genetically coded changes (Davies, 2010), it could be supposed that interactions between PGRs, stress-induced responses and genotype-specific PGRs sensitivity coordinate microspore reprogramming and regulate the final efficiency of ME. The analysis of recent findings obtained with the use of modern analytical techniques brought some new insights into hormonal regulation of microspore reprogramming and the initiation of embryogenic development.

The results of extensive analysis of phytohormone content changes after exposure to various ME-inducing stress treatments in anthers of three highly recalcitrant legume species were presented by Lulsdorf et al. (2012). It was revealed that the most common response was increased level of IAA-asparagine, a putative IAA metabolite. Of the various cytokinins, only *cZR* increased after the application of stressors.

In *B. napus*, the level of various auxin forms depends significantly on the sample source (leaves, flower buds, isolated microspores) and temperature regimes during the growth of donor plants ($10^\circ\text{C}/18^\circ\text{C}$). For the first time Dubas et al. (2012, 2013a) showed that IBA prevailed in isolated microspores and its level could be reduced by low temperature. Interestingly, the

combination of low temperature and heat shock reversed this effect. IAA level tends to change in a similar manner to IBA, both in responsive and recalcitrant genotypes. Based on these data, it could be concluded that noticeable changes in the level of both auxins forms caused by stress treatments are important for ME.

Similarly, low temperature ME-initiating treatment (3 weeks at 4°C) meaningfully changed PGRs homeostasis in several DH lines of triticale (Žur et al., 2015). Accumulation of IAA, IBA, *cZ*, *cZR*, and ABA together with a decrease in *tZ* content was observed in all studied genotypes. It was discovered that as result of cold treatment anthers of highly responsive triticale genotypes were characterized by higher concentrations of IBA, *cZ*, *tZ*, *cZR*, and lower amount of IAA and KN-like compound in comparison with recalcitrant ones. However, the effects of exogenously applied ABA, PCIB and TIBA suggest that none of the studied PGRs acts alone in the determination of embryogenic competency. An important prerequisite for effective ME seems to be a specific PGR homeostasis – lower auxin rate in comparison with cytokinins and ABA, and lower cytokinin/ABA ratio.

Genetically or/and environmentally determined changes in PGR sensitivity at least partially explain the importance of the timing of hormonal treatment/application. For example, Wassom et al. (2001) showed that modification of maize anther culture medium with various PGRs (ABA, GA₃, ancymidol, or fluridone) was ineffective in comparison to donor plant treatments, where these substances were pipetted into whorls of field-grown plants 3 days before tassel harvest. Similarly, Liu et al. (2002a,b) and Zheng et al. (2003) demonstrated stimulation of ME in wheat by a combination of high temperature tillers pre-treatment with their ‘inducer chemical formulation.’ It seems that altered PGR homeostasis preceding microspore isolation and transfer to *in vitro* culture triggers changes important for effective ME initiation.

Summary

The data presented above indicate why standard culture medium optimization using the traditional ‘one-factor-at-a-time’ and ‘trial-and-error’ techniques, which require a considerable amount of time and effort, can sometimes be completely ineffective. As a large number of important crop species, cultivars or genotypes still remain highly recalcitrant to ME, only a much more precise recognition of molecular/physiological/metabolical background that favors the initiation of embryogenic development could bring any substantial progress. As hormonal homeostasis seems to be one of the most important factors determining cell embryogenic competency, only a more comprehensive approach leading to the recognition of the mechanisms controlling the process could break the barrier of ME recalcitrancy.

Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00424/abstract>

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Early embryo achievement through isolated microspore culture in *Citrus clementina* Hort. ex Tan., cvs. ‘Monreal Rosso’ and ‘Nules’

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Microspore embryogenesis is a method of achieving complete homozygosity from plants. It is particularly useful for woody species, like *Citrus*, characterized by long juvenility, a high degree of heterozygosity and often self-incompatibility. Anther culture is currently the method of choice for microspore embryogenesis in many crops. However, isolated microspore culture is a better way to investigate the processes at the cellular, physiological, biochemical, and molecular levels as it avoids the influence of somatic anther tissue. To exploit the potential of this technique, it is important to separate the key factors affecting the process and, among them, culture medium composition and particularly the plant growth regulators and their concentration, as they can greatly enhance regeneration efficiency. To our knowledge, the ability of meta-Topolin, a naturally occurring aromatic cytokinin, to induce gametic embryogenesis in isolated microspores of *Citrus* has never been investigated. In this study, the effect of two concentrations of meta-Topolin instead of benzyladenine or zeatin in the culture medium was investigated in isolated microspore culture of two genotypes of *Citrus*. After 11 months of isolated microspore culture, for both genotypes and for all the four tested media, the microspore reprogramming and their sporophytic development was observed by the presence of multinucleated calli and microspore-derived embryos at different stages. Microsatellite analysis of parental and embryo samples was performed to determine the embryo alleles constitution of early embryos produced in all tested media, confirming their origin from microspores. To our knowledge, this is the first successful report of *Citrus* microspore embryogenesis with isolated microspore culture in *Citrus*, and in particular in *Citrus clementina* Hort. ex Tan, cvs. ‘Monreal Rosso’ and ‘Nules’.

Keywords: citrus breeding, gametic embryogenesis, homozygosity, isolated microspore culture, meta-Topolin

Introduction

Biotechnology methods can be used to enhance the efficiency of traditional breeding programs. Gametic embryogenesis is a biotechnological tool employed in both basic and applied research. Immature gametes, opportunely induced, can deviate from the normal gametophytic developmental pathway toward the sporophytic one. The sporophytic pathway leads to the production of haploid organisms (Hs), with the gametic chromosome number (n instead of $2n$), or doubled haploids (DHs), haploids that underwent, spontaneously or induced, chromosome duplication, becoming homozygous at all *loci*. Gametic embryogenesis techniques and particularly microspore embryogenesis, are efficient methods for obtaining homozygous individuals. They can be used for important breeding applications such as mutation, selection, genetic analysis, transformation, and gene sequencing (Germanà et al., 2013).

Developing homozygous lines is very important in crop improvement programs, particularly for woody plants characterized by long reproductive cycles, a high degree of heterozygosity, large size, and, sometimes, by self-incompatibility (Germanà, 2006, 2009, 2011a,b; Segù-Simarro, 2010). Woody plants are considered recalcitrant species. Few studies reported successful and efficient microspore embryogenesis protocols for woody species (Höfer, 2004; Ramírez et al., 2004; Barany et al., 2005; Bueno et al., 2005, 2006; Germanà, 2006, 2007, 2009, 2011a; Chiancone et al., 2013; Rodríguez-Sanz et al., 2014; Blasco et al., 2015).

Among the woody recalcitrant fruit producing species, *Citrus*, ranks first worldwide, with 126 million tons of fruit produced during 2013 (FAOSTAT Database, 2014). Clementine is believed to be a 'Mediterranean' mandarin \times sweet orange hybrid. Particularly, the group of Clementine cultivars is the most representative of the Spanish Citrus industry because of their quality and acceptance by the consumers. Especially, the cv. 'Nules' is one of the most cultivated clementine and 'Monreal Rosso' (MAR) was obtained by gamma rays mutation at the Research Center for the Citrus and the Mediterranean crops (CRA-ACM, Acireale, CT, Italy). Due to its economical importance, clementine is of great interest to breeders.

Among the *Citrus* microspore embryogenesis reports to date (Germanà et al., 1994, 2005; Germanà, 1997, 2007; Germanà and Reforgiato Recupero, 1997; Germanà and Chiancone, 2003), only one examined isolated microspore culture in several *Citrus* species (lemon, orange, clementine, sour orange, grapefruit) and a related genus (*Poncirus*; Germanà et al., 1996).

Since the first studies of Nitsch (1974) on *in vitro* isolated microspore cultures of *Nicotiana*, considerable research has been done on developing protocols for different species for increasing the frequency of embryogenesis via isolated microspore culture (Dunwell, 2010; Prem et al., 2012). Although anther culture is often the method of choice for DH production in many crops, because of its higher efficiency and simplicity, the isolated microspore culture technique provides a better way to investigate the processes of pollen embryogenesis at the cellular, physiological, biochemical, and molecular levels. However, it

requires better equipment and more skill than anther culture (Nitsch, 1977; Reinert and Bajaj, 1977; Germanà, 2011a). Also isolated microspore culture avoids the regeneration from somatic anther tissue (Ferrie and Caswell, 2010; Germanà, 2011a,b).

Numerous endogenous and exogenous factors affect the embryogenic response of immature gametes in culture (Smykal, 2000; Wang et al., 2000). Genotype, physiological status and growth conditions of donor plants, stage of gamete development, pre-treatment of the flower buds, culture media and conditions of incubation, and their interactions, are all factors that greatly affect the cell response to the *in vitro* culture (Germanà, 2011a,b).

There is no single standard condition or protocol for obtaining plant formation by isolated microspore culture. Microspores of different species and cultivars within a species can have much different requirements for embryogenic development. For these reasons studies of increasing microspore embryogenesis efficiency, focused on detecting the influence of growth regulators on anther culture and isolated microspore culture in *Citrus* spp. and other fruit crops (Germanà et al., 1996, 2006, 2011; Höfer et al., 1999; Germanà and Chiancone, 2003; Höfer, 2004; Bueno et al., 2005, 2006; Chiancone et al., 2006; Padoan et al., 2011).

Meta-Topolin (mT), a naturally occurring aromatic cytokinin, considered an alternative to benzyladenine (BA), zeatin (ZEA), and kinetin (KIN) in plant tissue culture (Aremu et al., 2012), has been used to increase *in vitro* plant propagation efficiency of several species including *Citrus* (Niedz and Evens, 2011). To our knowledge, this alternative cytokinin has not been used to induce microspore embryogenesis by anther or isolated microspore cultures. Esteves et al. (2014) recently tested meta-Topolin in the regeneration medium of isolated microspore culture of recalcitrant barley genotypes. It increased embryo differentiation into green plants by 2.9-fold.

This study investigated the effect of meta-Topolin as a substitute for benzyladenine or zeatin in the culture media used for generating embryos of *Citrus clementina* Hort. ex Tan., cultivars 'Monreal Rosso' and 'Nules' when using gametic embryogenesis via isolated microspore culture method.

Materials and Methods

Plant Material and Pollen Developmental Stage

Flower buds were harvested in April from trees of *C. clementina* Hort. ex Tan., cvs. 'Monreal Rosso' (MAR) and 'Nules', grown in a collection orchard (Campo d'Orléans, Palermo 38°N) of the Università degli Studi di Palermo, Italy. Microspore developmental stage was determined in one anther per flower bud size by 4', 6-diamidino-2-phenylindole (DAPI) staining. Anthers from buds of different sizes were squashed in a few drops of DAPI solution (1 mg/mL) and observed under a fluorescent microscope (Zeiss, Axiophot, Germany). For further experiments, only flower buds of the size containing anthers bearing microspores at the

uninucleated/vacuolated stage (3.5–4.0 mm), were selected for culture.

Flower Bud Sterilization, Microspore Isolation, and Culture

As pre-treatment, flower buds were placed in darkness at 4°C for 1 week. Around 80 flower buds were surface sterilized by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion for 20 min in 25% (v/v) commercial bleach (about 0.5% active chlorine in water) and then rinsed three times with sterile distilled water. Anthers were carefully separated from stamens and put in sterile 0.4 M mannitol solution until the isolation protocol, which was performed following the procedure reported by Kumlehn et al. (2006), with little modifications. Particularly, anthers were used as explants, instead of the entire flowers and the density gradient step was skipped. Isolated microspores were cultured at the concentration of 100,000 microspores per mL. A volume of 1.0 mL was placed into each 3001-type Petri dishes (35 mm × 10 mm, BD Biosciences).

All Petri dishes were put at 26 ± 1°C in the dark for the first 30 days, and then placed under cool white fluorescent lamp (Philips TLM 30W/84, France), with a photosynthetic photon flux density of 35 μmol m⁻² s⁻¹ and a photoperiod of 16 light hours.

Media Composition

For the culture, the medium (referred as medium P) previously employed in experiments on *Citrus* microspore embryogenesis through isolated microspore culture was used (Germanà et al., 1996; Table 1). In this medium, among the other plant growth regulators, several cytokinins are present, particularly BA, ZEA, KIN. To study the effect of mT, it was added in substitution of BA or ZEA at the same concentration (respectively, media: PmT/BA, PmT/ZEA) or at a concentration 10 times higher (respectively, media: PmT/BA10, PmT/ZEA10).

In particular, for the experiments the following media were tested:

- (1) PC (control medium): 0.5 mg/L of BA and 0.5 mg/L of ZEA; (Germanà et al., 1996);
- (2) PmT/BA: PC medium without BA + 0.5 mg/L mT;
- (3) PmT/ZEA: PC medium without ZEA + 0.5 mg/L mT;
- (4) PmT/BA10: PC medium without BA + 5.4 mg/L mT;
- (5) PmT/ZEA10: PC medium without ZEA + 5.6 mg/L mT.

Seven replicates for each medium were used, thirty five Petri dishes per cultivar.

Early embryos obtained were transferred onto different solid media (Table 2) in the attempt to obtain their germination.

TABLE 1 | Media used for ‘Monreal Rosso’ and ‘Nules’ isolated microspore culture.

Components	Media				
	P	PMT/BA	PMT/ZEA	PMT/BA10	PMT/ZEA10
Per liter					
N6 Chu Salts	1X	1X	1X	1X	1X
N&N vitamins	1X	1X	1X	1X	1X
Galactose	18 g	18 g	18 g	18 g	18 g
Lactose	36 g	36 g	36 g	36 g	36 g
Ascorbic acid	500 mg	500 mg	500 mg	500 mg	500 mg
Myoinositol	5 g	5 g	5 g	5 g	5 g
Biotin	500 mg	500 mg	500 mg	500 mg	500 mg
Thiamine	5 mg	5 mg	5 mg	5 mg	5 mg
Pyridoxine	5 mg	5 mg	5 mg	5 mg	5 mg
Coconut water	100 mL	100 mL	100 mL	100 mL	100 mL
Casein	500 mg	500 mg	500 mg	500 mg	500 mg
Serine	100 mg	100 mg	100 mg	100 mg	100 mg
Glycine	2 mg	2 mg	2 mg	2 mg	2 mg
Glutamine	800 mg	800 mg	800 mg	800 mg	800 mg
Malt extract	500 mg	500 mg	500 mg	500 mg	500 mg
2,4-D	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg
GA ₃	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Kinetin	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Zeatin	0.5 mg	0.5 mg	–	0.5 mg	–
Thidiazuron	0.5 mg	0.5 mg	0.5 mg	0.5 mg	0.5 mg
Benzyladenine	0.5 mg	–	0.5 mg	–	0.5 mg
Meta-Topolin	–	0.5 mg	0.5 mg	5.4 mg	5.6 mg
pH	5.8	5.8	5.8	5.8	5.8

P, Germanà et al. (1996); N6 Chu salts, Chu (1978); N&N vitamins, Nitsch and Nitsch (1969).

TABLE 2 | Solid media tested for embryo germination.

Components	Media				
	E	EE	E/ZEA	E/TDZ	MS/TDZ
Per liter					
MS salts	1X	1X	1X	1X	1X
MS vitamins	1X	1X	1X	1X	1X
Sucrose	30 g	30 g	30 g	30 g	30 g
Ascorbic acid	500 mg	500 mg	500 mg	500 mg	–
Malt extract	500 mg	500 mg	500 mg	500 mg	–
GA ₃	1 mg	2 mg	1 mg	1 mg	–
Zeatin	–	–	1 mg	–	–
NAA	0.02 mg	0.02 mg	0.02 mg	0.02 mg	–
Thidiazuron	–	–	–	1.0 mg	1.0 mg
Agar	8.5 g	8.5 g	8.5 g	8.5 g	8.5 g
pH	5.8	5.8	5.8	5.8	5.8

MS, Murashige and Skoog (1962); GA₃, gibberellic acid; NAA, α-naphthaleneacetic acid.

Evaluation of the Microspore Response In Vitro, Data Processing, and Statistical Analysis

Petri dishes containing isolated microspores in cultures were weekly observed by an inverted microscope (Zeiss) and a binocular microscope (Leica). Samples of isolated microspores were stained with DAPI and observed by a fluorescence microscope (Zeiss, Axiophot, Germany) to monitor their *in vitro* development, once a month, every month, during the culture. After 7 months of culture, per each medium, 400 microspores DAPI-stained (four replicates with around 100 microspores each) were observed, by a fluorescence microscope (Zeiss, Axiophot, Germany). Different structural features have been observed and registered: microspores uninucleated, binucleated with two equal-size nuclei that had just started the sporophytic pathway, trinucleated, tetranucleated, and multinucleated. Moreover, after 11 months of *in vitro* culture, the number of calli and embryos produced per each Petri dish was registered, using a binocular microscope. These values were used to calculate means. Statistical analysis was carried out using SYSTAT 13 software. Two factors were considered: “Cultivar” and “Culture medium,” and differences between them were tested by two-way analysis of variance (ANOVA), at $p \leq 0.05$ level. Tukey’s test was, then, used to separate means.

Fixation and Processing for Microscopic Analysis

In vitro cultures containing microspores and microspore-derived structures were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), overnight, at 4°C. After fixation, microspore culture samples were embedded in gelatin, washed in PBS, dehydrated through an acetone series, infiltrated and embedded in Technovit 8100 acrylic resin (Kulzer, Germany), at 4°C, as previously described by Solís et al. (2008). Staining solution of 0.075% toluidine blue in water, was applied on Technovit semithin sections (1 μm) for 10–15 min. After rinsing

and drying, preparations were mounted in Eukitt and observed under bright field for structural analysis in a light microscope Zeiss 68105 equipped with a Leica Microsystems DFC420C digital camera.

Allelic Pattern Detection by SSR Analysis

The allelic pattern was checked on the embryos obtained from *C. clementina* cultivar ‘Monreal Rosso’ and ‘Nules’ isolated microspore culture. DNA was extracted from leaves of the mother plant and from the embryos obtained by *in vitro* culture and collected from the medium by an insulin needle. The samples were frozen in liquid nitrogen and ground using steel beads and a Tissuelyser (QIAGEN®, Germany). DNA extraction was performed as described by Doyle and Doyle (1987). The parent DNA was resuspended in 60 μL TE buffer (Tris-EDTA, pH 8.00) and then diluted to 10 ng/μL. Embryo DNA was resuspended in 25 μL TE.

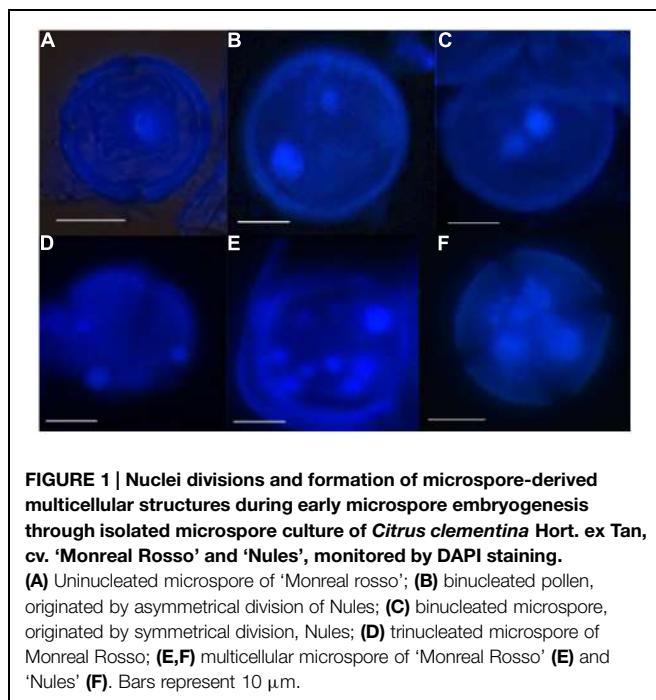
Ten microsatellite loci isolated by Novelli et al. (2006) from *C. sinensis* and by Froelicher et al. (2008) from *C. reticulata* were preliminarily screened on the DNA from the leaves and one was selected for its heterozygosity in the parental genotype: CCSM147 (Novelli et al., 2006). This locus was used for assessing the allelic pattern of the embryos.

Polymerase chain reactions (PCRs) were performed in two steps in a total volume of 10 μL containing 3 μL DNA (corresponding to 30 ng of DNA for the parent plants), 0.25 U of KAPA Taq DNA polymerase (KAPABIOSYSTEMS, Wilmington, MA, USA) 1 μL of 10X PCR buffer, 200 μM nucleotide mix and 0.5 μM of each primer. PCR conditions were as follows: an initial denaturation step at 95°C for 3 min followed by 34 cycles of denaturation (30 s at 95°C), annealing (45 s at 55°C), and extension (90 s at 72°C). The final elongation step was at 72°C for 30 min. Four μL of the product from the first amplification were then used as template for a second PCR, carried out for 28 cycles with the same conditions of the first one.

Polymerase chain reaction products were then analyzed on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Data were processed using GeneMapper Software (ver. 4.0; Applied Biosystems) and alleles were defined by their size in base pairs, by comparison with the standard size (GeneScan-500 LIZ, Applied Biosystems).

Results

Using the above methods allowed facilitated observation of the entire microspore embryogenesis process in clementine isolated microspore culture. Monitoring of the culture samples by DAPI staining (to show the nuclei) revealed that initially microspores of both genotypes were mainly uninucleated/vacuolated (**Figure 1A**) This is the developmental stage reported as being the most responsive for embryogenesis induction in clementine (Ramírez et al., 2003) and many other woody and herbaceous species (Germanà and Chiancone, 2003; Germanà et al., 2011; Solís et al., 2008; Prem et al., 2012). It was possible to observe that some microspores did not show any change in the nuclei number or shape. In other microspores the nucleus



started to divide. This rarely occurred asymmetrically, i.e., following the normal gametophytic pathway (Figure 1B). In most binucleate microspores, the two nuclei are similar in size and shape (Figure 1C), indicating their origin by a symmetric division. This type of division is considered the first step of the sporophytic pathway followed by the reprogrammed microspores in microspore embryogenesis (Germanà, 2011a,b). Many microspores followed this pathway and underwent subsequent divisions, so that, later trinucleated (Figure 1D), tetranucleated and multinucleated microspores (Figures 1E,F) were detected in DAPI-stained squash preparations.

The structural organization of these microspores and multinuclear structures observed in the cultures were analyzed on semithin sections (Figure 2). Samples of the *in vitro* culture were fixed and processed for further microscopical analysis. At culture initiation, the microspores exhibited the typical architecture of the vacuolated microspores, with one nucleus located at the periphery and a central vacuole (Figure 2A). At later stages, in toluidine blue-stained sections, developing microspores exhibited differential features, some of them showing two nuclei with similar size and organization, and dense cytoplasms (Figures 2B,C), in contrast with the two different nuclei of the bicellular pollen developed *in vivo*. These two-celled structures indicated that the microspores *in vitro* underwent a symmetrical division and switched from their gametophytic developmental pathway toward proliferation; the result of the first embryogenic division of the microspore still exhibiting the exine wall (Figures 2B,C). Some dead (empty) microspores with irregular shapes were also observed in the cultures, together with larger multicellular structures (Figure 2D). They were elongated structures formed by more or less polygonal cells showing one nucleus and low-dense cytoplasm and vacuoles, and separated by

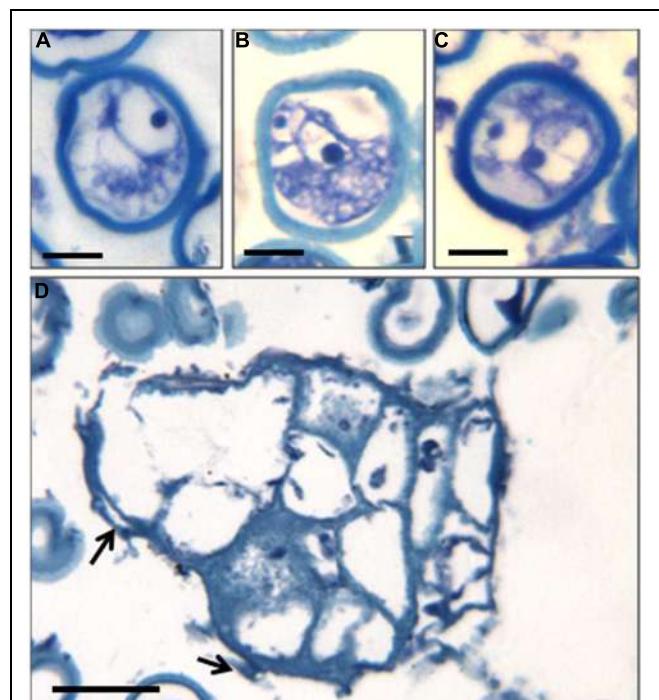


FIGURE 2 | Cellular structural organization at early microspore embryogenesis through isolated microspore culture of *C. clementina* Hort. ex Tan, cv. 'Monreal Rosso' and 'Nules.' Toluidine blue-staining of resin sections observed under bright field microscopy. (A) Vacuolated microspore at the beginning of the culture, 'Monreal Rosso'; (B,C) Two-celled microspores, 'Nules'; (D) Microspore-derived multicellular structure (in the center) and some dead microspores (at the top), 'Monreal Rosso.' Bars represent, in (A–C): 10 µm, in (D): 50 µm.

straight cell walls (Figure 2D). At the periphery of some of these multicellular structures, remnants of the exine, could be found (arrows in Figure 2D). These multicellular microspore-derived structures or proembryos resembled those found in other woody and herbaceous species. The evolution of the *in vitro* system described here, from two-cell and multicellular microspores to large multicellular structures or proembryos indicated that the reprogramming of the microspore and the first steps of the embryogenic pathway were achieved.

Results recorded after 7 months of microspore culture, and their statistical analysis are reported in Table 3. No statistically significant differences were detected among treatments of the percentages of uninucleated and binucleated microspores. Moreover, for both cultivars, the percentage of uninucleated microspores with no division was rather high (41.2% for MAR and 46.7% for 'Nules'). For the trinucleated microspores, a significant interaction was recorded between the two factors, "Cultivar" and "Culture medium," in which the main factor inducing variability was "Cultivar." Actually, the medium in which mT replaced ZEA at the same concentration, induced the highest response in MAR (19.1%) and the worst in 'Nules' (5.6%; data not shown).

The primary factor influencing induction of multinucleated microspores was "Culture medium." Tukey's test evidenced

TABLE 3 | Influence of cultivar and medium composition on two clementine cultivars, 'Monreal Rosso' and 'Nules', isolated microspore development, after 7 months (uninucleated, binucleated, trinucleated, multinucleated microspores) and 11 months (calli and embryos) of culture.

Factors		Uninucleated microspores (%)	Binucleated microspores (%)	Trinucleated microspores (%)	Multinucleated microspores (%)	Calli/Petri dish* (n°)	Embryos/Petri dish (n°)
Cultivar	Monreal Rosso	41.2 a	30.7 a	14.2 a	13.9 a	3.7 a	1.0
	Nules	46.7 a	32.3 a	9.7 b	11.2 a	2.9 a	1.5
Cultivar		0.088	0.359	0.002	0.548	0.090	
Medium	PC	41.9 a	30.8 a	11.5 a	15.9 a	4.0 ab	1.2
	PmT/BA	43.1 a	27.3 a	13.2 a	16.4 a	4.4 a	1.4
	PmT/ZEA	41.4 a	32.8 a	12.4 a	13.5 ab	2.5 ab	1.0
	PmT/BA10	44.7 a	34.2 a	13.0 a	8.2 b	2.4 b	1.4
	PmT/ZEA10	48.6 a	32.6 a	9.7 a	9.1 ab	3.2 ab	1.4
Culture medium		0.615	0.153	0.465	0.007	0.019	—
Cultivar × Culture medium		0.208	0.381	0.024	0.383	0.254	—

Two-way ANOVA, Tukey's test, $p \leq 0.05$.

*Average number of calli recorded per each medium and per each cultivar (seven Petri dishes/medium/cultivar).

PC (control medium), 0.5 mg/L BA and 0.5 mg/L ZEA (Germanà et al., 1996); PmT/BA, PC medium without BA + 0.5 mg/L mT; PmT/ZEA, PC medium without ZEA + 0.5 mg/L mT; PmT/BA10, PC medium without BA + 5.4 mg/L mT; PmT/ZEA10, PC medium without ZEA + 5.6 mg/L mT.

Per each factor and per each column, values followed by different letters are statistically different.

that the control medium (PC) and PmT/BA induced a statistically higher percentage (15.9 and 16.4% respectively) of multinucleated microspores, while the mT/BA10 medium the lowest (8.2%). For the other tested media, the percentages of multinucleated microspores were intermediate between the reported values (**Table 3**).

After 5 months of culture, binocular microscope observations revealed new structures: light brown calli (**Figure 3**) that increased in quantity and volume during the culture. A statistical analysis of number of calli per Petri dish after 11 months of culture, demonstrated that the culture medium was the also primary factor that influenced the microspore response of this

parameter. As with multinucleated microspores, the PmT/BA and PmT/BA10 media treatments produced statistically significant differences between the average number of calli/Petri dish (4.4 and 2.4, respectively; **Table 3**).

Together with calli, the formation of globular embryos was detected: they were pearl white and round (**Figure 4A**). During the culture, the round embryos elongated, often with a suspensor-like structure (**Figures 4B,C**). This kind of structure has not previously observed in the microspore-derived embryos obtained through *Citrus* anther culture.

Embryo production was observed for both cultivars and for all media tested. This is the first report of gamete-derived embryos obtained by isolated microspore culture in *Citrus*. Differences were recorded between the cultivars, with the 'Nules' cultivar showing a higher average number of embryos/Petri dish regenerated than in MAR (1.5 vs. 1.0; **Table 3**). However, while the two cultivars responded differently to the five different media, it appears that the higher concentration of mT, replacing BA or ZEA, was not detrimental for the embryo induction. The best responses were induced for MAR in the media PC and PmT/BA10 (1.3) and for 'Nules' in the media PmT/BA and PmT/ZEA10 (1.8; data not shown).

The results of the analysis at the SSR locus CCSM 147 showed a clear amplification: while the parental genotype was heterozygous, the allelic pattern of the embryos showed a single allele, shared with the parental genotype (**Figure 5**). This result is a first step in confirming the origin of the embryos from the 'Monreal Rosso' and 'Nules' gametophyte, although it was not yet possible, due to their small size, to check the ploidy condition of the embryos (either haploid or double haploid).

The embryos obtained were transferred from liquid to different solid media to achieve germination and plantlet production. After 12 months of trials with several media (**Table 2**), no germination was observed in microspore-derived embryos, probably due to dormancy caused by immaturity. Physiological and biochemical aspects of these microspore-derived embryos should be investigated to determine if the



FIGURE 3 | Microspore-derived callus of 'Monreal Rosso' in the PmT/BA medium.

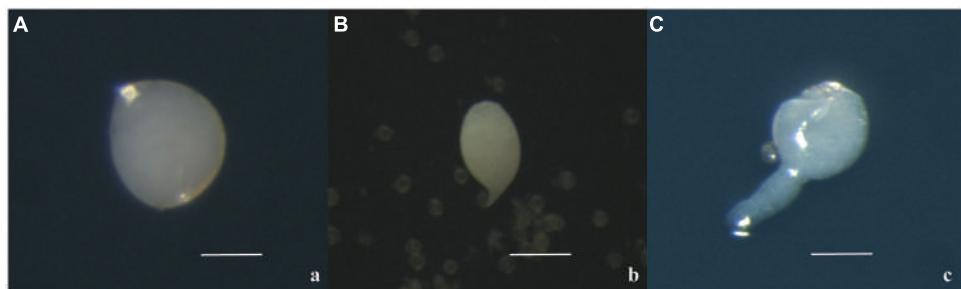


FIGURE 4 | Microspore-derived embryos of 'Nules' (A), 'Monreal Rosso' (B) and 'Nules' (C). Bars represent 100 μ m.

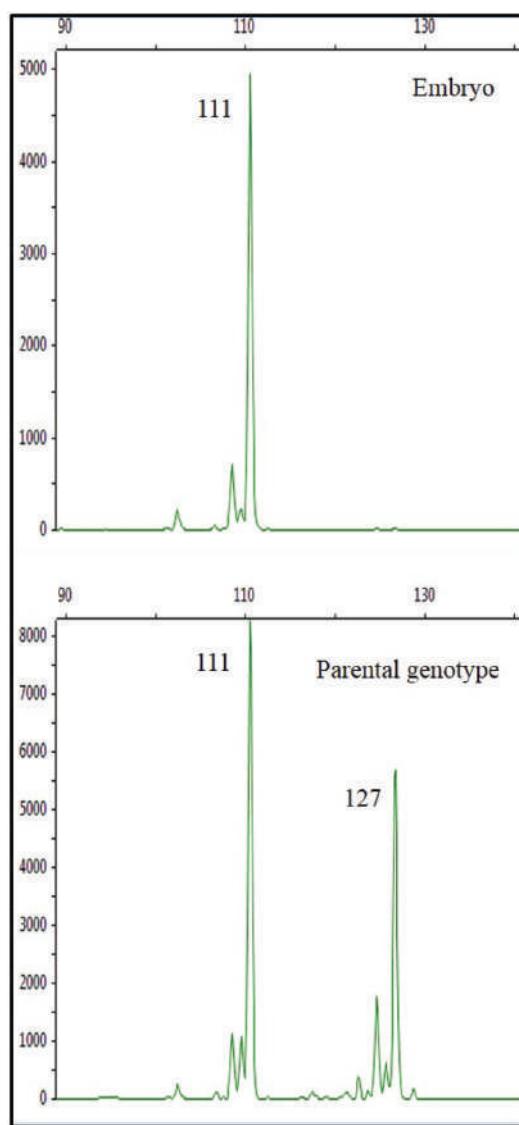


FIGURE 5 | Amplicons of the SSR locus CCSM147 in the embryo (top) and in the parental genotype (bottom) cv. 'Monreal Rosso.' Values in box beside each peak represent the allele size (bp). The allelic pattern of the embryo shows a single allele, shared with the parental genotype.

lack of endosperm, medium composition or dormancy prevent germination. Further investigations are in progress to obtain embryo conversion testing different factors, such as exposure to cold temperature and/or drying.

Discussion

The great potential of haploids and gametic embryogenesis in woody plant breeding has been well-demonstrated. Haploids can improve the efficiency and the speed of laborious and time-consuming traditional breeding methods. While *in vitro* isolated microspore culture is a standard breeding method in many crops, such as *Brassicaceae* and cereals, this technique has not been exploited in fruit crops because the induction frequency is low, plant recovery is difficult and response is highly genotype-dependent (Höfer et al., 1999; Bueno et al., 2005, 2006). Earlier work with isolated microspore culture of several *Citrus* species (lemon, orange, clementine, sour orange, and grapefruit) and a related genus (*Poncirus*) have been reported by Germanà et al. (1996). Multi-nucleated structures, pseudobulbs and small proembryos, were obtained but which failed to develop significantly.

In *C. clementina* Hort. ex Tan., the gametic embryogenesis process through isolated microspore culture, is slower than in other herbaceous and woody species, requiring up to 5 months for the first callus tissue or embryos. However, the microspores continued regenerating embryos and calli for 11 months in culture. In *Brassica* isolated microspore culture, first embryos are usually observed within 2 weeks of culturing (Barro and Martin, 1999), and in wheat after 9–12 days (Hu and Kasha, 1999). In fruit tree crops, apple embryo regeneration through isolated microspore culture, was observed after 8–12 weeks (Höfer et al., 1999). In olive, Bueno et al. (2005) reported the first pro-embryos after 4 weeks.

The media supplemented with mT showed microspore switching from the gametophytic to the sporophytic pathway as well as the PC medium. However, it appears that the response to mT in the media, as reported in numerous experiments, is genotype-dependent. For example, 'Nules' embryo production seems be favored by mT addition. Possibly mT replaces both BA and ZEA, at the same concentration, giving rise to embryo regeneration rate comparable to that of the control. In

'Nules', replacing BA with mT 10-fold more concentrated was not beneficial for the induction of multinucleate microspores and calli. However, for embryo induction, adding a higher concentration of mT as replacing BA did not affect the regeneration rate.

The results here reported indicate that meta-Topolin can be employed not only in *Citrus* micropropagation (Niedz and Evens, 2011), but also in the microspore embryogenesis process. The effect of mT on microspore embryogenesis induction could be due to its anti-senescence activity and plant growth stimulation activity. Other cytokinin-like compounds, such as polyamines (PAs), considered potent senescence inhibitors (Altman et al., 1977; Kaur-Sawhney et al., 1980) and implicated in several plant growth and development processes (Torrigiani et al., 1989; Bagni and Tassoni, 2001), improved the embryogenic callus production through anther culture in *C. clementina* Hort. ex Tan. (Chiancone et al., 2006). To understand how anti-senescence substances influence microspore embryogenesis induction could facilitate understanding the mechanisms beyond this phenomenon as well as being used to increase the efficiency of breeding programs.

Actually, an effective regeneration system through isolated microspore culture could facilitate male gametophytic selection (MGS) in *Citrus*. This strategy would allow early genotype screening for selection of desirable alleles on pollen grains (Clegg et al., 1978; Hormaza and Herrero, 1996; Ravikumar et al., 2007). With respect to the sporophytic selection, the MGS has advantages for selecting among very high numbers of haploid individuals in a small space (Soleimani et al., 2010), allowing selection also of recessive characters and mutations, without the interference of dominance. Furthermore, as pollen is the result of genetic recombination, possibly new allelic combination and mutations can be selected for physiological and biochemical characteristics by applying stress during microspore culture (Evens et al., 1990).

Conclusion

The characteristics of angiosperm pollen (haploidy, small size, great number, totipotency) make it very useful in biotechnology as immature microspores can be manipulated to improve the efficiency, rapidity, and precision of plant breeding methods. The *in vitro* culture of immature microspores is a good way to recover

homozygosity via embryogenesis in higher plants. The potential value of isolated microspore culture in higher plants is obvious. However, a well-defined and efficient procedure of regeneration through microspore embryogenesis is necessary.

The results here presented are a major step in understanding *C. clementina* Hort. ex Tan. microspore embryogenesis. Actually, this is first report of regeneration of microspore-derived embryos through isolated microspore culture of the two clementine cultivars 'Monreal Rosso' and 'Nules.' Additional investigations are needed to optimize the medium composition and increase the regeneration rate. Studies to promote the development of obtained embryos and recover plantlets from them are now in progress.

Author Contributions

BC statistically analyzed the data and wrote the first draft of the manuscript. AA, MK, and VG performed the experiments. BC and MK contributed to the design of the work. IB and PT processed the samples, elaborated, and interpreted the results of the microscopic analysis. DM and RB processed the samples, analyzed, and interpreted the results of the molecular marker analyses. MG designed the research and coordinated the project, drafted and revised the manuscript, and is corresponding author. All authors collaborated in the revising of the manuscript. All authors read and approved the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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