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Departamento de Genética

TESIS DOCTORAL

Key factors involved in stress-induced microspore embryogenesis in barley and rapeseed: DNA methylation, arabinogalactan proteins and auxin

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Madrid, 2016

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CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

CENTRO DE INVESTIGACIONES BIOLÓGICAS

LABORATORIO DE BIOTECNOLOGÍA DEL POLEN DE PLANTAS CULTIVADAS

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KEY FACTORS INVOLVED IN STRESS-INDUCED MICROSPORE EMBRYOGENESIS IN BARLEY AND RAPESEED: DNA METHYLATION, ARABINOGALACTAN PROTEINS AND AUXIN

Ph.D. thesis

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FDO. DRA. PILAR SÁNCHEZ TESTILLANO FDO. DRA. M. CARMEN RISUEÑO ALMEIDA

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A mi querido padre,

Tú me animaste a terminar este trabajo

Te echo de menos mucho

إلى أبي الغالي,

أنت من شجعني على إنهاء هذا العمل

إشتقت إليك كثيرا

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RESUMEN/ABSTRACT

RESUMEN

FACTORES CLAVE IMPLICADOS EN LA EMBRIOGÉNESIS DE MICROSPORAS INDUCIDA POR ESTRÉS EN CEBADA Y COLZA: METILACIÓN DEL DNA, PROTEÍNAS DE ARABINOGALACTANOS Y AUXINA

INTRODUCCIÓN

La embriogénesis de microsporas es un proceso *in vitro* en el que la microspora o polen inmaduro, mediante la aplicación de un tratamiento de estrés se reprograma y abandona su ruta de desarrollo gametofítico para iniciar la ruta embriogénica, dando lugar a embriones y plantas haploides y doble-haploides. Este proceso es de gran interés básico y aplicado en biotecnología y mejora vegetal para la obtención rápida de nuevas variedades, sin embargo aún tiene importantes limitaciones en su explotación por su baja eficiencia en muchas especies de interés económico. La limitación en la aplicación de este proceso es debida a que los mecanismos de inducción y progresión de la embriogénesis de microsporas no están todavía completamente dilucidados. La monocotiledónea *Hordeum vulgare* (cebada) y la dicotiledónea *Brassica napus* (colza) son especies modelo para este proceso, en las cuales se induce embriogénesis directa en cultivos de microsporas aisladas en medio líquido, mediante tratamientos de estrés con diferentes temperaturas.

OBJETIVOS Y RESULTADOS

En esta tesis doctoral, se ha inducido la embriogénesis en cultivos de microsporas aisladas mediante tratamientos de estrés a temperaturas diferentes en las dos especies ya mencionadas, a 32ºC en colza, y a 4ºC en cebada. Estudios previos de nuestro grupo han sugerido algunos factores que podrían estar implicados en la iniciación y

progresión de la embriogénesis de microsporas, los cuales se han estudiado en esta tesis. Se han analizado, mediante un abordaje multidisciplinar en las dos especies, cebada y colza, tres de estos factores: 1) la metilación del DNA, 2) las proteínas de arabinogalactanos (AGPs) y 3) la auxina endógena. Se han comparado los resultados en las dos especies con objeto de caracterizar los mecanismos implicados en la embriogénesis de microsporas que son comunes en especies monocotiledóneas y dicotiledóneas. Se ha estudiado la dinámica de estos factores durante el proceso de embriogénesis de microsporas, así como su función mediante el análisis del efecto de diversos inhibidores de estos factores sobre la inducción y eficiencia de embriogénesis. Además, se han analizado algunos de ellos durante el desarrollo del polen, para identificar los cambios asociados con el cambio de programa de desarrollo hacia la embriogénesis de microsporas. Los resultados han indicado que estos factores pueden ser utilizados como marcadores tempranos de la embriogénesis de microsporas. Puesto que se han encontrado resultados similares en las dos especies analizadas, con independencia del estrés inductor, estos informarían sobre mecanismos generales implicados en el proceso que podrían extenderse a otras especies.

Se ha estudiado **la dinámica de metilación global del DNA** durante la embriogénesis de microsporas y desarrollo gametofítico en *H. vulgare*, los resultados se han comparado con los datos recientemente obtenidos por nuestro grupo en *B. napus*. La cuantificación de los niveles de metilación global del DNA y los ensayos de inmunofluorescencia de 5-metil-deoxicitidina (5mdC) han mostrado niveles bajos de metilación del DNA en microsporas y un alto incremento a lo largo del desarrollo gametofítico y la maduración del polen. La señal de 5mdC aumenta en los núcleos generativo y espermáticos, mientras que el núcleo vegetativo muestra una señal más baja. Después del estrés por frío y la inducción de embriogénesis de microsporas, se observan niveles bajos de metilación del DNA y una señal débil de 5mdC en los núcleos de las microsporas embriogénicas y los proembriones de 2 – 4 células, lo que indica que se produce una hipometilación global del DNA durante el cambio del

programa de desarrollo y primeras divisiones embriogénicas en cebada, como se había demostrado en colza. En etapas posteriores del desarrollo, la metilación del DNA global es más alta en embriones en desarrollo y aumenta durante la maduración de los embriones. Estos datos sugieren que la metilación del DNA es fundamental para la formación del embrión y juega un papel en la regulación de la expresión génica durante la embriogénesis de microsporas.

También se ha analizado en esta tesis **los efectos de la 5-azacitidina (AzaC)**, un inhibidor de la metilación del DNA, sobre el inicio y progresión de la embriogénesis de microsporas en las dos especies, colza y cebada. Los tratamientos con AzaC disminuyeron los niveles de metilación global del DNA y favorecieron el inicio de la embriogénesis de microsporas, aumentando la proporción de embriones multicelulares tempranos en comparación con los cultivos no tratados. Por el contrario, este agente afectó al desarrollo en las etapas posteriores, dificultando la progresión de la embriogénesis de microsporas. En conjunto, los resultados indican que la demetilación del DNA por AzaC promueve la iniciación de la embriogénesis de microsporas, mientras que la diferenciación del embrión requiere metilación del DNA de novo y es inhibida por la AzaC.

Se ha estudiado la presencia, distribución y **dinámica de las proteínas de arabinogalactano (AGPs)** durante la embriogénesis de microsporas en *B. napus* y *H. vulgare*, mediante el empleo de un abordaje multidisciplinar utilizando anticuerpos monoclonales para AGPs (LM2, LM6, JIM 14, JIM 13 y MAC 207). Los resultados han mostrado que las AGPs se inducen durante la embriogénesis de microsporas y que el bloqueo de AGPs con el reactivo de Yariv inhibe el proceso, en las dos especies. Esto indica que las AGPs juegan un papel clave en el desarrollo del embrión de microsporas. Diversos epitopos de AGPs se localizaron en las paredes celulares y pequeños “spots” citoplásmicos, lo cual sugiere una producción activa y secreción de AGPs durante la reprogramación de la microspora y el desarrollo del embrión. También, se han analizado los patrones de distribución de AGPs durante el desarrollo

del polen en *B. napus* y los resultados han indicado que varias AGPs están relacionadas con la maduración y germinación de polen. Por el otro lado, se ha analizado el patrón de expresión del gen *BnAGP Sta 39*-4 en *B. napus*. Los resultados han mostrado un aumento en la expresión del gen *Sta 39-4* después del inicio de la embriogénesis de microsporas, lo que sugiere que las AGPs se inducen con el cambio de programa de desarrollo, constituyendo marcadores tempranos de la embriogénesis de microsporas. Estos resultados revelan nuevas evidencias sobre el papel de las AGPs endógenas como posibles moléculas reguladoras del proceso.

En esta tesis, también se ha estudiado **la dinámica de la auxina endógena** durante la reprogramación de la microspora a embriogénesis en *H. vulgare*, los resultados se han comparado con los obtenidos recientemente por nuestro grupo en *B. napus*. Los datos obtenidos en esta tesis han revelado cambios en la distribución de auxina durante la embriogénesis de microsporas en cebada, sistema modelo de monocotiledóneas. Los resultados han mostrado que la auxina aumenta durante las primeras etapas de la embriogénesis de microsporas, acumulándose en las células de los proembriones tempranos, hallazgos que son consistentes con los resultados previos de nuestro grupo sobre la distribución de auxina en la embriogénesis de microsporas de las dicotiledóneas *B. napus* y *Quercus suber*. Se ha analizado también, en este trabajo, el efecto del ácido 1-naftilftalámico (NPA), inhibidor del transporte polar de auxina, y del inhibidor de la acción de la auxina, ácido P-chlorophenoxyisobutytric (PCIB), en la embriogénesis de microsporas de cebada. El análisis ha revelado que el tratamiento con NPA disminuye la cantidad de embriones multicelulares producidos después de la inducción por estrés, en comparación con los cultivos no tratados. Los tratamientos con PCIB inhiben completamente la reprogramación de las microsporas a embriogénesis. Estos resultados indican que el transporte polar de auxina y la acción de auxina tienen un papel clave en la iniciación y progresión de la embriogénesis de microsporas en cebada, como también se ha demostrado en colza, lo que sugiere un papel general de la auxina en el proceso.

CONCLUSIONES

En conclusión, los resultados de esta tesis doctoral han revelado la implicación de la metilación del DNA, las AGPs y la auxina en la iniciación y progresión de la embriogénesis de microsporas, posiblemente con funciones comunes en especies dicotiledóneas y monocotiledóneas. Los nuevos datos aportados sugieren que la inducción de embriogénesis de microsporas requiere hipometilación del DNA, inducción de AGPs y de auxina, independientemente del estrés inductor y de la especie. Nuestros resultados podrían abrir nuevas vías para el diseño de nuevas estrategias biotecnológicas para la producción de doble-haploides más eficiente en programas de mejora de cultivos hortícolas y cereales.

El trabajo de esta tesis ha dado lugar a tres publicaciones en revistas científicas incluidas en el Journal Citation Report (JCR) y un manuscrito en preparación. Estas publicaciones son las siguientes:

1. **El-Tantawy, A.A.;** Solís, M.T.; Risueño, M.C. and Testillano, P.S. (2014). Change in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley. ***Cytogenetic Genome Research*****,** 143 (1-3), 200-208.

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4. **El-Tantawy, A.A.;** Solís, M.T.; Risueño, M.C.; Testillano, P.S. (2015). Auxin increase and distribution, and effects of auxin inhibitors on microspore embryogenesis initiation and progression in barley. ***In preparation***.

ABSTRACT

KEY FACTORS INVOLVED IN STRESS-INDUCED MICROSPORE EMBRYOGENESIS IN BARLEY AND RAPESEED: DNA METHYLATION, ARABINOGALACTAN PROTEINS AND AUXIN

INTRODUCTION

Microspore embryogenesis represents a unique system of single cell reprogramming in plants in which a highly specialized cell, the microspore, by specific stress treatment, switches its fate towards an embryogenesis pathway forming haploid embryos and producing haploid and doubled haploid plants. This process is a potent biotechnological tool for obtaining doubled haploids in many crops to be used in plant breeding programs. The application of this process has important limitations because the mechanisms of microspore embryogenesis induction and progression, which involve proliferation and differentiation events, are not yet completely elucidated. The monocot *Hordeum vulgare* (barley) and the dicot *Brassica napus* (rapeseed) are model systems for the process in which direct embryogenesis is induced, via different temperature stress treatments, in isolated microspores cultured in liquid media.

OBJECTIVES AND RESULTS

In this PhD thesis, isolated microspore cultures were induced by different temperatures in two species, by 32ºC in rapeseed, and by 4ºC in barley. Previous studies of our group suggested some factors that would be involved in the microspore embryogenesis initiation and progression. Three of these factors have been analysed in the two species by a multidisciplinary approach: 1) DNA methylation, 2) Arabinogalactan proteins (AGPs) and 3) Endogenous auxin. The results were

compared in the two species in order to characterize common mechanisms involved in microspore embryogenesis in monocot and dicot species. We studied their dynamics during the process of microspore embryogenesis, as well as their function by analysing the effect of inhibitors for these factors on the embryogenesis induction and efficiency. In addition, some of them were also analysed during pollen development to identify changes associated with the change of developmental program towards microspore embryogenesis. The results indicated that these factors can be used as early markers of microspore embryogenesis. Since they are found in the two species analysed, independently of the inductor stress, they would inform on general mechanisms involved in the process that could be extended to other plant species.

In this thesis, the **dynamics of global DNA methylation** was studied during microspore embryogenesis and gametophytic development in *Hordeum vulgare*, and the results were compared with those recently reported by our group in *B. napus*. The quantifications of global DNA methylation levels and the 5-methyl-deoxycytidine (5mdC) immunofluorescence assays showed low levels of DNA methylation in microspores and a high increase along gametophytic development and pollen maturation. The 5mdC signal increased in the generative and sperm nuclei whereas the vegetative nucleus showed lower signal. After cold stress and microspore embryogenesis induction, low levels of DNA methylation and weak signal of 5mdC were observed in nuclei of responsive microspores and 2 – 4 cell proembryos, indicating that a global hypomethylation occurs during the change of the developmental program and first embryogenic divisions in barley, as it was reported in rapeseed. At later developmental stages, the global DNA methylation was high in developing embryos and it increased during embryo maturation suggesting that DNA methylation is critical for embryo formation and plays a role in the regulation of gene expression in microspore embryogenesis.

This thesis also analysed **the effects of 5-azacytidine (AzaC)**, a DNA methylation inhibitor, on microspore embryogenesis initiation and progression in the two species, rapeseed and barley. AzaC treatments decreased global DNA methylation levels and favoured the initiation of microspore embryogenesis, increasing the production of multicellular embryos compared with untreated cultures. In contrast, this agent impaired the progression of microspore embryogenesis, at later stages. Taken together, the results indicate that DNA demethylation by AzaC promotes microspore embryogenesis initiation, while embryo differentiation requires *de novo* DNA methylation and is prevented by AzaC.

The dynamics of the presence and distribution of **arabinogalactan proteins (AGPs)** were studied during microspore embryogenesis in *Brassica napus* and *Hordeum vulgare*, by employing a multidisciplinary approach using monoclonal antibodies for AGPs (LM2, LM6, JIM 14, JIM 13 and MAC 207). The results showed that AGPs were induced during microspore embryogenesis and the blocking of AGPs with Yariv reagent impaired the process, in both species. This indicates that AGPs play a key role in microspore embryo development. AGPs epitopes were localized on cell walls and cytoplasmic spots suggesting an active production and secretion of AGPs during microspore reprogramming and embryo development. The distribution patterns of AGPs were also analysed during pollen development in *Brassica napus* and the results indicated that several AGPs are also related to pollen maturation and germination. It was also analysed the expression pattern of the *BnAGP Sta – 4* gene in *B. napus*. The results showed an increase in the expression of the *Sta 39 – 4* gene after microspore embryogenesis initiation suggesting that AGPs are induced with the switch of developmental program, constituting early markers of microspore embryogenesis. These results provide new evidence of the role of endogenous AGPs as potential regulating molecules of the process.

In this thesis, **the dynamics of endogenous auxin** was studied during the microspore reprogramming and embryogenesis in the monocot *H. vulgare*, and the results were

compared with those recently reported by our group in *B. napus*. The results obtained in this thesis provide information about the changes of auxin distribution during microspore embryogenesis in barley, a model system of monocot species. The results showed that auxin increased during early stages of microspore embryogenesis, findings that are consistent with previous results of our group on auxin distribution in microspore embryogenesis of *Brassica napus* and *Quercus suber*. It was also analysed, in the present work, the effect of N-1-naphthylphthalamic acid (NPA), inhibitor of polar auxin transport, and *P*-chlorophenoxyisobutytric acid (PCIB) auxin action inhibitor, on microspore embryogenesis. The analysis revealed that NPA treatment decreased the number of multicellular embryos, after the induction by stress, compared with untreated cultures; PCIB treatments completely inhibited the responsive microspores to reprogram to embryogenesis. These results indicate that polar auxin transport and auxin action have a key role in initiation and progression of microspore embryogenesis in barley, as it was reported in rapeseed, suggesting a common general role of auxin in the process.

CONCLUSION

In conclusion, the results in this PhD thesis revealed the involvement of DNA methylation, AGPs and auxin in microspore embryogenesis initiation and progression, possibly with common roles in dicot and monocot species. The results suggest that the induction of microspore embryogenesis requires DNA hypomethylation, AGPs induction and auxin accumulation, independently of the inductor stress and the species. Our results could open a way to design new biotechnological strategies for improving doubled-haploid production in breeding programs of horticulture and cereal crops.

The work of this thesis has given rise to three publications in scientific journals included in the Journal Citation Report (JCR) and a manuscript in preparation. These publications are the following:

1. **El-Tantawy, A.A.;** Solís, M.T.; Risueño, M.C. and Testillano, P.S. (2014). Change in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley. ***Cytogenetic Genome Research*, **143 (1-3), 200-208.

2. Solís, M.T.*; **El-Tantawy, A.A.*;** Cano, V.; Risueño, M.C. and Testillano, P.S. (2015). 5-azacytidine promotes microspore embryogenesis initiation by decreasing global DNA methylation, but prevents subsequent embryo development in rapeseed and barley, ***Frontiers in Plant Science*, **6: 472.

   * Both authors contributed equally.

3. **El-Tantawy, A.A.;** Solís, M.T.; Costa, M.L.; Coimbra, S.; Risueño, M.C. and Testillano, P.S. (2013). Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*. ***Journal of Plant Reproduction*, **26 (3): 231-243.

4. **El-Tantawy, A.A.;** Solís, M.T.; Risueño, M.C.; Testillano, P.S. (2015). Auxin increase and distribution, and effects of auxin inhibitors on microspore embryogenesis initiation and progression in barley. ***In preparation***.

GENERAL INTRODUCTION

1. SEXUAL REPRODUCTION IN ANGIOSPERMS

Sexual reproduction is the fusion of male with female gametes to produce a 2n diploid zygote that will form an embryo and this in turn, a new plant. Its importance is that in the zygote parental roles are combined, resulting genetically different to each of the parents (Figure 1). This type of reproduction allows variation by recombination of characters, which facilitates natural selection. The origin of gametes from somatic cells occurs at certain times of the life cycle of plants and there must be a reduction division called meiosis producing from each mother cell four daughter cells with chromosome number halved (gametic number). If this does not happen and the gametes have the same number of chromosomes as somatic or vegetative cells, the number of chromosomes would double with each fertilization (Meinke 1994: Fehér 2015).

1. a. Alternation of generations

The life cycle of vascular plants has alternation of two generations in which reproductive cells develop in the plant body (Figure 1). The generation that produces spores is called sporophyte ("sporophytic generation"), whose cells have chromosome number 2n. The generation that produces gametes is called gametophyte ("gametophytic generation"), and they are small plants whose cells have chromosome number n. The flowers in the sporophytes consist of modified leaves that produce two types of spores: microspores (male) and megaspores (female). The gametophytes have independent life, are parasitic plants, heterotrophic, which live at the expense of the sporophyte (Cocucci 1969). According to the type of produced gametes, there are microgametophyte (male) and megagametophyte (female). Accompanying cells with nourishing functions help these alternate generations during early development of spores and gametes (Esau 1982; Raghavan 1986; Meinke 1995; Purves et al 1998). Recent data reveal a two-way relationship between early reproductive cells and their companion cells involving complex epigenetic and signalling networks determining cell number and fate during these processes (Feng et al 2013; Solís et al 2014).

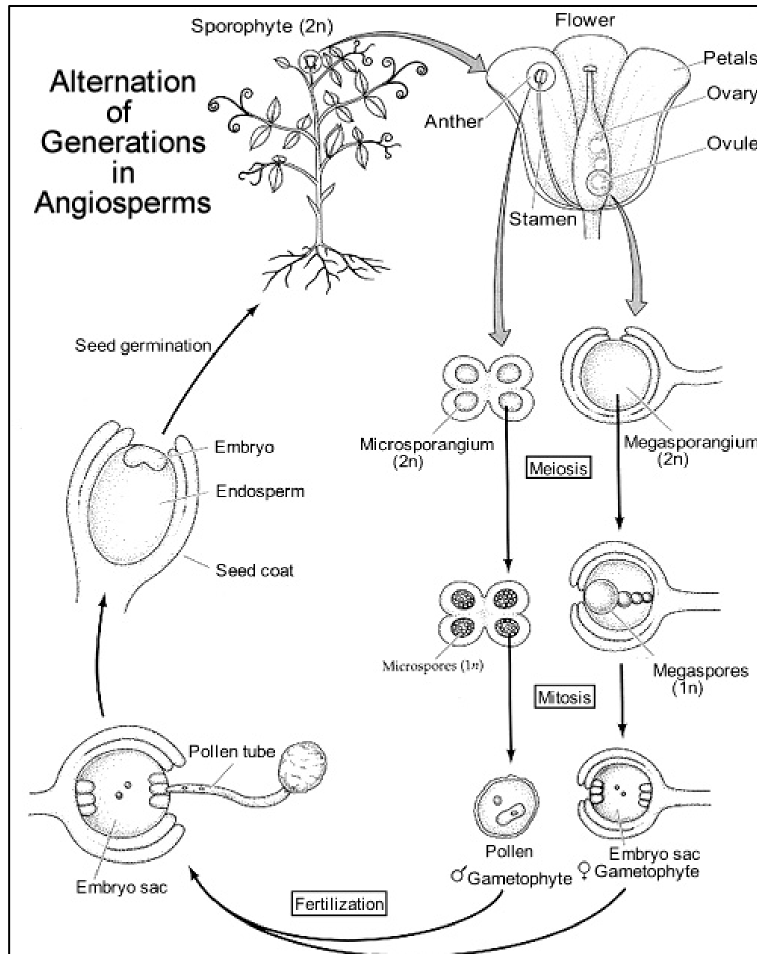

Figure 1: Representation of the haplo-diplod generations in Angiosperms (Pisabarro and Ramírez, University of Navarra)

1. b. Microsporogenesis and microgametogenesis in Angiosperms

In the pollinic sac of anthers, the diploid sporogenous cells differentiate as microspore mother cells (meiocytes) which undergo meiotic division to form four haploid microspores (tetrad) enclosed by the callose envelope (Konyar et al. 2013). At the beginning of meiosis, the microsporocytes are interconnected by wide cytoplasmic connections which are formed by expansion of plasmodesmata, making the entire mass of the meiocytes of the microsporangium a coenocyte, with rapid transport and distribution of nutrients that allow the synchronization of the meiocytes

in the meiotic prophase. Thus, this continuity is responsible for either synchronous meiosis within microsporangium (Risueño et al 1969).

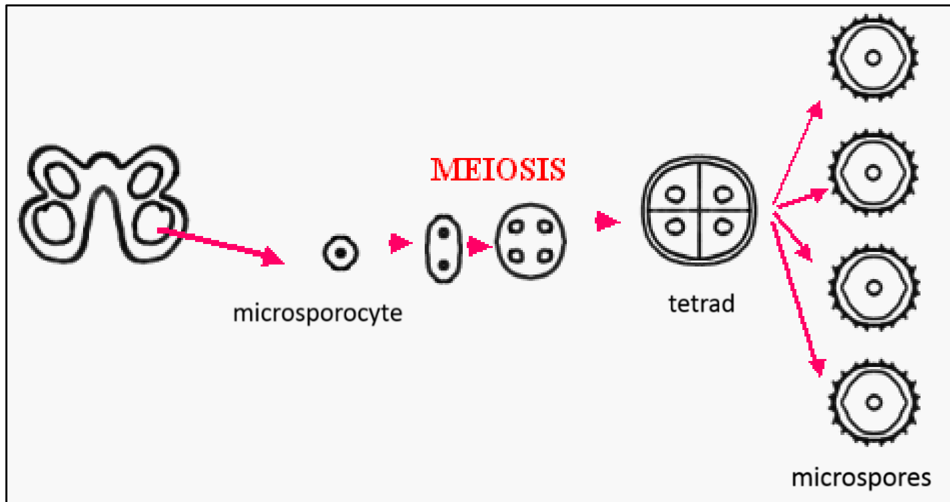

Figure 2: Microsporogenesis in Angiosperms (Arbo 2001)

Meiosis is a special cell division, specific to eukaryotic organisms whereby four haploid daughter cells are produced from a single diploid parent cell (Villeneuve and Hillers, 2001; Hamant et al., 2006; Mezard et al., 2007). This reductive division is achieved by a single round of DNA replication followed by two rounds of chromosome segregation and cell division (meiosis-I and meiosis-II) (Harrison et al. 2010). Upon completion of meiosis, each microspore is isolated from the others, surrounded by a special callose wall without plasmodesmata. RE cisterns regions locate where eventually the aperture will be formed. The size increase where there is no RE under the plasmalemma and the cellulose wall primexine deposits. The increased size and cell wall deposition occur while the microspores are still included in callose, and then continues till callose dissolves (Figure 2) (Risueño et al 1969).

Each unicellular microspore undergoes a mitotic division (Figure 3), resulting in the formation of the pollen grain with two different cells: a large vegetative cell and a small lenticular cell namely generative cell applied against the wall of microspore.

The function of the vegetative cell in the pollen grain is to extend a pollen tube to transport the two sperm cells to the embryo sac for fertilization. Because the vegetative cell is much larger and more metabolically active than sperm cells, it has been assumed that the vegetative cell also might supply various components to sperm cells, but there was no direct evidence to support this hypothesis. Recently using deletion and promoter exchange constructs, it has been provided direct evidence that the vegetative cell provides protein-encoding transcripts to sperm cells, highlighting a previously unidentified role of the vegetative cell (Jianga et al 2015).

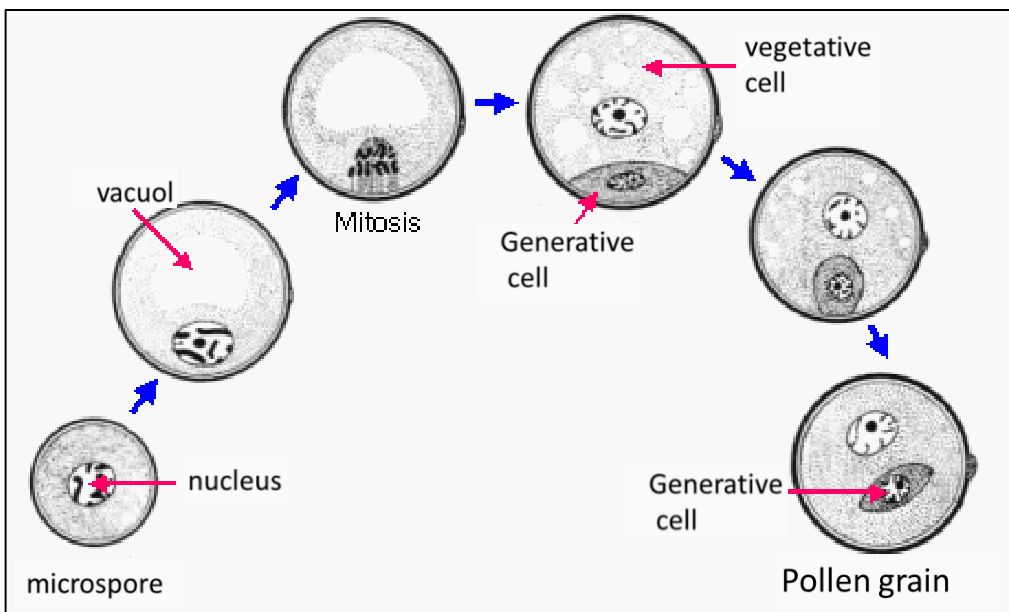

Figure 3: Microgametogenesis. Microspore gives rise to pollen grain, the macrogametophyte (Maheshwari 1950)

Thus, in addition its known role in transporting sperm cells during pollen tube growth, the vegetative cell also contributes transcripts to the sperm cells. Then, the generative cell undergoes a division (the second mitosis happens) and produces 2 cells: the male gametes, which are naked, they do not form cell wall (Figure 4). This division may still occur in the pollen sac or just after pollen germination; therefore, the pollen grain can be bicellular (generative cell + vegetative cell) or tricellular

(vegetative cell + 2 gametes) (Figure 4). Each mature pollen grain is the male gametophyte, ie the plant produces gametes, reduced to only two cells.

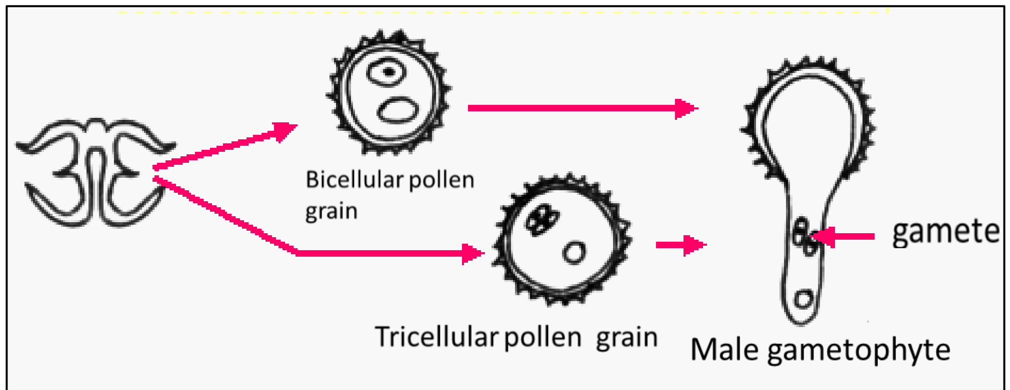

Figure 4: Microgametophyte (male) and polinic tube germination (Arbo 2001)

1. c. Megasporogenesis and megagametogenesis in Angiosperms

During megasporogenesis, the diploid megaspore mother cell undergoes meiosis and gives rise to four haploid nuclei. Angiosperms exhibit three main patterns of megasporogenesis, referred to as monosporic, bisporic, and tetrasporic (Figure 5). The three types differ mainly in whether cell plate formation occurs after these divisions, thus determining the number of meiotic products that contribute to the formation of the mature female gametophyte. Subsequently, three megaspores undergo cell death (Figure 5) (Maheshwari and Biswas 1970).

During megagametogenesis, the functional megaspore gives rise to the mature female gametophyte (Battaglia 1971). Initially, the megaspore undergoes one or more rounds of mitosis without cytokinesis, resulting in cell wall divided cytoplasm female gametophyte. Subsequently, cell walls form around these nuclei, resulting in a cellularized female gametophyte. During a third mitosis, phragmoplasts and cell plates form between sister and nonsister nuclei, and soon thereafter, the female gametophyte cells become completely surrounded by cell walls. During cellularization, two nuclei, one from each pole (the polar nuclei), migrate toward the

center of the developing female gametophyte and fuse together either before or upon fertilization of the central cell (Cocucci 1900; Maheshwari 1950; Willemse and van Went 1984; Haig 1990; Huang and Russell 1992; Drews et al. 1998).

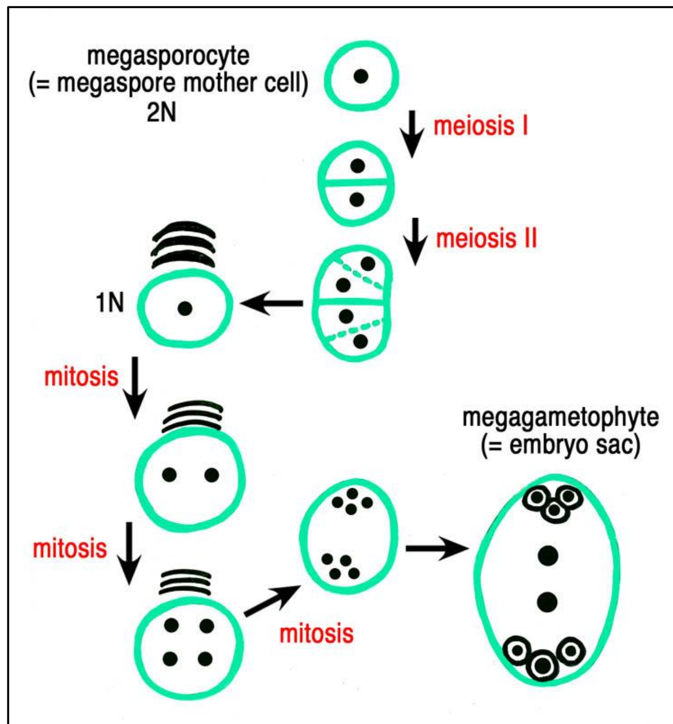

Figure 5: Megasporogenesis and Megagametogenesis. Megaspore divides and originates the Embryo Sac which is the Megagametophyte (female) constituted by 7 cells and 8 nuclei, n (Wiegand, 1900)

1. d. Double fertilization in Angiosperms

Double fertilization is a defining feature of reproductive development in the most evolutionarily successful and diverse group of plants, the angiosperms (Raghavan 1986; 2003; Purves at al 1988). The fertilization process begins with the germination of pollen grains on the stigma, developing the pollen tube, which grows through a process of cell wall synthesis in the tip, conducted by dictyosomes small vesicles that provide the wall contents of hemicellulose, pectic substances and the membrane that stretches the plasmalema (Risueño et al. 1968). On top, callose plugs, which seal on empty old parts, are formed. Many grains reach the stigma and germinate, but only

one of each fertilizes the egg cell. The tube grows on tissue cells transmitter digesting the cell walls middle lamina and continues to develop on the transmitter ovarian tissue, reaching the egg cell, which penetrates usually by micropyle.

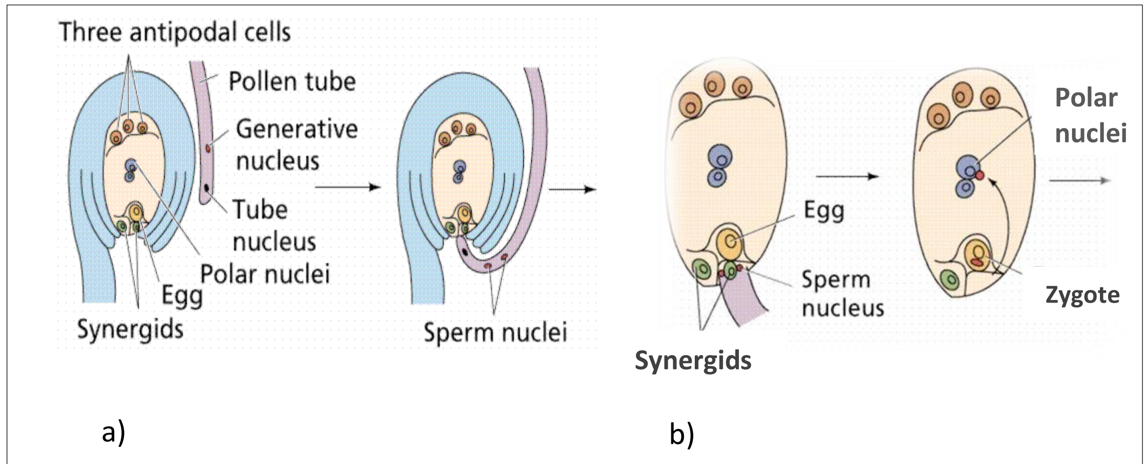

Figure 6: Several stages of the double fertilization process of the egg and polar nuclei by the two sperm cells in Angiosperms, a) Pollen tube grows style toward the ovules in the ovary, b) and once it reaches one of the synergids toward the ovule (egg cell) in the Embryo Sac or megametophyte one sperm cell fuses with the egg forming the zygote, the second sperm fuses with the two polar bodies located in the center of the sac, producing the nutritive triploid endoderm (adapted from Purves et al. 1998)

The eggs of some species have a micropylar viscous exudate that contains protein and carbohydrates that serve as a nutritious source and to guide the pollen tube. The cytoplasm, vegetative cell nucleus and gametes are found in the apical part of the pollen tube. The pollen tube contacts the embryonic sac in the filar apparatus of the synergid, and then a pore is formed at the tip of the tube so that it can discharge its contents into the cytoplasm of the synergid (Franssen-Verheijen & Willemse, 1993). This receives the gametes and part of its cytoplasm. One gamete penetrates the egg cell and the other sperm cells combine with the two polar nuclei of the large central cell of the megagametophyte. The haploid sperm and haploid egg combine to form a diploid zygote, whereas the other sperm and the two haploid polar nuclei of the large central cell of the megagametophyte form a triploid nucleus (triple fusion) (Figure 6). The large cell of the gametophyte will then develop into the endosperm, a

nutrient-rich tissue which provides nourishment to the developing embryo. The ovary, surrounding the ovules, develops into the fruit, which protects the seeds and may function to disperse them (Berger et al. 2008). The two central cell maternal nuclei (polar nuclei) that contribute to the endosperm, arise by mitosis from the same single meiotic product that gave rise to the egg. The maternal contribution to the genetic constitution of the triploid endosperm is double that of the embryo.

This process, called "double fertilization", is characteristic of angiosperms and represents an evolutionary advance (Raghavan 1986; Harada et al 2010; Weijers et al 2005). From pollination to fertilization it may take 12-48 hours, most common, or 14 months as in *Quercus*. (Johri et al. 1984). Characterization of the genes and their protein products has provided evidence for a predominant effect of maternal gametophytic genes and silencing genes during double fertilization. This is due to genetic imprinting producing differential gene expression which refers to differential activity of alleles inherited from the egg and sperm. Zygotic genome activation occurs following a period of transcriptional quiescence after fertilization (Baroux et al 2001, Lee et al 2014).

2. ZYGOTIC EMBRYOGENESIS

Zygotic embryogenesis is a process that can produce embryos from the zygote cell which is formed in the fertilized ovule. After a first asymmetric division, embryogenesis proceeds and differentiates into tissues and embryogenic organs such as shoot and root apical meristems (Meinke 1994; Suárez and Bozhkov 2008; Harada et al. 2010; Dodeman et al 1997).

The plant embryogenesis begins with an asymmetric cell division, resulting in a smaller apical cell, which will develop into an embryo proper, and a basal cell that develops into the suspensor by successive divisions. The suspensor anchors the embryo to the endosperm and serves as a nutrient conduit for the developing embryo

(Souèges et al 1914; 1919; Jurgens et al 1991; Park and Harada 2008; Goldberg et al. 1994; Meinke 1995). The smaller apical cell results the embryo by itself, thus, further cell divisions lead to form the globular embryo, in which the first cell differentiation is observed in the embryogenic epidermis or protoderm (Suárez and Bozhkov 2008). The hypophysis forms at the top of the suspensor, therefore, the embryo cell undergoes three divisions, two vertical divisions and one transverse division to form eight cells arranged in two layers, epibasal (terminal) and hypobasal (near the suspensor).

2. a. Development of dicotyledonous embryo

In a typical dicot, the zygote elongates and then divides by a transverse wall into two unequal cells (Schulz and Jensen, 1969) giving arise a basal cell (BC) and a terminal cell (TC) (Figure 7 A, B). Basal cell divides transversely and latter divides longitudinally forming the reverse -T shaped proembryo of 4 cells (Figure 7 C-E). Each of the two terminal cells divides by a vertical wall lying at right angles to the first to form quadrant stage (Figure 7 J). The quadrant cells divide by transverse walls giving rise to octant stage (Figure 7 K, L). Of this octant lower four cells form “stem tip and cotyledons” and upper four form hypocotyl. All the eight cells undergo periclinal divisions differentiating an outer “dermatogen and inner layer of cells” (Figure 7 M, N). The cells of dermatogen divide anticlinally to give rise to epidermis of embryo, while the inner cells by further divisions, give rise to the “ground meristem and procambial system of the hypocotyl” and “cotyledons”. At this time, the two upper cells of four-celled proembryo (Figure 7 D) divide to form a row of “6-10 suspensor cells” (Figure 7 F-K) of which the uppermost cell (V) becomes swollen and vesicular to form the “haustorium”. The lower cell (h) functions as “hypophysis” which divide to give rise to eight cells. The lower four of these form the “root cortex initials”. The upper four form the “root cap and root epidermis”. At first, the embryo is globular, later heart-shaped and constituted by two cotyledon primordia. The hypocotyl as well as cotyledons elongates soon forming the torpedo shaped embryo.

Thus, a developed embryo of dicotyledons has an embryonal axis differentiated into plumule, two cotyledons and radicle. Afterwards, the ovule becomes curved like horse-shoe (Figure 8).

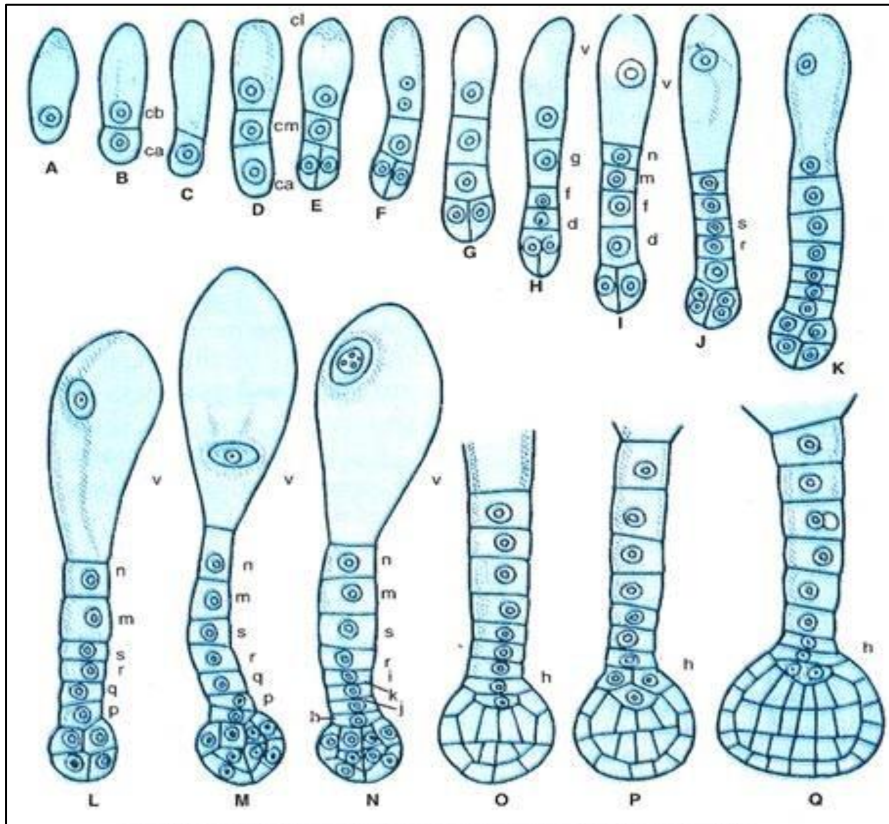

Figure 7: Early stages of development of a dicot embryo. *Capsella bursa-pastoris* (Crucifera) A-Q Early stages showing the successive divisions to globular stage (Souèges 1914).

The epibasal cells will eventually form the two cotyledons and the plumule whereas the hypobasal cells will produce the hypocotyl except its tip (Schulz and Jensen, 1969; Goldberg et al. 1994). The root meristem will be formed from derivatives of both basal and apical cells of two-cell embryo. The hypophyseal region, which is derived from the uppermost cell of the suspensor, is incorporated into the embryo proper, giving rise to part of the root apex, its initial cells and the ground meristem initial cells. In addition, the procambium tissue, which is in the central portion of the

embryo, is contributed by the apical cell with the ground meristem. The shoot apical meristem is determined in the early globular embryo before the cell cleavage that delineates the protoderm. Later, numerous divisions occur leading to an increase in size of the cotyledons and the shaft, to produce heart and torpedo-shaped embryos (Gilbert 2000; Souter and Lindsey 2000; Kawashina and Goldberg 2009). The cells in the apical region in the embryo are induced to proliferate and finally the mature cotyledonary embryo is formed (Figure 8).

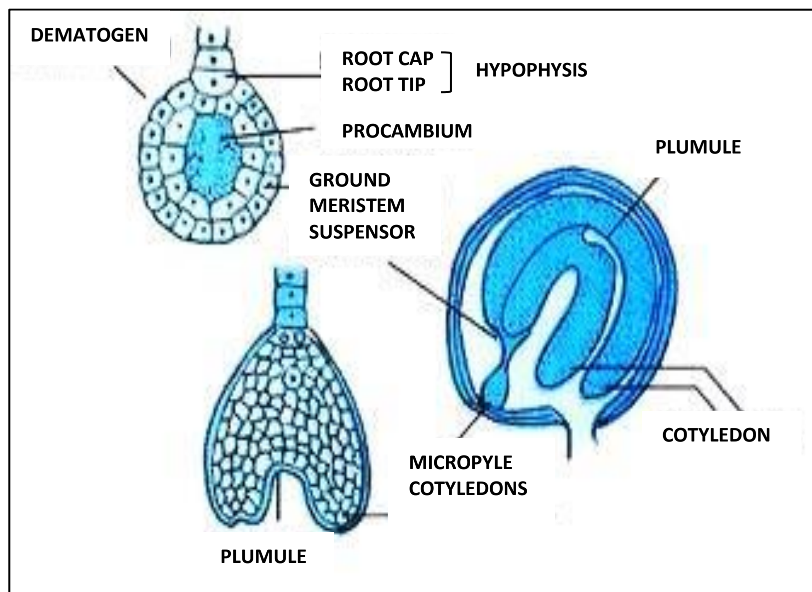

Figure 8: Advanced stages of development of a dicot embryo. *Capsella bursa-pastoris* (Crucifera). Globular embryo, Heart shaped (cordate) embryo, Horse shoe shaped embryo, showing the initial cells of the diferent tissues (dermatogen, procambiun, plumula, cotyledon etc.) (Souèges 1919).

2. b. Development of monocotyledonous embryo

Monocotyledonous embryo has several differences in the developmental stages regarding dicotyledons but there are no essential differences in the early cell divisions of proembryo. Typical embryogenesis in monocots are described in two main models, “type Sagittaria” and “type grass family’’ (Gramineae) (Raghavan 1986; Rudall 1997). In both types (Figure 9), firstly zygote or oospore elongates and then divides

transversally to form a three-cell stage proembryo, basal, middle and terminal cells. Larger basal cell at the micropylar region does not divide more and is transformed directly to form a large suspensor (vesicular cell), that it may function as haustorim. Terminal cell, by a number of divisions at various planes forms a single cotyledon. The middle cell undergoes repeated transverse and vertical divisions, differentiating into few suspensor cells, radicle, plumule and hypocotyl.

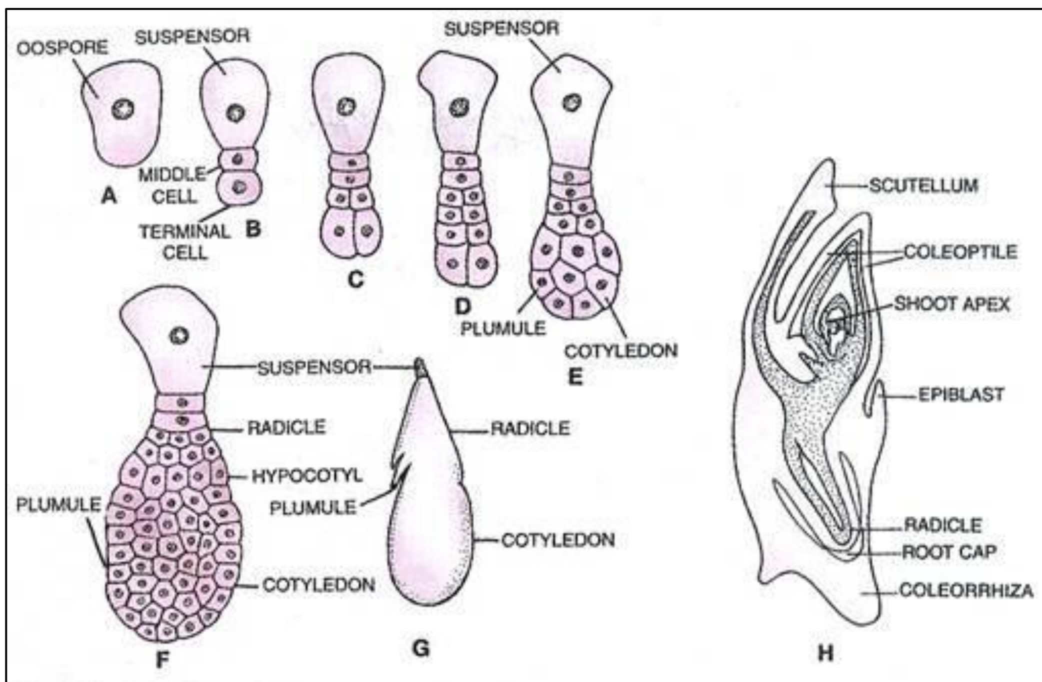

Figure 9: A-G, Stages of developmental monocot embryo. H, a monocot embryo (Wiegand 1914)

The embryos of monocotyledons have only one cotyledon which in grass family (Gramineae) is called scutellum (Figure 9) and is situated towards the lateral side of embryonal axis. And at the lower end of this embryonal axis there is the radicle and root cap enclosed in the coleorhiza. The region of axis above the attachment of scutellum is called epicotyl. It has as shoot apex and few leaf primordia enclosed in a hollow foliar structure called coleoptile (Wiegan1914; Maheshwari1950). And the epiblast represents rudiments of second cotyledon (Figure 9).

2. c. Factors involved in embryogenesis

Embryogenesis is a critical stage in the life cycle of higher plants (Meinke 1994; 1995; Harada et al. 2010; Harada 1999). At the end of the zygotic embryogenesis, the maturation of the embryo occurs; this developmental stage is characterized by accumulation of storage carbohydrates, lipid and proteins. Later, the water content will be reduced followed by cessation of metabolism. These processes are regulated by numerous factors, including phytohormones, enzymes and other substances related to embryogenesis (Mayer et al. 1998; Fehér 2015; Weijer et al. 2002).

Molecular and genetic studies on the component elements of double fertilization have focused on the identification of mutants of *Arabidopsis thaliana* that display developmental patterns in the seed resulting in autonomous endosperm development and even partial embryogenesis in the absence of fertilization.

Large numbers of genes must be expressed in a highly coordinated manner to ensure that the single cell zygote develops into an organized, multicellular structure capable of surviving desiccation and germinating to produce a viable seedling (Wiliams and Maheshwari 1986; Meinke 1995; Weijers and Jurgens 2005; Harada et al. 2010; Solís-Ramos et al 2012).

Zygote undergoes a series of differentiation events, leading to the formation of a mature embryo. Establishment of the major embryogenic organs and shoot and root apical meristems occur though partitioning events along the apical–basal axis, and many of these events are guided by the hormone auxin. Auxin and other plant hormones appear to play critical roles in inducing embryogenic competence (Jurgens et al 1991; Jurgens and Meyer 1994). Imprinting zygotic genome activation occurs in embryos during the first few days after fertilization (Gerhing 2013; Grimanelli and Grossniklaus 2011; Garcia-Aguilar and Guillmor 2015).

A bias in gene expression happen due to the genetic imprinting which causes the expression of a gene to be dependent on its parent of origin, due to differential

epigenetic modifications established during male and female gametogenesis (reviewed in Gehring 2013). Hundreds of imprinted genes have been discovered in the endosperm, and a few have been described in the embryo.

3. ZYGOTIC EMBRYOGENESIS *VERSUS* SOMATIC EMBRYOGENESIS

The zygote is not a unique cell in angiosperms to produce an embryo; a wide range of somatic cells can be induced to produce embryos either as part of the normal life cycle or through experimental manipulation. Thus, somatic cells can be induced to deviate from their normal fate and develop into embryos in a process called “somatic embryogenesis”. Embryogenesis can arise from isolated somatic cells either naturally, as has been observed in Kalanchoe, where somatic embryos form spontaneously on the edge of leaves or from microspore-pollen in anthers, as described in Datura (Guha and Maheshwari 1964) or in vitro after experimental induction (de Vries et al. 1988).

The developmental stage is of prime importance to enable the transition from somatic to embryogenic cells. The acquisition of embryogenic competence involves an induction phase for which there is no direct counterpart in zygotic embryogenesis. The zygote is intrinsically embryogenic. On the contrary, somatic embryogenesis requires the induction of embryogenic competence in cells which are not naturally embryogenic (Sliwinska and Bewley 2014; Fehér 2015). Molecular and genetic analyses have been performed to identify the mechanisms underlying the sequence of events during plant zygotic and somatic embryogenesis (Dodeman et al. 1997; Solis-Ramos et al. 2012; Weijers et al. 2005; Bueno et al. 1992; Rodriguez et al. 2015).

Somatic embryogenesis has long been studied, but the mechanisms underlying the change of this cell reprograming are still unclear (Williams and Maheshwari 1986; Harada et al. 2010; Sliwinska and Bewley 2014; Fehér 2015). Microspore derived–embryos after defined stress are a very useful system to study the events that accompany the change of the program from the somatic to embryogenic cell and to

identify mechanisms of embryo initiation and progression. Especially relevant are the processes of reprogramming, totipotency acquisition and embryogenic competence that operate in cell specification in plants as well as embryo development and polarity, establishment of the major embryonic organs and shoot and root apical meristems, etc. Zygotic and somatic embryogenesis represent parallel developmental programs in which cells acquire embryogenic cell fate and develop into mature embryos (Harada et al. 2010; Weijers et al. 2005; Rodríguez et al. 2015).

For a long time, somatic embryogenesis has been studied in cultures of carrot (*Daucus carota* L.) (Komamine et al, 1990) and alfalfa (*Medicago sativa* L.) (Dudits et al, 1991). Several authors originally described the latter two systems using their own terminology, (De Jong et al. 1993). Suspension cultures are often described as undifferentiated; nevertheless, unorganized is probably a better term since in many cultures subcellular populations retain features associated with specific differentiated cell types. The term 'embryogenic cell' would be limited to cells which have achieved the transition from a somatic cell to a stage where no further external stimuli are required to produce a somatic embryo (Komamine et al. 1990; Zimmerman 1993).

For instance, in carrot, the usual strategy to induce an embryogenic cell suspension consists in exposing explants to a high auxin concentration, then to transfer cells to an auxin-free medium which triggers somatic embryo formation. Cells able to undergo embryo development generally appear as proembryogenic masses (PEM) composed of dense cytoplasmic small cells (Halperin, 1966). It is important to note that in most carrot embryogenic cultures, the percentage of cells which are actually embryogenic is rather low, typically 1-2% (de Vries et al. 1988; Zimmerman 1993).

There exist at least three ways to induce somatic embryo development from *in vitro* cultured plant cells (Solís-Ramos et al 2013) as they are: (1) *in vitro* fertilization, (2) *in vitro* somatic embryogenesis and (3) *in vitro* microspore embryogenesis. Therefore, zygotic and somatic embryogenesis represents parallel developmental programs in which cells acquire embryogenic cell fate and develop into mature embryos.

4. MICROSPORE EMBRYOGENESIS

Microspore embryogenesis is a type of haploid embryogenesis which is induced by stress treatment from *in vitro* culture of anthers or isolated microspores which can divide to develop into embryogenic pathway to produce and regenerate doubled haploid plants (Reynolds 1997; Palmer and Keller 2005; Maluszynski et al. 2003; Forster and Thomas 2005).

To understand the microspore embryogenesis it is important to know the haploidy in the plants. The term haploid is used to indicate plants that have one set of gametic chromosomes (n) while the diploid plants have two sets of chromosomes (2n). The first haploids were indicated by Belling and Blakeslee in (1922) who studied the chromosomes behaviour in *Datura sp.* This was the beginning to investigate several species and cultivars developed as doubled haploids (DH) (Pintos et al. 2007; 2013; Palmer et al. 2005; Maluszynski et al. 2003; Thomas et al. 2003). It is important to notice that the doubled haploids (n+n) are homozygous because they actually originate from haploids but their chromosomes are doubled, whereas the diploids (2n) are not homozygous as their two chromosomes come from two different chromosome sets (Testillano, et al. 2004; Kasha and Maluszynski 2003).

The doubled-haploid plants are used widely to obtain isogenic lines and new varieties as well as important biotechnological tools in plant breeding; the production of these plants occurs by induction of haploid embryogenesis (Palmer and Keller 2005; Forster et al. 2005; Maluszynski et al. 2003). The production of haploid plants present four main methods:

- **Parthenogenesis:** The development of embryo from the egg cell without fertilization (Mohan et al. 1996). It can be induced by irradiate the inactivated pollens with gamma rays (Froelicher et al. 2007; Grouh et al. 2011) or with chemical treatments (Kielkowska et al. 2014; Klush and Virmani 1996).

- **Wide hybridization crosses:** The hybridization occurs after the crossing between two parents from the same species, called intraspecific hybridization, or from different species, called wide hybridization. In the later, followed by the fertilization, the chromosomes of one of the parent plants are eliminated during seed development (Mishra and Goswami 2014; Wędzony et al. 2009; Forster and Thomas 2005; Sharma et al. 1995) and thus, the haploid embryo should be rescued to make in vitro culture (Devaux 2003; Hayes et al. 2003). This method to produce doubled haploid plants succeeded in many plants especially the cereals (Murovec and Bohanec 2012; Laurie and Bennett 1988; Verma et al. 1990) but the reduction of fertility in DH plants has been reported by Riera-Lizarazu et al. 1996.

- **Gynogenesis:** It is possible to produce haploid embryos through culturing the unfertilized ovary (method called Ovule culture). The ovule can be isolated from the flower bud and placed in appropriate media (Mukhambetzhanov 1997; Nikolova and Alexandrova 2001; Mishra and Goswami 2014). On the other hand, the success of this process depends on the species, this culture shows very low potential in some plant families (Touraev et al. 2001; Alan et al. 2003).

- **Microspore embryogenesis:** On the contrary of the gynogenesis, this process occurs by inducing the microspores to form embryos through anther or isolated microspore culture by stress treatments (such as heat, cold, osmotic treatment and starvation). The microspores can reprogram, divide and proliferate to initiate embryogenesis, with the suitable conditions (Ferrie and Caswell 2011; Seguí-Simarro et al 2011; Kasha and Maluszynski 2003).

The phenomenon of microspore embryogenesis has been investigated by many scientists (Reynolds 1997; Palmer and Keller 2005). Guha and Maheshwari, in 1964, discovered the induction of microspore embryogenesis when they cultured the anthers of *Datura innoxia* and they proved that the microspore, which forms the pollen grain through the normal gametophytic pathway, can be induced to give rise haploid embryos and then haploid plants. Later, the first embryogenic induction from isolated microspore cultures was described by Nitsch and Norreel in 1973, with the same species; they cultured the microspores in liquid medium after pre-treatment of the flower buds for 48 hours at 3 ˚ C. About 40 years later, this process has been induced in many species and the scientists investigated the activity of the microspore to follow the embryogenic pathway in various species (Winarto and Teixeira de Silva 2011; Palmer et al. 2005; Maluszynki et al. 2003).

4. a. The developmental pathway of microspore embryogenesis

The vacuolated microspore is the most appropriate stage to change the program by application of stress and then to switch from the gametophytic development to embryogenic development. The microspores divide symmetrically producing new structures called multicellular embryos which further differentiate to form the embryos (Figure 10).

In response, to the stress treatment, there are changes in the gene expression and in the organization of the cell structure of the microspore which affect the nucleus and the cytoplasm (Touraev et al. 1996a; González-Melendi et al. 1995; Solís et al. 2012). It has been indicated that the early genes are very important to initiate the microsporogenesis whereas the late genes motive the microspore mitosis during microgametogenesis to form pollen grain, in maize and barley (Pulido et al. 2009; Bedinger and Ederton 1990). Although the changes in gene expression and proteins during microspore embryogenesis in different species have been described by many reports (Solís et al. 2012; Bárány et al. 2010a; Rodríguez-Serrano et al. 2012; Bárány et al. 2010b; Prem et al. 2012; Rodríguez-Sanz et al. 2014a; Rodríguez-Sanz et al.

2014b; Solís et al. 2014; Testillano et al. 2013; Germaná et al. 2006), the regulating mechanisms are still unknown.

Numerous reports indicated that for analyzing the process, the isolated microspore culture is the best way to induce the vacuolated microspore to develop into embryos compared to the anther culture in which microspore embryogenesis occurs in the anther locule causing difficulty to make analysis of the early stages of embryogenesis (Reynolds 1997), but this does not prevent that the choice of the method to induce the pollen embryogenesis will depend on the species and genotype (Palmer and Keller 2005). The induction of microspore embryogenesis has been reported in near 140 species, included dicots and monocots (Malusztnski in 2003).

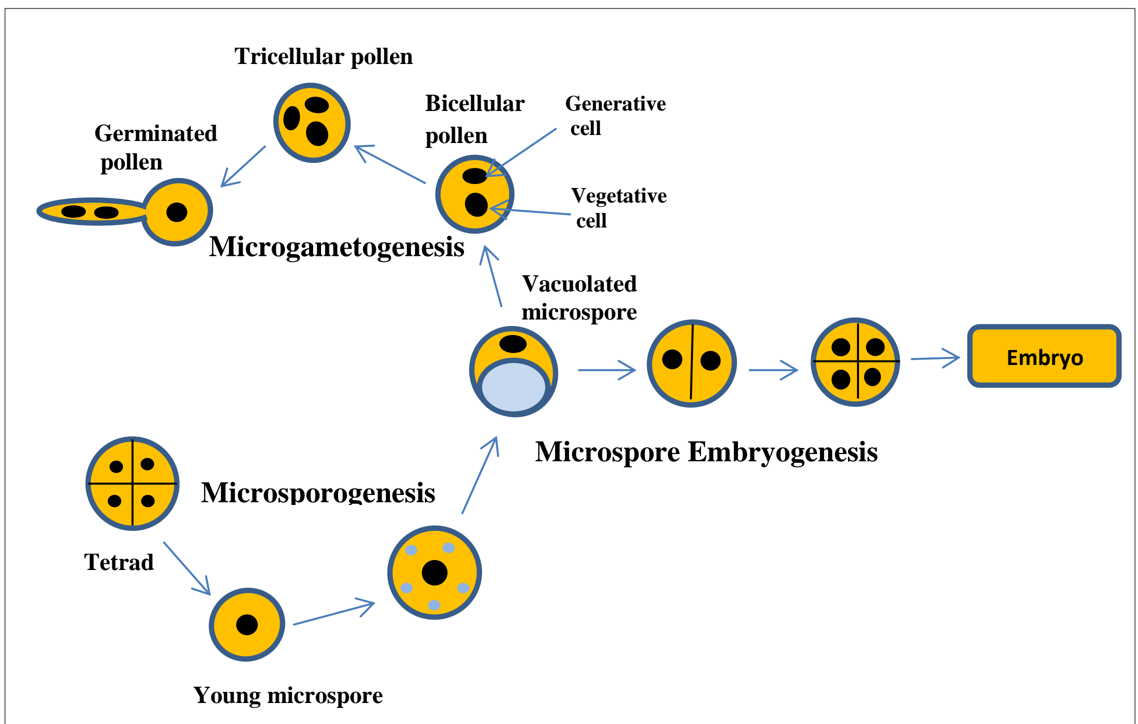

Figure 10: Scheme of pollen development (microsporogenesis and microgametogenesis) and microspore embryogenesis.

4. b. Factors affecting microspore culture

There are several endogenous and exogenous factors that affect the response of microspore embryogenesis induction. The most important factors are: (1) the growth

condition of donor plants, (2) the selected microspore stage, and (3) the stress inductive treatment.

The donor plant conditions play a very critical role in the response of microspore cultures. The growth conditions such as temperature, humidity and photoperiod control the efficiency of microspores to respond to the stress treatment, as demonstrated in many species (Cheng et al. 2013; Maluszynski et al. 2003; Palmer et al. 2005; Rynolds 1997; Prem et al. 2012). Several works indicated that the suitable temperature of the growth chambers is about 16 to 18 ˚C under 15 to 18 hours photoperiod with 60 – 70 % humidity in cereal plants (Kasha et al. 2003; Gupta et al. 2007; Winarto et al. 2011; Zheng 2003; Zapata-Arias 2003); but the optimal conditions depend on each species to grow healthy.

The stage of microspore development is the most important factor in the microspore embryogenesis induction. The vacuolated microspore is the most sensitive stage to induce embryogenesis in various species as noticed by many investigators (Telmer et al. 1992; Gonzalez-Melendi et al. 1995; Germaná et al. 2011; Pulido et al. 2001). Different reports found that the vacuolated microspore contains a nucleus with a chromatin pattern and nucleus organization typical of active cell for transcription (Seguí-Simarro et al. 2011; González-Melendi et al. 1995).

The third factor affecting microspore embryogenesis induction is the stress inductive treatments. The vacuolated microspores can be induced to embryogenesis by low or high temperature, depending on the species (Tyagi et al. 1979; Maluszynski et al. 2003). The cold treatment with 4 ˚C for long term (20 – 28 days) is the most common method in cereal plants (Ayed et al. 2010; Kasha et al. 2003; Zheng et al. 2003). During pretreatment, the microspores are induced to the embryogenic pathway by fragmentation of vacuole and block the first haploid mitosis to produce pollen grain (Khound et al. 2013; Kasha et al. 2001a, b). The heat treatment (32.5 ˚C) is the most common method for embryogenic induction in many horticultural species such as rapeseed, broccoli, pepper and tobacco (Custers 2003; Touraev and Heberle-Bors

2003; Prem et al. 2012; Kasha et al. 2003a; Kasha et al 2003b; Zheng et al. 2003; Bárány et al. 2005). Also, carbohydrate starvation and osmotic stress have been used as stress treatments for inducing the microspores to embryogenesis in different species and many cereals (Touraev et al. 1997; Lulsdorf et al. 2011; Zheng 2003).

Other important factors are the composition of the culture media, which includes macro and micro nutrients to feed the microspores, and sucrose as a source of energy in dicot plants, or maltose in cereal crops (Klíma et al. 2008; Castillo et al 2000). Growth regulators such as auxins and cytokinins are important phytohormones included in the medium of some microspore cultures (Esteves et al. 2014; Bárany et al. 2005; Kumlehn et al. 2006), but there are some species that do not require any growth regulators in the medium to induce the microspore embryogenesis, such as rapeseed (*Brassica napus*) and tobacco (*Nicotiana tabacum*) (Touraev et al. 1996b; Custers 2003; Touraev and Heberle-Bors 2003; Pechan et al. 1988; Prem et al. 2012).

4. c. The application of microspore embryogenesis in plant breeding

Swanson et al. 1987 used the microspore embryogenesis of *Brassica napus* to produce the doubled haploids plants by *in vitro* culture. This work and others encouraged scientists to investigate the applications of this method for improving various agricultural crops (Huang and Keller 1989; Jähne and Lärz 1995; Babbar 2004; Maluszski et al. 2003; Zheng 2003; Palmer et al. 2005; Pink et al. 2006; Germaná et al. 2006; Forster et al. 2007; Xu et al. 2007; Ferrie and Caswell 2010; Grouh et al. 2011; Kiełkowska et al. 2014).

The microspore embryogenesis become a biotechnological tool in plant breeding for value in crops and double-haploid (DH) protocols, since it can produce isogenic lines and genetic variability rapidly, as well as propagation of selected genotypes in species with long reproductive cycles or low seed production, through obtaining double-haploid plants. It can produce seedling rapidly and can obtain plants free from any

disease or viruses (Islam and Tuteja 2012). DH protocols are the fastest way to get homozygous plants which can reduce the time and costs of producing cultivars.

Although DH production is now widely used in many seed companies, this process still has significant limitations in their exploitation since there are one or more bottlenecks that need to be overcome before an efficient system can be established for a specific crop or genotype. The major bottlenecks in DH production are the low efficiency of haploid embryo induction and, consequently, very low number of seedlings (Germaná 2006), and in cereals, the high frequency of albino plants (Torp and Andersen 2009). The use of microspore embryogenesis has been extended for many economic crops (Ferrie et al. 2011; Seguí-Semarro et al. 2011). The efforts to reveal the mechanism of regulation of microspore embryogenesis process have been performed basically in the *in vitro* isolated microspore system in the species considered as models for this process such as *Brassica napus* and much less is known in other plant species. In this PhD thesis, the microspore embryogenesis has been studied by using the system of *in vitro* isolated microspore culture of two different plant species *Brassica napus* and *Hordeum vulgare*. The study has been conducted to analyze some key factors involved in the process: DNA methylation, arabinogalactan proteins (AGPs) and endogenous auxins.

5. PLANT SYSTEMS TO STUDY MICROSPORE EMBRYOGENESIS

In this thesis, the microspore embryogenesis is studied in two species, rapeseed (*Brassica napus*) as the best model in dicots, in this species, microspores can be reprogrammed by a stress treatment of high temperature without using growth regulators, and barley (*Hordeum vulgare*) as a model to study this process in monocots, in barley, the process is induced by low temperature treatment.

5. a. Rapeseed (*Brassica napus* L.)

Rapeseed (*Brassica napus*), belongs to the family *Brassicaceae*, it is a herbaceous annual plant that has stems of around 150 cm in length, with branches in the axils of

the largest leaves on the stem, and each terminates in an inflorescence which is an elongated raceme. The root hairs have an average length around 0.58 to 1.16 mm depending on the cultivars (Wees et al. 2015). The flowers are bright-yellow, clustered at the top and open upwards from the base of the raceme (Figure 11). This plant can be used as forage for animals, especially livestock, human consumption, and in the pharmaceutical industry (Friedt and Snwdon 2009). Also, biodiesel produced from the rapeseed oil that can be used in new vehicle engines.

Yield in rapeseed encouraged the plant breeders that used the heterosis in hybrid breeding leading to increase in the production levels. The average heterosis percentage is about 30 % for vegetative biomass, 17.5 % for seeds per pod. Leaves, pods, and other above-ground green tissues are able to photosynthesize as ‘source’ organs, while seeds are storage organs which serve as the ‘sink’ for photosynthetic products. (Luo et al. 2015). The rapeseed has higher requirements for nitrogen, phosphorus and sulphur than cereals and other plants (Bloem and Haneklaus 2002).

Figure 11: Rapeseed plant (*Brassica napus*)

The medical uses of rapeseed oil is limited as it contains a high level of erucic acid (50%), which can damage to cardiac muscle, and glucosinolates, which make less nutrients for animals. Therefore, Canadian scientists used traditional plant breeding program to produce another cultivar of rapeseed plants called canola which could produce oil with less erucic acid (2%) to avoid the harmful of rapeseed oil. Canola oil

is very healthy for human use; it contains very low saturated fats and the highest levels of plant sterols making it one of the recommended treatments to reduce the risk of heart disease. Moreover, canola oil is rich in vitamin E, which is used as an antioxidant, and in mono-unsaturated fatty acids producing high calories.

The isolated microspore culture in rapeseed has firstly been reported by Licher in (1982) producing high embryo yields without hormones in the culture media (Swanson et al., 1987; Polsoni et al., 1988; Keller et al. 1987). The system could generate haploid and doubled haploid plants (Prem et al. 2008; Prem et al. 2012; Ferrie and Caswell 2011; Möllers et al. 1994). Many investigations proved that in rapeseed, high temperature stress treatment, applied to fresh vacuolated microspores, can induce the production of abundant embryos, making this plant a model in dicots for studying the pollen embryogenesis induction (Hause et al. 1993; Zhao et al. 1996; Custers et al. 2001).

In the genus *Brassica*, the application of isolated microspore culture techniques were found in cauliflower *B. oleracea* L. *var. capitata* (Cao et al. 1990), Broccoli *B. oleracea* L. *var. italic* (Takahata and Keller 1991). The successful isolated microspore culture of *Brassica napus* has been achieved by Lichter in (1982). The heat treatment is one of the most important factors for the development of isolated microspore in culture where the heat treatments switch symmetric divisions and give a high embryogenesis efficiency compared to low temperature system (Prem et al. 2012; Duijs et al. 1992; Ferrie and Caswell 2011) although it may be replaced by other stresses such as low levels of irradiation, ethanol, and colchicine (Pechan and Keller 1989; Zhao et al. 1996).

Recently, it has been reported that the temperature at 32.5˚C is the optimal temperature to trigger microspore embryogenesis, and then, for the formation of multicellular structures or embryo-like-structures in *Brassica napus* (Zeng et al. 2015; Gu et al. 2003). However, the optimum temperature treatment to induce the microspore embryogenesis varies with different species and cultivars of *Brassica*. For

example it has been reported that the temperature at 30.5˚C for 48 hours followed by incubating at 25˚C continuously was the best temperature treatment to induce the isolated microspore culture in *B.oleracea* CV. 'Kemeh and Garung' (Winarto et al. 2011) whereas 32˚C for 1 day and 25˚C continuously were the best treatment for microspore culture in *B. oleracea* (Dias and Correia 2002). A new microspore embryogenesis system under low temperature (18˚C) has been performed by our group in *Brassica napus* and it has been found that the microspores divided symmetrically to form the pro-embryos but the embryo yield was lower compared to heat treatment (Prem et al. 2012).

In this thesis, isolated microspore cultures of *Brassica napus* were subjected to 32˚C; microspores were isolated from donor plants grown at low temperatures which contain a high proportion of microspores at optimum metabolic state, as described by Prem et al. (2012). This in vitro system flower buds in sizes ranged from 3.0 to 3.9 mm, collected from donor plants of the genotype The microspores were isolated from the buds after sterilize them in 5.0% bleach for 20 minutes. Later, the isolated microspores were pestled in NLN-13 medium containing 13% sucrose and cultured under 32ºC. A few days after the culture initiation, the responsive vacuolated microspores divided symmetrically forming multicellular embryos or proembryos which subsequently gave rise to early globular embryos after several divisions. After observing the globular/heart shape embryos, cultures were transferred to 25˚C. Around 30 days after culture initiation, mature cotyledonary embryos were formed (Figure 12).

Numerous reports indicated that for analysing the process, the isolated microspore culture is the best way to induce the vacuolated microspore to develop into embryos compared to the anther culture in which microspore embryogenesis occurs in the anther locule causing difficulty to make analysis of the early stages of embryogenesis (Reynolds 1997), but this does not prevent that the choice of the method to induce the pollen embryogenesis will depend on the species and genotype (Palmer and Keller

2005). The induction of microspore embryogenesis has been reported in near 140 species, included dicots and monocots (Malusztnski in 2003).

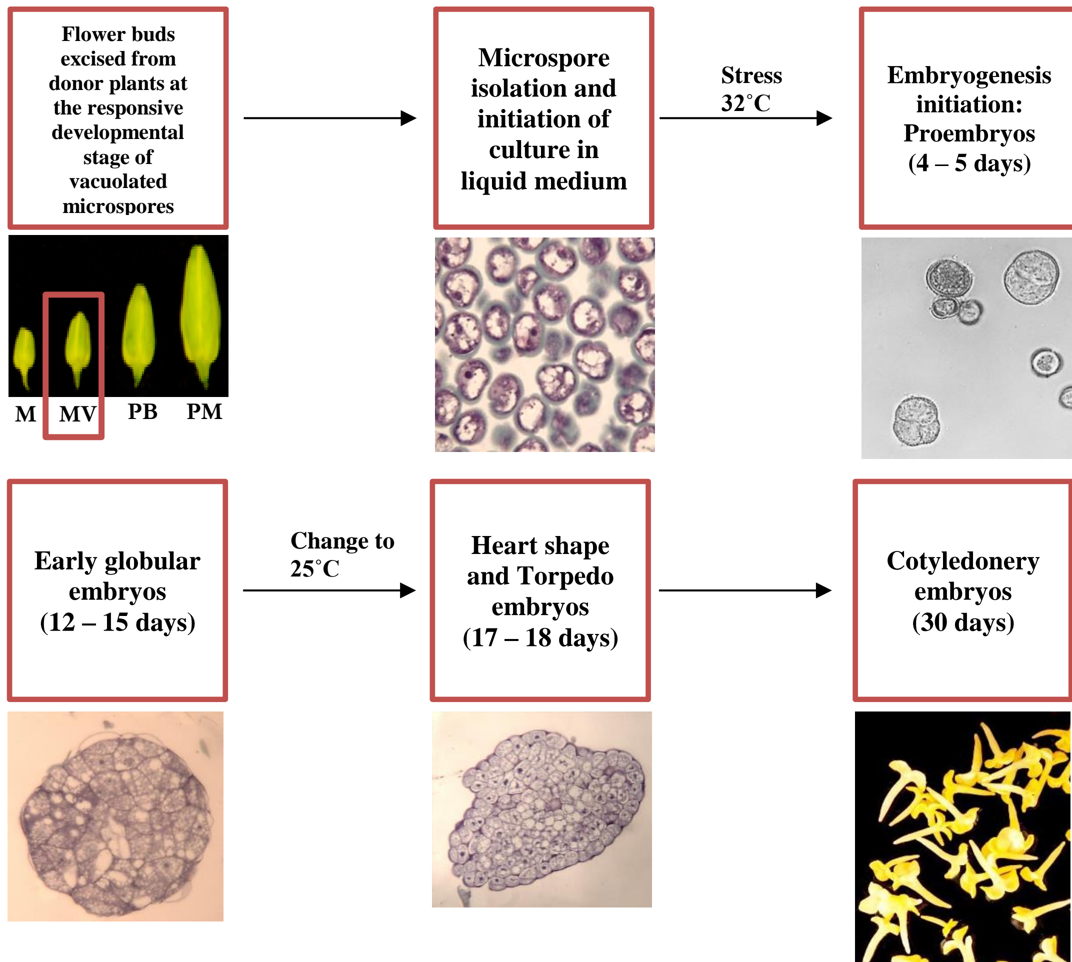

Figure 12: Scheme of stress-induced microspore embryogenesis *in vitro* system in *Brassica napus* (Prem et al. 2012)

5. b. Barley (*Hordeum vulgare* L.)

Barley (*Hordeum vulgare*), member of the true grass family *Poaceae*, is one of the most important cereal crops in the world. It is an annual plant that has erect stems (60 – 120 cm) with alternated leaves which have about 25cm long and 1.5 cm wide. The spikes are formed at the top of the plant and can rise up to 20 cm long. The spike has flowers arranged in three or two sides of a flattened rachis (Figure 13). Barley seeds

locate in cylindrical spikes composed of rachis each with 3 spikelets. Each spike produces 20–60 grains. (Briggs 1970; Fernando et al. 1992; Gomez-Macpherson 2001).

Barley is used as a traditional food for humans and animals by several centuries, its flour has been used instead of wheat to make bread (Bukantis and Goodman 1980) because it is rich in vitamin E, and amino acids like lysine. Barley contains enough starch that can be converted into more sugars than other cereals. Moreover, this process could be stopped when brewing beer which is produced from the first germinated barley, from which the malt is extracted and dried. The malted barley is used to make products including candies and beverages or it may be further processed by fermentation, to convert the sugars into alcohol to brew beer (Baum and Bailey 1990).

Figure 13: Barley plant (*Hordeum vulgare* L.)

Haploid plant can be produced by isolated microspore culture as well as by anther culture, where the microspore can be induced directly or indirectly to form haploid embryos. In barley, microspore embryos are not treated with any agent to double the chromosome numbers leading to regenerate doubled haploid plants since this species shows a long rate of spontaneous diploidization (Jacquard et al. 2003). Many scientists and plant breeders studied the barley microspore culture as a model tool for

the improvement of cultivars and to produce homozygous lines (Ramírez et al. 2001; Rodríguez-Serrano et al. 2012; Castillo et al. 2000; Daghma et al. 2012; Esteves et al. 2014; Kasha et al. 2001a; Kasha et al. 2001b; Li and Devaux 2003; Pulido et al. 2005).

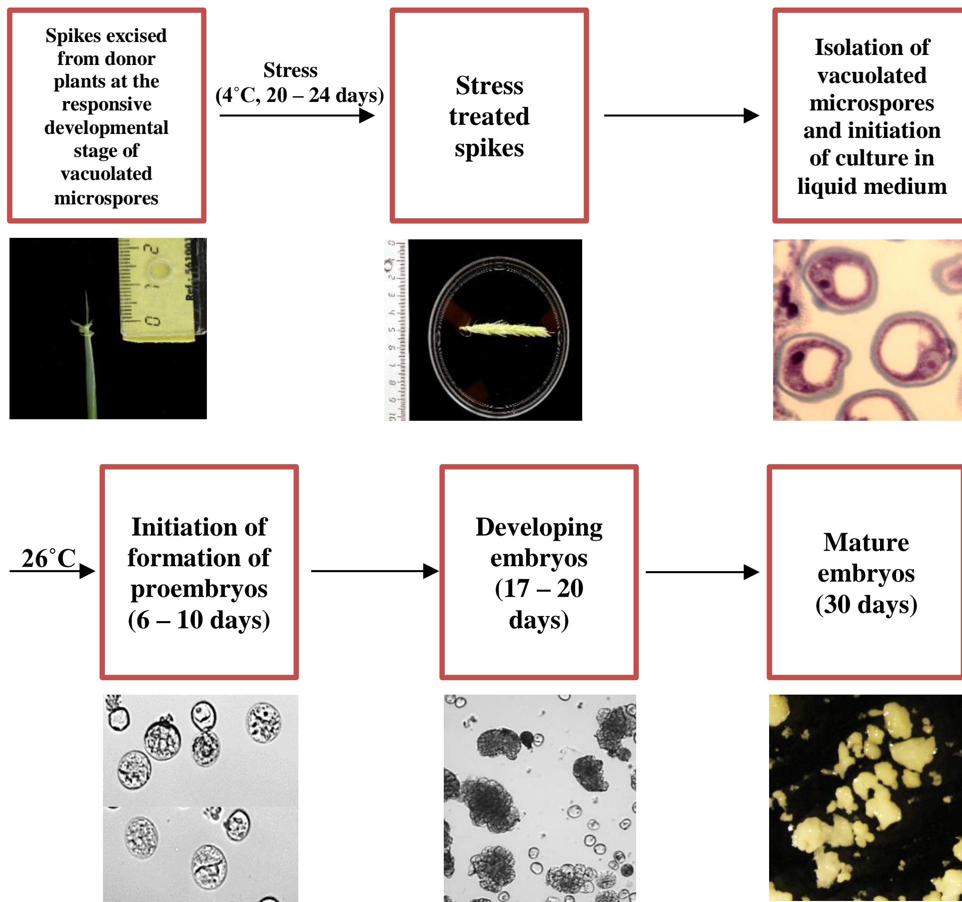

Figure 14: Scheme of stress-induced microspore embryogenesis *in vitro* system in *Hordeum vulgare* (Rodríguez-Serrano et al. 2012)

In vitro microspores culture in barley can be induced to change from their gametophytic development and switch to embryogenesis under cold stress treatments leading to form haploid embryos and homozygous breeding lines in a short period time. Therefore, isolated microspores are considered as ideal target cells for in vitro selection and transformation.

Many scientists have shown that cold stress treatments play a very important role in the microspore embryogenesis induction in monocots. Some scientists tried to explain the effect of cold pre-treatment on the microspore development in different species. It has been reported that cold treatment may delays the degeneration of anther wall tissues leading to protect the microspores within, from toxic compounds released by the degenerating maternal tissues (Ayed et al. 2010). The microspores in the anthers under low temperature disconnect from the tapetum thereby switching from gametophytic pathway to embryogenic development. Moreover, it has been reported that cold treatment of anthers can increase the content of free amino acids suggested that the microspore needs to undergo in preparation for the induction of embryogenesis (Kaushal et al. 2014; Khatun et al. 2012; Silva 2012).

In this thesis, isolated microspore cultures and embryogenesis induction in barley were performed in the following protocol (Rodríguez-Serrano et al. 2012). Spikes containing microspores at the vacuolated stage were collected from donor plants grown at 18ºC and surface sterilized by immersion in bleach at 5% for 20 min. The sterilized spikes were pre-treated at 4ºC for 23–24 days to stimulate embryogenic development. Later, the microspores were isolated by blending in 20 ml 0.4 M mannitol and cultured in KBP medium (Kumlehn et al. 2006) and then, incubated in 25ºC. After four days, the responsive microspores had divided and produced multicellular embryos. Over the following days in culture, globular, transitional, scutellar, and coleoptilar monocot embryos were formed (Figure 14).

6. EPIGENETIC MODIFICATIONS

Epigenetics is the study of heritable changes in gene activity and expression that occur without alteration in DNA sequence. Epigenetics describes the study of dynamic alterations in the transcriptional potential of a cell (Goldberg et al. 2007; Bird 2007). The non-genetic alterations are regulated by two main epigenetic mechanisms: DNA methylation and histone modifications. Thus, the epigenetic

marks can regulate the structure and function of chromatin (Ay et al. 2014; Jenuwein and Allis 2001; Ozanne and Constancia 2007). Functionally, the epigenetic modifications can be markers to study the change of expressed genes without altering the underlying DNA sequence (Capell and Berger 2013; Muñoz-Najar and Sedivy 2011; Handy et al. 2011; Seo et al. 2014). Therefore, these markers can interpret the genome under the influence of physiological factors.

Epigenetics modifications have been found to regulate gene activity and expression during development and differentiation of the cells (Kubota et al. 2012; Kawashima and Berger 2014; Barber and Rastegar 2010; Jaenisch and Bird 2003; Moore et al. 2013). In plants, the epigenetic marks play an important role in different physiological process such as acclimation (Correia et al. 2013), embryonic development (Rodríquez-Sanz et al. 2014), the senescence (Ay et al. 2014; Woo et al. 2013), and the flowering (Müller and Goodrich 2011). Epigenetic marks can be transmitted to the next generation; the DNA in the chromatin will be replicated and sorted into daughter cells during cell division and epigenetic modifications can be inherited, providing the ability to the plant to adapt to the surrounding environment and regulate developmental processes (Houben et al. 2014; Probst et al. 2009; Bird 2007; Budhavarapu et al. 2013). Several works indicated that the epigenetic changes such as DNA methylation and the post-translational histone modifications can control the growth of plant tissue during zygotic embryogenesis and play an important role in the initiation of somatic embryogenesis processes (Gonzalez-Sanchez et al. 2014; Pérez et al. 2015; Nic-Can et al. 2013; Mahdavi-Darvari et al. 2015; Miguel and Marum 2011; Smertenko and Bozkov 2014; Shibukawa et al. 2009; Ikeuchi et al. 2013).

In microspore embryogenesis, the knowledge on the genetic control of the process and the possible involvement of external factors regulating embryo growth and development is still scarce. Little is known about the mechanisms that induce the dedifferentiation of a single somatic cell into a totipotent embryogenic cell. Several

reports have related totipotency of cells to an open chromatin conformation, these cells are characterized by large nuclei and homogenous euchromatin (Grafi et al. 2011). There is increasing evidence that numerous processes of development and differentiation in both plants and animals are accompanied by chromatin remodelling (Kouzarides 2007). Stress-induced plant cell reprogramming involves changes in global genome organization, the epigenetic modifications being key factors of genome flexibility (Arnholdt-Schmitt 2004; Solís et al. 2012).

6. a. DNA Methylation

DNA methylation is an epigenetic mark that is found when a methyl group is added to the nucleotide cytosine (Figure 15). In plants, the cytosine can be methylated at CG, CHG and CHH sites where H can be any nucleotide except guanine (G), as indicated by Capuano et al. (2014) in *Arabidopsis thaliana*. DNA in plants is highly methylated in comparison with animals, 5-methylcytosines are located in symmetrical and non-symmetrical CG and CHG sequences (Kovarik et al. 1997; Goubely et al. 1999). Cytosine residues are methylated by DNA methyltransferases (Jin et al. 2011; Ryazanova et al. 2013; Feng and Jacobsen 2011). DNA methylation has been suggested to control plant growth, plant development, regulating gene expression and DNA replication (Mirouze and Paszkowski 2011; Meijón et al. 2009; 2010).

DNA methylation constitutes a prominent epigenetic modification of the chromatin fiber which is locked in a transcriptionally inactive conformation leading to gene silencing (Köhler et al. 2012). Generally, open chromatin increases the accessibility of the genome to the transcription machinery, while closed chromatin represses gene expression by limiting the accessibility (Reyes 2006; Kouzarides 2007). The past decade revealed exciting findings on epigenetic mechanisms controlling developmental processes specific to flowering plants: the determination of the sporogenic fate during development, the differentiation of gametes within multicellular gametophytes, and the distinction of the 2 male gametes involved in double fertilization (Twell 2011).

Recent studies of our group have demonstrated epigenetic changes during plant developmental processes and after microspore induction to the sporophytic pathway leading to embryogenesis in different plant species, such as *Brassica napus* (Solís et al. 2012; Testillano et al. 2013) and *Quercus suber* (Rodriguez-Sanz et al. 2014a).

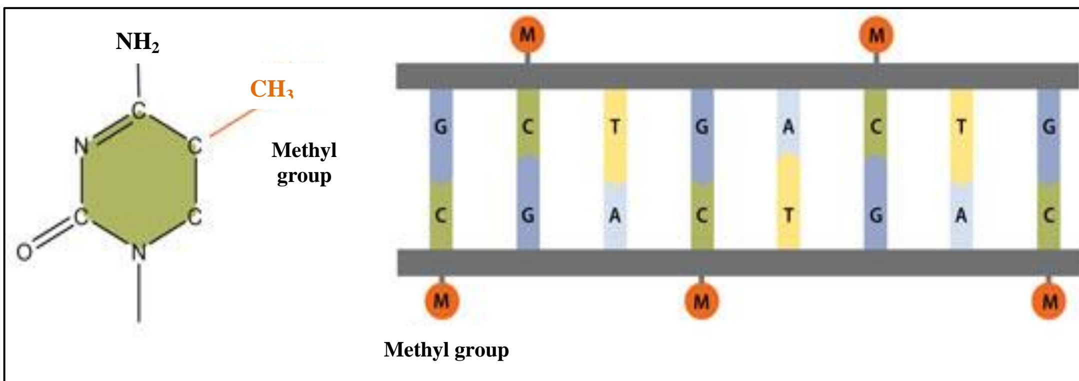

Figure 15: DNA methylation. It consists on the addition of a methyl group (M) to the DNA base cytosine (Bagga 2012)

5-Azacytidine (AzaC), known as a chemical analogue structurally similar to 5-cytosine (Figure 16) has been used as a demethylating agent since it cannot be methylated when incorporates to DNA, leading to the inhibition of DNA methyl-transferase activity and therefore to genomic DNA hypomethylation (Razin and Friedman 1981; Pedrali-Noy et al. 2001; Santos and Fevereiro 2002; Yang et al. 2010; Fraga et al. 2012; Teyssier et al. 2014). It has been reported the effect of AzaC treatment on chromosome behaviour, cell structures and nucleologenesis in different plant species (Castilo et al. 1999; Vorontsova et al. 2004). However, there are not reports, until the present thesis on the effect of AzaC on microspore embryogenesis initiation and progression.

In this thesis, the dynamics of global DNA methylation levels and distribution patterns were analysed during microspore reprogramming to embryogenesis in comparison with pollen development in barley (*Hordeum vulgare*). On the other hand, the effect of AzaC on microspore embryogenesis induction and progression, as

well as on global DNA methylation, nuclear distribution and chromatin organization, have been analysed in the two plant species, *Brassica napus* and *Hordeum vulgare*.

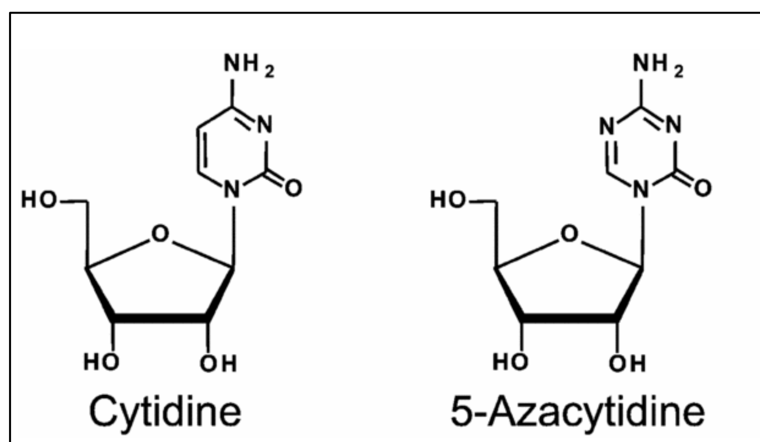

Figure 16: Structure of cytidine and 5-azacytidine

7. ARABINOGALACTAN PROTEINS (AGPs)

Arabinogalactan proteins (AGPs) are a type of proteins found in cell walls, plasma membranes, extracellular secretions and the apoplastic space; they are highly glycosylated hydroxyproline-rich glycoproteins (Coimbra and Pereira 2012; Maurer et al. 2010; Pennell et al. 1991; Nothnagel 1997) and these proteins present a high degree of heterogeneity with carbohydrate sequences (Ellis et al. 2010; Renard et al. 2012). AGPs belong to a large family of proteoglycans that contain about 1–10% (w/w) protein and 90–99% (w/w) carbohydrate (Pereira et al. 2015; Ling et al. 2012). The proteins are rich in hydroxyproline/ proline, alanine, serine and threonine whereas the carbohydrate is usually in form of polysaccharide chains type II arabino-3,6-galactans (AGs) (Showalter et al. 2010; Borderies et al. 2004).

The complexity of AGPs arises from the heterogeneity of the glycosylated protein backbone containing arabinogalactan chains with their peripheral carbohydrates (Showalter 2001; Seifert and Roberts 2007). The distribution of these proteins in all

plant kingdom pushed the scientists to study the biological roles for AGPs in plant growth and food applications (Pereira et al. 2014; Renard et al. 2012).

7. a. The structure and functions of AGPs

AGPs are formed by three main structures: the protein core, the carbohydrate and the glycosylphosphatidylinositol (GPI) anchor. The polypeptide chains of AGPs in the protein backbones start their translation by the N-terminal and they contain C-terminal GPI anchor after the translation of mRNA (Ellis et al. 2010). It is worth mentioning that the mature protein core consists of the amino acids Pro/Hyp, Ala, Ser and Thr which can repeat the genomes sequence to produce AGPs glycomodules (Coimbra and Pereira 2012; Renard et al. 2012; Fragkostefanakis et al. 2012). In general, the genes encoding proteins predicted to have AGPs glycomodules are classified in four classes: (1) classical AGPs which contain the central Pro/Hyp-rich domain decorating between the N-Terminal signal peptide and the C-Terminal GPI addition sequence, (2) the lysine-rich AGPs which contain Lys-rich module. (3) AG peptides which have protein backbones of less amino acids residues numbers than classical AGPs, (4) fasciclin-like AGPs (FLAs) which are a class of chimeric AGPs because they contain one or two fasciclin domains and typical glycosylation AGP modules, as described in *Arabidopsis thaliana* (Ellis et al 2010; Pereira et al. 2015).

The carbohydrate part forms polyssacharide chains, type II arabinogalactan (AGs) chains *O*-glycosidically linked to Hyp residues on the protein backbone. The AGs chains have *B*-(1→3)-galactopyranose linked Gal that substituted at C(O)6 by galactosylside chains with oligosacharide chains terminated in Ara, Rha, GlcA and Gal residues producing a neutral glycan chain, as indicated in Figure 17 (Brecker et al. 2005; Coimbra et al. 2009; Johnson et al. 2003; Ling et al 2012; Bento et al. 2014).

GPI anchors have been characterized in few plants compared to animals but many AGP proteins have been shown to be GPI anchored, that plays a very important role in classical AGPs (Schultz et al. 1998; Coimbra and Pereira 2012). GPI anchors have

a massively conserved trimannosyl-glucosamine tetrasaccharide called core glycan that contain C-Terminal GPI-addition linked to ethanolamine phosphate (Sarder and Showalter 2007; Oxley and Bacic 1999).

Specific monoclonal antibodies (mAbs) have been widely used as a tool in the study of AGPs; they can bind to AGP-specific carbohydrate epitopes. The setting-up of monoclonal antibodies directed against different AGP epitopes and cell wall polymers

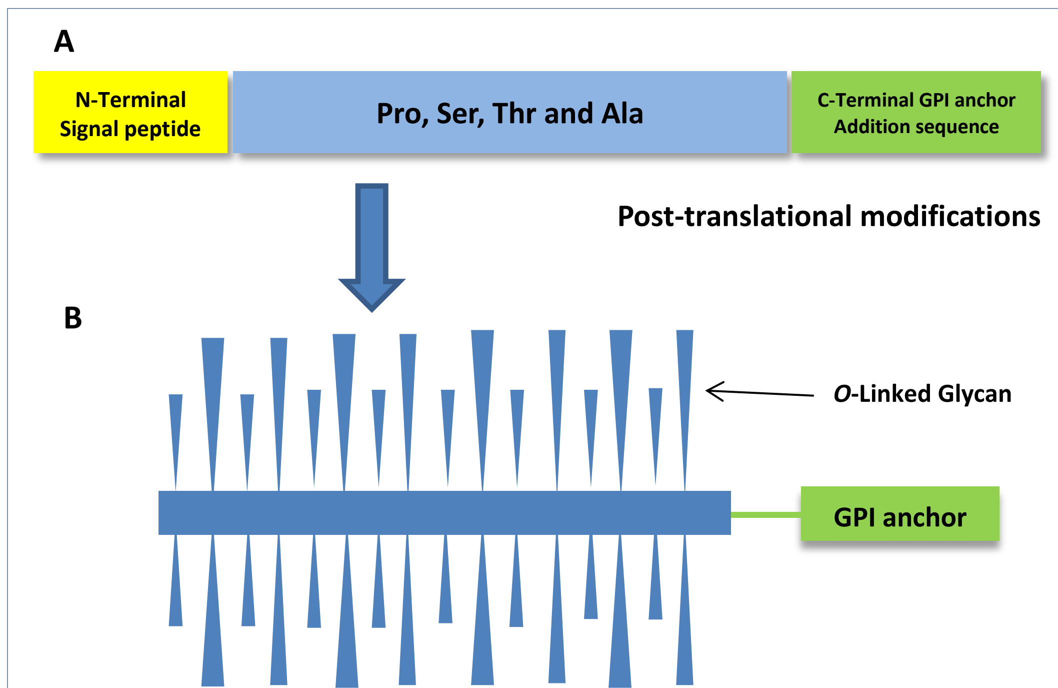

Figure 17: Scheme of the molecular structure of a classical AGPs. (A) Immature polypeptide, and (B) Mature proteoglycan with attached GPI anchor (Coimbra and Pereira 2012).

has facilitated the analysis of the complex cell wall structure and its dynamics during plant developmental processes. These anti-AGP monoclonal antibodies have been used to unravel the involvement of specific epitopes in controlling cell growth and morphogenesis. Increasing evidence has linked AGPs to many processes involved in plant growth and development, including somatic embryogenesis (Chapman et al. 2000; Thompson and Knox 1998; van Hengel et al. 2001), pollen grain development (Coimbra et al. 2009; Levitin et al 2008; Pereira et al. 2006) and pollen tube growth (Costa et al. 2013; Cheung et al. 1995; Wu et al. 2001). Despite the information

gained in recent years with respect to AGPs, their precise functions have not yet been elucidated (Seifert and Roberts 2007).

7. b. AGPs during pollen development and microspore embryogenesis

The distribution of AGPs during the pollen gametophytic pathway has been analysed in *Arabidopsis* by using specific monoclonal antibodies (mAbs) that binds to AGP carbohydrate epitopes. Coimbra and Pereira (2012) indicated that AGPs play an important role during pollen development. In *Arabidopsis thaliana*; they analyzed the stages of gametophytic pathway with the JIM8, JIM13, LM2 and MAC207 anti-AGP antibodies, and reported various labelling patterns in the generative and vegetative cells of pollen grains. Szczuka et al. (2013) supported these results in the same species. Moreover, the anti-AGP mAbs labelled the pollen intine of *Pinus bungeana* (Fang et al. 2008) and *Arabidopsis thaliana* (Jia et al. 2015).

The information of endogenous AGPs during microspore embryogenesis is very limited compared to zygotic embryogenesis (Zhong et al. 2011) and/or somatic embryogenesis (Portillo et al. 2012). There are some investigations that opened gates to discuss the distribution and the importance of AGPs during pollen embryogenesis; they suggested that maybe AGPs stimulate the cultured isolated microspores to form the embryos, as indicated by Yuan et al. (2012) in white cabbage and, Borderies et al. (2004) in maize.

Yariv reagent is a chemical compound consist on [1,3,5-tri(p-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene] (Figure 18) that was initially developed as carbohydrate antigen for the purification of anti-glycoside antibody and sugar-binding protein (Yariv et al., 1962, 1967; Kitazawa et al. 2013; Paulsen et al. 2014). It then turned out that Yariv phenylglycosides specifically precipitate AGPs. Yariv is widely used for staining, detection, quantification and purification of AGPs. It has been reported that by adding Yariv reagent to the culture medium, it binds to and aggregates AGPs and therefore, AGP action is blocked negatively, affecting embryogenesis (Tang et al.

2006). There are glucosyl-Yariv (β Glc-Y), utilized to bind and aggregate AGPs, and Manosyl-Yariv (β Man-Y) reagents (used normally as control since it does not bind AGPs). Yariv treatment causes the perturbation of different physiological process in plant (Kitazawa et al. 2013). The application of Yariv reagent to cultured cells of

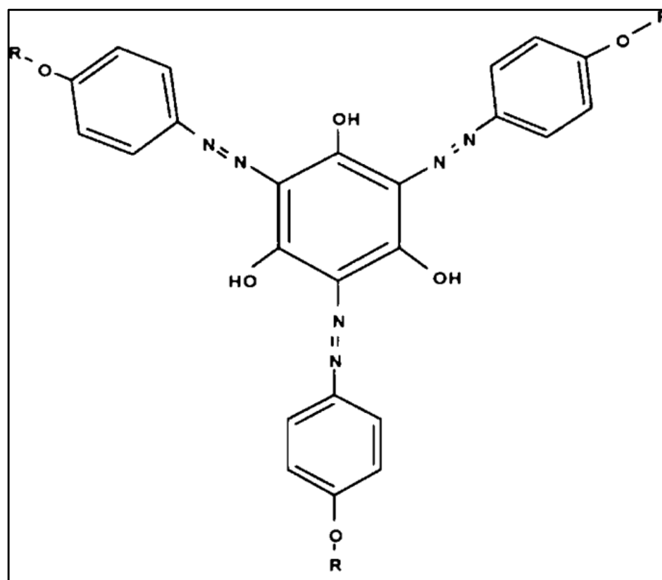

Figure 18: Chemical structure of Yariv reagent. R is a β glucosyl-Yariv reagent (β Glc-Y), and it is a β-D-Manosyl residue in β-Manosyl-Yariv reagent (β –Man-Y).

Arabidopsis thaliana (Gao and Showalter 1999) and *Nicotiana tabacum* (Sarder and Showalter 2007) reduced the embryogenesis indicating that AGPs have a role in the process; it was suggested that this role is related to the orientation of cortical and the polymerization of F-actin. Although Yariv phenylglycosides have been used to study AGPs over 40 years, the mechanisms of Glucosyl Yariv reagent that selectively and non-covalently binds to AGPs are not fully understood (Van Holst and Clarke 1985). Previous work has shown that the addition of Glucosyl Yariv to living cells disturbs AGP function. Manosyl Yariv reagents differ from Glucosyl Yariv reagents only in the isomerization of the hydroxyl group at carbon atom 2 of the sugar, which prevents their binding to AGPs. Manosyl Yariv thus provides an excellent control in studies of AGPs using βGlcY (Yariv et al., 1967; Nothnagel, 1997). In the present study, βGlcY and βManY reagents were used to examine the function of AGPs, yielding insight

into the possible roles of these proteins in microspore embryogenesis and plant development.

The interaction of βGlcY reagent with certain AGPs depends on both the state of Yariv reagent in solution and the structural and chemical composition of the AGPs. The reaction is usually performed in 1 % aqueous solution and the precipitated complex can be dissociated by adding either dimethylformamide (DMF) or by adding NaCl solution to 10 % (w/v). It has been reported that only β-D or α-1-linked glycopyranosyl Yariv reagents can make the complex formation, so that the saccharide moiety is implicated in the binding reaction (Paulsen et al. 2014). In addition, the OH group at C $(O)_2$ must be in the d-gluco configuration and the diazo-group which substitutes the phenylglycoside must be at the C4 position of the phenyl ring (Jermyn, 1978; Jermyn & Yeow, 1975). Little is known about the structure of the site on AGPs to which the β-D-Glc Yariv reagent binds. Conventional glycoside haptens do not compete for binding, although some flavonol glycosides present in crude tissue extracts may prevent binding between AGPs and the β-d-Glc Yariv reagent (Jermyn, 1978). There is some evidence that the binding site on AGPs is in a Hyp-rich domain and that it involves both the protein backbone as well as part of the galactan backbone of the AG (Gleeson and Jermyn 1979; Jermyn and Yeow 1975). Enzymatic digestion of AGP with subtilisin removed over 80% of the protein but left a Hyp-rich core which retained the capacity to bind β-d-Glc Yariv reagent (Gleeson and Jermyn 1979). AGs (protein free) from larch (Larix decidua) and the Hyp-containing AG-peptide from *Lolium multiflorum* do not bind the Yariv reagent, suggesting the need for some protein in the binding. Surprisingly, a carrot (*Daucus carota*) AGP which lacks Hyp binds the β-d-Glc Yariv reagent (Baldwin et al. 1993). In an exciting new development Kitazawa et al. (2013) conducted a series of experiments that concluded that β-Glc Yariv binds specifically to (1→3) β-D-galacto-oligosaccharides of DP > 5 but not to (1→6) β-D-galacto-oligosaccharides. However, their findings are not always consistent with those described above where the protein component is important for binding.

In this thesis, the presence and distribution of AGPs were studied during pollen development and in *in vitro* induced microspore embryogenesis in rapeseed and barley plants, by means of a multidisciplinary approach, combining immunocytochemical, biochemical and molecular techniques. Dot blot assays were carried out, along with immunofluorescence and confocal laser scanning microscopy (CLSM) analyses with several monoclonal antibodies for AGPs: JIM 13, JIM 14, MAC207, LM2 and LM6, the latter reacting with AGP arabinan epitopes which are also present in pectins. Analysis of expression pattern of the *BnAGP Sta* 39-4 gene (Gerster et al. 1996) by quantitative real-time PCR (qPCR) was also performed. On the other hand, the Yariv reagents were used to perturb AGPs during microspore embryogenesis in the both two species, and their effects on induction and embryo development were analysed in the two species, rapeseed and barley.

8. AUXINS

Auxins are a class of plant hormones or plant growth regulators (PGR) which play a critical role in plant growth and development (Prasad and Dhonukshe 2013; Himanen et al. 2002). Auxin is a phytohormone whose chemical structure more abundant *in vivo* is the indole-3-acetic acid (IAA), as demonstrated by Went and Thimann in 1937.

Auxin is a molecule present in all parts of the plant, with different concentrations. This molecule coordinates the development of plant organs such as leaves (Reed et al. 1998), stem (Ding and Friml 2010), and root (Zhao and Hasenstein 2010). In addition, auxin promotes cell elongation of the plant which changes the plant wall plasticity making it easier for the plant to grow upwards (Keuskamp et al. 2010). Auxin also prevents some physiological processes such as the shoot apical dominance (Ann Dun et al. 2006), and fruit senescence (Ellis et al. 2005). On the other hand, IAA plays a major role in the formation of embryo; the distribution and transport pattern of auxin during zygotic embryogenesis lead to the mature embryo to initiate

the formation of shoots and roots (Basu et al. 2002; Fischer et al. 1997; Hutchinson et al. 1996; Palovaara et al. 2010; Vondráková, et al. 2011; Hua Su and Zhang 2009; Pasternak et al. 2002).

The auxin biosynthesis (Figure 19) occurs mainly through the tryptophan (Trp) pathway, from which several pathways have been postulated: the indole-e-acetamide (IAM) pathway, the indole-3-pyruvic acid (IPA) pathway, the tryptamine (TAM) pathway, and the indole-3-acetaldoxime (IAOX) pathway (Mano and Nemoto 2012; Mashiguchi et al. 2011). It has been found that IAM and IPA pathways are widely distributed in the plant kingdom whereas the IAOX has not been found in plants except *Brassicaceae* plants (Mano and Nemoto 2012; Zhao 2011).

The role of auxins during plant embryogenesis has been demonstrated in *Arabidopsis sp.* in which dynamic changes in auxin flux within cells are mediated by PIN proteins (PIN-formed family of efflux auxin transporters) causing polar auxin transport (Chen et al. 2010; Petrásek and Friml 2009). PIN genes regulate asymmetric auxin distribution during zygotic embryogenesis (Feng and Jacobsen 2011; Palovaara and Hakman 2009; Huang et al. 2014). The expression of PIN genes causes the accumulation of auxin in specific regions of the embryo; it has been also found that the polar localization of epidermal PIN proteins in the globular embryo result in auxin maxima leading to cotyledon initiation (Chen et al. 2010).

Recent results in our research group in pollen development and microspore reprogramming, indicated that the endogenous auxin increased after stress-induced microspore embryogenesis and during embryogenesis progression in *Brassica napus* and *Quercus suber* (Rodríguez-Sanz et al. 2014a; Rodríguez-Sanz et al. 2014b) but the information in monocot species is not available.

In general, the regulation of auxin in plants occurs by polar auxin transport (PAT) which supports specific spatial auxin distribution leading to the responses of plant to the environment and plant growth (Van Berekel et al. 2013; Estrelle 1998;

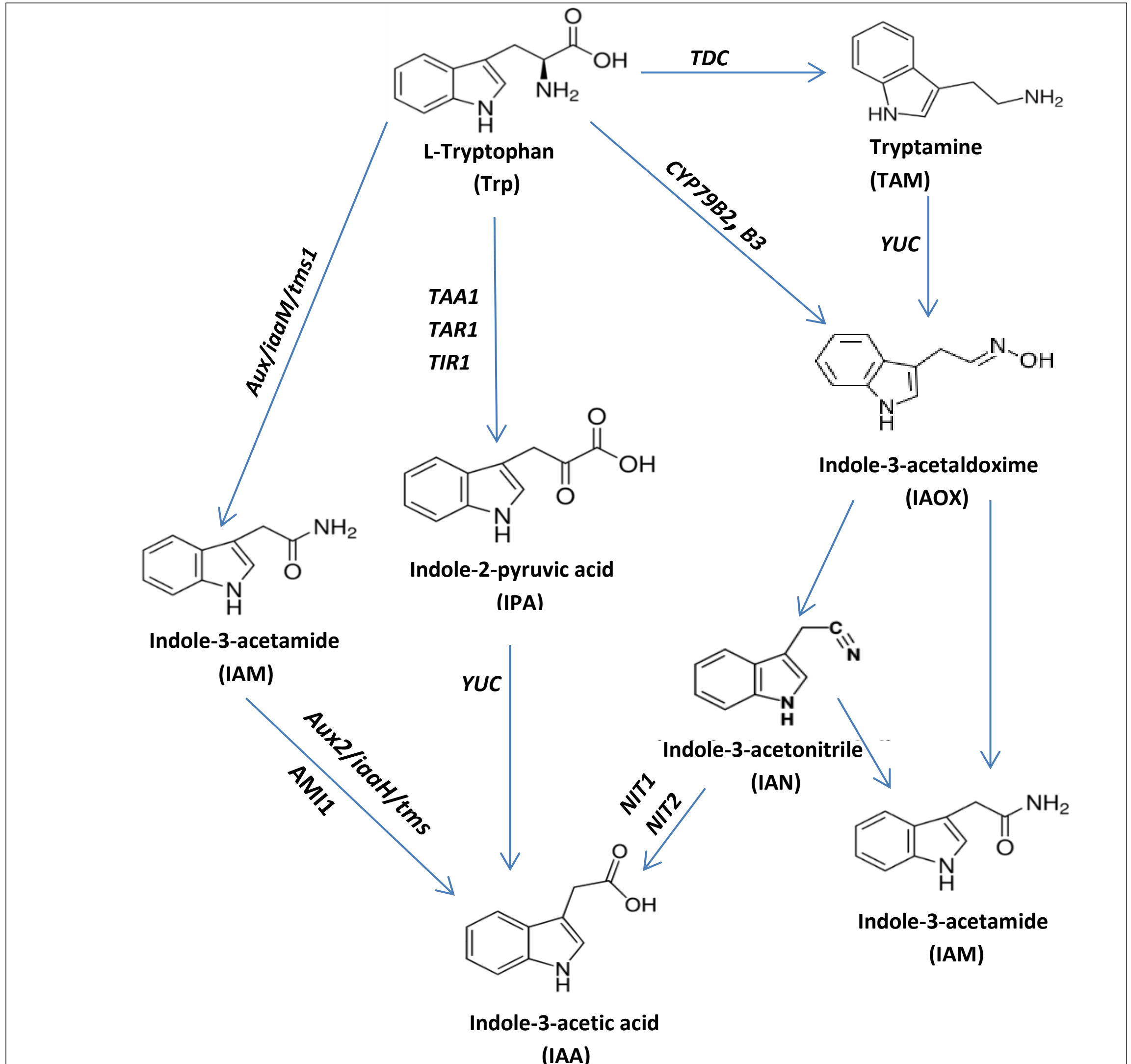

Figure 19: Scheme of Indole acetic acid (IAA) biosynthesis (Zhao 2014).

Michniewicz et al. 2007). The polar auxin transport can be blocked by some inhibitors such as N-1-naphthylphthalamic acid (NPA) which can inhibit the flux of auxin from cell to cell through interaction with PIN proteins (Hakman et al. 2009).

The effect of NPA has been studied in somatic embryogenesis in several species (Liu et al. 1993; Hadfi et al. 1998). NPA leads to abnormal formation of somatic embryos during their growth; it has been observed that there is a difference in frequency between shoots and roots development, as demonstrated by Fischer et al. (1997) in wheat, and Palovaara et al. (2010) in Norway spruce. In addition, it has been reported

that NPA increases the expression of PIN-like gene significantly (Shen et al. 2010; Hakman et al. 2009). Forestan and Varotto (2011) indicated that NPA causes auxin accumulation in epidermis of scutellum in the mature embryo of maize, leading to failure of symmetric development and proliferating abnormal vasculature.

There are only a few studies of the effect of NPA during microspore embryogenesis, compared to somatic embryogenesis. Our group found in *Brassica napus* and *Quercus suber*, that the development of embryos was reduced when the microspore cultures were treated with NPA in the medium, indicating that the transport of endogenous IAA and its biosynthesis are involved in microspore embryogenesis initiation and development in these two dicot species (Rodríguez-Sanz et al. 2014a; 2014b). There are only a few studies of the effect of NPA during microspore embryogenesis, compared to somatic embryogenesis. Our group found in *Brassica napus* and *Quercus suber*, that the development of embryos was reduced when the microspore cultures were treated with NPA in the medium, indicating that the transport of endogenous IAA and its biosynthesis are involved in microspore embryogenesis initiation and development in these two dicot species (Rodríguez-Sanz et al. 2014a; 2014b).

Although the studies on auxin in monocot are still scarce, some investigations on maize and rice have reported that the effect of NPA on the plant morphology and the sensitivity in both dicots and monocots are different (Mcsteen 2010).

PCIB (P-chlorophenoxyisobutiric acid) is an auxin inhibitor which inhibits auxin action leading to the blocking of the physiological effects of the auxin (Oono et al. 2003; Xie et al. 2000). PCIB does not affect polar auxin transport once it does not react with PIN proteins. The idea of using of PCIB as an auxin inhibitor derived from its structural similarity with auxin; it competes with auxin action at the auxin receptor, therefore, affecting the auxin-induced responses (Oono et al. 2003). PCIB may play a role in clarifying the mechanisms of auxin perception and signal transduction and their importance in plant growth and development (Trebitsh and

Riov 1987; Hutchinson et al. 1996; Zhang et al. 2011). However, the information about the mechanism of PCIB-mediated inhibition of auxin action is still scarce. Some scientists used the PCIB treatment to study some physiological processes such as the gravitropic response (Oono et al. 2003), the flower opening (Van Doorn et al. 2012) and root growth (Tamás et al. 2012). Also, it has been found that PCIB inhibited the early auxin gene expression in *Arabidopsis thaliana* (Oono et al. 2003) while, it did not inhibit the IAA response in corn coleptile (Peters and Felle 1991).

PCIB treatment in cultured media has been used to study the somatic embryogenesis, many results indicated that PCIB decreases the embryonic response and the formation of pro-embryonic masses in somatic embryogenesis (Fujimura and Komamine 1979; Hutchinson et al. 1996), while some other investigations found that PCIB treatment increased the percentage of calluses and did not affect morphogenesis in *Ipomoea sp.* (Kobayashi and Bouwkamp 1994; Chée and Cantliffe 1989). The response of plant to the PCIB treatment depends on the concentration of the solutions; Zhao and Hasentein (2010) reported different effects of this inhibitor on root growth when they treated the *Linum usitatissimum* and *Arabidospis thaliana* seeds with PCIB in various concentrations and they found that there was no effect with the highest concentration used. On the other hand, Zhang et al. (2011) found that PCIB treatment increased the number of embryos after the induction of the microspores of *Brassica rapa* whereas Rodríguez-Sanz et al. (2014b) found the opposite effect with *Brassica napus*.

In this thesis, the endogenous auxin distribution has been analyzed in microspore embryogenesis of *Hordeum vulgare* by using anti-IAA immunofluorescence and confocal analysis. Moreover, the effects of auxin transport inhibition and action on the induction of microspores have been analyzed by NPA and PCIB treatments.

OBJECTIVES

OBJECTIVES

In this thesis, the general objective is to study the involvement of several factors (DNA methylation, AGPs and auxin) on defined stages of microspore embryogenesis induced by different stresses in two plant species (*Hordeum vulgare* and *Brassica napus*), in order to identify general mechanisms involved in the regulation of the process, to improve the efficiency of the induction and embryo progression. The specific objectives are the following:

1. **Characterization of changes in global DNA methylation levels and nuclear distribution patterns during *in vitro* microspore embryogenesis initiation and progression, and *in vivo* pollen development in *Hordeum vulgare***

a) Quantification of global DNA methylation levels at specific developmental stages of pollen development and microspore embryogenesis

b) *In situ* localization of methylated DNA (5dmC) and characterization of its nuclear distribution patterns during pollen development and microspore embryogenesis

2. **Determination of the effects of the DNA demethylating agent 5-azacytidine (AzaC) on microspore embryogenesis induction efficiency, in *Hordeum vulgare* and *Brassica napus***

a) Quantification of the effects of short AzaC treatments on cell death and embryogenesis induction (percentage of proembryos formed)

b) Quantification of the effect of short AzaC treatments on DNA methylation levels

c) Characterization of changes in methylated DNA, analysis of nuclear distribution patterns and chromatin decondensation degree induced by short AzaC treatments

d) Quantification of the effects of long AzaC treatments on microspore embryogenesis progression and embryo production yield

3. **Characterization of Arabinogalactan proteins (AGPs) temporal profiles and distribution patterns during microspore embryogenesis and pollen development; effects of their blocking on embryogenesis induction, in *Hordeum vulgare* and *Brassica napus*.**

a) Temporal profiles and *in situ* localization patterns of several groups of APGs with different glycan epitopes (LM2, LM6, JIM13, JIM14, MAC207) during pollen development and microspore embryogenesis

b) Analysis of gene expression of *BnAGP-Sta 39-4,* AGP gene identified in *Brassica napus*, during pollen development and microspore embryogenesis

c) Determination of the effects of the blocking of AGPs by Yariv reagents on microspore embryogenesis initiation and progression

4. **Analysis of endogenous auxin dynamic and effects of its inhibition during microspore embryogenesis in *Hordeum vulgare***

a) Identification of auxin levels and accumulation sites patterns during microspore embryogenesis

b) Effects of the inhibition of polar auxin transport, by NPA, and auxin action, by PCIB, in microspore embryogenesis induction and progression.

5. **Comparative analysis of the dynamics of DNA methylation, AGPs and auxin, and effects of their respective inhibitions on defined stages of microspore embryogenesis induced by different stresses in two plant species (rapeseed and barley).**

RESULTS

PUBLICATION I

Changes in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley

Reference:

El-Tantawy, A.A., Solís, M.T., Risueño, M.C., & Testillano, P.S. (2014). Changes in DNA methylation levels and nuclear distribution patterns after microspore reprogramming to embryogenesis in barley. *Cytogenet Genome Res, 143(1-3)*, 200-208.

Publication I:

CHANGE IN DNA METHYLATION LEVELS AND UNCLEAR DISTRIBUTION PATTERNS AFTER MICROSPORE REPROGRAMMING TO EMBRYOGENESIS IN BARLEY

ABSTRACT

The microspore can be induced *in vitro*, under specific stress treatments, to deviate from its gametophytic development and to reprogram towards embryogenesis, becoming a totipotent cell and forming haploid embryos which can further regenerate homozygous plants for production of new isogenic lines, an important biotechnological tool for crop breeding. DNA methylation constitutes a prominent epigenetic modification of the chromatin fibre which regulates gene expression. Changes in DNA methylation accompany the reorganization of the nuclear architecture during plant cell differentiation and proliferation, however, global DNA methylation and genome-wide expression patterns relationship is still poorly understood.

In this work, the dynamics of global DNA methylation levels and distribution patterns have been analyzed during microspore reprogramming to embryogenesis and during pollen development in *Hordeum vulgare*. Quantification of global DNA methylation levels and 5-methyl-deoxy-cytidine (5mdC) immunofluorescence has been conducted at specific stages of pollen development and after reprogramming to embryogenesis, to analyze the epigenetic changes that accompany the change of developmental programme and cell fate.

Results showed low DNA methylation levels in microspores and a high increase along pollen development and maturation; an intense 5mdC signal was concentrated in the generative and sperm nuclei whereas the vegetative nucleus exhibited lower DNA methylation signal. After the inductive stress treatment, low methylation levels

and faint 5mdC signal were observed on nuclei of reprogrammed microspores and 2-4 cell proembryos. This data revealed a global DNA hypomethylation during the change of the developmental programme and first embryogenic divisions, in contrast with the hypermethylation of generative and sperm cells of the male germline accomplished during pollen maturation, suggesting an epigenetic regulation after microspore embryogenesis induction. At later embryogenesis stages global DNA methylation progressively increased, accompanying embryo development and differentiation events, like in zygotic embryos, supporting that DNA methylation is critical for the regulation of microspore embryogenesis gene expression.

INTRODUCTION

After specific stress treatments, the *in vitro*-cultured microspore at the vacuolated developmental stage (González-Melendi et al. 1995), can reprogram and initiate an embryogenesis program producing multicellular embryos. Homozygous plants can further regenerate from the embryos for producing new isogenic lines, which are important biotechnological tools for crop breeding. The microspore, after the external stress signals, becomes a totipotent cell because it can develop into an embryo and subsequently an entire plant, therefore acquiring the potential and embryogenic competence to give rise to all the cell types, tissues and organs that make up the plant body (Verdeil et al. 2007, Grafi et al. 2011). In barley, microspore embryogenesis has been induced in microspore *in vitro* cultures by cold and starvation stress treatments (Kasha et al. 2001; Coronado et al. 2005; González-Melendi et al. 2005, Rodríguez-Serrano et al. 2012).

Isolated microspore cultures constitute very convenient systems to analyze the subcellular mechanisms underlying cell reprogramming, totipotency acquisition and subsequent embryo formation. In recent years, increasing amounts of information have reported the presence of genes and molecules controlling early embryogenic events, but knowledge on the genetic control of the process and the possible

involvement of external factors regulating embryo growth and development is still scarce (El-Tantawy et al. 2013). Little is known about the mechanisms that induce the dedifferentiation of a single somatic cell into a totipotent embryogenic cell. Several reports have related totipotency of cells with an open chromatin conformation characterized by large nuclei and homogenous euchromatin (Grafi et al. 2011). There are increasing evidences that numerous processes of development and differentiation in both plants and animals are accompanied of chromatin remodeling (Kouzarides 2007). Stress-induced plant cell reprogramming involves changes in global genome organization, being the epigenetic modifications key factors of genome flexibility (Arnold-Schmitz 2004, Solís et al. 2012).

DNA methylation constitutes a prominent epigenetic modification of the chromatin fiber which is locked in a transcriptionally inactive conformation leading to gene silencing (Kohler et al. 2012). Generally, open chromatin increases the accessibility of the genome to transcription machinery, while closed chromatin represses gene expression by limiting the accessibility (Reyes 2006, Kouzarides 2007). The past decade revealed exciting findings on epigenetic mechanisms controlling developmental processes specific to flowering plants: the determination of sporogenic fate during development, the differentiation of gametes within multicellular gametophytes, and the distinction of the two male gametes involved in double fertilization (Twell 2011). Recent studies have demonstrated epigenetic changes during plant developmental processes and after microspore induction to the sporophytic pathway conducting to embryogenesis (Solís et al. 2012, Testillano et al. 2013). However, the knowledge of the DNA methylation regulation during microspore embryogenesis is very limited.

In this work, the dynamics of global DNA methylation levels and distribution patterns was analyzed during microspore reprogramming to embryogenesis in comparison with pollen development in *Hordeum vulgare*. Our results revealed a global DNA hypomethylation during the change of developmental program and the first

embryogenic divisions, in contrast with the hypermethylation of the generative and sperm cells of the male germline accomplished during pollen maturation, as well as in the embryo cells during embryo differentiation. These results suggest an epigenetic regulation after microspore embryogenesis induction and subsequent embryo development supporting the idea that DNA methylation is critical for the regulation of microspore embryogenesis gene expression.

MATERIAL AND METHODS

Plant material and growth conditions

Winter barley cultivars, *Hordeum vulgare* L. cv. Igri were used as donor plants. Seeds were germinated in soil for 1 month at 4ºC. After that, they were grown at 12ºC 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 18ºC.

Microspore isolation and culture

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 4ºC 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).

Processing for 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, washed in PBS, dehydrated in an acetone series, embedded in Historesin Plus at 4º C and sectioned at 2 µm thickness using an ultramicrotome (Ultracut E Reichert). Semithin sections

were collected on slides, stained with toluidine blue and observed under bright field microscopy.

Immunofluorescence

Immunolocalization of 5-methyl-deoxy-cytidine (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 2 N 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. Sections were counterstained with 1 mg mL^{-1} DAPI (4',6-diamidino-2-phenylindole) for 10 min and analyzed by confocal microscopy (TCS-SP5, Leica). As negative controls, either DNA denaturation or the first antibody was omitted. Also, as negative control, immunodepletion was carried out by preblocking the antibody with 5mdC at 4°C overnight prior to immunofluorescence as described (Testillano et al. 2013).

Quantification of global DNA methylation

Genomic DNA was extracted from microspores and mature pollen directly isolated from anthers, and from different microspore culture stages using a plant genomic DNA extraction kit (DNeasy Plant Mini, Qiagen) as described (Solís et al. 2013). A MethylFlash Methylated DNA Quantification Kit (Colorimetric) (Epigentek, NY) was used according to the manufacturer's instruction using 200 ng of genomic DNA for each sample (Li and Liu 2011, Testillano et al. 2013) for the quantification of the DNA methylation. Three biological and two analytical replicates per sample were taken. P-values were calculated using Student's t –test.

RESULTS

During the gametophytic development in vivo, the haploid microspore undergoes a long interphase with the formation of a large vacuole which pushes the nucleus to the

periphery forming the so-called vacuolated microspore (Fig. 1A). At this stage, the first asymmetric mitosis occurs originating the bicellular pollen (Fig. 1B) with two very different cells: the small generative cell immersed in the cytoplasm of the large vegetative cell. The generative nucleus ("Gn" in Fig. 1B) exhibits a highly condensed chromatin, while the vegetative nucleus ("Vn" in Fig. 1B) shows a less condensed chromatin and a large nucleolus. In barley, the second pollen mitosis occurs before anthesis forming the tricellular pollen grain (Fig. 1C) which contains the two small sperm cells (arrows in Fig. 1C) inside the vegetative cell. With the progression of pollen maturation the vacuole disappears and the cytoplasm is occupied by storage products and small vacuoles (Fig. 1C).

To induce the microspore embryogenesis process in barley, a cold stress treatment was applied to the vacuolated microspores which changed the gametophytic pathway to an embryogenic development (Rodríguez-Serrano et al. 2012). After the stress treatment, responsive microspores reprogrammed and divided symmetrically originating two-cell structures (Fig. 1D) with two nuclei of similar size and structural organization. After 4-6 days in culture, further divisions gave rise to multicellular embryos, still surrounded by the microspore wall, the exine (Fig. 1E) which later broke permitting a faster proliferation and the formation of larger multicellular embryos (Fig. 1F) at 9-10 days. As embryogenesis proceeded, more developed embryos were observed in 15 days of culture (Fig. 1G), and completely formed mature embryos were formed by 30 days (Fig. 1H, inset).

For the analysis of the changes in genomic DNA methylation during pollen development and microspore embryogenesis, the quantification of the percentage of methylated DNA was performed in selected stages of the two developmental pathways, the gametophytic and the embryogenic programs. Results revealed a very low proportion of methylated DNA in vacuolated microspores and a high increase

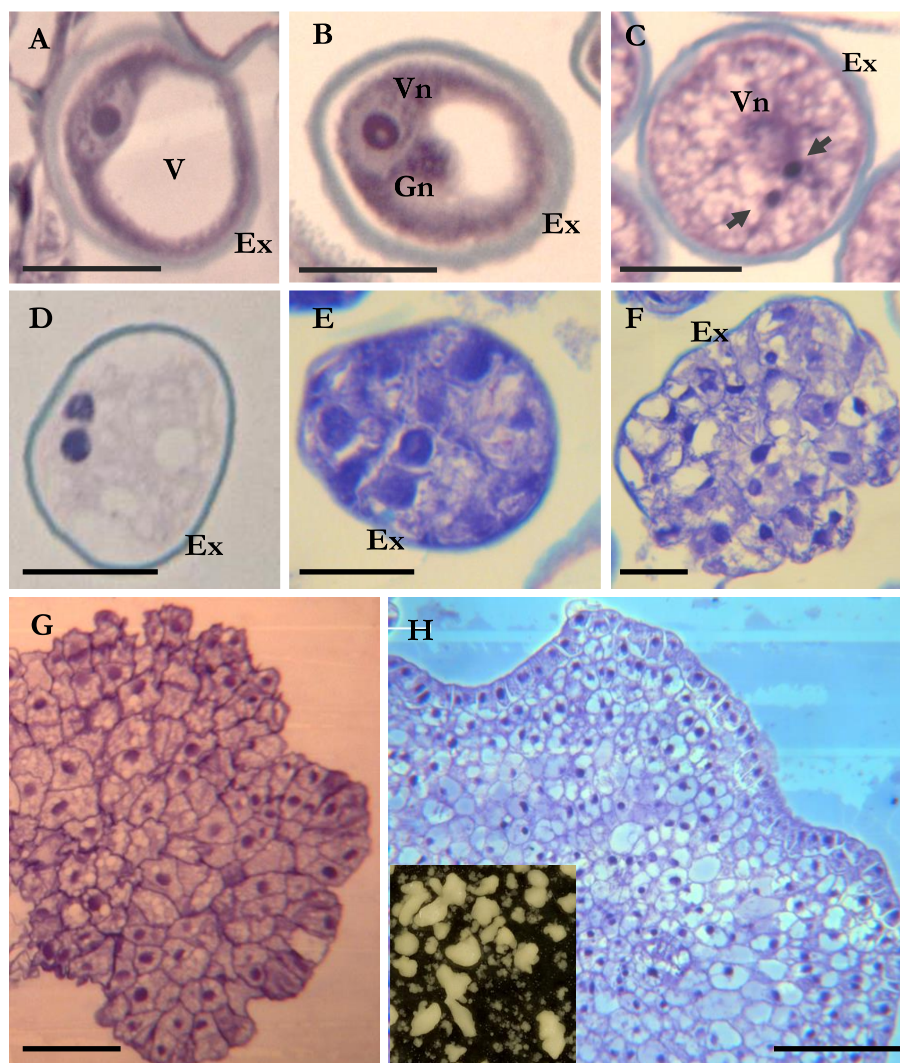

Figure 1: Main stages of pollen development and microspore embryogenesis in barley. Semithin sections, toluidine blue staining. (A-C) Male gametophytic development *in vivo*. (D-H) Microspore embryogenesis *in vitro*. (A) Vacuolated microspore. (B) Young bicellular pollen with the vegetative and generative cells. (C) Mature tricellular pollen with the vegetative nucleus and the two small sperm cells (arrows). (D) Two-cell structure after microspore embryogenesis induction. (E) Multicellular embryo still surrounded by the pollen wall, the exine. (F) Multicellular embryo just after the exine breakdown. (G) Developing embryo after 15 days in culture. (H) Mature embryo formed after 30 days in culture, region showing the peripheral cell layer of the protodermis, inset: panoramic view of several embryos in culture. Ex: exine, V: vacuole, Vn: vegetative nucleus, Gn: generative nucleus. Bars: A-F, 20 µm; G-H: 100 µm.

with the progression of the pollen development and maturation, reaching a much higher proportion in mature pollen (Fig. 2A). On the contrary, samples after the inductive stress containing reprogrammed microspores and 2-4 cell embryos showed low levels of DNA methylation (Fig. 2B). At later embryogenesis stages, DNA methylation levels progressively increased in developing embryos of 15 days, and mature embryos of 30 days (Fig. 2B). The global DNA methylation percentage of mature pollen was the highest, seven-fold more than vacuolated microspores (Fig. 2A), and near two-fold more than mature embryos (Fig. 2B).

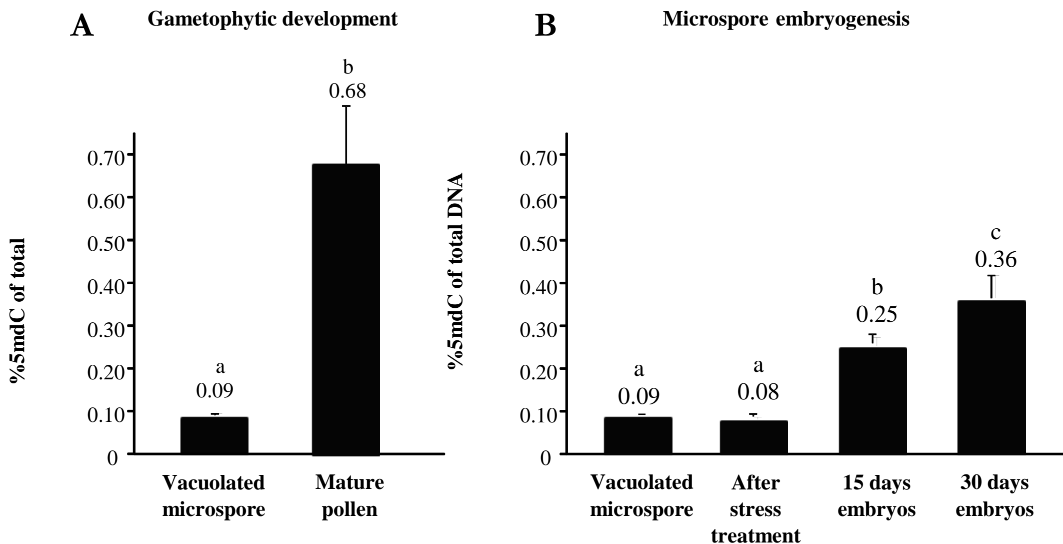

Figure 2: Quantification of global DNA methylation during pollen development and microspore embryogenesis. Histograms representing the mean values of 5mdC percentage of total DNA in different developmental stages of pollen gametophytic development (A) and microspore embryogenesis (B). Each column represents an average of three independent biological and two technical replicates per sample. Bars with different letters indicate developmental stages in which the mean percentage values are significantly different at $P < 0.001$.

Confocal microscopy analysis of the 5-methyl-deoxy-cytidine (5mdC) immunofluorescence assays revealed the nuclear distribution of methylated DNA and showed differences in the intensity and distribution pattern of the immunofluorescence signals at defined developmental stages. Confocal analysis were performed using the same laser excitation and sample emission capture settings for image acquisition in all immunofluorescence preparations, this procedure permitted

an accurate and reliable comparison between signals from cells at different developmental stages.

During the gametophytic development, the vacuolated microspore showed a faint fluorescence 5mdC signal distributed as very small spots over chromatin regions (Fig. 3A-D), the large central nucleolus appeared negative for 5mdC immunolocalization and for DAPI staining (Fig. 3B-D). After the first mitosis, the two nuclei of the bicellular pollen showed different 5mdC distribution patterns (Fig. 3E-H), the

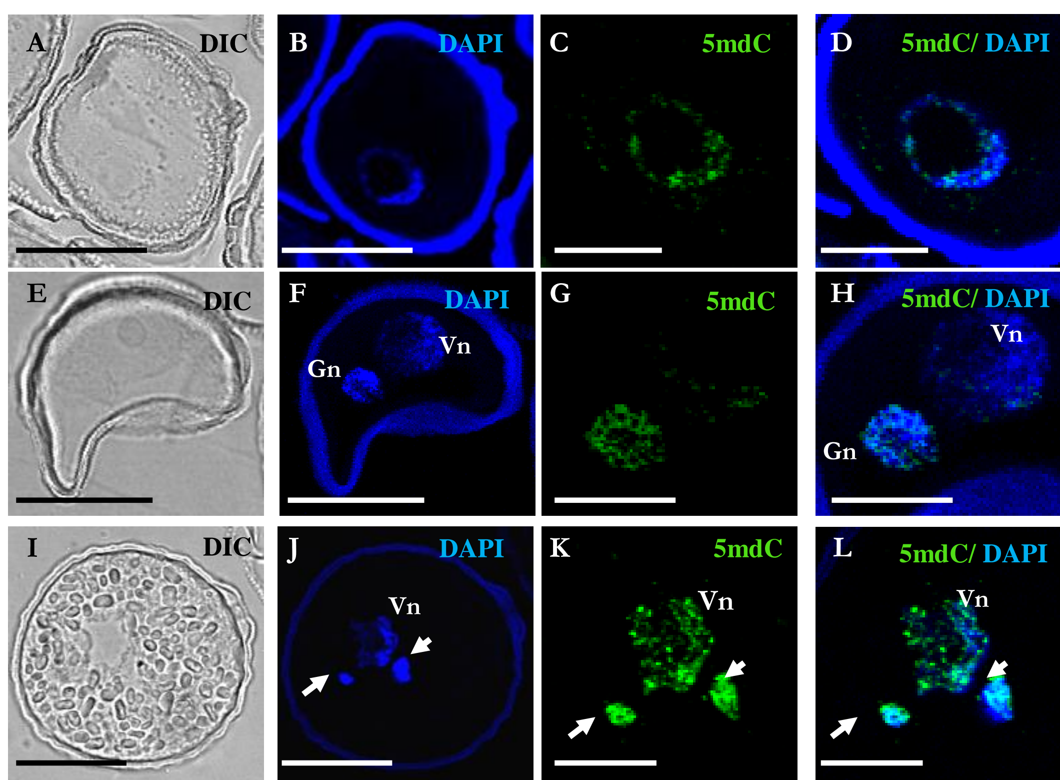

Figure 3: 5mdC immunolocalization during pollen development. A-D: Vacuolated microspore, E-H: Bicellular pollen, I-L: Mature tricellular pollen. A, E, I: Differential interference contrast (DIC) images of the cell structure. B, F, J: DAPI staining of nuclei (blue). C, G, K: 5mdC immunofluorescence (green), higher magnification. D, H, L: Merged images of DAPI (blue) and 5mdC immunofluorescence (green). The same structures are visualized under different microscopy modes from A to D, from E to H and from I to L. The exine showed unspecific autofluorescence under UV excitation in DAPI images. Vn: vegetative nucleus, Gn: generative nucleus, Arrows: sperm cells. Bars: A, B, E, F, I, J, 10 µm; C, D, G, H, K, L, 20 µm.

generative nucleus exhibited higher immunofluorescence signal than the vegetative one and the signal formed a thick reticulum covering the entire generative nucleus, whereas the vegetative nucleus showed very few or no signals (Fig. 3G, H). Finally, in mature tricellular pollen, 5mdC fluorescence was very intense on the two sperm nuclei, covering almost the whole nuclear areas; the vegetative nucleus, with a lobulated shape at this late developmental stage, showed 5mdC labeling as small spots on a thin reticulum throughout the nucleus (Fig. 3I-L).

The labeling pattern of 5mdC was different after the stress treatment for embryogenesis induction than in developing pollen grains. In samples collected just after the stress, some microspores still appeared uninucleated (Fig. 4A-D), while other microspores had already divided producing 2-4 cell structures or proembryos (Fig. 4E-H). Nuclei of the reprogrammed microspores and small proembryos showed low 5mdC fluorescence, with a diffuse signal (Fig. 4B-D) or forming a very thin reticulum (Fig. 4F-H) over the DAPI-stained nuclei. At later embryogenesis stages,

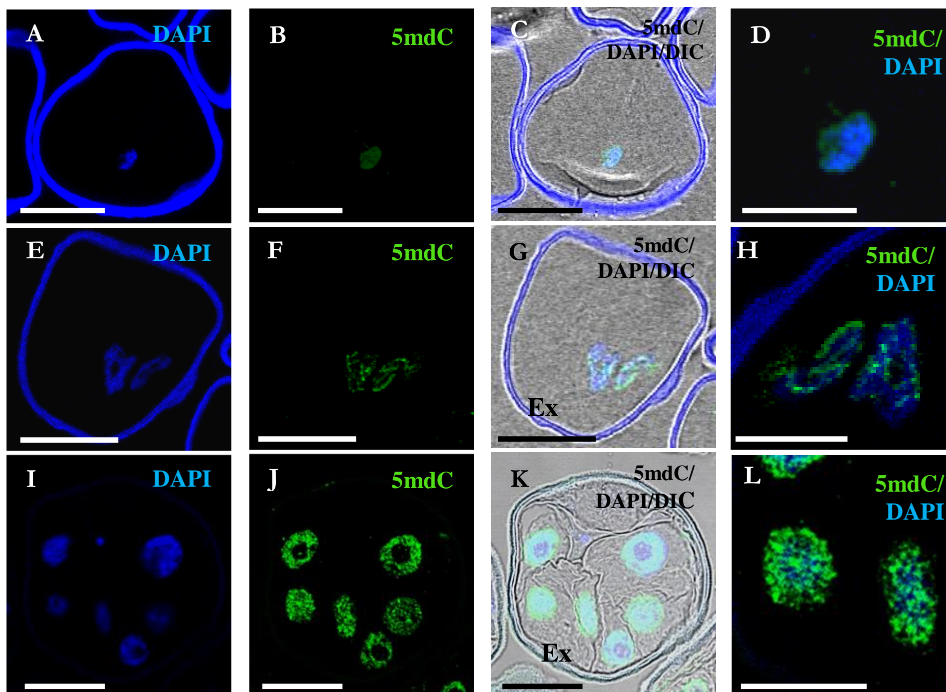

Figure 4: 5mdC immunolocalization during early stages of microspore embryogenesis. A-H: Vacuolated microspores and two-cell structures after embryogenesis induction. I-L: Multicellular embryo still surrounded by the exine. A, E, I: DAPI staining of nuclei (blue). B, F, J: 5mdC immunofluorescence (green). C, G, K: Merged images obtained by differential interference contrast (DIC) to show the cell structure, DAPI (blue), and 5mdC immunofluorescence (green). D, H, L: Higher magnification of DAPI (blue) and 5mdC (green) merged images. The same structures are visualized under different microscopy modes from A to D, from E to H and from I to L. The exine showed unspecific autofluorescence under UV excitation in DAPI images. Bars: A-C, E-G, I-K, 20 µm; D, H, L, 10 µm.

the multicellular embryos surrounded by the exine (Fig. 4I-L) and after the exine breakdown (Fig. 5A-C), were formed at 5-10 days after the stress treatment and exhibited large rounded nuclei. The cell nuclei of multicellular embryos showed different labeling intensities (Fig. 5 A - C), probably corresponding to nuclei at

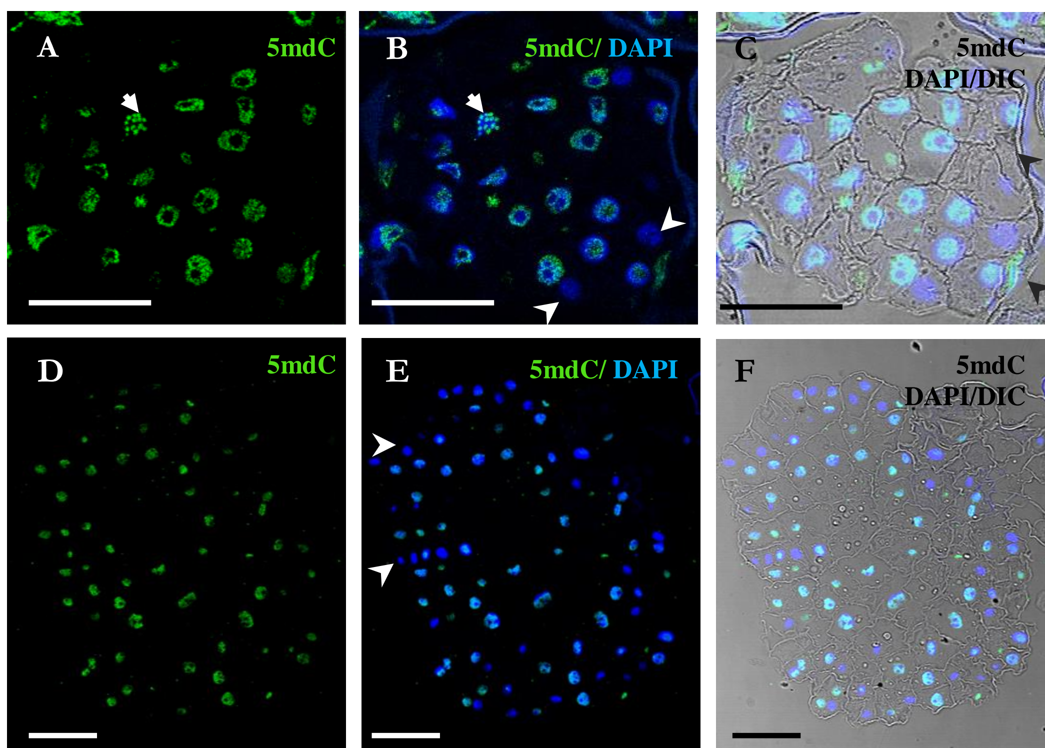

Figure 5: 5mdC immunolocalization during advanced stages of microspore embryogenesis. A-C: Multicellular embryo just after the exine breakdown. D-F: Developing embryo after 15 days in culture. A, D: 5mdC immunofluorescence (green). B, E: Merged images of DAPI (blue) and 5mdC immunofluorescence (green). C, F: Merged images obtained by differential interference contrast (DIC) to show the cell structure, DAPI (blue) and 5mdC (green) fluorescence signals. Arrows in A and B point mitotic chromosomes. Arrowheads in B and E point nuclei which did not show 5mdC labelling. Arrowheads in C point exine remnants still attached to the periphery of the multicellular embryo. The same structures are visualized under different microscopy modes from A to C, and from D to F. Bars: A-C, 25 µm; D-F, 75 µm.

different interphase periods of the cell cycle which presented different chromatin condensation states; the nucleoli did not show fluorescence in any case, appearing as dark rounded regions in both DAPI and 5mdC micrographs (Figs. 4I-K, 5A-C). A few interphasic nuclei did not show labeling (arrowheads in Fig. 5B) whereas other nuclei appeared with medium fluorescence intensity. Mitotic nuclei were observed in multicellular embryos and exhibited high fluorescence which covered the condensing chromosomes of prophasic nuclei (arrow in Fig. 5B) and chromosomes at different mitotic phases. With the progression of embryo development, larger embryos were formed and displayed a heterogeneous pattern of 5mdC labeling in the nuclei (Fig. 5D-F), most embryo cell nuclei showed fluorescence of different medium-high intensity and some nuclei did not show any detectable signal (Fig. 5D-F).

Immunofluorescence control experiments performed by eliminating either the denaturation treatment or the first antibody as well as immunodepletion experiments completely abolished the signal in all samples (figures not shown).

DISCUSSION

The results reported in this study illustrate for the first time the epigenetic changes, regarding DNA methylation, during pollen development and after embryogenesis induction in barley microspore cultures. The chromatin remodeling occurring during development of male gametes provide generative and sperm nuclei with a unique chromatin organization in a highly condensed state. The high increase of global methylation observed in the present work during pollen maturation in barley was associated with the heterochromatization that accompanied cellular differentiation in the most advanced stages of pollen development. In plants, the male germline is represented by the generative cell and the two sperm cells. Some reports have indicated the specific accumulation of epigenetic histone variants in the nuclei of the male germline (Ingouff et al. 2007), as well as a variable epialleles hypermethylation in the male germline (Borges et al. 2012). The results presented here in barley

revealed a differential genome-wide hypermethylation in the generative and sperm nuclei, while the vegetative nucleus remained hypomethylated after the first pollen mitosis.

The distribution of several histone modifications has been recently reported in the generative, sperm and vegetative nucleus of barley pollen (Pandey et al. 2013); in this report, marks associated with transcriptional activity, like active RNA Polymerase II, acetylated histone H3 in lysine 9, H3K9Ac, and tri-methylated histone H3 in lysine 27, H3K27me3 (Liu et al. 2010) appeared very low or absent in generative and sperm nuclei, whereas late microspore and vegetative nuclei exhibited higher labelling (Pandey et al. 2013). Our results on DNA methylation in pollen nuclei are in agreement with these findings and provide new evidences that gene silencing-related epigenetic modifications are enriched in the generative and sperm nuclei while activity-related marks are mainly localized in late microspore and vegetative nuclei. The repressive epigenetic mark di-methylated histone H3 in lysine 9, H3K9me2, has been found not only in the generative and sperm nuclei but also in the vegetative and microspore nuclei of barley pollen, and the activating mark di-methylated histone H3 in lysine 4, H3K4me2, has been also localized in microspore and all pollen grain nuclei (Pandey et al. 2013), the authors suggested that these modifications would be related to local changes in transcriptional activity and tissue-specific regulated genes and would be therefore present in nuclei of all stages of pollen development.

Low DNA methylation levels have been reported in microspores and vegetative nuclei of *Brassica napus*, as well as a punctuate 5mdC distribution nuclear pattern (Solis et al, 2012) corresponding to the scarce heterochromatin masses typical of this species (Seguí-Simarro et al 2011). In barley, the results revealed low DNA methylation and punctuate-thin reticulum distribution patterns of 5mdC in microspores and vegetative nuclei, in relation to the low condensed chromatin pattern of this monocot plant and with the high transcriptional activity reported for the vacuolated microspore (Testillano et al. 2000, 2005), the most responsive

developmental stage for embryogenesis induction in many species (González-Melendi et al 1995, Maluzinsky et al. 2003, Bárány et al. 2005, Prem et al. 2012).

Immunofluorescence assays showed no 5mdC signal over the nucleolus of vacuolated microspores. In the nucleolus, rRNA genes are transcribed, being the nucleolar architecture a clear reflect of the level of ribosome biosynthesis activity. In many organisms including plants, rDNA occurs in high copy numbers of genes which can be silent or transcribed depending on the activity state of the cell. Epigenetic changes involving DNA methylation and histone modifications have been reported to act in the dosage regulation of the number of active rRNA genes at any one time (Lawrence et al. 2004, Galetzka et al. 2006, Preuss et al. 2008, Bartova et al. 2010). In contrast with animal cells, plant cells do not usually exhibit a shell of perinucleolar heterochromatin where the silent portion of the repeated ribosomal genes and some telomeric and centromeric DNA regions were found (Bartova et al. 2010, Politz et al. 2013), only a very few condensed chromatin knobs containing inactive rDNA can be found at the nucleolar periphery (Testillano et al. 2005) and could correspond with some of the 5mdC spots found at the periphery of the nucleolus in the vacuolated microspore (Fig. 3C). In plants, low active nucleolus displayed small condensed chromatin masses, containing silent rDNA, at the nucleolar interior in the so-called heterogeneous fibrillar centers, whereas in very active nucleolus rDNA is distributed in a decondensed state throughout the nucleolar dense fibrillar component (Risueño and Testillano 1994, Testillano et al. 2005). The large nucleolus of the vacuolated microspore has been characterized with a typical organization of high transcriptional activity, corresponding to the G2 phase of the cell cycle (Risueño and Testillano 1994, González-Melendi et al. 1995, Testillano et al. 2005), the ribosomal chromatin being distributed in a decondensed state through the fibrillar component of the nucleolus, as revealed by rDNA in situ hybridization (Risueño and Testillano 1994, Testillano et al. 2005). In barley microspores, the nucleolus did not show 5mdC labeling indicating a decondensed state of ribosomal chromatin which correlates with an active rDNA transcription at this developmental stage.

In contrast with the microspore and vegetative nuclei, generative and sperm nuclei 5mdC signals were intense and distributed in wider nuclear regions covering the large heterochromatin masses that occupied the major part of the nuclear volume. In *Brassica napus*, hypermethylation of mature pollen was correlated with up-regulation of *BnMET1* methyl transferase, suggesting the involvement of MET1 in the methylation of generative nuclei (Solís et al. 2012), but no data is available in barley on the MET1 participation on epigenetic mechanisms during pollen development. The results obtained indicated an important change in global DNA methylation specifically in the male germline of barley, process probably contributing to the epigenetic inheritance after fertilization that has been reported in many plant species (Calarco et al. 2012).

Cell reprogramming by stress involves morphological and physiological changes as well as modifications in the genome organization and activity, as reported in dicot plants (Arnholdt-Schmitt, 2004; Miguel and Marum, 2011, Solis et al. 2012), the present study shows in a monocot species, that in contrast with the DNA methylation increase in the gametophytic development, microspore reprogramming to embryogenesis was associated with very low levels of global DNA methylation. Significant variations in global DNA methylation have been related to global changes of gene expression occurring during plant vegetative developmental processes (Meijón et al., 2010). The present data reveal an epigenetic change associated with the microspore reprogramming to a new developmental program and the first embryogenic divisions, epigenetic change that can be related to a global change of gene expression reported by transcriptomic analysis (Maraschin et al., 2006). Thus, our results indicate, for the first time in a monocot species, the existence of epigenetic changes after pollen embryogenesis induction that could be associated with the acquisition of embryogenic competence by the microspore and the ability to erase its gametophytic program and switch to a new cell fate.

At later stages of microspore embryogenesis, the results of the present study show a mild increase in global DNA methylation levels in multicellular embryos whose cells showed 5mdC localization patterns covering the nuclear volume with 5mdC signals of different intensity and distribution. It has been recently reported that nuclei of cycling cells of root meristems exhibited different signal intensities and distribution patterns of 5mdC immunofluorescence related to different interphase periods of the cell cycle presenting different chromatin condensation states (Testillano et al. 2013). Most cells of the early microspore-derived multicellular embryos were in active proliferation, as revealed in several monocot and dicot species (Testillano et al 2002, 2005, Bárány et al. 2005); in the present study, nuclei of young barley multicellular embryos presented distribution patterns of 5mdC which varied in intensity and localization, similar to the patterns related to the different chromatin condensation states observed in plant cycling cells.

The quantification of DNA methylation performed during advanced stages of microspore-derived embryo development revealed a gradual DNA methylation increase. Recent reports have shown an increase in global DNA methylation during the progression of in vitro somatic embryogenesis of the pineapple *Acca sellowiana* (Fraga et al. 2012), as well as during microspore embryogenesis of rapeseed (Solís et al. 2012). Since the progression of embryogenesis is accompanied by cell differentiation events, the results presented would indicate that the increase of global DNA methylation levels in microspore-derived embryos is related to the cellular differentiation, as found in other plant systems (Costa and Shaw, 2007, Solís et al. 2012). Differences in the distribution pattern of 5mdC between proliferating and differentiating plant cells have been established in various plant systems (Testillano et al. 2013, Solis et al. 2012), cells in differentiation showing much higher 5mdC labeling. The different 5mdC localization patterns found in cells of the advanced developing barley embryos could reflect different chromatin states of dividing cells and differentiating embryo cells.

During the zygotic embryogenesis, hypomethylation in the endosperm is accompanied by an extensive hypermethylation in the embryo (Köhler et al. 2012). This process has been suggested to ensure silencing of transposons and repetitive elements in the embryo and it occurs in monocots as well as in dicots, implicating an evolutionarily conserved mechanism of DNA hypermethylation in the embryo (Köhler et al. 2012). The results of the present work also revealed a hypermethylation process during microspore-derived embryo development in barley providing new evidences of analogous mechanisms acting in microspore embryogenesis and zygotic embryogenesis and supporting that DNA methylation is critical for the regulation of plant embryogenesis gene expression.

Taken together, the results presented revealed epigenetic changes that accompany the two pollen developmental programs analyzed: pollen maturation and microspore embryogenesis, in barley, suggesting the possible involvement of DNA methylation dynamics in regulating microspore embryogenesis induction and progression in a monocot species.

ACKNOWLEDGMENTS

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PUBLICATION II

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

Reference:

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***These authors have contributed equally to this work**

Publication II:

5-AZACYTIDINE PROMOTES MICROSPORE EMBRYOGENESIS INITIATION BY DECREASING GLOBAL DNA METHYLATION, BUT PREVENTS SUBSEQUENT EMBRYO DEVELOPMENT IN RAPESEED AND BARLEY

ABSTRACT

Microspores are reprogrammed by stress *in vitro* towards 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, 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.

Key words: 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 towards 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* (Rodriguez-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 4ºC. After that, they were grown at 12ºC 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 18ºC. 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 15 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 15ml 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 xg for 5 min at 4°C. The pellet was resuspended in 10mL 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: 20mm) 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 4ºC 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 x 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 xg 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 xg 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 x 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-azacytidine, 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 µM, 5 µM 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; thes 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 standard error of the means. 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 standard error of the means. 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 (Solis et al., 2014). A MethylFlash Methylated DNA Quantification Kit (Colorimetric) (Epigentek, NY) 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 15mL 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 5mdC of total DNA were calculated. The results were shown in histograms in which columns represented mean values and bars represented standard error of the mean. 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 4º C and sectioned at 2 μm thickness using an ultramicrotome (Ultracut 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 5-methyl-deoxy-cytidine (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 2 N 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 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 cacodilate 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, denaturated 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 *Brassica napus* (Prem et al., 2012) and *Hordeum vulgare* (Rodríguez-Serrano et al., 2012), as described in the Materials and Methods section. Vacuolated microspores (Figs. 1A, 1B, 2A, 2B), 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. 32ºC 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” (Figs. 1C, 1D, 2C, 2D). These proembryos (arrows in Figs. 1E, 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 (Figs. 1F, 1G), heart, torpedo (Fig. 1H) and cotyledonary embryos (Fig. 1I) were formed (Prem et al., 2012), while in barley microspore cultures globular, transitional, scutellar and coleoptilar monocot embryos (Figs. 2F-H) were developed (Rodríguez-Serrano et al., 2012).

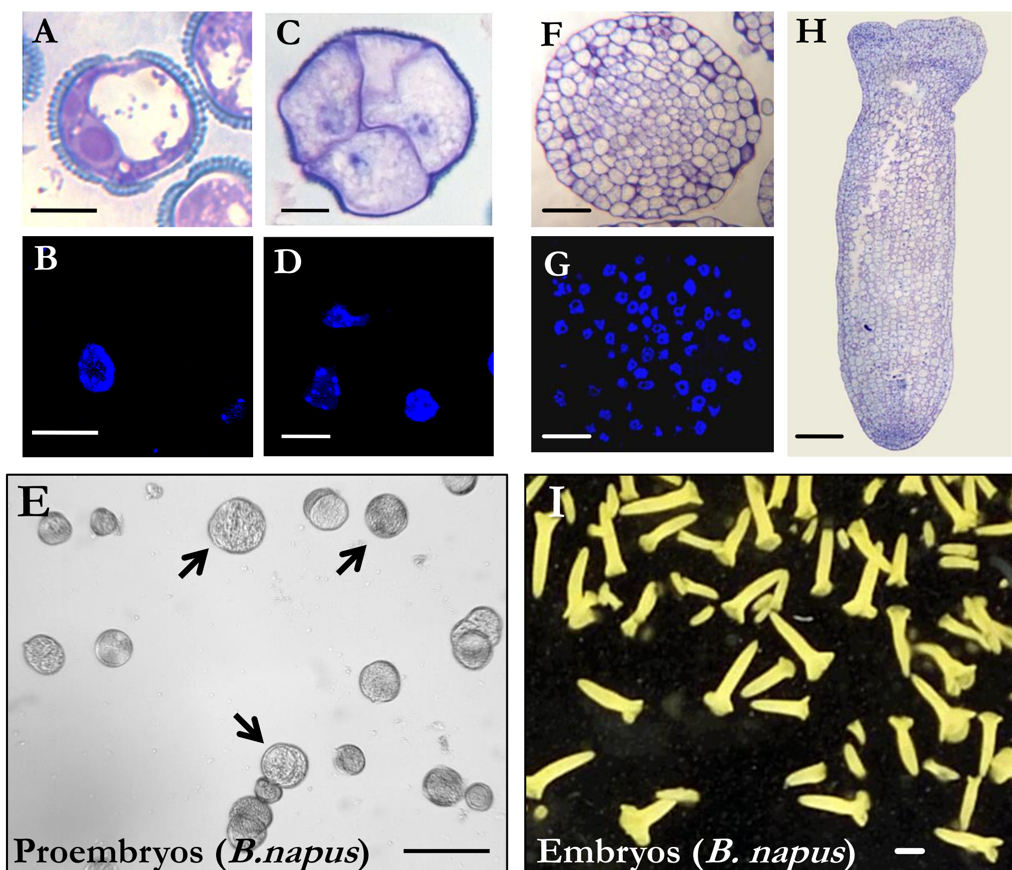

Figure 1: Microspore embryogenesis in *Brassica napus*. A, B: Vacuolated microspores at the beginning of the culture. **C, D:** Proembryos formed by 4 cells, still surrounded by the exine (the microspore wall). E: In vitro culture at the proembryo formation stage (4 days), proembryos are pointed by arrows. **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: 10µm, in E: 250µm, in **F, G:** 50µm, in H: 100 µm, in I: 1mm.

Firstly, different concentrations of AzaC, 2.5µM, 5.0 µM and 10µM, were tested during short treatments (4 days) on rapeseed 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 (Fig. 3A), present in cultures at the proembryo formation stage (Fig. 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 mcirospores (Fig. 3B). Microspore cultures treated with 2.5 μM and 5 μM AzaC showed a small but statistically significant reduction in cell death, in comparison with control cultures (Fig. 3B).

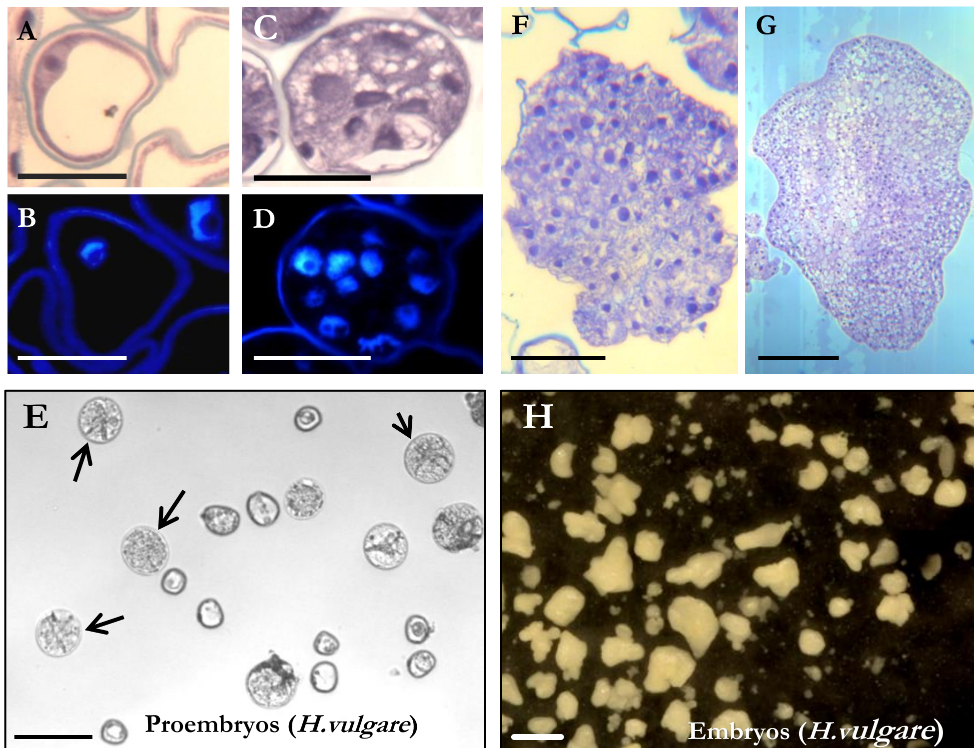

Figure 2: Microspore embryogenesis in *Hordeum vulgare*. A, B: Vacuolated microspores at the beginning of the culture**. C, D:** Proembryos formed by several cells, still surrounded by the exine (the microspore wall). E: In vitro culture at the proembryo formation stage (4 days), proembryos are pointed 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: 20μm, in C, D: 50μm, in **E:** 250μm, in **F, G:** 100μm, in **H:** 1mm.

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

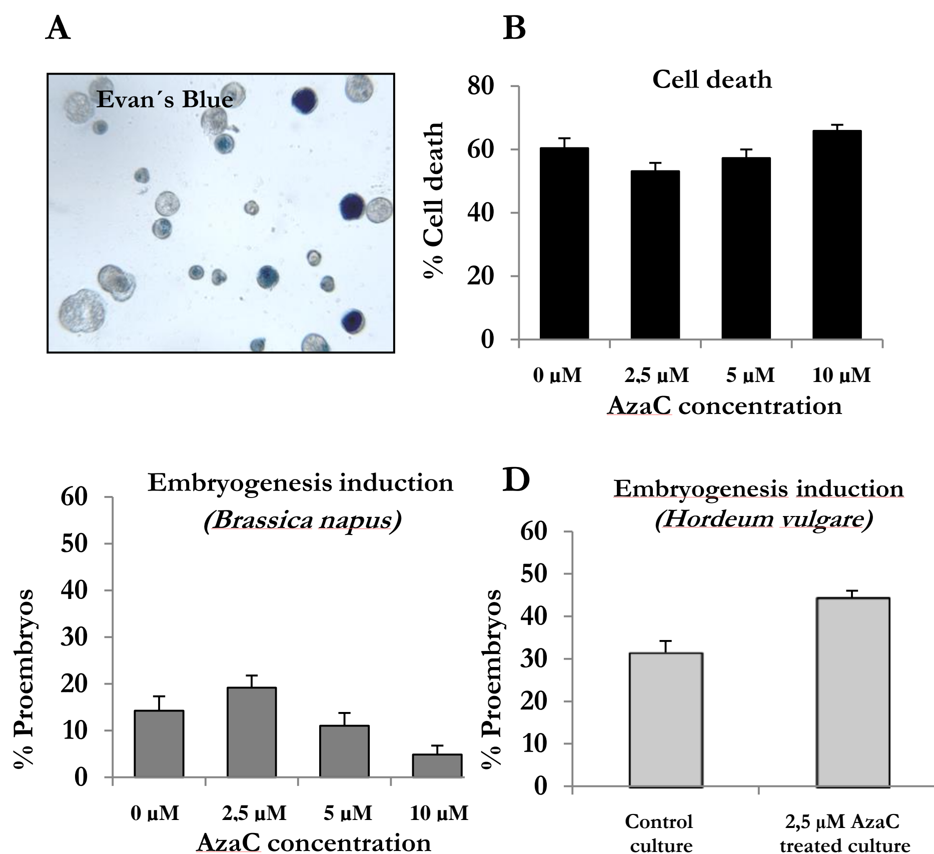

Figure 3: Effects of short AzaC treatment in microspore cultures on cell death and embryogenesis induction. A: Evan's blue staining to detect dead cells in microspore embryogenesis cultures of Brassica napus at the proembryo formation stage. The staining solution only enters into dead cells, which appeared blue. **B, C:** Quantification of the percentage of dead cells (B) and proembryos (C) in microspore cultures of B. napus at the proembryo formation stage, after short treatment (4 days) with AzaC at the concentrations of 0µM (control), 2.5µM, 5µM and 10µM. **D:** Quantification of the percentage of proembryos in microspore cultures of Hordeum vulgare, after short treatments (4 days) with AzaC at the concentrations of 0µM (control) and 2.5µM. Bar in A represents 100µm. In histograms (B-D), columns represent mean values and bars represent standard error of the means; asterisks indicate significant differences with the non-treated/control culture sample (Student's t-test at $P \leq 0.05$).

Short AzaC treatments were also applied to barley microspore cultures, at the concentration of 2.5 µM, 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 (Fig. 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 2.5µM 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 (Figs. 1E, 2E). Results showed significant decreases in global DNA methylation after the AzaC treatments in both plant species (Fig. 4). In *B. napus* microspore cultures treated by AzaC, DNA methylation levels reached only half of that in control cultures (Fig. 4A). In barley microspore cultures, the level of methylated DNA also diminished after AzaC treatment (Fig. 4B), but to a lesser extent than in rapeseed cells.

Immunofluorescence assays with 5-methyl-deoxy-cytidine (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 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 (Fig. 5A). The 5mdC immunofluorescence signal

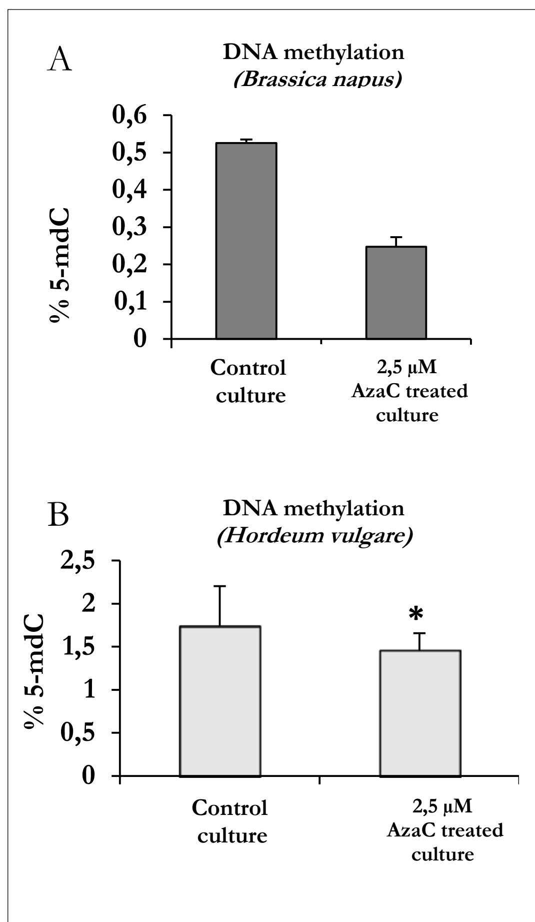

Figure 4: Effects of short AzaC treatment in microspore embryogenesis cultures on global DNA methylation levels. Quantification of global DNA methylation levels in control and 2.5 µM AzaC-treated cultures of Brassica napus (A) and Hordeum vulgare (B), at the proembryo formation stage. Columns represent mean values and bars represent standard error of the means of 5mdC percentage of total DNA. Asterisks indicate significant differences with the non-treated/control cultures (Student's t-test at P≤ 0.05).

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 (Figs. 5A', 5A''). In microspore cultures treated with 2.5µM AzaC, proembryos exhibited a cellular organization similar to that in control cultures (Fig. 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 (Figs. 5B', 5B'').

Barley microspore-derived proembryos, still surrounded by the exine, displayed numerous small cells with large nuclei and wavy cell walls (Fig. 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 (Fig. 5D). In control cultures, the 5mdC immunofluorescence signal was intense, covering the whole nucleus (Figs. 5C', 5C'') which also exhibited an intense fluorescence intensity by DAPI (Fig. 5C'). In proembryos developed in the presence of AzaC, the 5mdC immunofluorescence signal was less intense and was distributed over the entire nucleus (Figs. 5D', 5D'').

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.

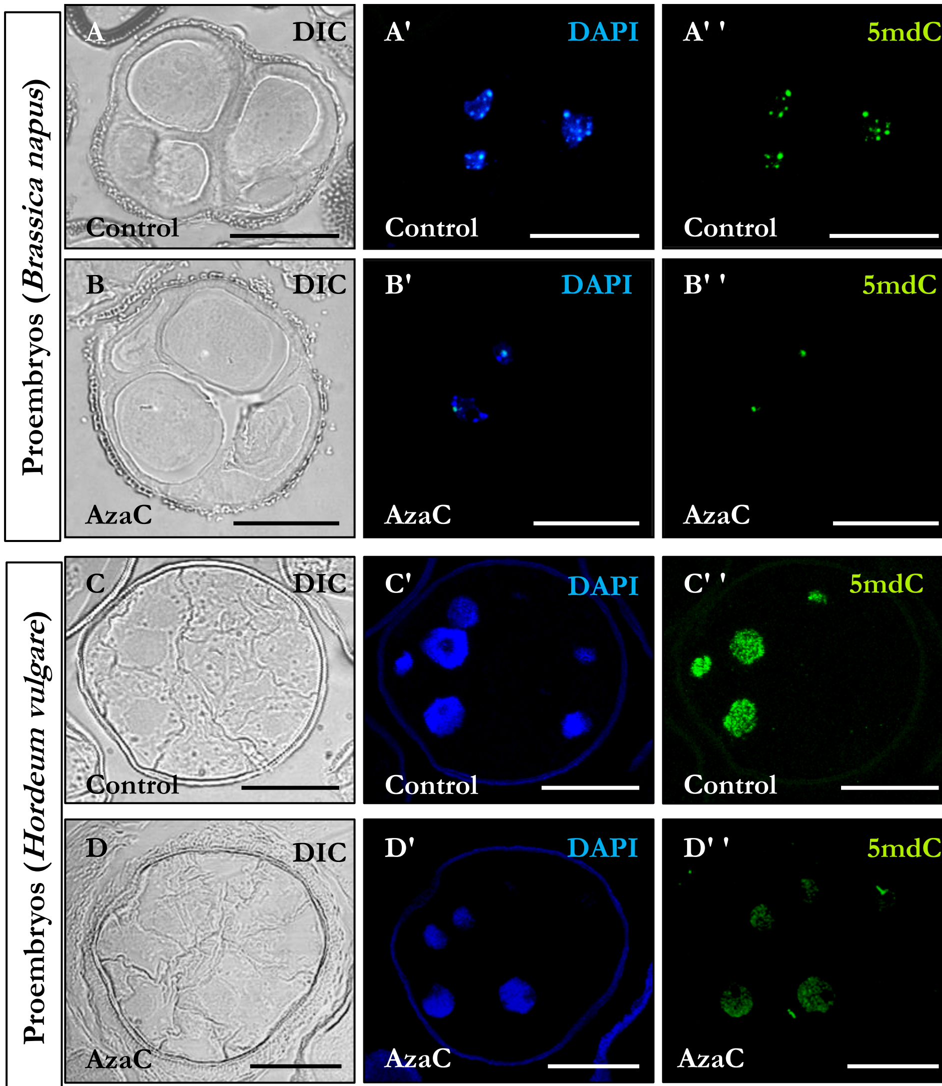

Figure 5: Distribution patterns of methylated DNA in microspore proembryos under control conditions and short AzaC treatment. 5mdC immunofluorescence and confocal laser scanning microscopy analysis in *Brassica napus* (A, B) and *Hordeum vulgare* (C, D) microspore proembryos of control (A, C) and 2.5µM AzaC-treated (B, D) cultures. A, B, C, D: Nomarsky's differential interference contrast (DIC) images of the proembryo structure. A', B', C', D': DAPI staining of nuclei (blue). A'', B'', C'', D'': 5mdC immunofluorescence (green). The same structures are visualized under different microscopy modes in A-A'', B-B'', C-C'' and D-D''. The exine showed unspecific autofluorescence under UV excitation in some DAPI images (C', D'). Bars represent 20µm.

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 (Figs. 6, 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 (Fig. 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 (Fig. 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 (Fig. 6B'). After the treatment with AzaC, proembryo nuclei showed a homogeneous chromatin distribution in both toluidine blue (Fig. 6C) and DAPI (Fig. 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 (Fig. 6D').

Transmission electron microscopy (TEM) analysis revealed the chromatin ultrastructural organization of rapeseed proembryo nuclei, which exhibited a very low condensed chromatin pattern (Fig. 6E) with a few isolated and electron dense condensed chromatin masses (arrows in Fig. 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 (Fig. 6E). 5mdC immunogold labeling

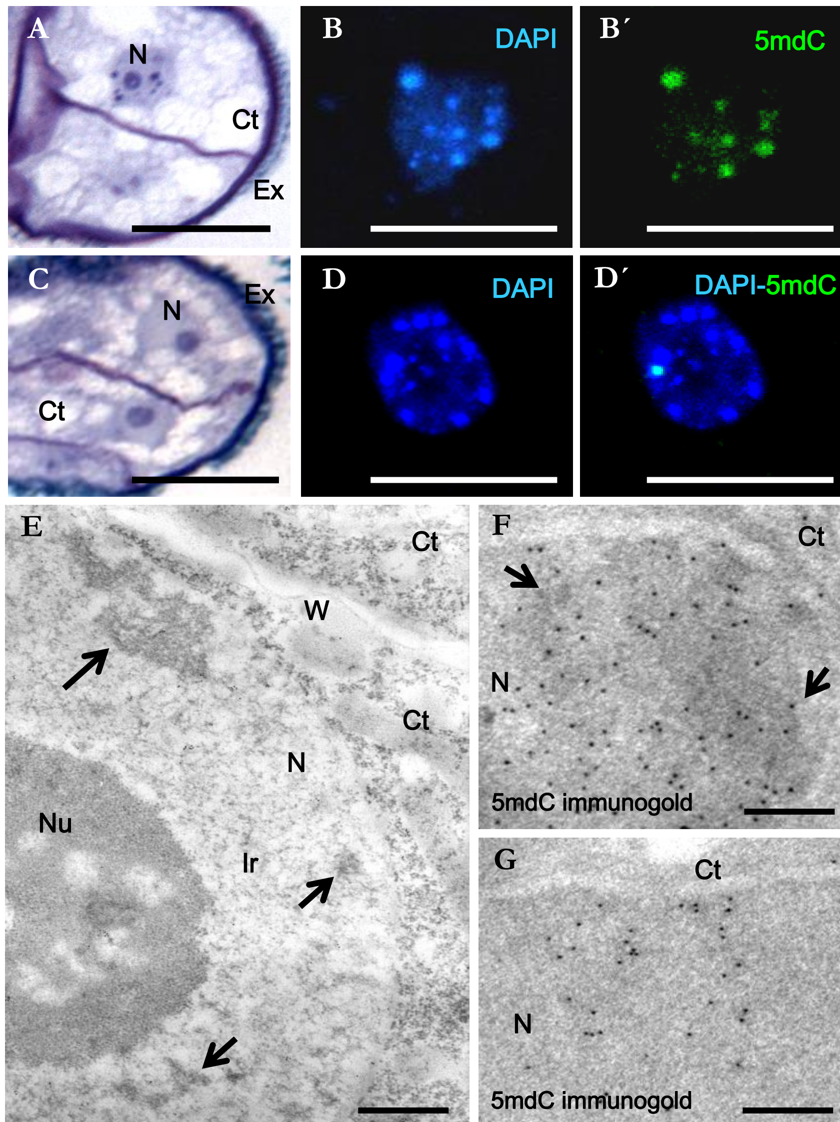

Figure 6: Chromatin condensation patterns and methylated DNA distribution in microspore proembryos of *Brassica napus*. A-D: High magnification light microscopy images of microspore proembryo nuclei in control (A, B, B') and 2.5µM AzaC-treated (C, D, D') cultures, observed after toluidine blue staining (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 and B', and in D and D'. **E-G**: Transmission electron microscopy 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 µm, in E: 0.5 µm, in F, G: 0.2 µm.

revealed the ultrastructural distribution of methylated DNA; numerous gold particles were found decorating the large condensed chromatin masses, while no labeling was observed in decondensed chromatin (Figs. 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 (Fig. 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 (Fig. 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 (Fig. 7C), indicating a less condensed chromatin pattern than in control samples. DAPI staining provided an intense fluorescence to proembryo nuclei of non-treated cultures (Fig. 7B) while nuclei of AzaC-treated proembryos showed less intense DAPI fluorescence (Fig. 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 (Fig. 7B'). AzaC-treated nuclei showed a less intense distribution pattern of 5mdC immunofluorescence (Fig. 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 (Figs. 7B, B', D, D').

Ultrastructural analysis by transmission electron microscopy showed the pattern of chromatin condensation in barley proembryo nuclei (Fig. 7E). High magnification electron micrographs showed heterochromatin patches distributed throughout the

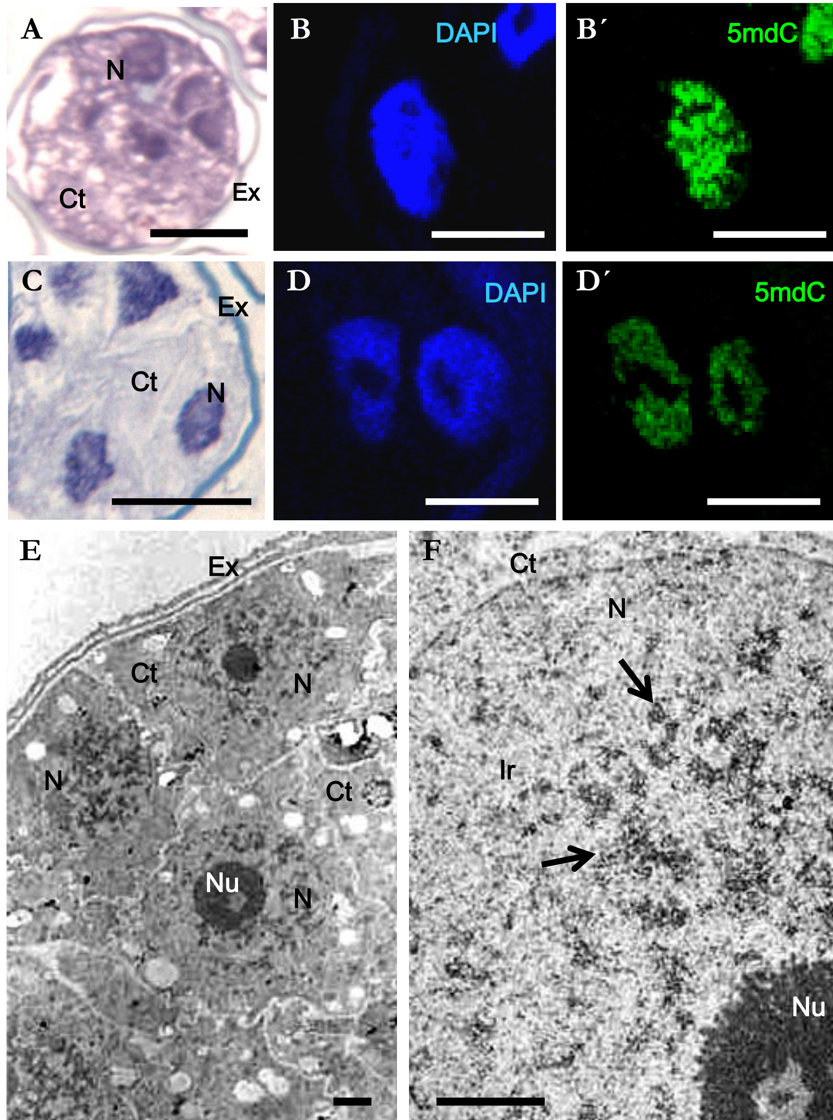

Figure 7: Chromatin condensation patterns and methylated DNA distribution in microspore proembryos of *Hordeum vulgare*. A-D: High magnification light microscopy images of microspore proembryo nuclei in control (A, B, B') and 2.5µM AzaC-treated (C, D, D') cultures observed after toluidine blue staining (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 and B', and D and D'. **E-F**: Transmission electron microscopy 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 µm, in B, B', D, D': 10µm, in E, F: 1 µm.

whole nucleus, connected by chromatin threads of different thicknesses (Fig. 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 interchromatin region that typically contained fibrillo-granular RNPs was less abundant in barley than in rapeseed proembryo nuclei (compare Figs. 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 (Fig. 1I) and late scutellar and coleoptilar embryos in barley (Fig. 2H). The results showed a very marked reduction of embryo production in 2.5µM AzaC-treated cultures in which only very few embryos were found in both species, in contrast with control cultures which exhibited numerous embryos (Figs. 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 (Figs. 8E, 8F).

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 (Figs. 9, 10).

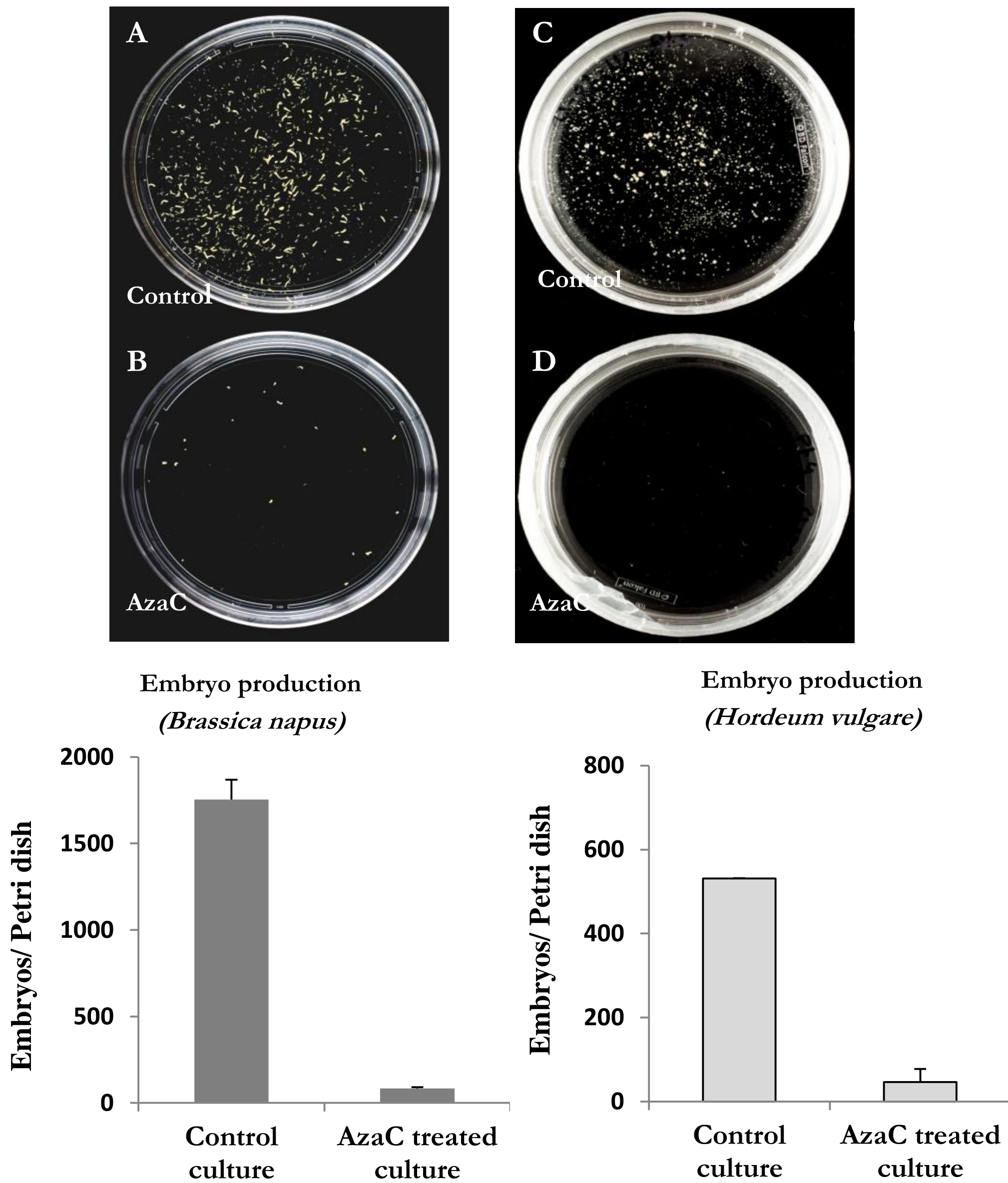

Figure 8: Effects of long AzaC treatment on embryo production yield. A-D: Plates showing the microspore-derived embryos produced in control (A, C) and 2.5µM AzaC-treated (B, D) cultures of B. napus (A, B) and H. vulgare (C, D), after 30 days. **E-F:** Quantification of the embryo production in control and 2.5µM AzaC-treated cultures of *B. napus* (E) and *H. vulgare* (F). In histograms (E, F), columns represent mean values and bars represent standard error of the means 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≤ 0.05)

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 (Fig. 9A, 10A). Later, the number of proembryos slightly increased until day 12, remained

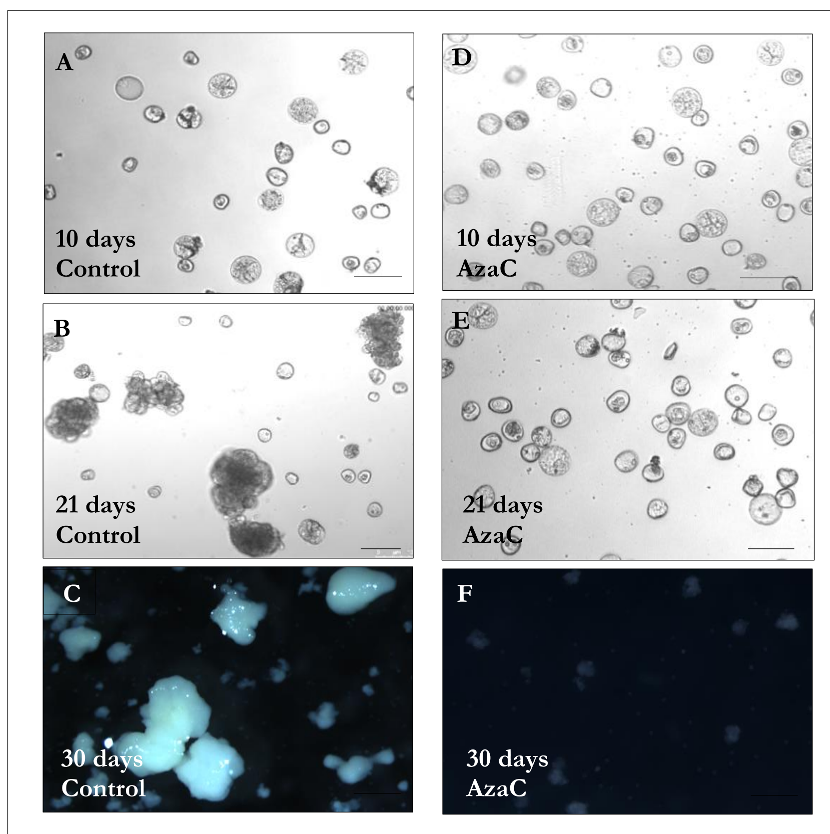

Figure 9: Progression of microspore embryogenesis in control and AzaC-treated cultures of barley. Micrographs of microspore cultures observed at different time points. **A-C**: Control cultures. **D-F**: 2.5µM AzaC-treated cultures. **A, D**: 10 day-old cultures showing typical rounded proembryos surrounded 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 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.

relatively stable for several more days and progressively decreased until day 21 (Fig. 9B, 10A). However, in AzaC-treated cultures, the proportion of proembryos at day 10 was significantly higher than in control cultures (Fig. 9D, 10A). During the following

days, the number of proembyos in AzaC-treated cultures progressively increased, until day 21 (Fig. 9E, 10A). The proembryos formed during long AzaC treatments showed similar morphology and size to the proembryos formed in non-treated cultures at early stages (Figs. 9A, 9D, 9E), 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.

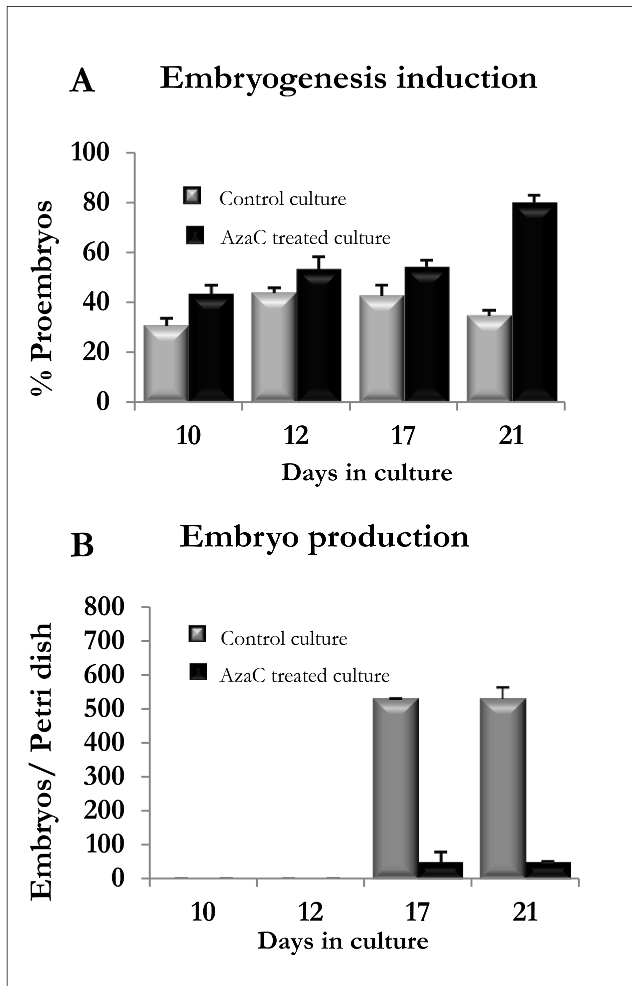

Figure 10: Effects of long AzaC treatment on microspore embryogenesis progression in barley. Quantification of the percentage of proembryos (A) and developing embryos (B) observed at different time intervals (10, 12, 17 and 21 days) during microspore embryogenesis progression in control (grey columns) and 2.5µM AzaC-treated (black columns) cultures of barley. Columns represent mean values and bars represent standard error of the means. Asterisks indicate significant differences with the non-treated/control culture sample at each time point, days in culture (Student's t-test at P≤ 0.05).

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 (Fig. 9B, 9C, 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) (Fig. 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 (Fig. 9E, 9F, 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-azacytidine (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.

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 2.5µM, 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 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;Tokuji et al., 2011;Fraga et al., 2012). In these previous studies, the range of concentration of AzaC has been very variable and high (from 10µM to 200µM). 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 µM, 5µM and 10µM, and their effects on cell death have been evaluated; the results of these analyses reveal that cultures with the lowest AzaC dose (2.5µM) 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, 2.5µM was the concentration selected for the treatments. Moreover, the quantification of global DNA methylation indicates that 2.5µM 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 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;Testillano et al., 2002;Bárány et al., 2005;Testillano 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 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;Testillano et al., 2002;Bárány et al., 2005;Testillano et al., 2005;Seguí-Simarro et al., 2011). *De novo* auxin

biosynthesis and accumulation has been recently reported in early microspore embryogenesis, from the first divisions (Rodriguez-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, González-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; Rodríguez-Sanz et al. 2014b; El-Tantawy et al. 2014).

Recently, it has been shown that the change of developmental program of the microspore towards 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* (Rodríguez-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 gamethophytic 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-azacytidine (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 [^{3}H]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;Testillano et al., 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 2.5μM 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;Costa and Shaw, 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 *Brassica napus (*Solís et al. 2012; Solís et al. 2014). This increase in *MET1* expression

correlated with the increase in global DNA methylation and heterochromatization events (Solís et al., 2012;Solis et al., 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.

CONCLUSIONS

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, 2.5μM AzaC for four 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.

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PUBLICATION III

Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*

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Publication III:

ARABINOGALACTAN PROTEIN PROFILES AND DISTRIBUTION PATTERNS DURING MICROSPORE EMBRYOGENESIS AND POLLEN DEVELOPMENT IN *Brassica napus*

ABSTRACT

Arabinogalactan proteins (AGPs), present in cell walls, plasma membranes and extracellular secretions, are massively glycosylated hydroxyproline-rich proteins that play a key role in several plant developmental processes. After stress treatment, microspores cultured *in vitro* can reprogramme and change their gametophytic developmental pathways towards embryogenesis, thereby producing embryos which can further give rise to haploid and double haploid plants, important biotechnological tools in plant breeding. Microspore embryogenesis constitutes a convenient system for studying the mechanisms underlying cell reprogramming and embryo formation. In this work, the dynamics of both AGP presence and distribution were studied during pollen development and microspore embryogenesis in *Brassica napus*, by employing a multidisciplinary approach using monoclonal antibodies for AGPs (LM2, LM6, JIM13, JIM14, MAC207) and analyzing the expression pattern of the *BnAGP Sta 39-4* gene. Results showed the developmental regulation and defined localization of the studied AGP epitopes during the two microspore developmental pathways, revealing different distribution patterns for AGPs with different antigenic reactivity. AGPs recognized by JIM13, JIM14 and MAC207 antibodies were related to pollen maturation, whereas AGPs labelled by LM2 and LM6, were associated with embryo development. Interestingly, the AGPs labelled by JIM13 and JIM14 were induced with the change of microspore fate. Increases in the expression of the *Sta 39-4* gene, JIM13 and JIM14 epitopes, found specifically in two-four cell stage embryo cell walls, suggested that AGPs are early molecular markers of microspore embryogenesis. Later, LM2 and LM6 antigens increased progressively with embryo

development and localized on cell walls and cytoplasmic spots, suggesting an active production and secretion of AGPs during *in vitro* embryo formation. These results give new insights into the involvement of AGPs as potential regulating/signalling molecules in microspore reprogramming and embryogenesis.

Key words: microspore culture, cell wall, *Sta39-4* gene, *Brassica napus,* AGP epitopes

INTRODUCTION

Arabinogalactan proteins (AGPs) with a key role in several plant developmental processes (reviewed in Seifert and Roberts 2007) are massively glycosylated hydroxyproline-rich glycoproteins that can be found in cell walls, plasma membranes and extracellular secretions. AGPs present a high degree of heterogeneity not only with respect to their protein part but also in their carbohydrate sequences and composition, which have been analysed by a combination of chemical studies and the use of antibodies (Knox 1997). In fact, one of the main tools in the study of AGPs has been the use of monoclonal antibodies that bind to AGP-specific sugar epitopes. The setting up of monoclonal antibodies directed against different AGP epitopes and cell wall polymers has facilitated the analysis of the complex cell wall structure and its dynamics during plant developmental processes. These anti-AGP monoclonal antibodies have been used to unravel the involvement of specific epitopes in controlling cell growth and morphogenesis. Increasing evidence has linked AGPs to many processes involved in plant growth and development, including somatic embryogenesis (Chapman et al. 2000, Thompson et al. 1998, van Hengel et al. 2001), pollen grain development (Coimbra et al. 2009, Levitin et al. 2008, Pereira et al. 2006) and pollen tube growth (Costa et al. 2013, Cheung et al. 1995, Wu et al. 2001). Despite information gained in recent years with respect to AGPs, their precise functions have not yet been elucidated (for review, see Seifert et al. 2007).

Microspore embryogenesis constitutes an intriguing system in which a cell, namely the microspore, has its gametophytic programme redirected towards an embryogenic pathway. After specific stress treatments *in vitro*, the microspore can reprogram itself during specific developmental stages, and initiate an embryogenesis programme that produces embryos from which haploid and double haploid plants, important biotechnological tools in plant breeding, can finally develop (review in Maluszynski et al. 2003). Isolated microspore cultures, in which microspores are separated from the anther tissues, constitute very convenient systems for studying the mechanisms underlying cell reprogramming and embryo formation. Although, in recent years increasing amounts of information have been reported on the presence of genes and molecules controlling early embryogenic events, knowledge of the genetic control of the process and the possible involvement of external factors regulating embryo growth and development is still scarce.

It has frequently been hypothesized that AGPs are sources of soluble signal molecules in the form of sugar chain fragments (Johnson et al. 2003, Schultz et al. 1998). Previous findings have revealed changes in cell wall components associated with cell reprogramming, and many of the molecular markers of somatic embryogenesis have also been found in cell walls. Exogenous AGPs are known to affect somatic embryogenesis in different ways (Portillo et al. 2012), namely, as stimulating factors for microspore embryogenesis (Yuan et al. 2012). Nevertheless, there is very little information on the presence and possible function of endogenous AGPs in microspore embryogenesis. On the other hand, it has been reported that by adding a Yariv reagent to the culture medium, a synthetic probe that binds to and aggregates AGPs, AGP action is blocked negatively, affecting embryogenesis (Tang et al. 2006) This would suggest that endogenous AGPs are involved in embryo development *in vitro*.

In this work, the presence and distribution of AGPs were studied during pollen development and in *in vitro* induced microspore embryogenesis in *Brassica napus,* by

means of a multidisciplinary approach, combining immunocytochemical, biochemical and molecular techniques. Dot-blot assays were carried out, along with immunofluorescence and confocal laser scanning microscopy (CLSM) analyses with several monoclonal antibodies for AGPs: JIM13, JIM14, MAC207, LM2 and LM6, the latter reacting with AGP arabinan epitopes which are also present in pectins. Analysis of the expression pattern of the *BnAGP Sta 39-4* gene (Gerster et al. 1996) by quantitative real time PCR (qPCR) was also performed.

Results showed the developmental regulation and well-defined localization of the studied AGP epitopes during pollen development and microspore embryogenesis, which revealed different distribution patterns. The dynamics of specific AGP epitopes (JIM13, JIM14, MAC207) were related to pollen maturation, whereas other epitopes were associated with the change of the microspore developmental programme (JIM13, JIM14) and with microspore-derived embryo differentiation (LM2, LM6), suggesting AGPs as potential regulating/signalling molecules involved in these processes.

MATERIAL AND METHODS

Plant material and microspore culture

Brassica napus L. cv. Topas donor plants were grown under controlled conditions at 15ºC day, 16h photoperiod, and 10°C night. Both isolated microspore culture and embryogenesis induction were performed by a 32°C treatment, as described by Prem et al. 2012.

Fixation and processing for microscopic analysis

Fresh samples from different culture times and anthers from flower buds at different stages of pollen development were collected and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.3, overnight at 4ºC and washed in PBS. Isolated microspores and small proembryos were embedded in gelatin and processed

as described by Solís et al., 2008. All samples were dehydrated in acetone series and embedded in Technovit 8100 resin (Kulzer, Germany) at 4°C. Semithin resin sections were placed on slides coated with APTES (3-aminopropyltriethoxysilane, Sigma) and stored at 4ºC until used for immunofluorescence. Some sections were stained with toluidine blue and examined under bright field microscopy for structural analysis.

Antibodies

The antibodies used in this study were rat monoclonal anti-AGPs: JIM13, JIM14, MAC207, LM2 and the anti-(1→5)-α-L-Arabinan LM6 (Plantprobes).

Immunofluorescence

Sections were blocked with 5% bovine serum albumin (BSA) in PBS and incubated with the primary antibodies JIM13, JIM14, MAC207, LM6 and LM2 for 1h in 1/5 dilution, except for LM2 which was used in 1/10 dilution in 1% BSA in PBS. After washing in PBS, the signal was revealed with the Alexa Fluor 488-labelled anti-rat antibody (Molecular Probes) diluted 1/25 in PBS for 45 min in the dark, as described by Testillano et al. 2012. Finally, sections were counterstained with 1mg/ml DAPI (4'.6-diamidino-2-phenylindole) for 10 min and analysed in a laser scanning confocal microscope (TCS-SP5, Leica). Negative controls were obtained by replacing the primary antibody with PBS.

Immuno-dot-blot assay

Proteins were extracted from 60 mg samples of cultures at different time points, and microspores and pollen grains were isolated from anthers according to sizes. Samples were homogenized in liquid nitrogen using a mortar and pestle, in 50 ml of buffer containing 50 mM Tris-HCl pH 7.2, 50 mM trans-1.2-diaminocyclohexane-N,N,N#N#-tetraacetic acid (CDTA), and 25 mM dithiothreitol. The resulting supernatant concentrations were determined according to Bradford, 1976 (Bio-Rad Protein Assay reagent) using Bovine serum albumin (BSA) as calibrator and all

samples were adjusted to a concentration of 0.5 mg/ml. For immuno-dot-blot assays, 5 µl aliquots of adjusted supernatants were applied to a nitrocellulose membrane (Millipore; Bedford, MA) and left to dry for 1 h according to Bárány et al. 2010. Strips were first stained for total protein detection with Ponceau red and the images of the stained dots were captured.

The membrane was incubated overnight at room temperature, with the primary antibodies (anti-AGPs JIM13, JIM14, MAC207, LM2 and the (1→5)-α-L-Arabinan LM6) diluted 1:100, except LM2 which was diluted 1:200, in the blocking buffer (2% powdered skimmed milk containing 0.05% Tween-20 in PBS), washed, and incubated for 1 h with alkaline phosphatase-conjugated anti-rat antibody diluted 1:1000 in the blocking solution. Finally, the epitopes recognized by the antibodies were revealed by treatment with a nitroblue tetrazolium, bromo-chloroindolyl-phosphate (NBT-BCIP) mixture.

Quantification of the relative intensity of the dot-blot signals was performed by using appropriate image analysis software. For the quantification, images of three replicates for each antibody and developmental stage were used. Mean values and standard deviations were calculated and the results showed in histograms. *P*-values were calculated using *Student's t* test.

Quantitative real-time PCR (qPCR)

RNA was isolated from the different culture and pollen samples at the different stages analysed, according to Solís et al. 2012. One microgram of total RNA was used for the RT reaction using the Superscript TM II reverse transcriptase enzyme (Invitrogen). The oligonucleotides used for Sta39-4 expression analysis were: 5´ GGCACCCTCAGCTGCTC 3´ and 3´ ATGGTCCATCAACAACCTCTG 5´ from the sequence of the Sta39-4 gene (L47352.1), one of the first pollen-specific putative AGP genes to be characterized in *Brassica napus* (Gerster et al. 1996).

cDNA was amplified using SsoAdvanced™SYBR®Green supermix on an iQ™5 Real-Time PCR Detection System (Biorad). All qPCR reactions were run in duplicate. Thermocycle settings were as follows: Initial denaturation of 30 s at 95°C, followed by forty cycles, each consisting of 5 s at 95°C, 30 s and 30 s at 56°C. After each run, a dissociation curve was acquired to check for amplification specificity by heating the samples from 65 to 95°C. Serial dilutions of cDNA were used to determine the efficiency curve of each primer pair according to Costa, et al. 2013. β-tubulin (TUB) and glyceraldehyde-3-phosphate dehydrogenase 2 (GAPDH.2) were used as internal reference genes. At the end of the PCR cycles, the data were analysed with the Bio-Rad CFX Manager 3.0 (3.0.1224.1015) (Biorad), using the Livak calculation method (Livak et al. 2001).

RESULTS

Temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis revealed by different antibodies

To analyse the presence and variations in AGPs during the developmental processes studied, a set of five monoclonal antibodies against AGPs: LM2, LM6, JIM13, JIM14 and MAC207, were used for dot-blot assays at selected key phases of both pathways, male gametophytic development and microspore embryogenesis. The selected stages of microspore-pollen development for analysis were “vacuolated microspore”, “young pollen” and “mature pollen”.

The vacuolated microspore is the responsive developmental stage for induction of embryogenesis, characterized by a large cytoplasmic vacuole which pushes the nucleus towards a peripheral location (Fig. 1a). The vacuolated microspore exhibited the inner thin wall or intine, surrounded by the outer sporopollenin pollen wall, the exine. During gametophytic development the vacuolated microspore underwent an

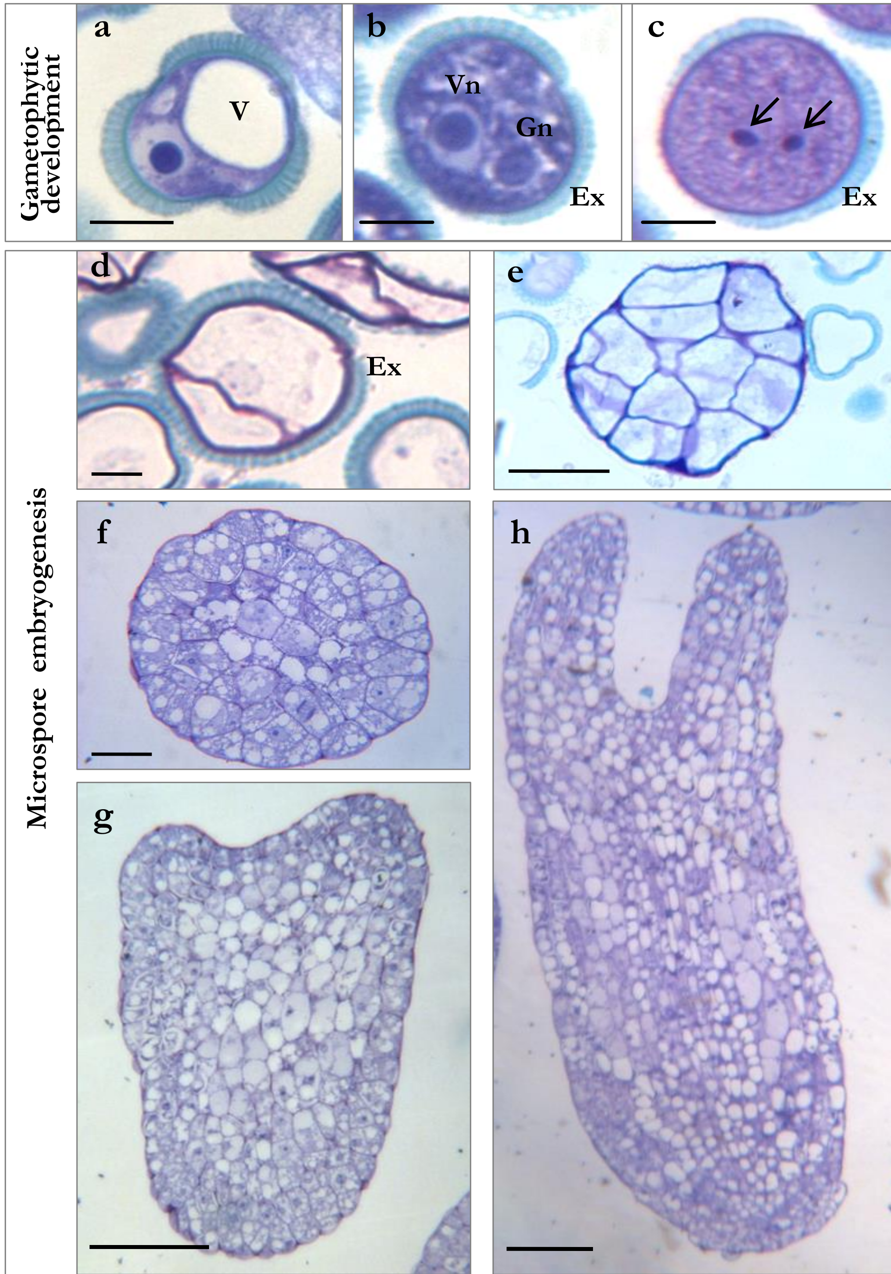


Figure 1: Main stages of male gametophytic development and microspore embryogenesis. Semithin sections, Toluidine Blue staining. (a-c) Male gametophytic development *in vivo*. (d-h) Microspore embryogenesis *in vitro*. (a) Vacuolated microspore. (b) Young pollen. (c) Mature pollen. (d) Two-celled embryo surrounded by the exine. (e) Small globular embryo. (f) Large globular embryo. (g) Embryo at the heart-torpedo transition. (h) Late torpedo embryo. Ex: exine, V: vacuole, Vn: vegetative nucleus, Gn: generative nucleus, Arrows in c point to sperm nuclei. Bars: a-d, 10µm; e, f, 20µm; g. h, 50µm.

asymmetric division leading to the formation of the young pollen grain (Fig. 1b) which is formed by the small generative cell inside the cytoplasm of the large vegetative cell. During later developmental stages, the generative cell divided forming the two sperm cells responsible for the double fertilization. The vegetative cell contains numerous starch granules and other storage products (Fig. 1c) which will be used as energetic and structural substrates during pollen tube growth and fertilization; this is the characteristic morphology of the mature pollen.

After the application of heat treatment for embryogenesis induction *in vitro*, the responsive vacuolated microspores divided symmetrically forming two-celled embryos (Fig. 1d) which subsequently gave rise, after several divisions, to early embryos (Fig. 1e), still surrounded by the exine. As embryogenesis progressed, the exine broke and embryo growth increased to form typical globular (Fig. 1f), heart-shaped and torpedo-shaped (Figs. 1g, h) embryos, structures which were found developing together in the same plates. Finally, cotyledonary mature embryos formed after approximately 30 days *in vitro*. The selected stages of microspore embryogenesis for analysis were "early embryos", "globular and torpedo embryos" and "cotyledonary embryos".

The results of the immuno dot-blot for the different antibodies on equal amounts of protein extracted from the selected developmental stages are shown in figures 2a, 3a and 4a. A relative quantification of the dot colour intensities for each stage and antibody was performed, results are illustrated in the histograms of figures 2b, 3b and 4b. The results identified three main labelling features which suggest three temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis. The AGP epitopes revealed by LM2 and LM6 antibodies increased progressively with microspore embryogenesis progression and reached their maximum level in cotyledonary embryos, while remaining scarce in microspore-pollen development (Fig. 2). Although the AGPs recognized by JIM13 and JIM14 antibodies showed low levels in microspores, they increased after microspore

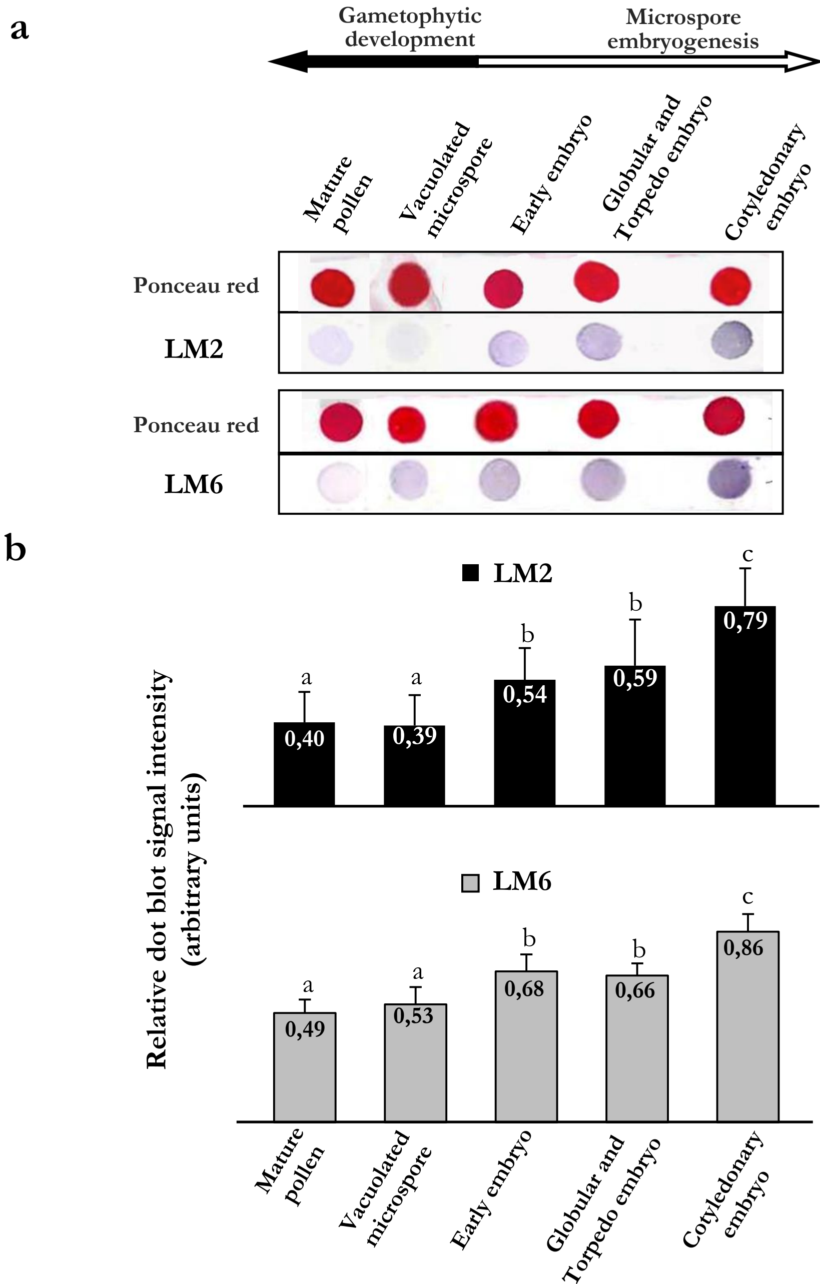


Figure 2: Temporal distribution patterns of LM2 and LM6 epitopes during male gametophytic development and microspore embryogenesis. a) Immuno dot-blot assays at different developmental stages of microspore-pollen development (vacuolated microspore and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immuno-dot-blot of the same strip is shown for each antibody. b) Histograms representing the mean values of relative dot colour intensities in arbitrary units, numbers in columns indicate mean values. Different letters indicate significant differences at P <0.001.

reprogramming in early embryos, but diminished during later stages of embryogenesis (Fig. 3). Conversely, JIM13 and JIM14 epitope levels were higher in mature pollen (Fig. 3). The MAC207 antibody showed a different temporal distribution pattern with low levels in microspores and all stages of microspore embryogenesis, but with a very high signal in mature pollen (Fig. 4).

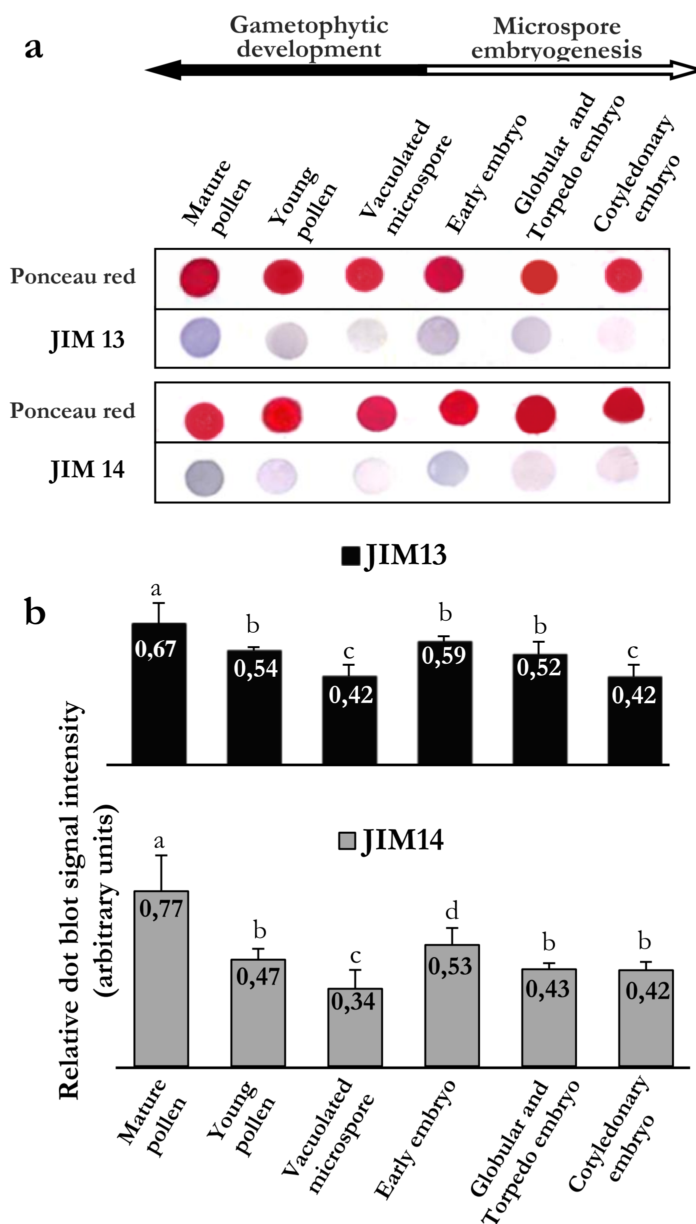


Figure 3: Temporal distribution patterns of JIM13 and JIM14 epitopes during male gametophytic development and microspore embryogenesis. a) Immuno dot-blot assays at different developmental stages of microspore-pollen development (vacuolated microspore, young pollen and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immuno-dot-blot of the same strip is shown for each antibody. b) Histograms representing the mean values of relative dot colour intensities in arbitrary units, numbers in columns indicate mean values. Different letters indicate significant differences at P <0.001.

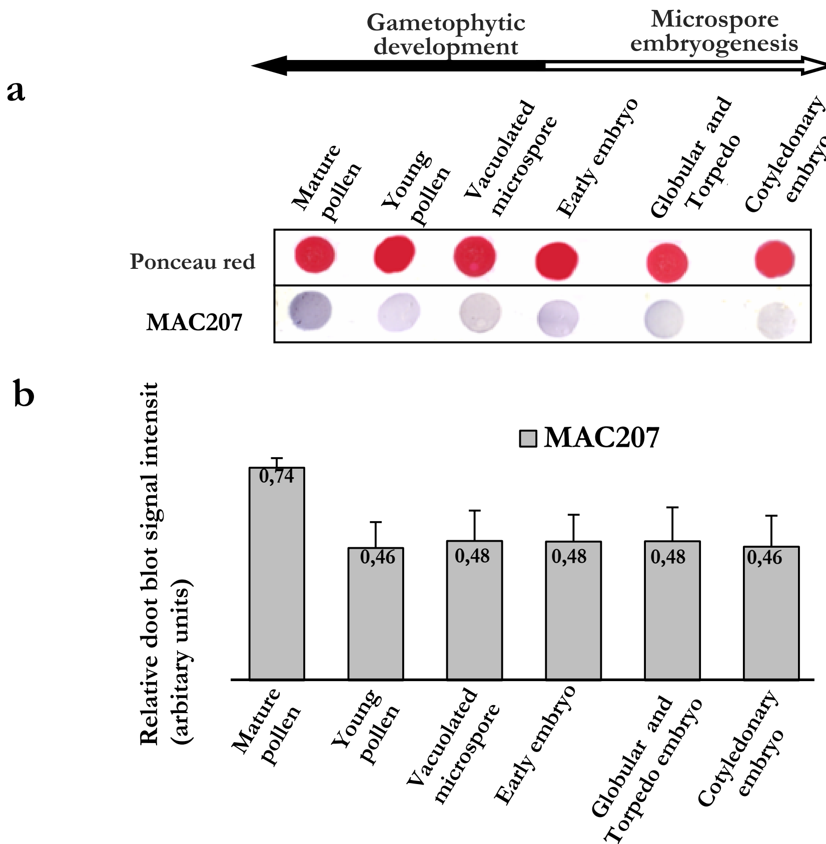


Figure 4: Temporal distribution pattern of MAC207 epitope during male gametophytic development and microspore embryogenesis. a) Immuno dot-blot assays at different developmental stages of microspore-pollen development (vacuolated microspore, and mature pollen) and microspore embryogenesis (early embryo, globular and torpedo embryo, cotyledonary embryo). Ponceau Red staining for total proteins and immuno-dot-blot of the same strip is shown. b) Histogram representing the mean values of relative dot colour intensities in arbitrary units, numbers in columns indicate mean values. Different letters indicate significant differences at P <0.001.

The above results indicated that the microspore programme change from gametophytic development to the embryogenic pathway involved variations in AGP expression. The different AGP epitopes showed different temporal patterns as follows: LM2 and LM6 epitopes were progressively induced during microspore embryogenesis progression (Fig. 2), JIM13 and JIM14 epitopes were induced only during early stages of embryogenesis and with pollen maturation (Fig. 3), and the MAC207 epitope was scarce during microspore embryogenesis but very abundant in the mature pollen stage (Fig. 4).

Subcellular localization of AGP epitopes during microspore-pollen development and microspore embryogenesis

Immunofluorescence and confocal analyses were performed on semithin resin sections of anthers and on *in vitro* microspore cultures at the selected “vacuolated microspore”, and “mature pollen” developmental stages of microspore-pollen development (Figs. 1a, c). The same analyses were carried out on microspore embryogenesis during the “early embryo”, “globular and torpedo embryo” and “cotyledonary embryo” stages (Figs. 1d-h). Merged images of a fluorescent green signal for the epitope and a blue signal for DAPI-stained nuclei were captured, as well as DIC images of the same microscopic field in order to reveal the structure.

LM2 and LM6 antibody labelling on microspores and pollen grains was low. In vacuolated microspores, LM2 labelling was also very low or absent on the intine (Figs. 5a, a’), and in young and mature pollen the labelling was not only very low but specifically localized on small regions of the generative cell wall (Figs. 5c, c’). A similar pattern of localization was observed for the LM6 antibody in vacuolated microspores (Figs. 5b, b’) and pollen grains (Figs. 5d, d’), but in this case, the labelling on the generative cell wall was higher (Fig. 5d’) though not specific to the wall. No significant labelling was found in other subcellular compartments.

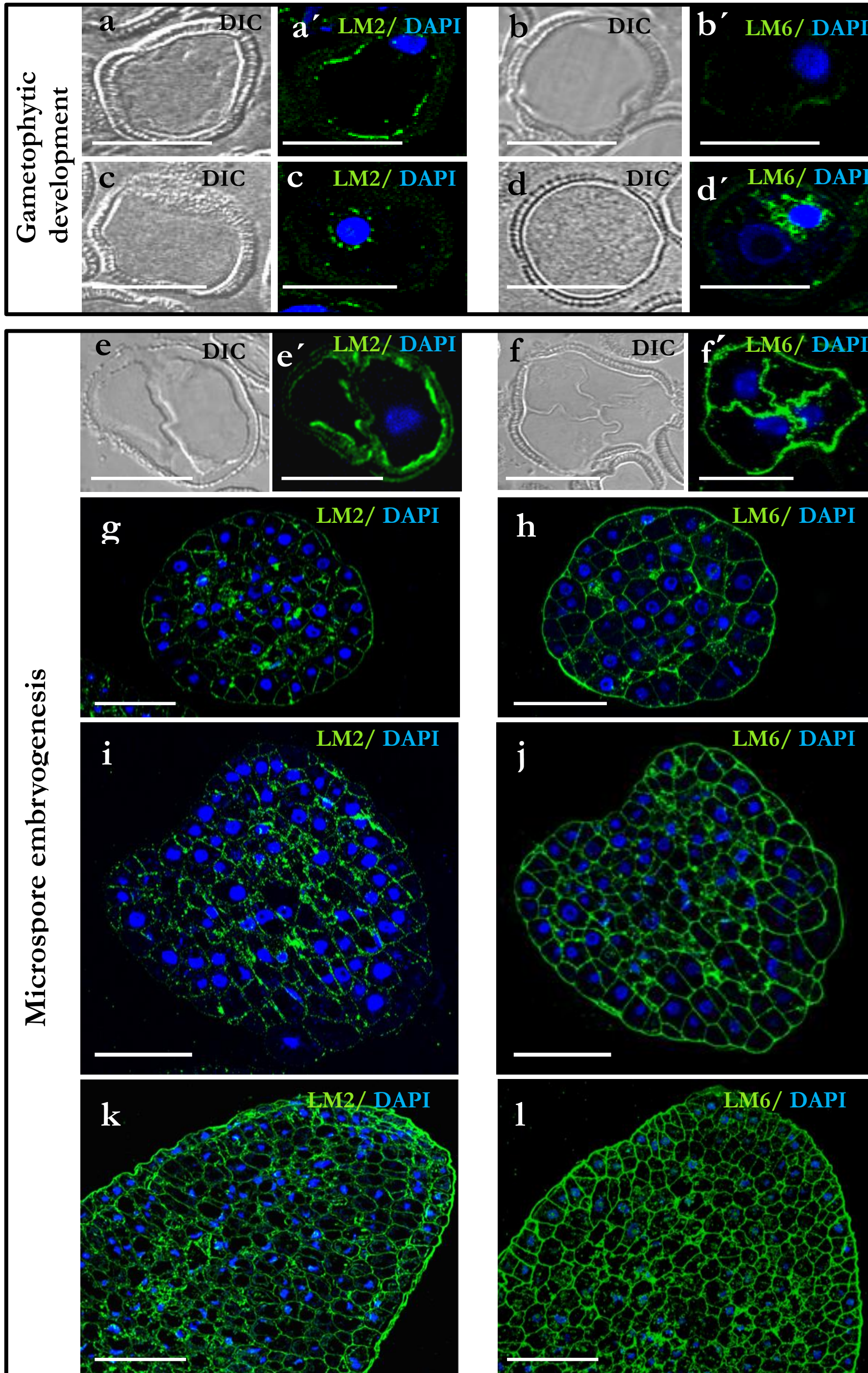


Figure 5: Immunolocalization of LM2 and LM6 epitopes during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (green) and DAPI staining of nuclei (blue). For some stages, a DIC image of the same section is shown to reveal the structure (left side for each pair of images). (a, a', b, b') vacuolated microspore, (c, c', d, d') mature pollen, (e, e', f, f') two- four-celled embryos confined by the exine, (g, h) small globular embryos, (i, j) heart-shaped embryos, (k, l) cotyledonary embryos. (a', c', e', g, i, k) immunofluorescence of LM2 antigen, (b', d', f', h, j, l) immunofluorescence of LM6 antigen. Bars: a-f, 20µm; g-j, 50µm; k-l, 75µm.

Contrary to the above, during microspore embryogenesis, LM2 and LM6 labelling was higher. In early embryos with few cells, surrounded by the exine, labelling with both antibodies was intense on the walls of every embryo cell (Figs. 5e, e', f, f'). During the later stages of embryogenesis, the LM6 antibody showed increasing immunofluorescence on the embryo cell walls of small globular, late globular and torpedo-stage embryos (Figs. 5h, j, l). In the case of the LM2 antibody, though labelling was lower in cell walls, it was also found on small cytoplasmic spots, resembling secretory vesicles, which were abundant in small globular (Fig. 5g), late globular, and heart and torpedo-shaped embryos (Fig. 5i). Cotyledonary embryos exhibited intense fluorescence signalling with both LM2 and LM6 antibodies (Figs. 5k, l).

The immunofluorescence assays with JIM13 and JIM14 antibodies showed a progressive increase in labelling as microspore-pollen developed, with low signalling on the intine of the vacuolated microspores (Figs. 6a, a', b, b') but higher signalling on mature pollen (Figs. 6c, c', d, d'). However, JIM13 and JIM14 epitopes exhibited different localization patterns in mature pollen. The JIM13 antibody highly labelled the cytoplasm and wall of the generative and sperm cells (Figs. 6c, c'), while the vegetative cell remained unlabelled. In contrast, JIM14 labelling was intense in the vegetative cytoplasm of mature pollen (Figs. 6d, d'), but the generative and sperm cells appeared negative.

During microspore embryogenesis, the most intense JIM13 and JIM14 antibody labelling was found on early embryos with only a few cells, whereas at later stages of embryogenesis there were considerably fewer signals. During the first stages, JIM13 labelling was intense in cell walls of early embryos surrounded by the exine (Figs. 6e, e'). JIM14 also labelled the cell walls of these early embryos (Figs. 6f, f') but displayed lower immunofluorescence intensity than JIM13. In small globular, late globular and torpedo stage embryos the labelling was lower and localized on small cytoplasmic spots which decreased progressively as the embryos developed (Figs. 6g-

j). In some globular embryos, JIM13 labelling was also found in a few discontinuous regions along the embryo cell walls (Fig. 6i).

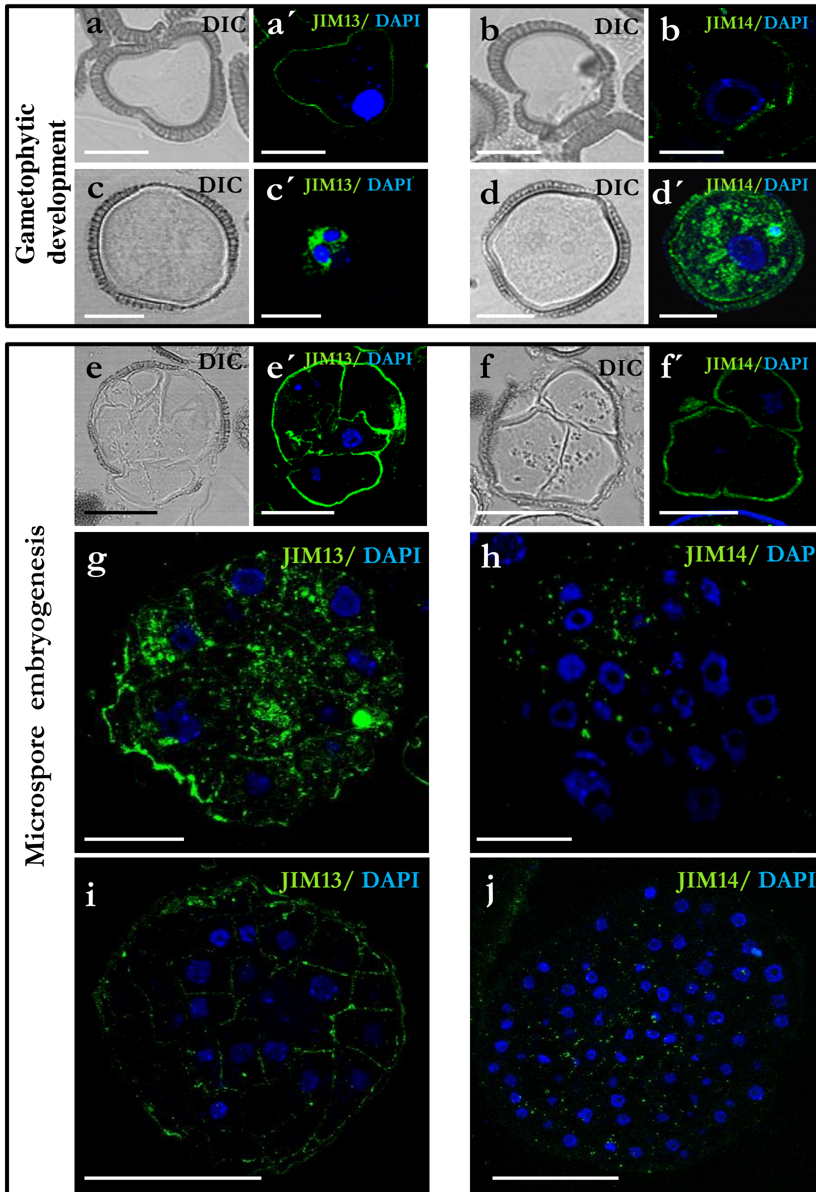
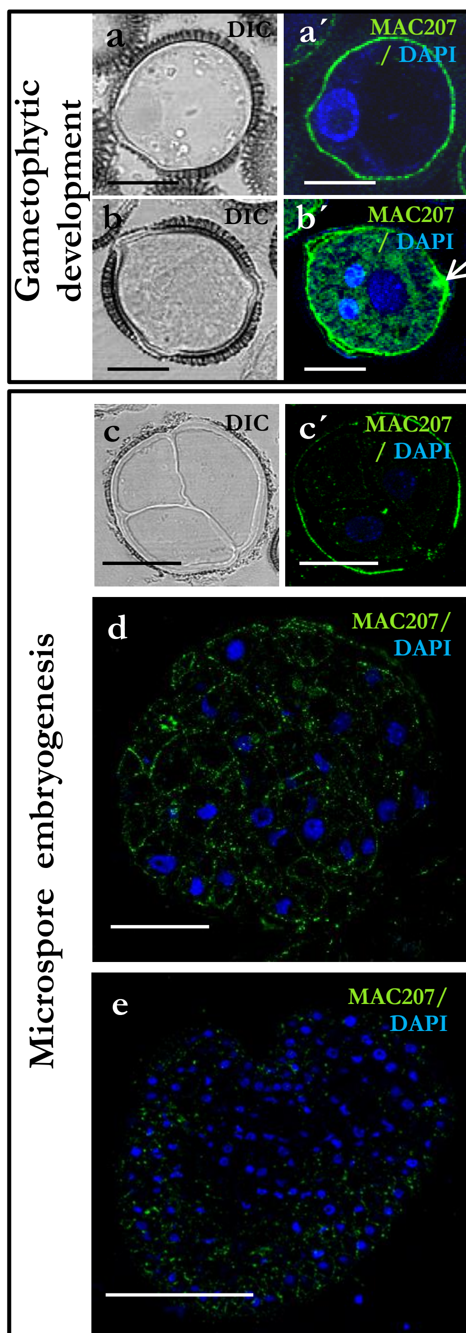


Figure 6: Immunolocalization of JIM13 and JIM14 epitopes during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (green) and DAPI staining of nuclei (blue). For some stages, a DIC image of the same section is shown to reveal the structure (left side for each pair of images). (a, a', b, b') vacuolated microspore, (c, c', d, d') mature pollen, (e, e', f, f') two- four-celled embryos confined by the exine, (g, h) small globular embryos, (i, j) late globular embryos. (a', c', e', g, i) immunofluorescence of JIM13 antigen, (b', d', f', h, j) immunofluorescence of JIM14 antigen. Bars: a-d, 10µm; e-h, 20µm; i-j, 50µm.



The labelling pattern of the MAC207 antibody throughout the two microspore pathways was different from the other antibodies. It provided high immunofluorescence signals at pollen development stages, but very low labelling was found during microspore embryogenesis (Fig. 7). Vacuolated microspores had intense fluorescence signalling on the intine (Figs. 7a, a'). MAC207 labelling increased with pollen development and mature pollen grains exhibited high fluorescence in the vegetative cytoplasm as well as on the intine (Figs. 7b, b'). In the thick intine of the apertural regions (arrows in Fig. 7b'), where the pollen tube will emerge and grow during germination, the labelling was more intense.

Figure 7: Immunolocalization of MAC207 epitope during male gametophytic development and microspore embryogenesis. Confocal merged images of immunofluorescence signal (green) and DAPI staining of nuclei (blue). For some stages, a DIC image of the same section is shown to reveal the structure (left side for each pair of images). (a, a') vacuolated microspore, (b, b') mature pollen, arrow points to the pollen aperture (c, c') two- four-celled embryos confined by the exine, (d) small globular embryo, (e) late globular embryo. Bars: a-b, 10µm; c-d, 20µm; e, 50µm.

However, MAC207 labelling was very scarce during microspore embryogenesis. Only the intine, which remained under the exine at the periphery of two-four cell stage embryos, displayed MAC207 antibody labelling (Figs. 7c, c'), whereas no labelling was found on the inner walls separating cells of two-four cell stage embryos (Fig. 7d). During later stages of embryogenesis, very low signalling appeared as small

cytoplasmic spots on globular (Fig. 7e) and torpedo-shaped embryo cells. Very low or no labelling was observed on cotyledonary embryos.

For all antibodies and developmental stages analysed, the negative controls avoiding the primary antibody did not show any labelling on any subcellular compartment (data not shown). Only the exine, which was clearly distinguished from the intine, along with the more external, thicker and decorated wall layer, exhibited low unspecific autofluorescence in negative controls and in some assays with antibodies.

Temporal expression pattern of Bn*AGP Sta 39-4* gene during microspore-pollen development and microspore embryogenesis

To obtain more information on the presence of AGPs and variations in their presence during microspore-pollen development and microspore embryogenesis, the expression of one of the scarce AGP genes identified in *Brassica napu* as Bn*AGP Sta 39-4* (Gerster, et al. 1996) was analysed by qPCR at the selected "vacuolated microspore" and "mature pollen" developmental stages of microspore-pollen development, and the "early embryo", "globular and torpedo embryo" and "cotyledonary embryo" stages of microspore embryogenesis.

The results showed an expression profile with significant changes during microspore-pollen development and microspore embryogenesis (Fig. 8). The expression value of the vacuolated microspore was considered as the unit of comparison for the other stages. *Sta 39-4* expression was highly induced during pollen development reaching its highest expression levels in mature pollen. During microspore embryogenesis, this AGP gene was up-regulated in the early stages of embryogenesis, exhibiting significantly higher levels in early embryos. Nevertheless, as embryogenesis progressed its expression decreased, being down-regulated at later stages of embryogenesis, in globular, torpedo and cotyledonary embryos (Fig. 8).

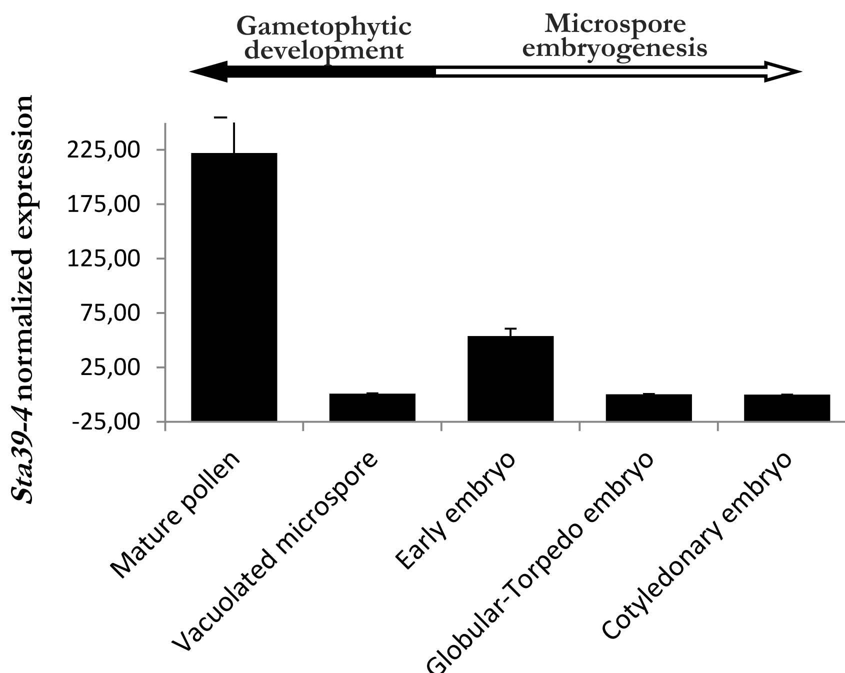


Figure 8: qPCR analysis of *BnAGP Sta39-4* gene expression during male gametophytic development and microspore embryogenesis. Quantification of real-time RT-PCR amplification products of *Sta39-4* mRNA transcripts at different stages of male gametophytic development (vacuolated microspore and mature pollen) and microspore embryogenesis (early embryos, globular and torpedo embryos, and cotyledonary embryos). Each bar represents an average of two independent reactions and technical replicates. Transcript levels were normalized to vacuolated microspore levels.

DISCUSSION

AGPs are regulated during the two microspore developmental pathways and their production accompanies pollen maturation and embryo development

The results presented in this paper have identified three main patterns of labelling which have indicated three temporal AGP distribution patterns during microspore-pollen development and microspore embryogenesis. The heterogeneous nature of the AGP family suggests that AGPs should have more than one specific role. Carbohydrate-directed monoclonal antibodies are estimated to bind 50 to 100 different AGP proteins (Ellis et al. 2010). RNA transcript analysis has shown that in many cases there is a clear gene expression pattern both in tissue location and the developmental stage for many individual AGPs, whereas in other cases, specific

AGPs are found in several tissue types (Ellis et al. 2010). *Sta 39-4* and *Sta 39-3* genes are among some of the scarce AGP genes isolated from *Brassica napus* (Gerster et al. 1996), and they were the first characterized pollen-specific putative AGP genes. The temporal pattern of Bn*AGP Sta 39-4* gene expression found during the two microspore pathways, with high expression levels on mature pollen and early embryos, resembled the patterns exhibited by JIM13 and JIM14 epitopes, suggesting that a certain group/family of AGPs could be involved in two different processes of the microspore pathways, namely, late gametophytic development, and microspore reprogramming and/or early embryogenesis. The patterns of LM2 and LM6 antibodies revealed that there were other groups of AGPs with increasing expression throughout microspore embryogenesis progression, therefore indicating that AGPs could be involved in other functions related to microspore-derived embryo development.

The involvement of AGPs in pollen ontogeny has been reported in several plant species (Coimbra et al. 2009, Coimbra et al. 2010, Pereira et al. 2006, Qin et al. 2007). Microarray data of Arabidopsis sperm cell transcriptome indicated that male gametes have a different gene expression from pollen grains (Borges et al. 2008). Different genetic, immunochemical and biochemical approaches have indicated a role for AGPs in pollen germination, pollen tube growth and male gamete function (Coimbra et al. 2010, Costa et al. 2013, Qin, et al. 2007). Our results have also indicated that AGPs are involved in pollen development in *B. napus* and suggest that different AGP families, grouped together by the same polysaccharide antigenic determinants, could act separately in two different processes. In these processes the AGPs localized in vegetative cytoplasms, intine and apertures (JIM14 and MAC207 epitopes) would be involved in pollen germination and pollen tube growth, whereas the AGPs localized in the generative and sperm cells (JIM13, LM2 and LM6 epitopes) would be related to the gamete function. Even though the AGP molecular mechanism of action is still unknown, AGPs specifically localized in generative and sperm cells have been linked with the signals that are necessary to direct these cells to

their targets inside the pollen tube (Coimbra et al. 2012). AGP epitopes localized in the intine and pollen tube wall, predominantly associated with the tip region, have been suggested as structural and/or control elements for germination through the modulation of water uptake (Coimbra et al. 2010).

In vitro embryogenic systems are influenced by numerous exogenous and endogenous factors, which can stimulate or inhibit development. Many studies have reported the positive effects on *in vitro* somatic and microspore embryogenesis by adding exogenous AGPs, normally those contained in gum arabic, to the culture medium (Pandey et al. 2012, Yuan et al. 2012). During *Brassica napus* microspore embryogenesis, which progressed without the addition of exogenous AGPs (Prem et al. 2012), AGPs progressively increased in embryo cells in line with development and differentiation. Some pectin epitopes, like JIM5 which recognize low-esterified pectins, were found to increase during microspore-derived embryo development, being especially abundant in the cell wall of differentiating embryo cells (Bárány et al. 2010, Solís 2012, Solís et al. 2012). LM6 can recognize a pentasaccharide of (1–5)-a-L-arabinans present in AGPs, but also present in the rhamnogalacturonan I domain of pectins (Willats et al. 1998). In the present work, LM2 and LM6 epitopes increased progressively in embryo cells along with development, but their localization patterns did not differ between proliferating and differentiating embryo cells, being distributed not only in cell walls but also in cytoplasmic spots, especially the LM2 antigen. Previous immunochemical studies have revealed that LM2 recognizes AGPs secreted by suspension-cultured carrot cells (Smallwood et al. 1996), and that this AGP epitope is associated with the subcellular elements of the secretory pathway within plant cells actively producing and secreting AGPs (Samaj et al. 2000). The presence of secreted AGPs in maize microspore and zygote cultures have been reported to be a stimulating factor for embryo development (Massonneau et al. 2005). Several reports have revealed that scavenging cellular AGPs, through the addition of a “Yariv” reagent to the tissue culture media, inhibit somatic embryogenesis in *Daucus carota* (Thompson and Knox 1998) and Cichorium hybrids

(Chapman, et al. 2000); whereas the exogenous addition of AGPs restores such potential, increasing somatic embryogenesis. These studies, among others, have suggested that secreted AGPs could be the extracellular matrix molecules that control and maintain plant cell fate during somatic embryogenesis (Pandey et al. 2012). Recent work with maize microspore cultures revealed that tunicamycin treatment, that blocks protein glycosilation and therefore secretion, inhibited microspore-derived embryo development, which was subsequently recovered by supplementation with a medium containing all the secreted factors from a well-developed microspore culture (Testillano et al. 2010). A role for AGPs has also been proposed in the initiation and maintenance of microspore embryogenesis (Tang, et al. 2006). The present results, with the localization of AGPs on cell walls and cytoplasmic spots which resembled elements of the secretory pathway, suggest an active production and secretion of AGPs during microspore-derived embryo formation and differentiation. This indicates a possible role for endogenous AGPs in sustaining/stimulating *in vitro* microspore embryogenesis, as reported in somatic embryogenesis.

Microspore reprogramming and early microspore embryogenesis involve AGP expression

Induction of embryogenesis in microspore cultures is a reliable and convenient model for investigating the mechanism of cell fate reprogramming and the onset of embryogenesis. Changes in various cell activities and the structural organization of subcellular compartments have been reported as accompanying the microspore reprogramming process in some herbaceous and woody species (Bárány et al. 2005, Seguí-Simarro et al. 2006, Solís et al. 2008, Solís, et al. 2012, Testillano et al. 2005). Information on the biological significance of a protein can be inferred from its differential presence in specific developmental stages, cell types and external conditions. Our results have revealed that a wide group of AGPs (those recognized by JIM13, JIM14, LM2 and LM6 antibodies) were induced and specifically localized in the cell walls of early embryos with just two or four cells which were formed by the

first embryogenic divisions of the microspore after reprogramming, whereas they were much less present or absent in the microspore before the programme changed. The qPCR analysis also showed high expression induction of the AGP gene *Sta 39-4,* specifically in the early microspore embryogenesis stages, in early embryos, followed by down-regulation during later embryogenesis stages. The specific expression of AGPs in two- four cell stage embryos and their localization in the newly formed embryo cell walls strongly suggests that AGPs are early molecular markers of microspore embryogenesis

The β-D-Glucosyl Yariv reagent (Yariv et al. 1962) is used in many studies to bind AGPs, thereby interfering with all AGP activity and thus indirectly inferring/elucidating their function. The addition of the Yariv reagent, that specifically reacts with AGPs, to microspore embryogenesis cultures disturbed microspore embryogenesis initiation in a concentration-dependent manner (Tang, et al. 2006). Recent studies of *in vitro* cultured zygotes and proembryos of tobacco have shown that AGP scavenging by the Yariv reagent affected the first zygotic divisions and proembryo pattern formation, indicating the involvement of AGPs in cell division and cell plate formation during the initial embryogenic divisions of the zygote (Yu and Zhao 2012). Furthermore, in tobacco zygote cultures, both immunofluorescence detection with the JIM13 antibody and staining with the Yariv reagent showed that AGPs were distributed in the new cell plate during normal *in vitro* zygotic division (Yu and Zhao 2012). In Arabidopsis zygotic embryogenesis *in vivo*, JIM13-labelled AGPs have been localized in the embryo proper at very early stages, but they gradually disappeared after the torpedo stage (Zhong et al. 2011). Our study of *Brassica* microspore embryogenesis provides new evidence of the specific association of AGPs with the newly-formed walls of two-four cell stage embryos suggesting their involvement in the first embryogenic divisions of the microspore, similar to zygote divisions.

In conclusion, our results have provided new data which indicate that different AGPs are involved in pollen maturation and germination, microspore reprogramming, early embryogenesis and embryo development. Further work will be needed to shed more light on the precise mechanisms of AGP action in these processes.

ACKNOWDLEGMENTS

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ANNEX 1 to publication III

AGPs are induced after microspore embryogenesis initiation in barley

Annex 1:

AGPs ARE INDUCED AFTER MICROSPORE EMBRYOGENESIS INITIATION IN BARLEY

Changes in AGPs content and distribution were also analyzed during microspore embryogenesis of barley, by immunofluorescence and immuno dot-blot, using several anti-AGPs monoclonal antibodies, and following the procedures previously described by us (El-Tantawy et al. 2013). Microspore embryogenesis induction in *Hordeum vulgare* was performed with the application of cold stress treatment.

Immunofluorescence with LM2 and LM6 antibodies and confocal analyses were performed on semithin resin sections of in vitro microspore cultures at the selected stages of ‘vacuolated microspore’, ‘multicellular embryos’, globular embryos’ and ‘coleoptilar monocot embryos’. Merged images of fluorescent green signal for the epitope and blue signal for DAPI-stained nuclei were captured. Vacuolated microspores did not show significant labelling with LM2 or LM6 antibodies (Figure 1 A, A’, G, G’). After embryogenesis induction, in early multicellular embryos surrounded by the exine formed by a few cells, the antibodies intensely labelled the walls of every cell (Figure 1 B, B’, H, H’). Later, labelling was also found on the cytoplasm of some cells in the late multicellular embryos with exine (Figure 1 C, C’, I, I’) and just after the exine breakdown (Figure. 1 D, D’, J, J’). During the late stages of embryogenesis, LM2 antibody signal was lower in cell walls (Figure 1 D, D’, E, F) compared to the signal of LM6 antibody in the same stages (Figure 1 J, J’, K, L). These results indicated that AGPs increased in embryo cells after embryogenesis initiation and were localized on cell walls and cytoplasmic spots, which resembled elements of the secretory pathway, suggesting an active production and secretion of AGPs during early microspore embryogenesis.

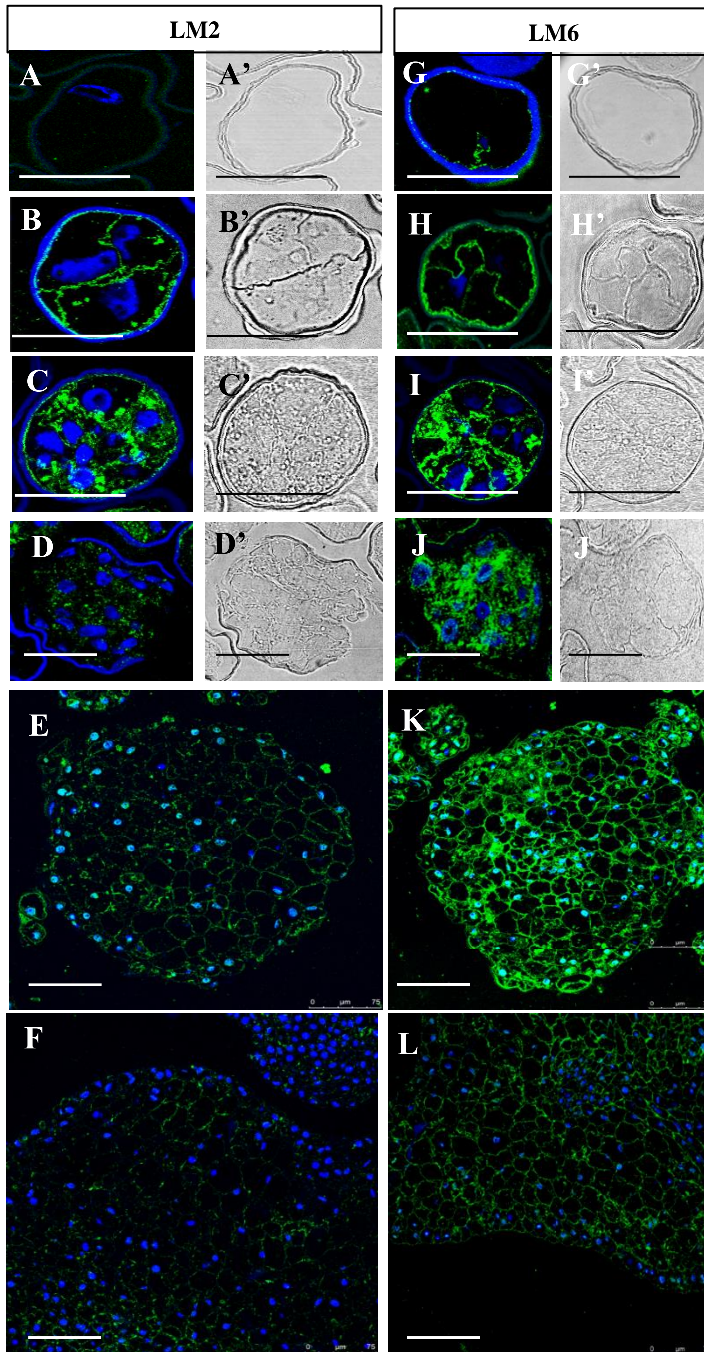


Figure 1: Immunolocalization of LM2 and LM6 epitopes during microspore embryogenesis in *Hordeum vulgare*. Confocal merged images of immunofluorescence signal (green) and DAPI staining of nuclei (blue). In some images, a DIC image of the same area is shown to reveal the structure (left side for each pair of images). (A,A',G,G') Vacuolated microspore. (B,B',H,H') 2-4 celled embryos. (C,C',I,I') Multicellular embryos surrounded by exine. (D,D',J,J') Multicellular embryos after break down of the exine. (E,K) Late globular embryos. (F,L) Coleoptilar embryos. (A,B,C,D,E,F) Immunofluorescence of LM2 antigen. (G,H,I,J,K,L) Immunofluorescence of LM6 antigen. Bars in A,B,C,D,G,H,I,J: 20 µm; in E,K: 50 µm; F,L: 75 µm.

Immuno dot-blot for the LM2 and LM6 antibodies was performed by extracting equal amounts of protein from the selected developmental stages (vacuolated microspores, multicellular embryos and coleoptilar embryos). The results showed that AGP epitopes revealed by LM2 and LM6 antibodies increased at early microspore embryogenesis (Figure 2) in comparison with vacuolated microspores, while at advanced stages, coleoptilar embryos, immunodot-blot signals for LM2 and LM6 decreased (Figure 2). The results of the dot-blot assays correlated with those of the immunofluorescence and indicated that AGPs are induced after microspore embryogenesis, in barley.

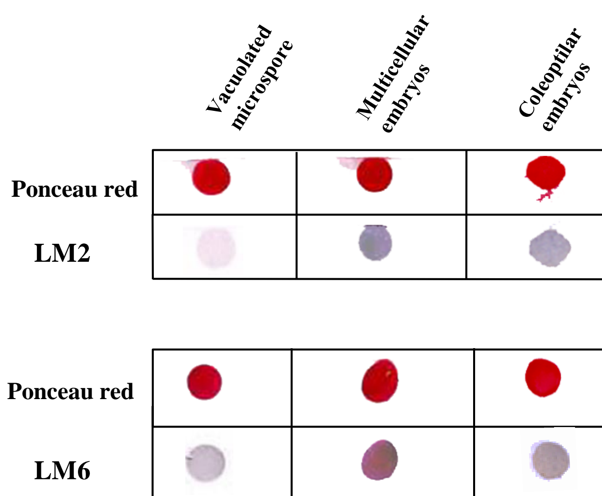


Figure 2: Immunodot-blot assays with LM2 and LM6 antibodies at different stages of microspore embryogenesis in *Hordeum vulgare*. Ponceau Red staining for total proteins and immunodot blot of the same strip is shown for each antibody.

The results in this work provided new data on AGPs dynamics during microspore embryogenesis of barley which correlate with those of AGPs obtained in rapeseed, showed in Publication III. Taken together, this data indicate that AGPs are induced in microspore embryogenesis initiation in the two species, rapeseed and barley, suggesting a common role of AGPs in both dicots and monocots species.

ANNEX 2 to publication III

The blocking of AGPs by Glucosyl-Yariv reagent impairs microspore embryogenesis initiation and progression in rapeseed and barley

Annex 2:

THE BLOCKING OF AGPs BY GLUCOSYL-YARIV REAGENT IMPAIRS MICROSPORE EMBRYOGENESIS INITIATION AND PROGRESSION IN RAPESEED AND BARLEY

Yariv phenylglycosides [1,3,5-tri(p-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene] are synthetic probes that were initially developed as carbohydrate antigens for the purification of AGPs (Yariv et al., 1962, 1967). Glucosyl-Yariv (βGlcY) is known to bind and aggregate AGPs, while Manosyl-Yariv (βManY) does not (Tang et al. 2006; Kitazawa et al. 2013; Paulsen et al. 2014). In this work, to analyse the effect of the blocking of AGPs over microspore embryogenesis, Yariv reagents were added to the isolated microspore culture medium in both *Hordeum vulgare* and *Brassica napus* in vitro microspore cultures, at the concentration of 30 µM which is one of the lower concentrations usually employed for in vitro treatments in plants suspension cultures (Gao and Showalter 1999; Zhong et al. 2011). Untreated cultures and βManY-treated cultures were used as controls.

The analysis of dead cells, identified by positive Evans blue staining (Figure 1), present in cultures of barley at the multicellular embryo stage, showed a slightly higher level of dead cells in βGlcY treated cultures compared to both control and βManY treated cultures, indicating the loss of viability in some microspores in βGlcY-treated cultures. Similar levels of cell dead were found in both untreated and βManY-treated cultures (Figure 1).

Microspore embryogenesis initiation and progression was evaluated in untreated and treated cultures in both species, barley (Figure 2, 3,4) and rapeseed (Figure 5, 6). After the stress treatment for embryogenesis induction, the responsive vacuolated microspores initiated the new program and formed multicellular embryos in untreated and βManY-treated cultures, and in lower proportion in βGlc-treated cultures in barley (Figure 2 A, E, I, 4 A) and rapeseed (Figure 5 A, D, G, 6 A). Later, the number

of multicellular embryos slightly increased and the exine was broken to form developing embryos in both untreated and βManY cultures (Figure 2 B, F, 4 A, 5 B, E, 6 A) whereas in the βGlcY-treated cultures the exine was not broken and the scarce proembryos formed did not progress in none of the two species (Figure 2 J, 4 B, 5 H). In untreated and βManY-treated cultures, cells proliferated and differentiated to form embryos of various sizes and shapes, after 17 days in barley or 15 days in rapeseed (Figure 2 B, F, 4 B, 6 A) concomitantly, the percentage of multicellular embryos in these stages decreased (Figure 4 A, 6 A).

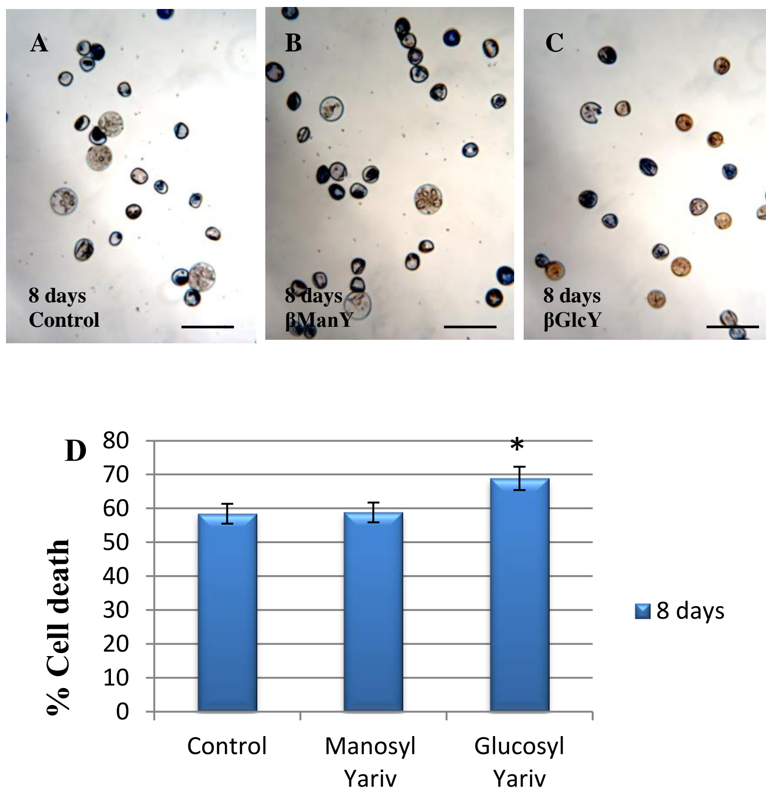


Figure 1: Effect of Yariv treatment on cell death during microspore embryogenesis in barley. Cell death detection by Evan's blue staining in microspore embryogenesis cultures of barley at the stage of multicellular embryos (A, B, C). The dead cells are stained in blue. The number of dead cells is lower in both control cultures (A) and βManY-treated cultures (B) than in βGlcY-treated cultures (C). (D) Quantification of the percentage of dead cells in 8 days-old cultures (control cultures and Yariv-treated cultures). Bars in (A – C) 100 µm. Columns represent mean values and bars represent SEM. Asterisks indicate significant differences with the control cultures sample (Student's *t*-test at $P \leq 0.05$).

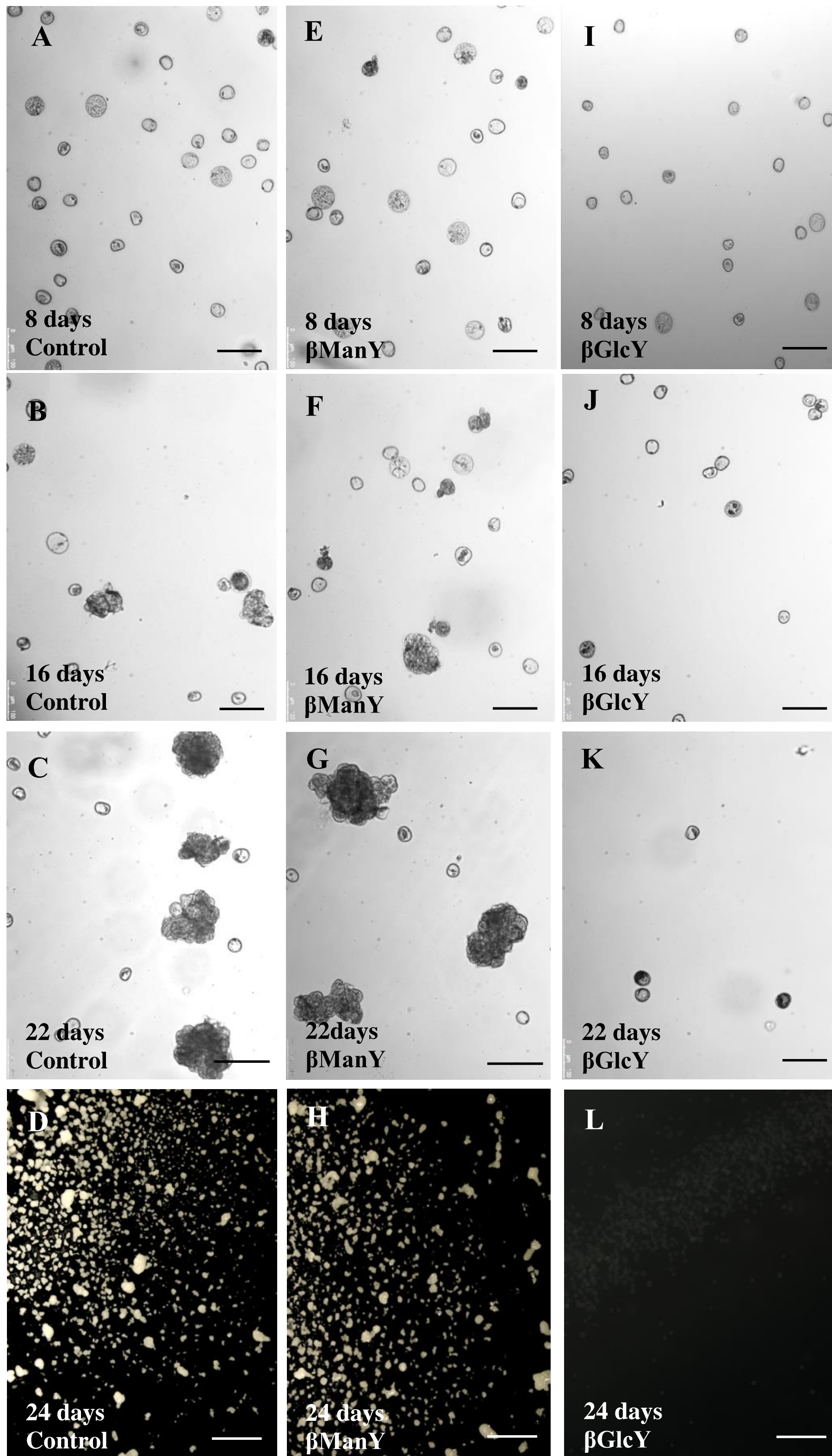

A
8 days
Control
E
8 days
βManY
I
8 days
βGlcY
B
16 days
Control
F
16 days
βManY
J
16 days
βGlcY
C
22 days
Control
G
22days
βManY
K
22 days
βGlcY
D
24 days
Control
H
24 days
βManY
L
24 days
βGlcY

Figure 2: Microspore embryogenesis induction and progression in untreated and Yariv-treated cultures of *Hordeum vulgare*. (A – D) Control cultures. (E – H) βManY-treated cultures. (I – L) βGlcY-treated cultures. (A,E,I) 8 day-old cultures showing multicellular embryos surrounded by the exine. High proportion of multicellular embryos are found in control cultures (A) and βManY-treated cultures (E), but there are low proportion of multicellular embryos in βGlcY-cultures (I). (B,F,J) 16 day-old cultures showing multicellular embryos with broken exine and some developing embryos. The proportion of these developing embryos in control (B) and βManY-treated cultures (F) is higher than in the βGlcY-treated cultures (J). (C,G,K) 22 day-old cultures showing larger developing embryos which were formed after the breakdown of the exine. The same proportion of these embryos is observed in both control (C) and βManY cultures (G), whereas the βGlcY-treated cultures stopped to form developing embryos (K). (D,H,L) General views of 24 day-old cultures showing embryos at advanced developmental stages (transitional and coleoptilar embryos) in both control (D) and βManY-treated cultures (H), whereas only multicellular embryos are present in βGlcY-treated cultures (L). Bars in A, B, C, E, F, G, I, J, K: 100 µm, in D, H, L: 175 µm.

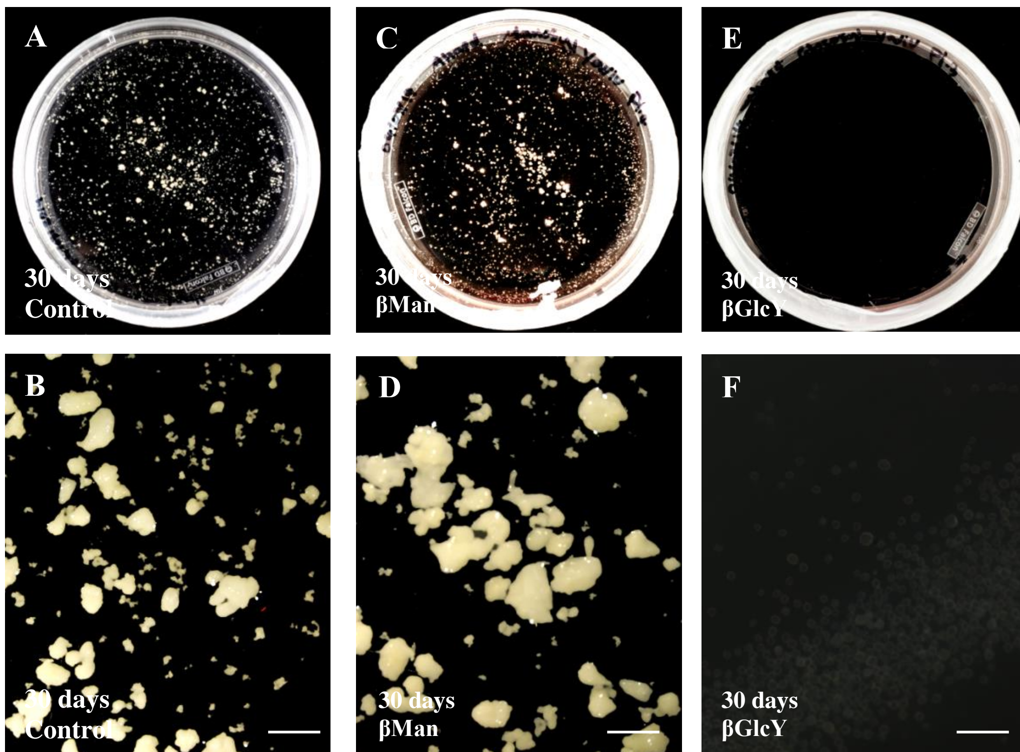


Figure 3: Effect of Yariv treatment on embryo production by microspore embryogenesis of *Hordeum vulgare*. Developing embryos are found in Control culture (A, B) and βMan Y-treated cultures (C, D), but no embryos are found in βGlc Y-treated cultures (E,F). Bars in B, D, F: 175 µm.

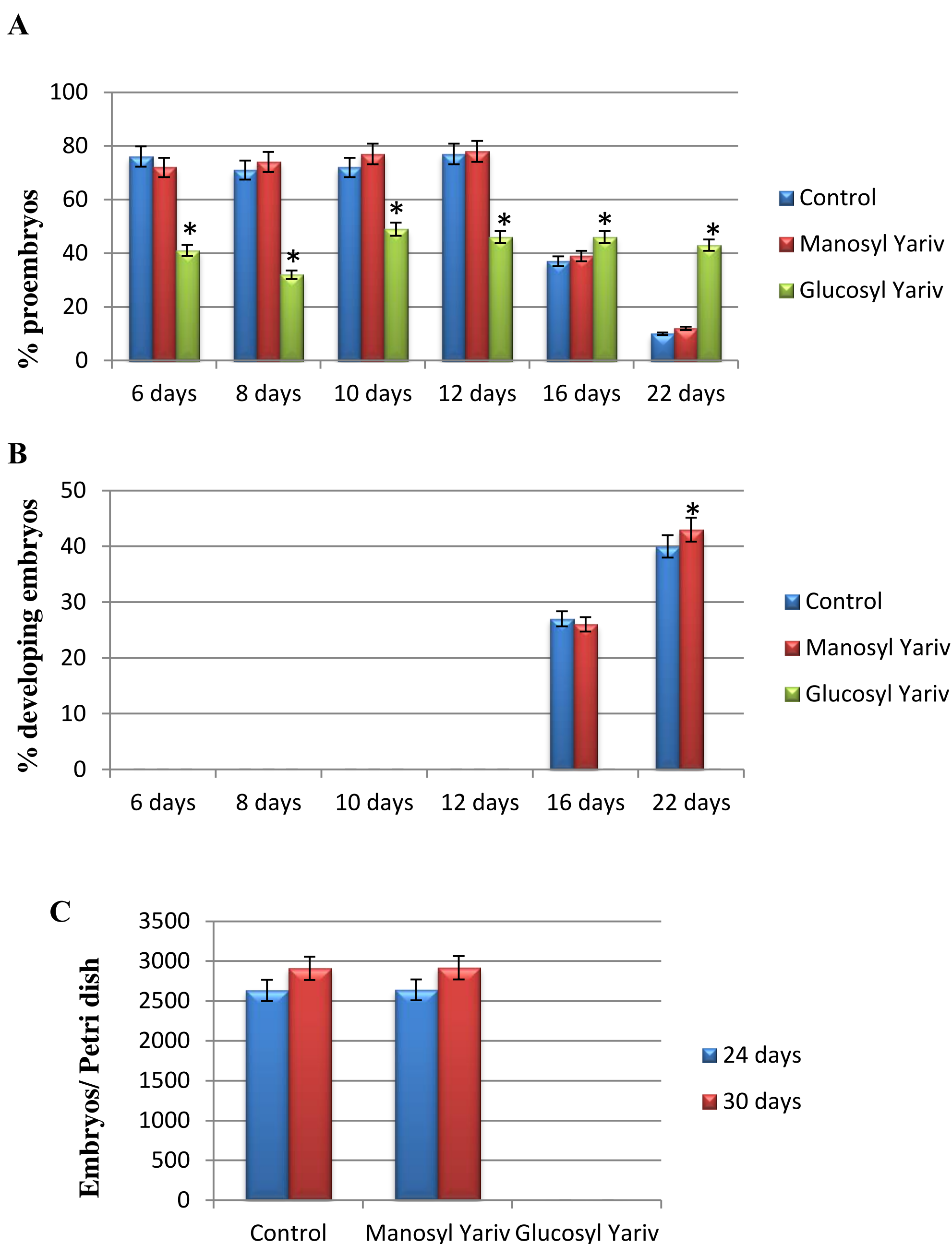


Figure 4: Quantification of the effect of Yariv treatment on microspore embryogenesis induction and progression in barley. (A – C) Quantification of microspore embryogenesis induction and progression in control (blue columns), βManY-treated (red columns) and βGlcY-treated cultures (green columns). (A) Quantification of percentage of proembryos (multicellular embryos with exine) at different time intervals (6,8,10,12,16 and 22 days) during microspore embryogenesis progression in control and Yariv-treated cultures. (B) Quantification of percentage of developing embryos at the same time intervals during microspore embryogenesis progression in control and Yariv-treated cultures. (C) Quantification of the embryo production in control and Yariv-treated cultures after 24 and 30 days. Columns represent mean values and bars represent SEM. Percentages of proembryos and developing embryos in (A) and (B), and total number of embryos per petri dish in (C). Asterisks indicate significant differences with the control culture at each time point (Student's *t*-test at $P \leq 0.05$).

As embryogenesis proceeded, in untreated and βManY-treated cultures of barley, larger developed embryos were found in significant proportions from day 22 (Figure 2 C, G, 4B) and they maintained high proportions by day 30 (Figure 2 D, H, 3 A – D, 4 C).

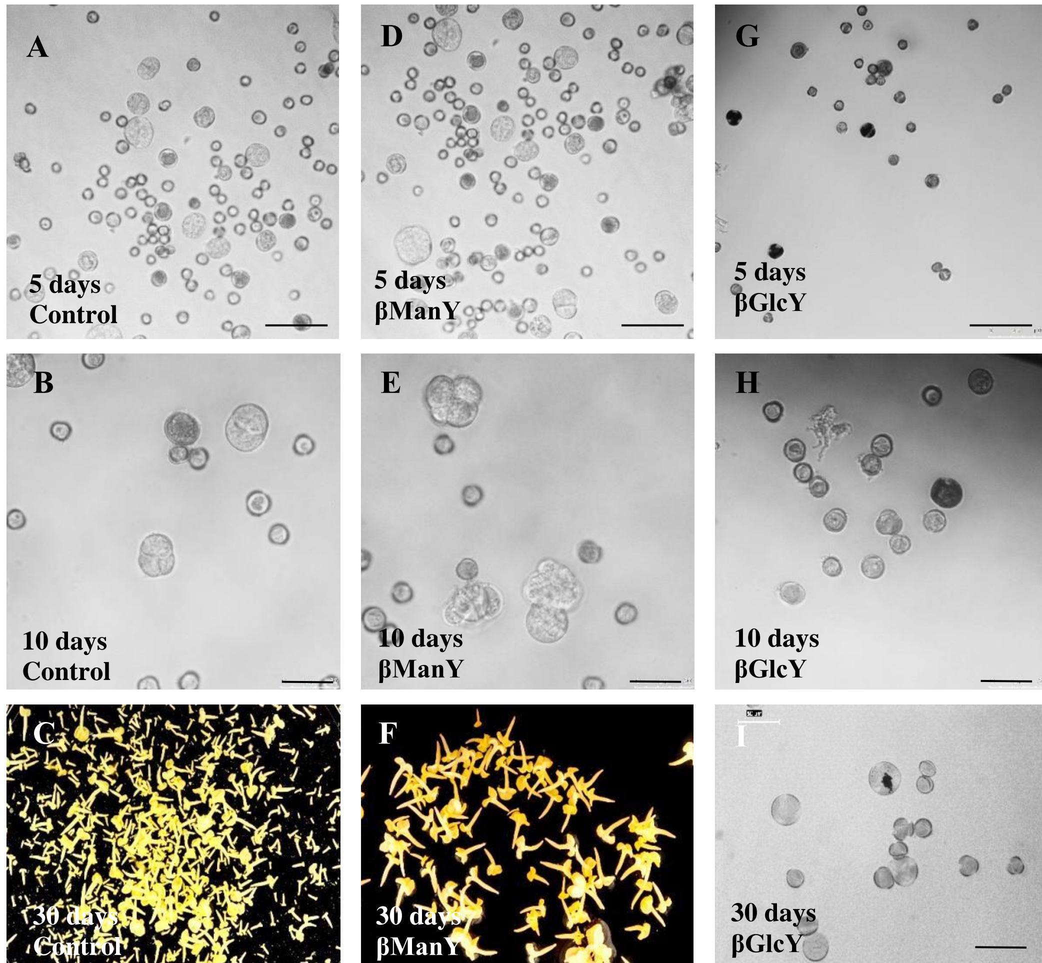
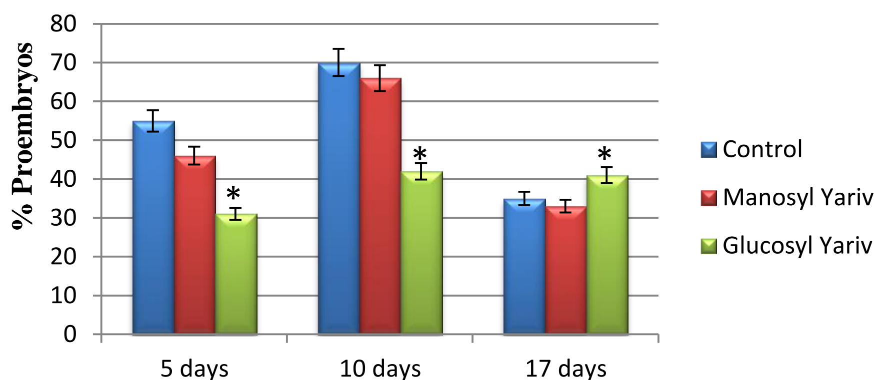


Figure 5: Progression of microspore embryogenesis in control and Yariv-treated cultures of *Brassica napus*. (A – C) Control cultures. (D – F) βManY-treated cultures. (G – I) βGlcY-treated cultures. (A,D,G) Multicellular embryos surrounded by the exine after 5 days of culture. High proportion of multicellular embryos are found in control (A) and βManY-treated cultures (D), but there is a lower proportion of multicellular embryos in βGlcY-cultures (G). (B,E,H) 10 day-old cultures showing multicellular embryos with broken exine and some globular embryos. Similar proportion of these embryos is observed in both control (B) and βManY-treated cultures (E), whereas the βGlcY-treated cultures still contain multicellular embryos with exine (H). (C,F,I) General views of 30 day-old cultures showing cotyledonary embryos in both control (C) and βManY-treated cultures (F), whereas only multicellular embryos are present in βGlcY-treated cultures indicating that the βGlcY blocked the microspore embryogenesis progression (I). Bars in (A,D,G) 100 µm, in (B,E,H,I) 50 µm.

In *Brassica napus*, embryos were observed in untreated and βManY-treated cultures from day 17 until day 30 (Figure 5 C, F, 6 B). By contrast, in βGlcY-treated cultures, the progression of embryogenesis was inhibited and developing embryos were not found at none of the time intervals analysed (Figure 5 I, G, B). The quantitative analyses of untreated and βManY- treated cultures showed similar high levels of embryo production, whereas in βGlcY-treated cultures no embryos were observed in neither of the two species (Figure 4 C, 6 B).

A



B

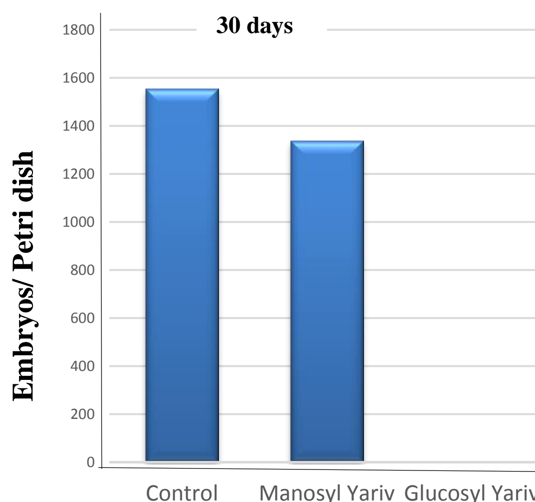


Figure 6: Quantification of the effect of Yariv treatment on microspore embryogenesis induction and progression in rapeseed. (A) Quantification of the percentage of proembryos observed at different time intervals (5, 10, 17 days) during microspore embryogenesis progression in control cultures (blue columns), βManY treated culutres (orange columns) and βGlcY treated cultures (gray columns). (B) Quantification of the embryo production in control cultures, βManY treated cultures and βGlcY treated cultures (30 days). Asterisks indicate significant differences with the control culture at each time point (Student's *t*-test at $P \leq 0.05$).

In conclusion, the βGlcY reagent produces similar effects on microspore embryogenesis in both in vitro systems, rapeseed and barley. This blocking agent of AGPs slightly reduces the viability of microspores, decreases the rate of embryogenesis initiation and completely inhibited the microspore embryogenesis progression. These results indicate that AGPs are involved in the development of microspore-derived embryos and are required for proper microspore embryo formation, suggesting that the Glucosyl Yariv-AGP interaction modulates the developmental fate of the early embryo, particularly inhibiting the maturation of embryos. Our results suggested that AGPs play a crucial role in the initiation of microspore embryogenesis and the progression of the embryogenic development, in monocots and dicots species.

PUBLICATION IV

Auxin increase and distribution, and effects of auxin inhibitors on microspore embryogenesis initiation and progression in barley

Reference:

El-Tantawy, A.A.; Solís, M.T.; Risueño, M.C.; Testillano, P.S. (2015). Auxin increase and distribution, and effects of auxin inhibitors on microspore embryogenesis initiation and progression in barley. *In preparation*.

Publication IV:

AUXIN INCREASE AND DISTRIBUTION, AND EFFECTS OF AUXIN INHIBITORS ON MICROSPORE EMBRYOGENESIS INITIATION AND PROGRESSION IN BARLEY

ABSTRACT

Auxins are hormones or plant growth regulators (PGR) which play a very important role in plant growth and development. The microspores cultured can be reprogramed in vitro, after specific stress treatments, to change its gametophytic development to reprogram towards embryogenesis forming haploid embryos, important biotechnological tools in plant breeding. Microspore embryogenesis is a convenient system for studying the cell reprogramming and embryo formation but little is known about the dynamics of auxin in this process; recent reports in dicot species supposed a key role of the hormone in microspore embryogenesis. In this work, the distribution of IAA was studied during microspore embryogenesis in the monocot *Hordeum vulgar*, induced by cold stress treatment, by performing immunofluorescence with anti-IAA-specific antibodies. We also analysed the effect of N-1-naphthylphthalamic acid (NPA) and α-(*P*-chlorophenoxy) (PCIB) inhibitors of auxin transport and action respectively, on microspore embryogenesis. Results showed that IAA increased and accumulated after embryogenesis induction in the cells of proembryos and globular embryos, indicating that auxin accumulation is associated with microspore embryogenesis initiation and progression and suggesting that microspore reprogramming may activate the auxin biosynthesis in barley. NPA, which inhibits polar auxin transport (PAT), impaired embryo development showing a decrease in the embryo yields, whereas PCIB, which inhibits auxin action, prevented the initiation of microspore embryogenesis, indicating that PAT and auxin action are required for microspore embryogenesis initiation and progression in monocot species. Our results indicate that auxin accumulation, transport and perception play an important role in

the initiation and progression of microspore embryogenesis in a barley, monocot species. This data suggests that auxin has a key role in the switch of the embryogenesis progression, independently of the stress treatment used for the induction.

INTRODUCTION

The microspores in culture, at the vacuolated microspore stage, can reprogram by stress treatments from the gametophytic development to an embryogenic pathway, a process named microspore embryogenesis (Prem et al. 2012; González-Melendi et al. 1995). After specific stress treatments, the microspore can divide to initiate the embryogenesis program producing multicellular embryos and then haploid embryos which regenerate homozygous plants with many applications for plant breeding (Maluszynski et al. 2003). The isolated microspore culture constitutes a very convenient system to study the mechanisms of embryogenesis initiation, nevertheless, the knowledge about the responsible factors of embryogenesis initiation through microspore culture is still limited. In barley, microspore embryogenesis can be induced in microspore *in vitro* cultures by cold and starvation stress treatments (Rodríguez-Serrano et al. 2012). Despite recent reports on microspore embryogenesis have reported some data on the epigenetic regulation (Solís et al. 2012; El-Tantawy et al. 2014), the role of arabinogalactan proteins (El-Tantawy et al. 2013; Testillano et al. 2010), the role of reactive oxygen species (ROS), nitric oxide (NO) and programmed cell death (Rodríguez-Serrano et al. 2012; Solís et al. 2014), and auxin biosynthesis (Rodríguez-Sanz et al. 2015), the knowledge on hormonal regulation of embryogenesis initiation and embryo development in established cultures are still scarce.

In angiosperms, the auxins are responsible for phototropism and apical dominance. The predominant form of auxin in vivo is indole acetic acid (IAA). They coordinate the development of leaves (Reed et al. 1998), stem and coleoptile growth (Ding and

Friml 2010; Prasad and Dhonukshe 2013), and lateral root (Zhao and Hasenstein 2010; Himmanen et al. 2002). In addition, auxins are widely used in plant propagation and tissue culture. Cuttings are routinely treated with auxins to induce root formation. In tissue culture, exogenous auxins are balanced with cytokinins for full morphogenesis. In addition, auxin promotes cell elongation of the plant which changes the plant wall plasticity making it easier for the plant to grow upwards (Keuskamp et al. 2010). Moreover, the distribution and transport pattern of auxin during zygotic embryogenesis lead to the mature embryo to initiate the formation of shoots and roots (Rademacher et al. 2012; Weijers et al. 2005; Palovaara et al. 2010; Vondráková, et al. 2011; Hua Su and Zhang 2009). The role of auxins during plant embryogenesis has been demonstrated in *Arabidopsis sp*. in which dynamic changes in auxin flux within cells are mediated by PIN proteins causing polar auxin transport (Chen et al. 2010; Petrásek and Friml 2009). PIN genes regulate asymmetric auxin distribution during zygotic embryogenesis. (Feng and Jacobsen 2011; Palovaara and Hakman 2009; Huang et al. 2014). The expression of PIN genes causes the accumulation of auxin in specific region of the embryo to initiate root; it has been also found that the polar localization of epidermal PIN proteins in the globular embryo result in auxin maxima leading to cotyledon initiation (Chen et al. 2010).

In general, the polar auxin transport (PAT) supports specific spatial auxin distribution leading to the responses of plant to the environment and plant growth (Van Berekel et al. 2013; Estrelle 1998; Michniewicz et al. 2007). The polar auxin transport can be blocked by some inhibitors such as N-1-naphthylphthalamic acid (NPA) which can inhibit the flux of auxin from cell to cell through interaction with PIN proteins (Hakman et al. 2009). The effect of NPA inhibitor has been studied in somatic embryogenesis in several species (Liu et al. 1993; Hadfi et al. 1998). NPA leads to abnormal formation of somatic embryos during their growth; it has been observed that there is a difference in frequency between shoots and roots development, as demonstrated by Fischer et al. (1997) in wheat, and Palovaara et al. (2010) in Norway spruce. In addition, it has been reported that NPA increases the expression of PIN-

like gene significantly (Shen et al. 2010; Hakman et al. 2009). Forstan and Varotto (2011) indicated that NPA causes auxin accumulation in epidermis of scutellum in the mature embryo of maize, leading to failure of symmetric development and the formation of abnormal vasculature. There are only a few studies of the effect of NPA inhibitors during microspore embryogenesis, compared to somatic embryogenesis. Our group found in *Brassica napus* and *Quercus suber*, that the development of embryos was reduced when the microspore culture was treated with NPA in the culture medium, indicating that the transport of auxin is involved in microspore embryogenesis initiation and development in these dicot species (Rodríguez-Sanz et al. 2014a; Rodríguez-Sanz et al. 2014b). Although the studies on auxin in monocots are still scarce, some investigations on maize and rice have reported that the effect of NPA on the plant morphology and the sensitivity in both dicots and monocots are different (Mcsteen 2010).

PCIB (P-chlorophenoxyisobutiric acid) is an auxin inhibitor which inhibits auxin action leading to the blocking of the physiological effects of the auxin induction (Oono et al. 2003; Xie et al. 2000). PCIB does not affect polar auxin transport once it does not react with PIN proteins. The idea of using of PCIB as an auxin inhibitor derived from its structural similarity with auxin; it competes with auxin action at the auxin receptor, therefore, affecting the auxin-induced responses (Oono et al. 2003). The use of PCIB has helped to clarify the mechanisms of auxin perception and signal transduction and their importance in plant growth and development (Trebitsh and Riov 1987; Hutchinson et al. 1996; Zhang et al. 2011). However, the information about the mechanism of PCIB-mediated inhibition of auxin action is still scarce. Some scientists used the PCIB treatment to study some physiological processes such as the gravitopic response (Oono et al. 2003), the flower opening (Van Doorn et al. 2012) and root growth (Tamás et al. 2012). Also, it has been found that PCIB inhibited the early auxin gene expression in *Arabidopsis thaliana* (Oono et al. 2003) while, it did not inhibit the auxin response in corn coleptile (Peters and Felle 1991). PCIB treatment in cultured media has been used to study the somatic embryogenesis,

several results indicated that PCIB decreases the embryogenic response and the formation of pro-embryogenic masses in somatic embryogenesis (Fujimura and Komamine 1979; Hutchinson et al. 1996), while some other investigations found that PCIB treatment increased the percentage of calluses and did not affect morphogenesis in *Ipomoea sp*. (Kobayashi and Bouwkamp 1994; Chée and Cantliffe 1989). Maybe the response of plants to the PCIB treatment depends on the concentration of the solutions; Zhao and Hasentein (2010) reported different effects of this inhibitor on root growth when they treated the *Linum usitatissimum* and *Arabidospsis thaliana* seeds with PCIB at various concentrations and they found that there was no effect with the higher concentration. On the other hand, Zhang et al. (2011) found that PCIB treatment increased the number of embryos after the induction of the microspores of *Brassica rapa* whereas Rodríguez-Sanz et al. (2015) found the opposite effect with *Brassica napus*.

In this work, the endogenous auxin distribution has been analyzed in microspore embryogenesis of *Hordeum vulgare* by using anti-IAA immunofluorescence and confocal analysis. Moreover, the effects of the inhibition of auxin transport and action on the embryogenesis induction of microspores have been analyzed by NPA and PCIB treatments.

MATERIAL AND METHODS

Plant Material and Growth Conditions:

Hordeum vulgare L. CV. Igri were used as a donor plants. Seeds were germinated in soil for one month at 4˚C, then, they were grown at 12˚C with a 12/12-hour light/dark cycle (10,000-16,000 lx) for one month in a plant growth chamber (relative humidity about 70%), and then, the seedlings are transferred to a greenhouse to grow under a controlled temperature at 18˚C.

Isolated Microspore Culture

Spikes containing microspores at the vacuolated stage were collected and surface sterilized by immersion in 5% bleach for 20 minutes followed by 3 – 4 washes with sterile distilled water. Cold stress treatment, to induce embryogenic development, was performed by exposing the spikes at 4˚C for 23 – 24 days. Isolation and culture of the microspores were performed as described by Rodríguez-Serrano et al. (2012) with a final density of 1.1 x 10^5 cells/ml in an appropriate volume of KBP medium (Kumlehn et al. 2006). The spikes were blended in 20 ml of precoold 0.4 M mannitol using Waring Blender (Eberbach, Ann Arbor, MI, USA), and the extracts were filtered through a 100 µm nylon mesh (Wilson, Nottingham, UK) into a vessel at 4˚C. The collected microspore suspension was transferred into a 50 ml Falcon-tube and centrifuged at 100 x g for 10 minutes at 4˚C. therefore, the supernatant was removed. After that, 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 x 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 x 10^5 cells per ml. The microspores were incubated at 25˚C in the dark.

Treatments of microspore culture with PCIB and NPA

A stock solution of PCIB at 100 µM in ethanol was prepared and adequate volumes was added to the KBP media for obtaining two concentrations, 5 µM and 10 µM, to parallel plates of the same culture after filtering with a sterile Ministart filter, (Sartorius Biotech), keeping some plates without PCIB as control.

NPA (Duchefa), auxin transport inhibitor, was added to the KBP media, using a stock of 0.1 M NPA in DMSO, after filtering with a sterile Ministart filter (Sartorius

Biotech). The solution was added to the culture media of parallel plates of the same cultures at two concentrations, 3 µ and 10 µM, keeping some plates without NPA as controls.

Quantification of the number of proembryos (Multicellular embryos still surrounded by exine) and mature embryos was performed at defined time points of the culture. Quantifications were carried out using stereomicroscope micrographs randomly obtained from control and treated cultures. Mean percentages of proembryos were calculated and total number of mature embryos per plate was counted. Pictures of plates of each treatment was taken. Differences between control and treated cultures were tested by Student's *t*-test at $P \leq 0.05$.

Fixation and Processing for Microscopic Analysis

Samples of microspore culture at different stages during microspore embryogenesis, were collected and fixed in 4% paraformaldehyde in 15% saccharose in phosphate buffered saline (PBS), pH 7.3, overnight at 4°C and washed in PBS. Isolated microspores and small proembryos were embedded in gelatin and processed as described by Solís et al. (2008). All the samples were dehydrated in an acetone series, embedded in Historesin Plus at 4°C and sectioned at 2 µm thickness using an ultramicrotome (Ultracut E, Reichert). Semithin resin sections were collected on slides and were stained with toluidine blue and observed under a bright-field microspore.

Cell Death Detection

Detection of dead cells in microspore embryogenesis was performed by Evan's blue staining (Rodríguez-Serrano et al. 2012) in control and NPA-treated cultures. Samples were incubated with a 0.25% (w/v) aqueous solution of Evan's blue for 30 min. Dead cells were stained by Evan's blue while the live cells were not stained. The quantification of dead and live cells was performed on random micrographs from two replicas and three independent samples of each culture treatment; mean percentages

of dead cells were calculated. The results were shown in histograms in which columns represented mean values and bars represented standard error of the means (SEM). Significant differences in the percentage of dead cells between non-treated cultures and NPA-treated cultures at different concentrations were evaluated by Student's *t*-test at $p \leq 0.05$.

IAA Immunofluorescence and Confocal Microscopy

Immunofluorescence was performed as previously described (Prem et al. 2012). Historesin semithin sections were placed on slides coated with APTES (3-aminopropyltriethoxysilane, Sigma). Sections were first blocked with 5% bovine serum albumin (BSA) in PBS and incubated with the anti-IAA mouse monoclonal antibodies (Sigma, Cat. n°: A 0855) for one hour at 1/100 dilution in 1% BSA in PBS. After washing in PBS, the sections were incubated for 45 min in darkness with Alexa Fluor 488-labelled anti-mouse IgG antibody diluted 1/25 in PBS, after that, nuclei were stained with 4.6-diamidino-2-phenylindole (DAPI), washed with PBS, mounted in Mowiol and examined in a confocal microscope (Leica TCS-SP2-AOBS, Vienna, Austria). 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 for image acquisition in all immunofluorescence preparations, allowing an accurate comparison among signals of samples from different development shapes and from control and treated cultures with NPA.

Images of the different stages of microspore embryogenesis in barley were captured by bright field, differential interference contrast (DIC) and IAA immunofluorescence (green), combined or not with DAPI fluorescence (blue), to identify the structures and to establish a dynamic sequence of events of the IAA distribution at the different developmental stages.

Negative controls of IAA immunofluorescence

Negative controls were performed either by replacing the first antibody by PBS or by immunodepletion assays. The anti-IAA antibody was incubated with a solution of 5mg/ml synthetic IAA at 4˚C overnight; the pre-blocked antibody solution was used as primary antibodies for immunofluorescence, following the same protocol and conditions described above.

RESULTS

IAA immunolocalization during microspore embryogenesis in barley

The haploid microspore undergoes a long interphase with the formation of a large vacuole which pushes the nucleus to the periphery forming the so-called vacuolated microspore (Fig. 1 A). Microspore embryogenesis was induced in isolated microspore cultures in barley by cold stress treatment (4˚C), after that, the vacuolated microspores changed the gametophytic pathway to an embryogenic development (Rodríguez-Serrano et al. 2012). Around six days after induction of the microspores, further divisions gave rise to proembryos or multicellular embryos, still surrounded by the exine (Fig. 1 B, F). The non-responsive microspores were also found in the culture with the proembryos or multicellular embryos which showed higher size (Fig.1 F). After a few more days, the exine broke down and proembryos proliferated forming larger multicellular embryos (Fig.1 C). More developed embryos were observed at 21 days of culture namely globular (Fig. D) and, transitional (Fig.1 E) embryos. The coleoptilar monocot embryos were completely formed after 30 days (Fig.1 G, H).

Immunofluorescence with anti-IAA-specific antibodies was performed on semithin resin sections of the selected stages of microspore embryogenesis: ‘vacuolated microspores’, ‘proembryos or multicellular embryos, and ‘globular’ embryos. Merged

images of fluorescent green signal for the IAA epitope and blue signal for DAPI-stained nuclei were captured, as well as DIC images.

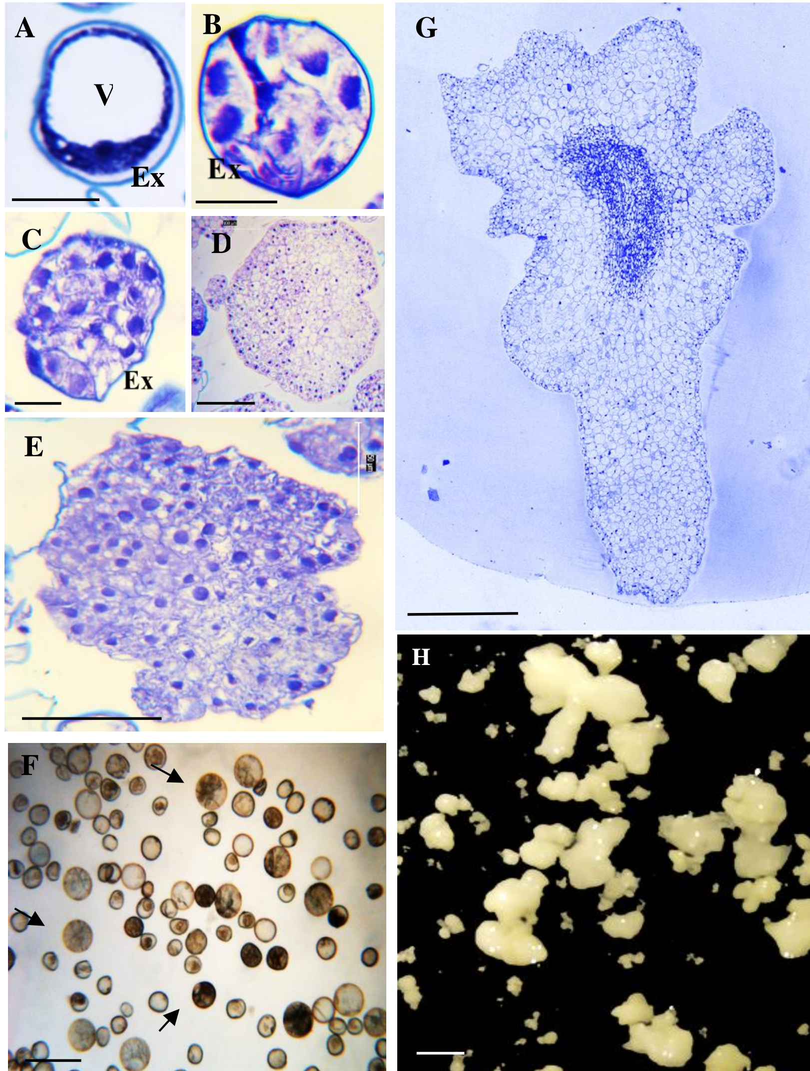


Figure 1: Stages of microspore embryogenesis in *Hordeum vulgare*. (A) Vacuolated microspore. **(B)** Multicellular embryo with exine. **(C)** Multicellular embryo without exine which is formed after 13 days. **(D)** Globular embryo. **(E)** Early transitional embryo. **(F)** In vitro culture after, 8 days, showing non responsive microspores and, proembryos which are pointed by arrows. **(G)** Mature coleoptilar embryo. **(H)** In vitro culture at the coleoptilar embryo formation stage (30 days), some embryos at earlier developmental stages (globular, transitional and scutellar embryos) are also present. **(A,B,C,E,G)** Semithin sections, toluidine blue staining showing general structure visualization. **(F,H)** General views of cultures observed under the stereomicroscope. Ex= Exine; V= Vacuole. Bars in **(A)** 20 µm, in **(B – D)** 50 µm, in **(D,G,H)** 100 µm, **(F)** 200 µm.

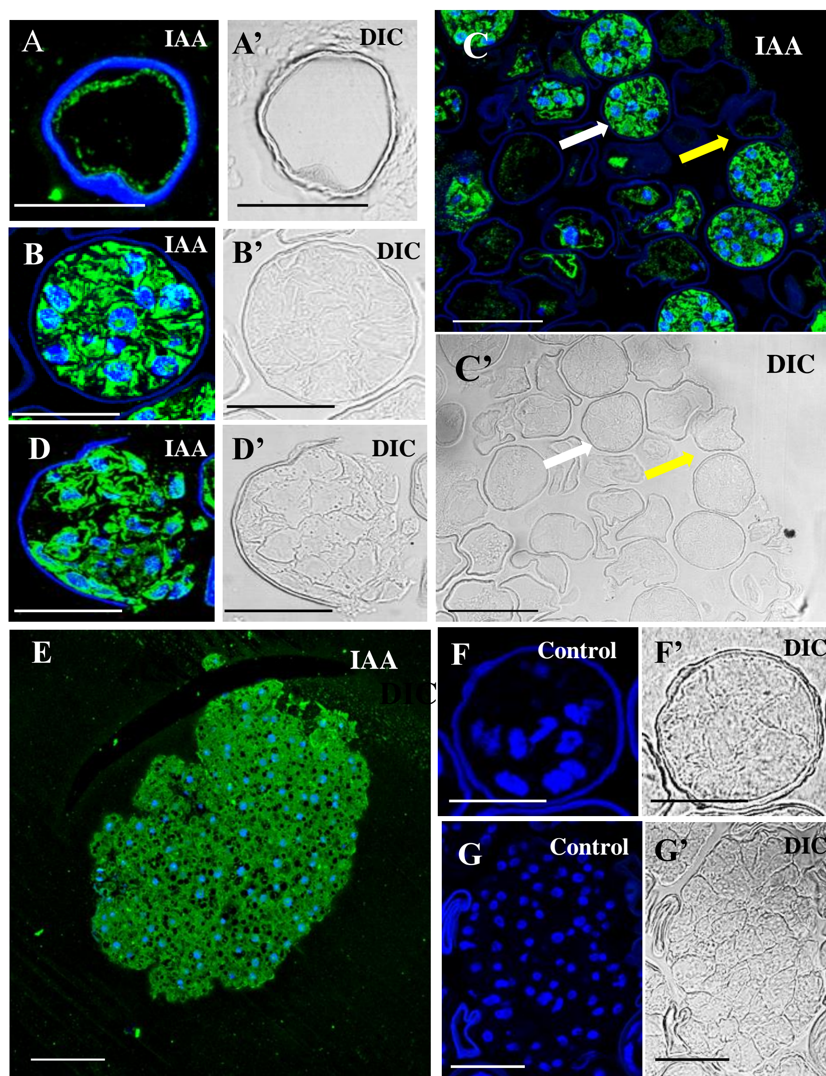


Figure 2: IAA immunolocalization during microspore embryogenesis. Confocal merged images of IAA immunofluorescence signal (green) and DAPI staining of nuclei (blue). DIC images of the same sections are shown to reveal the structure (right side for each pair of images). **(A, A')** Vacuolated microspore. **(B, B')** Multicellular embryo with exine. **(C, C')** Panoramic view of 10 days-old cultures observed under confocal microscope showing IAA immunofluoresnce labelling on cells of multicellular embryos (white arrow), while no signal is observed on non-responsive microspores (yellow arrow) also present in the culture. **(D, D')** Multicellular embryo with broken exine. **(E, E')** Late globular embryo. **(F, F', G.G')** Negative controls during microspore embryogenesis with no fluorescence signal in any region of the embryos. Bars in **(A, A', B, B', D, D', F, F', G, G')** 20 µm, in **(C, C', E)** 50 µm.

The assays of the IAA immunofluorescence during different developmental stages showed that vacuolated microspores exhibited a low IAA-fluorescence signal (Fig.2

A, A'). During microspore embryogenesis, IAA immunofluorescence revealed changes in the auxin accumulation pattern. Multicellular embryos with exine showed a very high IAA immunofluorescence signal being localized in the cytoplasm of every cell (Fig.2 B, B'). On the contrary, non-responsive microspores that were present in the culture with the multicellular embryos did not show any labelling with anti-IAA antibodies (Fig. 2 C, C'). After broking the exine, multicellular embryos without exine also showed IAA labelling (Fig.2 D, D'). Globular embryos showed an intense anti-IAA signal in the cytoplasm in every cell (Fig. 2 E).

Negative controls avoiding the first antibody or by immunedepletion experiments were carried out by pre-blocking the anti-IAA antibody; they showed no immunofluorescence signal in embryo cells at all developmental stages analysed supporting the specificity of the results of IAA immunofluorescence and the absence of autofluorescence in developing embryos (Fig.2 F, F', G, G').

Effect of polar auxin transport inhibition by NPA treatment on microspore embryogenesis induction and progression:

Two different concentrations of NPA, 3 and 10 µM were applied to *in vitro* microspore culture which are in the range of concentrations used for treatments to seedlings and *in vitro* embryogenesis systems of different species to inhibit polar auxin transport (Abrahamsson et al. 2012; Larsson et al. 2012). The effects of NPA treatment on cell death after 7 days (multicellular embryos) were analysed. Evan's blue staining was performed to identify death cells, which appeared blue, in control and NPA-treated microspore cultures (Fig. 3 A, B, C). The analysis showed that the percentage of dead cells in NPA-treated cultures were significantly higher (Fig. 3 B, C, D), in comparison with control cultures (Fig. 3 A, D). Cell death detected in control cultures could be originated by both the isolation and *in vitro* culture procedures and by the application of cold stress treatment on non-responsive microspores. The cell death percentages found in NPA-treated cultures were similar at the two concentrations used, 3 and 10 µM (Fig. 3 B, C, and D).

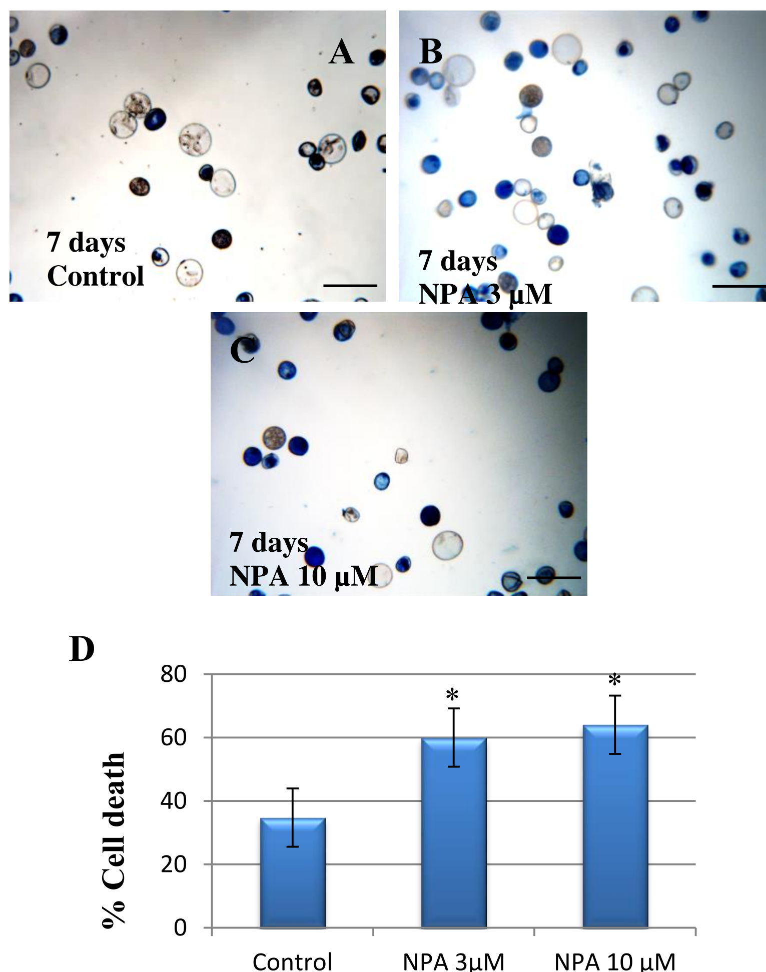


Figure 3: Effect of NPA treatment on cell death during microspore embryogenesis in barley. **(A,B,C)** Evan's blue staining to detect dead cells in microspore embryogenesis cultures of *Hordeum vulgare* at the multicellular embryo stage. The presence of dead cells (which are stained with blue) in control cultures is less than in NPA-treated cultures. The level of cell death in 3 µm NPA-treated cultures **(B)** is less than in 10 µm NPA treated cultures **(C)**. **(D)** Quantification of the percentage of dead cells after 7 days after the treatment with NPA at the concentrations 0 µm (control), 3 and 10 µm. Bars in **(A – C)** 100 µm. Columns represent mean values and bars represent SEM. Asterisks indicate significant differences with the non-treated/control culture sample (Student's *t*-test at $P \leq 0.05$).

To evaluate the effect of NPA on the progression of microspore embryogenesis, control and NPA-treated cultures were monitored under the stereomicroscope every few days until the stage in which the first coleoptilar embryos were observed. The number of multicellular embryos (still surrounded by the exine) and the number of developing embryos (after exine break down) found in control and NPA-treated cultures were quantified at each time interval. In control cultures, responsive microspores divided during the first days of culture and produced proembryos or

multicellular embryos (Fig.4 A) which reached a relevant proportion by 7 (4 A, 5 A) and 10 days (Fig. 5 A). Later, the quantity of multicellular embryos slightly increased until day 14 (Fig. 4 B, 5 A), remained relatively stable for several more days and progressively decreased in 21 (Fig. 5 A) and 25 day-old cultures (Fig. 4 C, 5 A) at these stages, most proembryos have developed into developing embryos (Fig. 4 C). Analysis of embryogenesis induction in NPA-treated cultures showed that the production of proembryos or multicellular embryos was lower compared to untreated cultures during the first days of cultures until 14 days (Fig. 4 E, I, F, J, 5 A). Moreover, from day 21st and later, NPA-treated cultures showed higher proportions of multicellular embryos than controls (Fig. 5 A), suggesting that most of them were stopped in development at this stage whereas in control cultures, they continued their development (Fig. 4 A – D, 5 A). In control cultures, after the exine breakdown, the embryogenesis progressed and further cell proliferation and differentiation occurred, leading to the formation of embryos with various sizes and shapes, the so-called 'developing embryos'. These developing embryos were found in significant proportions in control cultures from day 17 and maintained high proportions on day 21 and later, until day 30 (Figure 4 C, D, 5 B). Developing embryos were not found at earlier stages, during the first time points studied (10 – 12 days) (Fig. 5 B), when multicellular embryos were abundant in the cultures. In contrast, in NPA-treated cultures, the plates containing 3 and 10 µM NPA showed lower proportions of developing embryos than control cultures, similar for both concentrations (Fig. 4, G, K, H, L, 5 B).

To evaluate the effect of NPA on embryogenesis efficiency, the production of embryos was analysed in the *in vitro* cultures at the embryo production stage, after 30 days of culture initiation. The results showed a lower production of embryos in NPA-treated cultures compared to control (Fig.6 A, B, C, D). The NPA-treated cultures at 10 µM showed much lower embryo production (Fig. 6 C) than 3 µM NPA-treated cultures (Fig. 6 B, D).

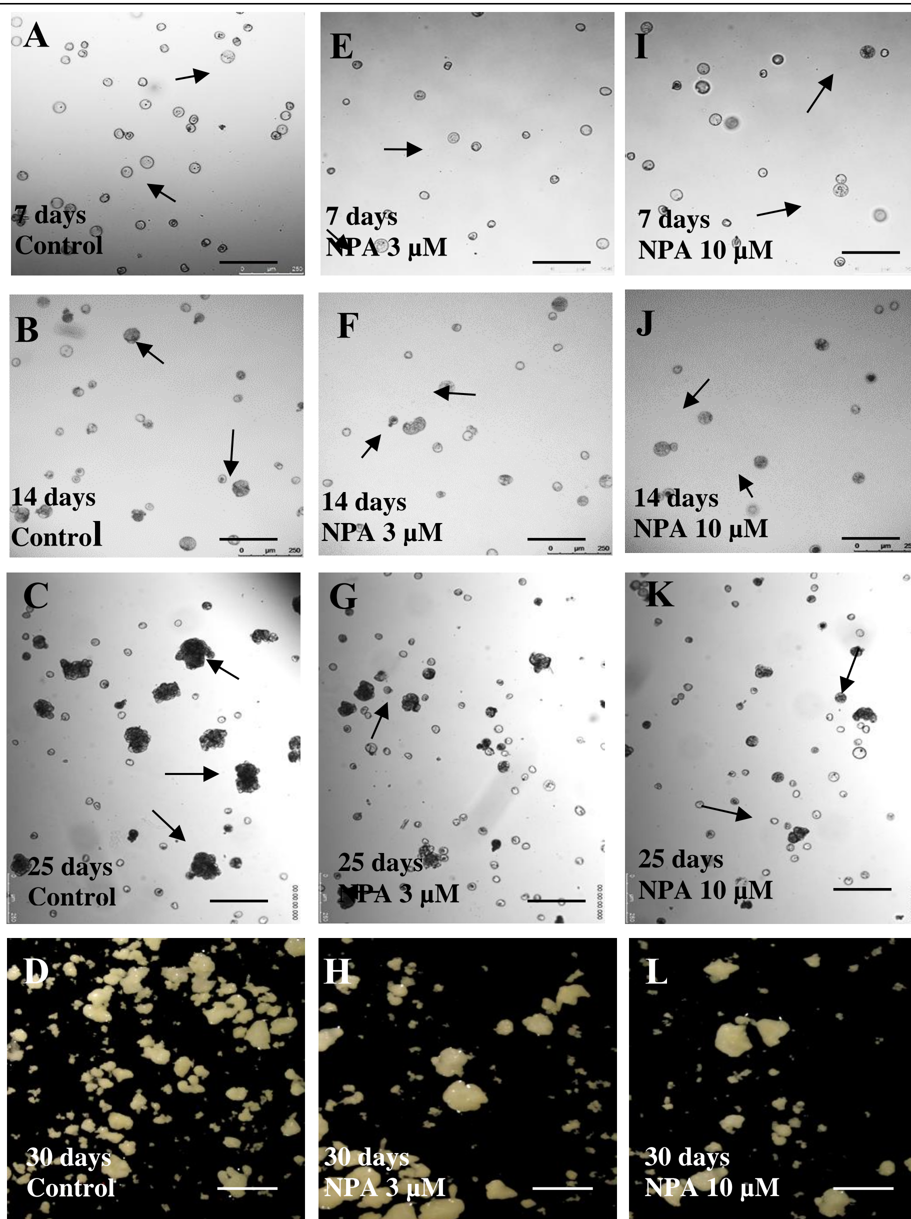


Figure 4: Progression of microspore embryogenesis in control and NPA-treated culture of *Hordeum vulgare*. (A – D) Control cultures. (E – H) 3 µM NPA-treated cultures. (I – L) 10 µM NPA-treated cultures. (A,E,I) 7 day-old cultures showing typical rounded multicellular embryos surrounded by the exine. High proportion in control cultures (A) is observed compared to NPA-treated cultures (E,I). (B,F,J) 14 day-old cultures showing multicellular embryos with broken exine to start to form embryos. The proportion of these embryos in control culture (B) is higher than in the NPA-treated cultures (F,J). (C,G,K) 25 day-old cultures showing developing embryos of different sizes which were formed after the breakdown of the exine. Higher proportion of these embryos is observed in control (C) is observed compared to NPA-treated cultures (G,K). (D,H,L) 30 day-old culture showing embryos at advanced developmental stages (transitional and coleoptilar embryos) which observed in both control (D) and NPA-treated cultures (H,L) but their density in control is higher than in NPA-treated cultures. The induction and progression of microspore embryogenesis in 3 µM NPA-treated cultures (E,F,G,H) is higher than in 10 µM NPA-treated cultures (I,J,K,L). Bars in (A,B,C,E,F,G,I,J,K) 250 µm, in (D,H,L) 175 µm.

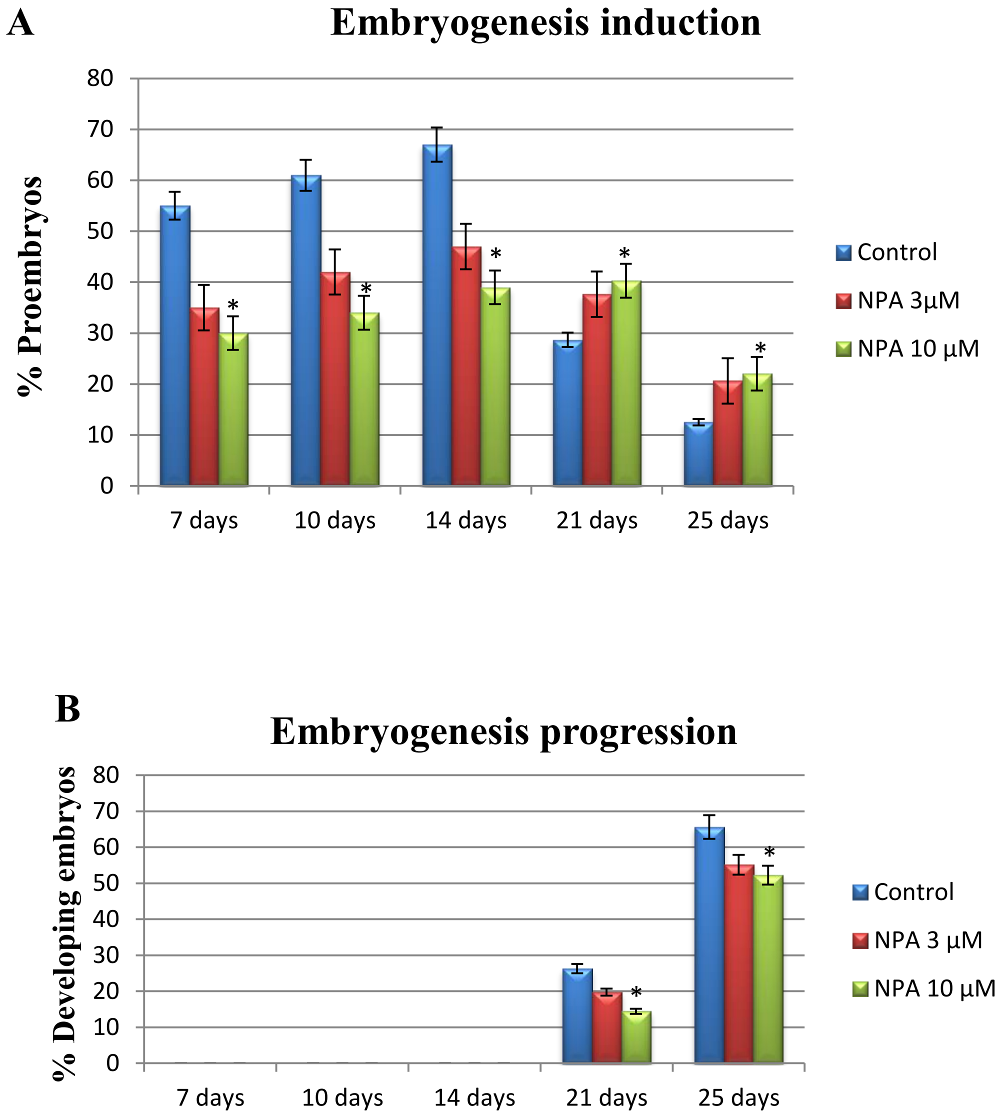
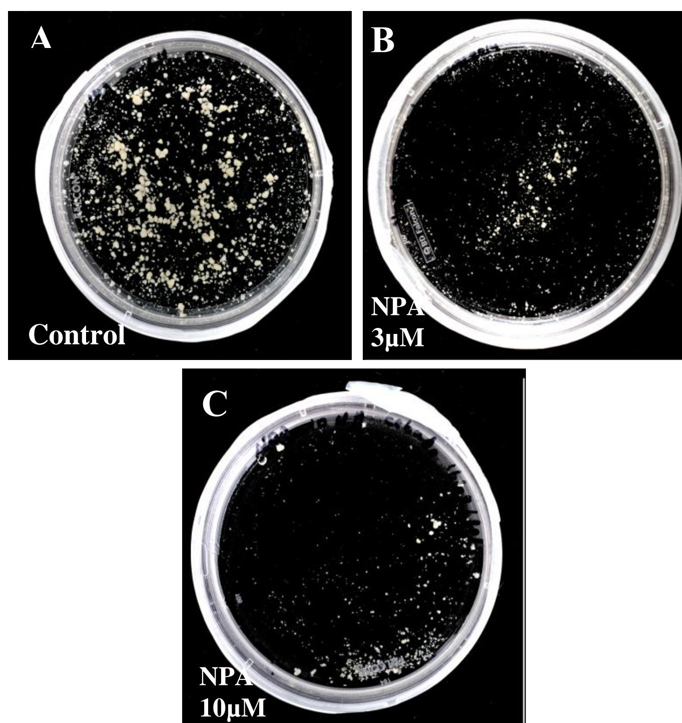


Figure 5: Effect of NPA treatment on microspore embryogenesis induction and progression in *Hordeum vulgare*. (A) Quantification of the percentage of proembryos (A) and developing embryos (B) at different time intervals (7, 10, 14, 21 and 25 days) during microspore embryogenesis progression in control (blue columns), 3 µM NPA-treated (orange columns) and 10 µM NPA-treated (grey columns) cultures of barley. Columns represent mean values and bars represent SEM. Asterisks indicate significant differences with the control culture sample at each time point (Student's *t*-test at $P \leq 0.05$).



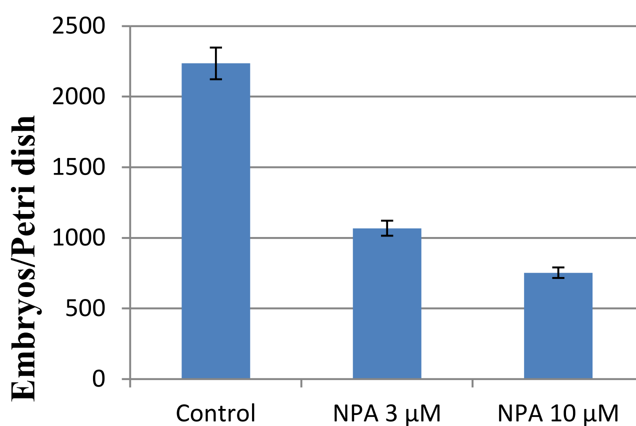


Figure 6: Effect of NPA treatment on embryo production. (A – C) Plates showing the microspore-derived embryos produced in control (A), 3 µM NPA (B) and 10 µM NPA treated (C) cultures of *Hordeum vulgare* after 30 days. (D) Quantification of the embryo production in control, 3 µM NPA and 10 µM NPA treated cultures, columns represent mean values and bars represent SEM of the total number of embryos per petri dish.

IAA immunofluorescence was performed on NPA-treated samples at early stages. In multicellular embryos and globular embryos, the IAA-immunofluorescence was

localized in the cytoplasm in every cell like in control cultures, but the signal was lower than in the untreated cultures (Fig.7).

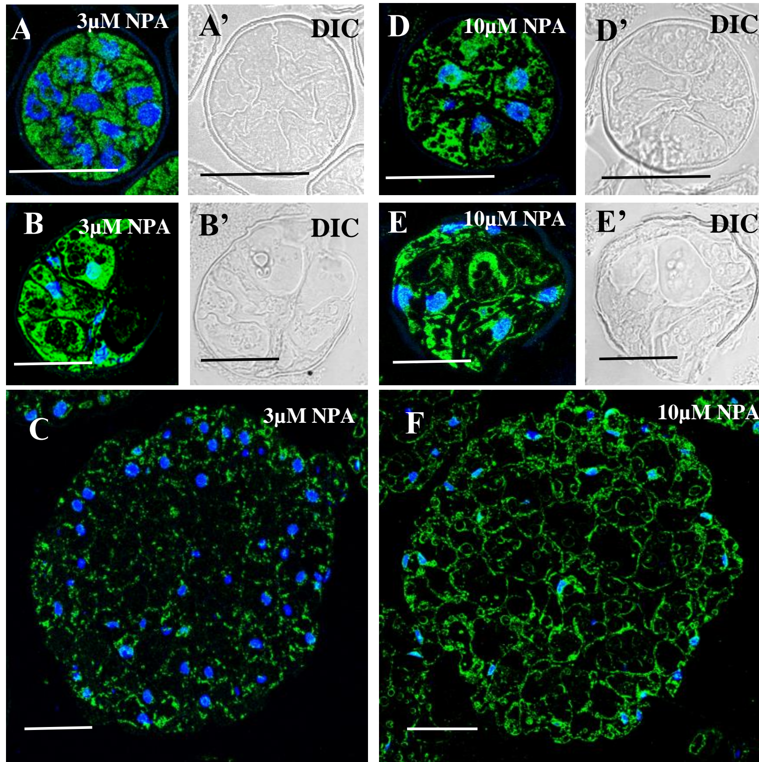


Figure 7: IAA immunolocalization in NPA-treated cultures of microspore embryogenesis. (A – F) IAA immunofluorescence of different microspore developmental stages in NPA-treated cultures at 3 µM. (A,A',B,B',C) and 10 µM (D,D',E,E',F). (A,A',D,D') Multicellular embryos surrounded with exine. (B,B',E,E') Multicellular embryos after exine breakdown. (C,F) Globular embryos. (A',B',D',E') Differential interference contrast (DIC) images to show the cell structure. Bars in (A,A',B,B', D, D', E,E') 20 µm, in (C,F) 50 µm.

Effects of inhibition of IAA action by PCIB on the microspore embryogenesis

The IAA action inhibitor PCIB was added to microspore cultures at different concentrations, 5 µM and 10 µM, and their effects on stress-induced microspore embryogenesis progression were studied by the analyses of embryogenesis induction (proembryos at early stages) and efficiency (mature embryos). The results showed that, in PCIB-treated cultures, the microspores did not respond to embryogenesis induction in comparison with the control cultures which produced numerous

proembryos or multicellular embryos (Fig.8 A, C, E), and mature embryos (Fig.8 B, D, E). Almost no proembryos and embryos were observed in the PCIB-treated cultures from day 0 until day 30 (Fig. 8 C, D, E, F), indicating that PCIB treatment completely blocked the development during microspore embryogenesis in barley.

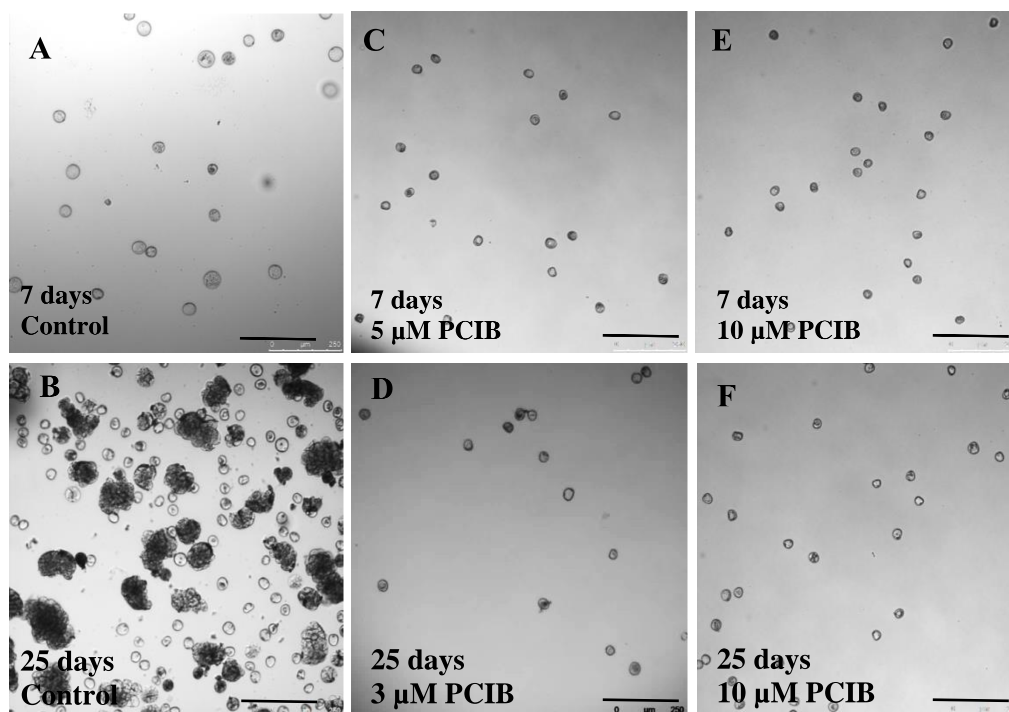


Figure 8: Effect of PCIB treatment on microspore embryogenesis induction of *Hordeum vulgare*.(A – B) Control culture. (C – D) 5 µM PCIB-treated cultures. (E – F) 10 µM PCIB-treated cultures. (A,C,E) 7 days-old cultures showing multicellular embryos surrounded by exine in control (A), whereas the microspores did not progress in PCIB-treated cultures (C,E). (B,D,F) 25 days-old cultures showing developing embryos of different sizes in control (B), whereas no embryos are found in PCIB-treated cultures (D,F). Bars in all these figures are 250 µm.

DISCUSSION

After induction of microspore embryogenesis, IAA accumulates in early embryos

The results in this work revealed changes in the auxin accumulation pattern during microspore embryogenesis. After embryogenesis induction, IAA immunolocalization assays in microspore cultures reveal a differential and significant increase of the cellular IAA endogenous levels in multicellular embryo cells in comparison with

vacuolated microspores before induction. In contrast, the non-responsive microspores, which do not follow the embryogenesis pathway, show no IAA immunofluorescence signal indicating the absence of new auxin biosynthesis. The confocal analysis of immunofluorescence with immunodepleted IAA antibody shows a complete absence of the immunofluorescence signal and an almost null cross-reactivity against other endogenous molecules such as conjugated IAA or other IAA-related compounds, indicating that the immunolocalization assays reveal the presence of free IAA.

The results presented here on microspore embryogenesis in barley induced by cold stress showed that IAA increased and accumulated in the cells of early multicellular embryos and during embryogenesis progression suggesting that microspore reprogramming probably activates the auxin biosynthesis. In *Brassica napus*, microspore embryogenesis is usually induced by heat treatments (32 ºC); in this system, Rodríguez-Sanz et al. (2015) found that the IAA immunofluorescence signal intensity increased in early microspore embryos and in globular embryos. In our laboratory, we have developed a new in vitro system of microspore embryogenesis induced at low temperature (18ºC) in *Brassica napus* (Prem et al. 2012). In this system, it has been reported that IAA also accumulated in the cells of early multicellular embryos. The results presented here in barley revealed the increase and accumulation of endogenous auxin in early microspore embryos after induction by cold treatments (4ºC), indicating that auxin accumulation does not depend on the temperature stress treatment used for the induction and initiation of embryogenesis.

Recent studies indicated that auxin is delivered from the suspensor to the embryo at early embryogenesis stages in Arabidopsis (Robert et al. 2013; Moller and Weijers 2009). It has been found that auxin accumulated in immature embryos especially at the root apex, ends of cotyledon primordia and at the hypohysis during zygotic embryogenesis (Ni et al. 2001). In maize, auxin concentrations increase at the onset of endreduplication and remain high throughout the seed development (Lur and Stter

1993). Recently, it has been suggested that the auxin is involved in positional signalling during aleurone development and specification (Forestan et al. 2010). Complete loss of endogenous auxin in the embryo might be lethal, confirming a key role of this phytohormone in the development of the embryo. Our results indicate that endogenous auxin increase and accumulation accompany the initiation of microspore embryogenesis in barley, like in dicot systems as *Brassica napus*, suggesting that auxin play a key role in the switch of the embryogenesis progression, independently of the stress treatment used for the induction.

The inhibition of polar auxin transport by NPA impairs microspore embryogenesis progression

Polar auxin transport (PAT) can be inhibited by NPA treatment and it has been reported that the role exerted by auxin in the regulation of plant growth depends on its characteristic polar transport (Locascio et al. 2014). In this work on barley microspore embryogenesis cultures, NPA was applied at the low concentrations of 3 and 10 µM (Esmon et al. 2006, Hakman et al. 2009, Peer et al. 2013, Abrahamsson et al. 2012, Larsson et al. 2012, Li et al. 2013). It has been reported that low concentrations of NPA (1 – 5 µM) can block the PAT required for establishment of embryonic apical-basal polarity and organogenesis (Geldner et al. 2001; Murphy et al. 2002; Friml 2003; Peer et al. 2013). The results in this research showed a decrease in the embryogenesis yield that was similar in the two NPA concentrations, indicating a key role for PAT in microspore embryogenesis in barley, as reported in the dicot species *Brassica napus* and *Quercus suber* (Rodríguez-Sanz et al. 2014; 2015).

The knowledge about the effect of NPA on cellular auxin accumulation patterns in monocots is still scarce. (Carraro et al., 2006). Recently, it has been reported that the auxin gradient may be responsible for differentiation of zygotic embryos in maize (Locascio et al. 2014). The switch from the apical to basal membrane localization of *ZmPIN1* proteins characterizes the coleoptilar stage and the following establishment

of an auxin flux from both the differentiated scutellum and shoot apical meristem which is responsible for the differentiation of embryonic roots.

In wheat, it has been concluded that diffusion low-active transport of auxin occurred in radially symmetrical embryos and the shift from radial to bilateral symmetry of the embryos is associated with a change in auxin distribution (Fischer-Iglesias et al. 2001). It has also been hypothesized the existence of a bidirectional polar transport of auxin toward the scutellum and the SAM from the root pole of the embryo (Fischer-Iglesias et al., 2001; Feng and Jacobsen 2011). The results presented here provide new evidence on the role of PAT in the microspore embryogenesis progression in a monocot species, barley.

The auxin action inhibitor PCIB prevents the initiation of microspore embryogenesis in barley

In this work, treatments with the inhibitor of the auxin response PCIB were performed (Xie et al. 2000) to address the involvement of auxin action in the progression of microspore embryogenesis. The results with PCIB-treated cultures showed the absence of embryos indicating that this treatment completely blocked the development of the microspores in which no cell division has been observed. In *Brassica napus*, the microspore cultures were treated with PCIB with several concentrations by our group, and the results showed that the PCIB treatment during the process greatly affected embryo development but it did not prevent 30 – 40 % of the microspores to divide and initiate (Rodríguez-Sanz et al. 2015). In contrast, our results in barley showed that the PCIB treatment prevented most of the microspores to initiate to form embryos indicating that auxin perception and response are highly required for the progression and initiation of microspore embryogenesis, also in monocots.

Plant growth regulators such as exogenous auxins or cytokinins were used to increase embryogenesis *in vivo*, but they showed different effects. In a previous study by Cao

et al. (1994), addition of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP) to NLN medium improved the embryo yield. Several developmental aberrations and defects were reported under NPA and PCIB treatments applied to developing zygotic embryos of several species (Hadfi et al. 1998).

Taken together, the data reported here indicate that auxin accumulation, transport and perception are needed for initiation and progression of microspore embryogenesis in a monocot species, barley.

GENERAL DISCUSSION

1. SIMILAR KEY FACTORS ARE INVOLVED IN MICROSPORE EMBRYOGENESIS OF MONOCOT AND DICOT SPECIES AND REVEAL GENERAL REGULATORY MECHANISMS

Microspore embryogenesis is a powerful method for crop breeding with limited applicability since its regulating mechanisms are still unknown. Specifically, the microspore embryogenesis has important limitations to improve many crops of economic interest, which show very low embryogenesis efficiencies. The mechanisms of microspore embryogenesis induction and progression, which involve proliferation and differentiation events, are still unclear. Investigations using model systems of microspore embryogenesis, like isolated microspore *in vitro* cultures of the monocot *Hordeum vulgare* and the dicot *Brassica napus*, have provided some information on the cellular processes underlying cell reprogramming and embryogenesis initials in each species (Prem et al. 2012; Seguí-Simarro et al. 2003; Satpute et al. 2005; Ferrie and Caswell 2011; Jacquard et al. 2009; Rodríguez-Serrano et al. 2012; Daghma et al. 2012; Dubas et al. 2014; Maraschin et al. 2006; Ramírez et al. 200; González-Melendi et al. 2005). These two 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. In this PhD thesis, isolated microspore culture was performed in both species to analyse the dynamics of several factors in order to characterize general regulatory mechanisms involved in microspore embryogenesis.

It has been reported that stress treatments are necessary to induce the microspore embryogenesis; the temperature is the most used stress since it can control the development of isolated microspores in culture, inducing symmetric divisions (the first sign of the change of developmental program) in many species including rapeseed and barley. In barley microspore culture, cold stress treatment at 4º C induces the responsive microspores to embryogenesis; in general, cold treatment is essential to induce the embryogenic response in the monocot species as it has been found in previous works in wheat (Khound et al 2013), maize (Bedinger and Edgerton

1990), and rice (Khatun et al. 2012). In contrast, in many dicot species, microspore embryogenesis has been induced by heat treatments, usually at 32ºC, as *Brassica napus* or *Quercus suber* (Prem et al. 2012; Bueno et al. 1997; Rodríguez-Sanz et al. 2014a). In this PhD thesis, for the first time, a comparative analysis between two microspore embryogenesis systems has been performed as a suitable approach to search factors involved and general mechanisms. Two systems of microspore embryogenesis of very different species (barley and rapeseed) induced by different temperatures (32ºC in rapeseed and 4ºC in barley) have been used to analyse the dynamics of various factors whose previous data from our group have suggested their involvement in microspore embryogenesis: DNA methylation, arabinogalactan proteins (AGPs) and endogenous auxin. The findings have revealed common dynamics of these factors for the two plant species, suggesting the existence of general regulating mechanisms which are common to different *in vitro* systems and plant species.

2. DYNAMICS OF GLOBAL DNA METHYLATION LEVEL AND DISTRIBUTION DURING MICROSPORE EMBRYOGENESIS

In this thesis, the dynamics of global DNA methylation was analyzed after reprogramming of microspore to embryogenesis, in barley. The results revealed epigenetic changes that accompany the two pollen developmental programs analyzed: pollen maturation and microspore embryogenesis, indicating for the first time in a monocot species the possible involvement of DNA methylation in regulating microspore embryogenesis induction and progression. Results in barley were in agreement with those recently reported by us in the dicot plants, rapeseed and cork oak (Solís et al. 2012; Rodríguez-Sanz et al. 2014a). Moreover, the results presented in this work provided new insights into the role of epigenetic modifications in microspore embryogenesis and suggested a potential benefit of epigenetic inhibitors, such as 5-azacytidine (AzaC), to improve the process efficiency in biotechnology and breeding programs of monocot and dicot crops.

2. a. The patterns of DNA methylation change during pollen development and microspore embryogenesis

In this work, analysis of the changes in genomic DNA methylation during pollen development and microspore embryogenesis was performed in barley. The approach involved the quantification of the percentage of methylated DNA and confocal microscopy of the 5mdC immunofluorescence assays. The results reported illustrate for the first time the epigenetic changes, regarding DNA methylation, during pollen development and after microspore embryogenesis induction in a monocot species, barley.

The chromatin remodelling during development of the plant male gametes provides the generative and sperm nuclei of the mature pollen grain with a unique chromatin organization in a highly condensed state. The high increase of global DNA methylation observed in the present work during pollen maturation in barley is related to the heterochromatization that accompanied the differentiation of cells in the most advanced stages of pollen development. Some investigations indicated the specific accumulation of histone variants in the nuclei of the male germline as well as a variable epiallele hypermethylation (Ingouff et al. 2007; Borges et al. 2012). During gametogenesis, the haploid genome of each gamete represents the allelic diversity of the diploid genome, and is the basis of the genetic and epigenetic variation in the germline and in following generations. Several reports indicated that the vegetative cell as a source of epigenetic information can be transmitted through the germline before fertilization (Slotkin et al. 2009; Calarco et al. 2012; Ibarra et al. 2012). The present study revealed a differential genome-wide hypermethylation in the generative and sperm nuclei, whereas the vegetative nucleus remained hypomethylated after the first pollen mitosis. The dynamics of several histone modifications in generative, sperm and vegetative nucleus of barley pollens has been reported by Pandey et al. (2013). They have observed that the most active histone modifications increased their expression in the vegetative nucleus as indicated by immunolabeling of active RNA

polymerase II, acetylated H3 in lysine 27 (AcH3K27), and three-methylated H3 in lysine27 (H3K27me3) (Pandey et al. 2013). These modifications were limited in the generative nucleus during the differentiation in bicellular pollen, while late microspores and vegetative nuclei exhibited higher labelling, indicating a role of these epigenetic marks in activating rather than suppressing gene expression (Pandey et al. 2013). Our results on DNA methylation in pollen nuclei of barley are in agreement with these findings and provide new evidence that gene silencing-related epigenetic modifications are enriched in the generative and sperm nuclei, while activity-related marks are mainly localized in late microspore and vegetative nuclei. The repressive epigenetic mark dimethylated histone H3 in lysine 9, H3K9me2, was found not only in the generative and sperm nuclei but also in the vegetative and microspore nuclei of barley pollen, and the activating mark dimethylated histone H3 in lysine 4, H3K4me2, was also localized in microspore and all pollen grain nuclei (Pandey et al. 2013). It has been suggested that these modifications would be related to local changes in transcriptional activity and tissue-specific regulated genes and would be therefore present in nuclei of all stages of pollen development.

Some reports have indicated that global DNA methylation levels increased during the differentiation and maturation of the pollen in several species (Zluvova et al. 2001; Meijón et al. 2009; Ribeiro et al. 2009). Nevertheless, the pattern of DNA methylation distribution has not been analysed during pollen development except in our previous report in rapeseed (Solís et al. 2012). Our immunofluorescence analyses showed low signal of 5mdC antibodies in the vacuolated microspores and vegetative nucleus, while an intense signal was found in the generative and sperm cells of barley. The increase in 5mdC is associated with the cell differentiation processes of the generative and sperm cells of pollen grains (Solís et al. 2012).

In the nucleolus, rRNA genes are transcribed, and the nucleolar architecture clearly reflects the level of ribosome biosynthesis activity. The DNA methylation plays an important role in regulating the number of active rRNA genes (Lawrence et al. 2004; Bártova et al. 2010; Gruumt and Pikaard 2003). Promoters of silenced genes are

heavily methylated and are allied with histone H3 dimethylated on lysine 9 whereas the promoters which are hypomethylated are associated with histone H3 trimethylated on lysine 4 (Lawrence et al. 2004). In contrast with animal cells, plant cells do not usually exhibit a shell of perinucleolar heterochromatin where the silent portion of the repeated ribosomal genes and some telomeric and centromeric DNA regions are found (Bártová et al. 2010; Poltiz et al. 2013). Very few condensed chromatin knobs containing inactive rDNA can be observed at the nucleolar periphery in plant cells (Testillano et al. 2005) and could correspond to some of the 5mdC spots that we found at the periphery of the nucleolus in the vacuolated microspore. In plants nucleoli, rDNA is distributed in the fibrillar component (Risueño and Testillano 1994; Testillano et al. 2005). The large nucleolus of the vacuolated microspore has been characterized with a typical organization of high transcriptional activity, corresponding to the G2 phase of the cell cycle (Risueño and Testillano 1994; González-Melendi et al.1995; Testillano et al. 2005), the ribosomal chromatin being distributed in a decondensed state through the fibrillar component of the nucleolus, as revealed by rDNA in situ hybridization (Risueño and Testillano 1994; Testillano et al. 2005). In this work, the nucleolus of barley microspores did not show 5mdC labelling, indicating a decondensed state of ribosomal chromatin which correlates with an active rDNA transcription at this developmental stage. 5mdC labelling in generative and sperm nuclei showed high signal and was distributed in wider nuclear regions, covering the large heterochromatin masses that occupy the major part of the nuclear volume. In *Brassica napus*, hypermethylation of mature pollen was correlated with up-regulation of *BnMET1* methyl transferase, suggesting that MET1 is involved in the methylation of generative nuclei (Solís et al. 2012), but no data is available in barley on the MET1 participation in epigenetic mechanisms during pollen development. These results indicate an important change, specifically in the global DNA methylation in the male germline of barley, a process probably contributing to the epigenetic inheritance after fertilization that has been reported in many plant species (Calarco et al. 2012).

In contrast with pollen development, microspore embryogenesis initiation shows very low levels of global DNA methylation, in barley. Recently, it has been shown in our laboratory that the microspore reprogramming to embryogenesis is accompanied by modifications in global DNA methylation which exhibits low levels after induction and early embryogenesis , in *Brassica napus* (Solís et al. 2012) and *Quercus suber* (Rodríguez-Sanz et al. 2014a). These results reveal epigenetic modifications which accompany the reprogramming of the microspore towards a new developmental program and the first embryogenic divisions. The DNA methylation changes observed in this thesis can be associated with microspore embryogenesis induction in monocot species, and would be related to the global change of gene expression reported in barley microspore embryogenesis by transcriptomic analysis (Maraschin et al. 2006). In contrast, at later stages of microspore embryogenesis, the global DNA methylation showed high levels. Cells of advanced embryos showed 5mdC signals of different intensity and distribution, in differentiating and proliferating cells; in differentiating embryo cells, the signal covered the nuclear volume indicating low transcriptional activity, as reported in other differentiated cells of several monocot and dicot species (Testillano et al. 2002; Bárány et al. 2005). Our study reported that nuclei of young barley multicellular embryos presented distribution patterns of 5mdC which varied in intensity and localization, similar to the patterns related to the different chromatin condensation states observed in plant cycling cells (Testillano et al. 2013). The quantification of DNA methylation performed during advanced stages of microspore embryogenesis revealed a gradual DNA methylation increase. Previous studies showed an increase in global DNA methylation during somatic embryogenesis progression in *Acca sellowiana* (Fraga et al 2012).

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, remodelling of the chromatin organization patterns have been characterized in several 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 sporophytic pathways followed by the microspore, permitting the identification of defined nuclear changes that occurred when the microspore is reprogrammed and switches to embryogenesis.

In conclusion, our results show epigenetic changes that accompany microspore embryogenesis and pollen development, suggesting the possible involvement of DNA methylation dynamics in regulating microspore embryogenesis induction and progression, for the first time in a monocot species.

2. b. The DNA demethylating agent AzaC favours microspore embryogenesis initiation

In this work, the effect of the demethylating agent 5-azacytidine (AzaC) on microspore embryogenesis and its potential application to improve induction and progression of the process was studied in the monocot barley (*Hordeum vulgare*) and the dicot rapeseed (*Brassica napus*). The results showed a positive effect of the short AzaC treatments on microspore embryogenesis induction, at low concentration of 2.5 μM, increasing the percentage of microspore-derived proembryos formed, in the two systems. It has previously been studied the effect of AzaC on *in vitro* systems of somatic embryogenesis and organogenesis by adding the agent in the culture medium. Generally, AzaC inhibits the methyltransferase (MTase, E.C.2.1.1.37) activity causing hypomethylation of DNA when included into DNA as a substitute to deoxycytosine (Juttermann et al. 1994). The compound is included in DNA mainly during replication and reparation processes (Brown 1989; Habu et al. 2001). Some

studies reported that AzaC-induced rDNA demethylation in the embryogenic line and arrested calli growth in the non-embryogenic line leading to negative effects of this 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 studies in which AzaC promoted organogenesis or somatic embryogenesis (Li et al. 2001; Belchev et al. 2004; Tokuji 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 µM). It has been reported that the toxic effect of AzaC increased with increasing its concentration (Juttermann et al. 1994; Teyssier et al. 2014). In addition, data on AzaC effects on early events of the process have not yet been analysed. In this thesis, lower concentrations of AzaC have been tested, 2.5, 5, and 10 µM, and their effects on cell death have been evaluated; the results of these analyses reveal that cultures with the lowest AzaC dose (2.5 µM) 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 µM AzaC significantly decreased the DNA methylation level of cells in microspore cultures of the two species studied, precisely at the same culture stage when we detected significant increases in proembryo formation. These results indicate that, in *B. napus* and *H. vulgare* AzaC-induced DNA hypomethylation promotes microspore embryogenesis initiation and formation of proembryos, a few days after culture initiation.

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 rapessed, 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.

AzaC would increase the efficiency of embryogenesis initiation by promoting DNA hypomethylation and chromatin decondensation of cells, may be stimulating reprogramming, totipotency acquisition, and early proembryo divisions. 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 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 of the hypomethylated NORs were larger, a sign of high transcriptional activity, as demonstrated by the increase of the rate of [^{3}H] 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.

2. c. Microspore-derived embryo differentiation involves DNA hypermethylation

DNA methylation has been implicated in regulating plant cellular differentiation (Kingham et al. 1998). It has been reported that loss of genome methylation is lethal in vertebrate embryos; plants are able to tolerate and survive, although pleiotropic

defects over generations are observed (Finnegan et al. 1996). In this thesis, it was analysed 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, long treatments of 2.5 μM AzaC were applied to microspore cultures. The results revealed that longer treatments prevented subsequent embryogenesis progression. The multicellular embryos 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.

It is known that differentiated cells acquire a stable and unique DNA methylation pattern that regulates tissue-specific gene transcription. The process 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 during terminal cell differentiation and this has been linked to increased levels of DNA methylation (Politz et al. 2013). It has been found that the pattern of *Arabidopsis* embryogenesis was not maintained in a significant fraction of embryos with mutations in the *MET1* and *CMT3* DNA methyltransferase genes (Xiao et al. 2006). Generally, in *Arabidopsis*, DNA hypomethylation is more likely to cause phenotypic defects due to improper gene expression (Bender 2004), such as the case of ectopic *FWA* expression and delayed flowering in *met1* mutant backgrounds suggesting that *met1-6* embryogenesis may be perturbed because hypomethylation and ectopic hypermethylation cause changes in gene transcription (Xiao et al. 2006; Soppe et al. 2000). The hypermethylation was associated with the heterochromatization that accompanies cell differentiation in advanced embryogenesis stages (Solís et al. 2012). In addition, the gene expression of *MET1* DNA methyltransferase has been reported to increase during differentiation of embryos originated from zygotes and microspores, in *Brassica napus* (Solís et al. 2012). The increase in *MET1* expression correlated with the increase in global DNA methylation and heterochromatization events during different developmental

processes like embryo cell differentiation and tapetum programmed cell death (Solís et al. 2012; 2014).

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.

3. ARABINOGALACTAN PROTEINS (AGPs) ARE INDUCED AFTER MICROSPORE EMBRYOGENESIS INITIATION

In this work, AGPs were analyzed during microspore embryogenesis and pollen development in both the dicot *Brassica napus* and the monocot *Hordeum vulgare*. Immunofluorescence and confocal laser microscopy analyses were performed using monoclonal antibodies for AGPs (LM2, JIM13, JIM14 and MAC207) and AGP arabinan epitopes (LM6). Dot blot assays and analysis of the expression patterns of the *BnAGP Sta 39 – 4* gene by quantitative real-time PCR were also performed.

The results showed different distribution patterns of AGPs during microspore embryogenesis and gametophytic development through the localization by the monoclonal antibodies. The dynamics of the AGP epitopes JIM13, JIM14 and MAC207 were related to pollen maturation while the AGP epitopes JIM13, JIM 14, LM2 and LM6 were associated with the induction of the microspore embryogenesis; LM2 and LM6 epitopes were also related to embryo differentiation.

3. a. AGPs show different patterns during microspore embryogenesis and pollen development.

Studies of mammalian glycoproteins/ proteoglycans showed the highly heterogeneous nature of the AGPs which suggests that AGPs have more than one specific role (Ellis

et al. 2010; Filmus et al. 2008; Schaefer and Schaefer et al. 2010). In the last years, AGPs have been reported to be involved in different plant developmental processes (Ellis et al. 2010; Seirfert and Roberts 2007).

In this thesis, immunofluorescence with several monoclonal antibodies for AGPs was performed in several pollen development stages (vacuolated microspores and mature pollens) and microspore embryogenesis stages (multicellular embryos, and early embryos, such as globular and heart shape embryos and cotyledonary embryos). The AGPs labelling was very low in the vacuolated microspores whereas in multicellular embryos, the signal was high on the cell wall of every proembryo cell, in both species, rapeseed and barley; additionally higher signal was observed in cytoplasmic spots, in barley with some AGP antibodies. These specific antibodies can bind to the complex structure of the carbohydrate epitopes of AGPs which have been useful in revealing the developmental dynamics of AGP glycan moiety and represent a diagnostic tool for AGPs (Coimbra and Pereira 2012; Ellis et al. 2010). The results in this thesis have revealed that a wide group of AGPs (those recognized by JIM13, JIM14, LM2 and LM6 antibodies) were induced after microspore embryogenesis induction and were specially localized in the cell walls of embryos with just two or four cells, which were formed by the first embryogenic divisions of the microspore after reprogramming; in contrast, AGPs were much less present or absent in the microspore before the programme changed.

Recently, in our laboratory, some pectin epitopes, like JIM5 which recognize low-esterified pectins, were found to increase during microspore-derived embryo development, being especially abundant in the cell wall of differentiating embryo cells in pepper and cork oak (Bárány et al. 2010a; Rodríguez-Sanz et al. 2014a). LM6 can recognize a pentasaccharide of (1 – 5) – a – L – arabinans present in AGPs, but also present in the rhamnogalacturonan I domain of pectins which may be covalently attached in cell wall (Willats et al. 1998; Jones et al. 1997). Willats et al. (1998) indicated that the usefulness of LM6 as a probe for the localization of arabinan epitopes was assessed by tissue printing of lemon fruits which are known to contain

linear arabinans. They found that LM6 antibody reacted with tissue prints of lemon fruit and revealed important information regarding the distribution of the arabinan epitope. Immunolocalization of arabinan in embryos, seeds, and seedlings revealed that arabinans can accumulate in developing and mature embryos, but disappear during germination and seedling establishment (Gomez et al. 2009). Recently, it has been indicated that arabinans are abundant in walls of embryo cell and undergo extensive degradation during germination in *Arabidopsis thaliana* (Gomez et al. 2009), *Prunus dulcis* (Dourado et al. 2004), *Brassica napus* variety Casino (Eriksson et al. 1996) and *Gledisia triacanthos.* (Navarro et al. 2002). On the other hand, it has been shown that the cell wall of coleoptilar embryos in barley has high levels of pectic arabinose (Gibeaut et al. 2005). In this thesis, LM2 and LM6 epitopes increased in embryo cells during the microspore embryogenesis progression, in barley and rapeseed, being distributed not only in cell walls but also in cytoplasmic spots, especially the LM2 antigen.

The involvement of AGPs during pollen development has been reported in several plant species (Coimbra et al. 2010; Coimbra and Pereira 2012; Ellis et al. 2010; Qin et al. 2007; Pereira et al. 2015). It has been found that AGPs were newly synthesized and increased in concentration during pollen germination (Castro et al. 2013). In *Trithuria submerse*, anti-AGP antibodies labelled the anthers and the intine wall, the latter signal was associated with pollen tube emergence (Costa et al. 2013). In *Quercus suber*, pollen-expressed AGP genes were correlated with microgametogenesis progression using anti-AGP antibodies (Costa et al. 2014). Coimbra and Pereira (2012) documented that there are differences in the pattern of distribution of specific AGP sugar epitopes during pollen development in *Arabidopsis thaliana*. On the other hand, anti-AGP antibodies have labelled the generative and sperm cells, but no labelling was observed in the vegetative cell (Coimbra and Pereira 2012). Our results in the pollen grain of *B.napus*, showed that the two sperm cells are strongly labelled by anti-AGP antibodies. The specific labelling of the generative cell was also reported in rapeseed (Pennell et al. 1991), tobacco (Li et al. 1995) and *B.*

campestris (Southworth and Kwiatkowski 1996). Microarray data of Arabidopsis sperm cell transcriptome indicated that male gametes have a different gene expression from pollen grains (Borges et al. 2008). Different genetic, immunochemical and biochemical approaches have indicated a role for AGPs in pollen germination, pollen tube growth and male gamete function (Coimbra et al. 2012; Costa et al. 2013; Qin et al. 2007).

Our results in this thesis have indicated that AGPs could play a role in pollen development in *B. napus* and suggested that different AGP families, grouped by the same polysaccharide antigenic determinants, could act separately in different processes. The AGPs localized in vegetative cytoplasms, intine and apertures (JIM14 and MAC207 epitopes) would be involved in pollen germination and pollen tube growth, whereas the AGPs localized in the generative and sperm cells (JIM13, LM2 and LM6 epitopes) would be related to the gamete function. Even though the AGP molecular mechanism of action is still unknown, AGPs specifically localized in generative and sperm cells have been linked with the signals that are necessary to direct these cells to their targets inside the pollen tube (Coimbra and Pereira 2012). AGP epitopes localized in the intine and pollen tube wall, predominantly associated with the tip region, have been suggested as structural and/or control elements for germination through the modulation of water uptake (Coimbra et al. 2010).

Sta 39-4 and Sta 39-3 genes are among some of the scarce AGP genes isolated from *Brassica napus* (Gerster et al. 1996), and they were the first characterized pollen-specific putative AGP genes. The temporal pattern of Bn*AGP Sta 39-4* gene expression found in this thesis during the two microspore pathways in rapeseed, with high expression levels on mature pollen and early embryos, resembled the patterns exhibited by JIM13 and JIM 14 epitopes, suggesting that a certain group/family of AGPs could be involved in different processes of the two microspore pathways, pollen development and microspore embryogenesis. Other groups of AGPs, those revealed by LM2 and LM6 antibodies, showed increasing expression throughout microspore embryogenesis progression, in both barley and rapeseed species, therefore

indicating that AGPs could be involved in other functions related to microspore-derived embryo development. The qPCR analysis also showed high expression induction of the AGP gene *Sta 39-4*, specifically in the early microspore embryogenesis stages, in early embryos, followed by down-regulation during later embryogenesis stages. The specific expression of AGPs in multicellular embryos and their localization in the newly formed embryo cell walls strongly suggest that AGPs are early molecular markers of microspore embryogenesis.

The results in this PhD thesis have revealed that a wide group of AGPs (those recognized by JIM13, JIM14, LM2 and LM6 antibodies) were induced and specifically localized in the cell walls of early embryos with just two or four cells which were formed by the first embryogenic divisions of the microspore after reprogramming, suggesting a role for AGPs in the initiation of the new developmental programme.

3. b. The blocking of AGPs by Yariv reagent impairs microspore embryogenesis initiation.

To analyse the possible function of AGPs in microspore embryogenesis, in this PhD thesis, we studied the effect of Yariv reagent, which blocks AGPs, on the initiation and progression of the process. It has been reported that AGPs are involved in the interactions between different cell types in suspension cultures and that they act indirectly on somatic embryo development (Kreuger and van Holst 1995; Thompson and Knox, 1998; Chapman et al., 2000). β-glucosyl Yariv reagent (βGlcY), are synthetic probes that bind to and aggregate AGPs whereas β-D-Mannosyl Yariv (βManY) differs only in the isomerization of the hydroxyl group at carbon atom 2 of the sugar, which prevents their binding to AGPs. βManY thus provides an excellent control in studies of AGPs using β GlcY (Yariv et al., 1967; Tang et al. 2006). Our results indicated that βGlcY reagent inhibited the progression of the embryogenesis after reprogramming of microspores by heat stress treatment in rapeseed and cold stress treatment in barley. It has been found that high concentration of βGlcY reagent

can completely inhibit the microspore embryogenesis initiation in rapeseed (Tang et al. 2006).

Yariv phenylglycosides are very helpful to explore AGP function as the treatment with βGlcY causes the perturbation of various physiological processes in plants. Application of βGlcY to the cell culture in *Arabidopsis thaliana* induced programmed cell death, indicating that AGPs are involved in the determination of cell fate (Gao and Showalter 1999). In tobacco cell culture, the Yariv treatment has revealed a possible role of AGPs in the orientation of cortical microtubules and the polymerization of F-actin (Sardar et al. 2006). In our work, the percentage of dead cells in barley, identified by positive Evans blue staining, was higher in βGlcY treated cultures compared to both control and βManY treated cultures, indicating a loss of viability of some microspores by the βGlcY treatment.

It has been reported that the addition of Yariv reagent to the tissue culture media, inhibits somatic embryogenesis in *Daucus carota* (Thompson and knox 1998) and Cichorium hybrids (Chapman et al. 2000); whereas the exogenous addition of AGPs restored such potential, increasing somatic embryogenesis. Recent studies of in vitro cultured zygotes and proembryos of tobacco have shown that AGP scavenging by the Yariv reagent affected the first zygotic divisions and proembryos pattern formation, indicating the involvement of AGPs in cell division and cell plate formation during the initial embryogenic divisions of the zygote (Yu and Zhao 2012). Furthermore, in tobacco zygote cultures, both immunofluorescence detection with the JIM13 antibody and staining with the Yariv reagent showed that AGPs were disturbed in the new cell plate during normal in vitro zygotic division (Yu and Zhao 2012). The results of this thesis on the quantification of microspore embryogenesis initiation and progression, in rapeseed and barley showed that the βGlcY reagent highly reduced the multicellular embryo formation and prevented further development of embryos. On the contrary, microspore embryogenesis induction and progression were not inhibited and there was no effect on viability in untreated or in βManY-treated cultures,

indicating that the effect of βGlcY treatment was dependent of its capacity to bind and block AGPs.

Recent work with maize microspore cultures revealed that tunicamycin treatment, that blocks protein glycosylation and therefore secretion, inhibited microspore-derived embryo development, which was subsequently recovered by supplementation with a medium containing all the secreted factors from a well-developed microspore culture (Testillano et al. 2010). Several molecules, including AGPs, have been found to be secreted from cells to the culture medium during maize microspore embryogenesis (Massonneau et al. 2005). A role for AGPs has also been proposed in the initiation and maintenance of microspore embryogenesis (Tang et al. 2006). The results presented in this thesis, with the localization of AGPs on cell walls and cytoplasmic spots, which resembled elements of the secretory pathway, suggest an active production and secretion of AGPs during microspore-derived embryo formation and differentiation. This indicates a possible role for endogenous AGPs in stimulating *in vitro* microspore embryogenesis, as reported in somatic embryogenesis. The study of microspore embryogenesis in both *Brassica napus* and *Hordeum vulgare* in this PhD thesis provides new evidence of the association of AGPs with the newly formed walls of proembryo cells suggesting their involvement in the first embryogenic divisions of the microspore, similar to zygote divisions.

4. AUXIN IS INVOLVED IN MICROSPORE EMBRYOGENESIS INITIATION AND PROGRESSION

Auxins play very important roles in embryo formation and development, despite that, knowledge of the molecular mechanism of auxin during early stages of embryogenesis is still limited (Moller and Weijers 2009; Rademacher et al. 2012). The differential auxin distribution pattern is the most important feature of this phytohormone. In the early stages of zygotic embryogenesis of *Arabidopsis thaliana*, auxin is first weakly distributed in the apical cell derived from the first division of the zygote (Hua Su and Zhang 2009). Afterwards, auxin accumulates in the proemebryo

and the incipient cotyledon primordia in the heart-shape stage (Friml et al. 2003; Jenik et al. 2007). It has been reported that auxin signal is very low in peripheral callus and within initiating primordia flanking meristems during shoot regeneration of Arabidopsis (Gordon et al. 2007). Auxin accumulation occurred in apical cells of the somatic embryo, indicating that auxin gradients were re-established in the shoot apical meristem (SAM) of somatic embryos (Hua Su and Zhang 2009). In this study, it was analysed the auxin dynamics during microspore embryogenesis in *Hordeum vulgare*, a process induced by cold stress treatment (Rodríguez-Serrano et al. 2012). The results were compared with previous works of our group in *Brassica napus* that studied the dynamics of auxin during microspore embryogenesis which was induced by heat treatment without adding any plant growth regulator (PGRs) in the culture medium (Prem et al. 2012; Rodríguez-Sanz. et al. 2015).

4. a. Endogenous auxin increases and is accumulated in early microspore embryo cells.

The effects of plant growth regulators (PGRs) supplemented to the nutrient media has been studied in various in vitro systems (Mousaavizadeh et al. 2010). In vitro secondary metabolites synthesis and somatic embryogenesis were affected by the combination of nutritional molecules and growth regulators, included auxins such as 2,4-D, in the medium (Mousaavizadeh et al. 2010; Raghavan et al. 2005; Bárány et al. 2005; Elhiti and Stasolla 2011). Nevertheless, there is little information on the presence and function of endogenous auxin over in vitro plant systems. It has been found a differential and significant increase in the IAA endogenous levels in the early proembryos cells of *B. napus* (Prem et al. 2012; Rodríguez-Sanz et al. 2015).

Our results in barley microspore embryogenesis showed that IAA increased and accumulated in the cells of multicellular embryos and early embryos (globular and transitional embryos) suggesting that microspore reprogramming probably can activate the auxin biosynthesis. Recently, it has been indicated that auxin is delivered from the suspensor to the embryo at early embryogenesis stages in *Arabidopsis*

(Robert et al. 2013; Moller and Weijers 2009). In maize, auxin concentrations increase at the onset of endoreduplication and remain high throught the seed development (Lur and Setter 1993). Complete loss of endogenous auxin in the embryo might be lethal, confirming a key role of this phytohormone in the embryo development. Our results indicated that endogenous auxin increase and its intracellular accumulation accompanies the initiation of microspore embryogenesis in barley, induced by cold stress, like in dicot systems as *B. napus* that are induced by heat stress, suggesting that auxin plays a key role in the switch of the embryogenesis progression, independently of the stress treatment used for the induction.

4. b. Auxin transport and action are required for microspore embryogenesis initiation and progression

The inhibitors of auxin transport (NPA) and auxin action (PCIB) have been studied during plant development in some species (Fischer et al. 1997; Forestan et al. 2012; Scanalon 2003; Hadfi et al. 1998; Oono et al. 2003; Kaneyasu et al. 2007; Zhao and Hasenstein 2010). It has been reported that the treatments with NPA and PCIB inhibited the root development in *Arabidopsis thaliana* (Oono et al. 2013; Reed et al. 1998). In this thesis, the results with these inhibitors showed a decrease in the embryogenesis yield when NPA was added to the microspore culture media, whereas PCIB treatment prevented the microspores to initiate embryogenesis indicating a key role for the polar auxin transport (PAT) and auxin action in microspore embryogenesis in barley.

In the angiosperms, the auxins are transported through the vascular cambium from the shoot towards the root apex (Goldsmith 1977); it also mediates short-range auxin movement in different tissues (Petrásik and Friml 2009). During zygotic embryogenesis, NPA induced a number of specific abnormal phenotypes during zygotic embryo development in wheat (Fischer et al. 1997). Nevertheless, the information about the influence of NPA on cellular auxin accumulation patterns is still limited, especially in monocot species (Carrato et al. 2006). It has been recently

reported that the auxin gradient probably plays a role in differentiation of zygotic embryos in maize (Locascio et al. 2014). In wheat, it has been shown that the diffusion low-active transport of auxin occurred in radially symmetrical embryos and the shift from radial to bilateral symmetry of the embryos is associated with a change in auxin distribution (Fischer-Iglesias et al. 2001). The results presented in our work provide new evidence on the role of PAT during microspore emrbyogenesis progression in a monocot species, barley.

Although the molecular mechanism of PCIB has not been completely elucidated, it has been reported that PCIB can block the auxin receptors, without any effect on the transport (Oono et al. 2003). The addition of exogenous auxins such as 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) to the microspore culture media improved the embryo yield in some species (Baillie et al. 1992). Our group previously studied the effect of NPA and PCIB during *Brassica napus* microspore embryogenesis, induced by heat stress treatment, without adding plant growth regulators (PGRs) (Rodríguez-Sanz et al. 2015). This report indicated that PAT and auxin action play a critical role in microspore embryogenesis progression (Rodríguez-Sanz et al. 2015). Several studies using the auxin transport inhibitor (NPA), which inhibits auxin binding to PIN protein carriers, have observed that it interferes with the normal development of zygotic embryos and *in vitro* somatic embryogenesis in several plant species (Moller and Weijers 2009; Abrahamsson et al. 2012). In our work, IAA immunolocalization has been performed in different embryo stages after NPA treatment. Our results showed lower IAA signal than in controls indicating that PAT is involved in the establishment of auxin accumulations in barley microspore embryogenesis. Other works have indicated the role of PAT during post-embryonic development in maize (Forestan and Varotto 2012).

Some investigators have discussed that the different plant architecture between monocots and dicots depends on the differentiation of structures whose development is controlled by auxins (Forestan and Varotto 2012; Locascio et al. 2014; McSteen 2010). Moreover, several developmental aberrations and defects were reported under

NPA and PCIB treatments applied to develop zygotic embryos of various species (Hadfi et al. 1998). Taken together, the data reported here indicate that auxin accumulation, transport and action are required for initiation and progression of microspore embryogenesis in a monocot species, barley.

5. KEY FACTORS INVOLVED IN MICROSPORE EMBRYOGENESIS ARE COMMON AND INDEPENDENT OF THE INDUCTOR STRESS AND THE SPECIES

In this thesis, we have performed a comparative analysis of the developmental patterns of microspore embryogenesis in two species, the monocot *Hordeum vulgare* and the dicot *Brassica napus*, in which the microspore reprogramming was induced by different stress treatment, i.e., 32º C for *B. napus* and 4º C for *H. vulgare*. In these two plant systems, vacuolated microspores are the most responsive developmental stage for embryogenesis induction, as reported in various species of both taxa, monocots and dicots (González-Melendi et al. 1995; Bárány et al. 2005; Testillano et al. 2002). After induction, the early stages of proembryo development are similar, however, the progression of the process is different in rapeseed and barley, since it mimics the zygotic embryo development of dicots and monocots respectively. In rapeseed, globular, heart, torpedo, and cotyledonary embryos were formed (Prem et al. 2012), whereas, in barley microspore cultures globular, transitional, scutellar, and coleoptilar monocots embryos were developed (Rodríguez-Serrano et al. 2012). Embryos of type-grass (graminea) monocots are strikingly different from those of dicots because of the presence of only one cotyledon.

We have characterized the dynamics (expression, localization, temporal profiles) during microspore embryogenesis, in rapeseed and barley, of several key factors or regulatory elements like DNA methylation, AGPs and auxin, factors in which previous data of our group suggested a possible involvement in the process. We also analysed the effect of inhibitors of these elements on the microspore embryogenesis.

Interestingly, despite the differences in the microspore embryogenesis inductive treatments and its progression, the comparative analysis of the results obtained in the two species together with two previous recent reports of our group (Solís et al. 2012; Rodríguez-Sanz et al. 2015) provided similar key factors at the early stages which indicated a similar dynamics of the three elements characterized in both rapeseed and barley microspore embryogenesis (Figure 1). In vacuolated microspores before induction, low levels of DNA methylation, AGPs and auxin were found in both, the monocot *Hordeum vulgare* and the dicot *Brassica napus* (Figure 1). After the inductive stress, multicellular embryos kept low levels of DNA methylation whereas it progressively increased during microspore embryogenesis progression (Figure 1). All AGPs tested increased after induction, in proembryos. The AGP epitopes LM2 and LM6 progressively increased from globular to cotyledonary embryos whereas JIM13 and JIM14 epitopes were induced only at early stages of embryogenesis but slightly decreased during embryogenesis progression (Figure 1). Endogenous auxin was low in vacuolated microspores but rapidly increased after embryogenesis induction, keeping high levels during embryo development (Figure 1).

Many reports have shown that the progression of plant embryo at cell differentiation is associated with DNA hypermethylation, and polar auxin accumulations (Xiao et al. 2006). It has been found that the DNA methylation level increased during embryogenesis after adding 2,4-D, on the tissue culture medium of *Cucurbita pepo* (Legrand et al. 2007). Moreover, changes in the chromatin structure associated with DNA methylation, in presence of 2,4-D have been related to cell reprogramming in somatic embryos in *Arabidopsis thaliana* (Karami et al. 2009; Feher 2013). Our group has shown that pectin esterification, and endogenous auxin increased concomitantly with the DNA hypomethylation at early stages of the microspore embryogenesis in *Quercus suber* (Rodríguez-Sanz et al. 2014a). Similar dynamics of DNA methylation and auxin were observed in this thesis during barley microspore embryogenesis initiation and progression; these results are also in agreement with the recent findings in *Brassica napus* microspore embryogenesis of the dynamics of

DNA methylation (Solís et al. 2012) and auxin (Rodríguez Sanz et al. 2015). Arabidopsis mutants in DNA methyltransferases *met1* and *cmt3* showed reduced DNA methylation levels, incorrect patterns of cell divisions, polarity and auxin gradients, finally producing aberrant embryos (Xiao et al. 2006) and it has been suggested that DNA methylation regulates *de novo* shoot regeneration by modulating auxin signalling in *Arabidopsis* (Li et al. 2011).

AGPs play a role in plant developmental processes that are regulated by phytohormones as it has been recently reported in carrot, where the addition of exogenous AGPs to the protoplasts increased cell division in a manner similar to that of auxin (Van Hengel 2001), while the binding of AGPs by βGlcY in carrot suspension cells suppressed cell division (Thompson and Knox 1998).

		Microspore embryogenesis					
		Before induction (Vacuolated microspore)		**After induction** (Proembryos and early embryos)		**During embryogenesis progression** (From globular to developed embryos)	
Key factors		**Rapeseed**	**Barley**	**Rapeseed**	**Barley**	**Rapeseed**	**Barley**
DNA methylation		(+/ -) (1)	(+ / -)	(+/-) (1)	(+ / -)	(+++) (1)	(+++)
Auxin		(+ / -) (2)	(+ / -)	(+++) (2)	(+++)	(++) (2)	(++)
AGPs	JIM13, JIM14 epitopes	(-)	n.a.	(++)	n.a.	(+)	n.a.
	LM2, LM6 epitopes	(-)	(-)	(++)	(++)	(+++)	(+++)

Figure 1: Summary of the level of DNA methylation, auxin and AGPs during microspore embryogenesis initiation and progression in rapeseed and barley. (-) absence; (+/ -) low level; (+) mid; (++) high; (+++) very high levels. n. a.: not analysed. (1) Solís et al. 2012, (2) Rodríguez-Sanz et al. 2015.

Further work will be necessary to elucidate whether DNA hypomethylation could modulate auxin expression and/ or signalling which could in turn influences AGPs induction or action during microspore embryogenesis.

To investigate the possible functions of these three factors in the process, in this PhD thesis, the effect of inhibitors of them such as 5-azacytidine (AzaC) which inhibits DNA methylation, Yariv reagent which binds AGPs, N-1-naphthylphthalamic acid (NPA) that inhibits polar auxin transport, and *P*-chlorophenoxyisobutytric acid (PCIB) which inhibits auxin action, were analysed. The results were similar in both species, rapeseed and barley, and are summarized in figure 2.

	Effect on microspore embryogenesis			
	Initiation		Progression	
Inhibitors	**Rapeseed**	**Barley**	**Rapeseed**	**Barley**
AzaC (DNA methylation inhibitor)	(+++)	(+++)	(-)	(-)
NPA (Auxin transport inhibitor)	n. a.	(-) low effect	(-) (1)	(-) low effect
PCIB (Auxin action inhibitor)	n. a.	(-) strong effect	(-) (1)	(-) strong effect
βGlcY (AGPs blocking)	(-)	(-)	(-)	(-)

Figure 2: Overview of the effect of some inhibitors on microspore embryogenesis initiation and progression in rapeseed and barley. (-) negative effect, significant decrease of the proembryos (initiation) or developing embryos (progression); (+++) positive effect, significant increase of the proembryos (initiation). n. a.: not analysed. (1) Rodriguez-Sanz et al. 2015.

AzaC increased the number of multicellular embryos after stress-induced microspore embryogenesis in both species. Glucosyl-Yariv, NPA and PCIB treatments inhibited the acquisition of totipotency of the microspores to reprogram towards

embryogenesis, being the effect of PCIB stronger since it completely blocked the proembryos formation (Figure 2).

Taken together, the results indicate that these factors, DNA methylation, auxin and AGPs, play a key role in microspore reprogramming, embryogenesis initiation and progression in the two species, suggesting common regulating mechanisms which could be extended to other crop plants.

CONCLUSIONS

CONCLUSIONS

1. The reprogramming of the microspore to embryogenesis involves the change of global DNA methylation levels and nuclear distribution patterns, in comparison with pollen development, in barley.

2. During pollen development and maturation, global DNA methylation levels progressively increase, and methylated DNA is concentrated in the highly condensed chromatin of the generative and sperm nuclei, in barley.

3. Microspore reprogramming and embryogenesis initiation is associated with global DNA hypomethylation and nuclear chromatin decondensation of early proembryo, while embryogenesis progression and embryo differentiation involves the increase of global DNA methylation levels and chromatin condensation, in barley.

4. Short treatments with the demethylating agent 5-azacytidine (AzaC) promote microspore reprogramming and embryogenesis initiation, producing DNA hypomethylation and chromatin decondensation, in rapeseed and barley.

5. Long treatments with AzaC prevent microspore-derived embryo development, indicating that DNA methylation is required for embryo differentiation, in rapeseed and barley.

6. The groups of Arabinogalactan proteins (AGPs) that contain the glycan epitopes JIM13, JIM14, LM2 and LM6, and the AGP gene *Sta 39-4* are induced after microspore embryogenesis initiation, in rapeseed. The groups of AGPs that contain the epitopes LM2 and LM6 are also induced after

microspore reprogramming and embryogenesis initiation in barley. In both species, AGPs localize in cell walls of early proembryos.

7. The groups of AGPs that contain the glycan epitopes MAC207, JIM13 and JIM14 are associated with pollen development at maturation stages, in rapeseed.

8. Blocking of AGPs by Yariv reagent impairs initiation and progression of microspore embryogenesis, in rapeseed and barley, indicating a key role of AGPs in the process.

9. Auxin levels increases after microspore embryogenesis induction, at the first embryogenic divisions and accumulate in cells of early proembryos, in barley.

10. Auxin action and polar auxin transport are required for microspore embryogenesis progression, since they are respectively prevented by the specific inhibitors PCIB and NPA, in barley.

11. The induction of microspore embryogenesis requires key factors as DNA hypomethylation, AGPs induction and auxin accumulation, independently of the inductor stress (4°C or 32°C) and the species (the monocot barley or the dicot rapeseed), indicating general regulatory mechanisms of the process.

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