Calcium and cell wall dynamics during microspore embryogenesis and doubled haploid production in rapeseed and eggplant

Alba Rivas Sendra

Thesis submitted in fulfillment of the requirements for the degree of Doctor in Biotechnology

Supervisor: Dr. Jose M. Seguí Simarro

June 2017

June 2017

Thesis layout and cover design: Alba Rivas Sendra and André Arôxa
Watercolor drawings: Alba Rivas Sendra

Acknowledgements

En primer lugar, quiero dar las gracias especialmente a Jose María, que me ha guiado en este proceso desde el primer momento. Me ha impulsado a crecer y, cuando no era posible, me ha traído un banquito para que pudiera alcanzar siempre más alto. Con tu ayuda me he convertido en una investigadora y todo lo que he aprendido me acompañará para siempre. Aunque a veces ponernos de acuerdo ha sido un reto, eso representa la esencia misma de la investigación y la ciencia, y lo agradezco sinceramente. Los retos siempre nos inspiran, nos obligan a aprender, a mantenernos activos y a tener menos miedo de las soluciones creativas. Y de esto es mejor tener los bolsillos llenos.

También quiero expresar mi sincero agradecimiento a Kim Boutilier. Ella me abrió sus puertas el primer día, y las mantiene abiertas desde entonces. No solo aprendí cosas nuevas de los mejores expertos, sino que la estancia en su laboratorio me permitió ver un mundo diferente, encontrar un segundo hogar, sembrar y recoger muchísimo amor expatriado increíblemente verdadero, y darme cuenta de que son las cosas más diminutas (como puede ser una pequeña bici rosa) las que llenan nuestras vidas de felicidad.

Familia, amigos, amigos que se convierten en familia, todos habéis tenido un papel en la parte de mí que queda reflejada en este libro. Los que siempre habéis caminado a mi lado, los que aparecisteis un día y ya nunca os marchasteis, los que acabáis de llegar pero es como si siempre hubieseis estado aquí, los que me dais la bienvenida cuando vuelvo a casa, donde quiera que mi casa esté, e incluso aquellos que solo me acompañaron durante una etapa... Todos vosotros habéis ayudado a dar forma a la persona que soy hoy. No hay un momento del que me arrepienta, un beso que desee nunca haber dado, una palabra que preferiría no haber oído. Ha sido un largo camino, pero esto es solo el comienzo. Las olas siguen llegando a la playa, las células siguen creciendo en nuestras placas de cultivo, y la vida, como el espectáculo, debe continuar. Lo único importante es ser feliz. Siempre hacia delante y apuntando alto, retroceder nunca, rendirse jamás (Corey Yuen, 1986).

First of all, I specially want to thank Jose María, who has guided me through this life-changing process since the very beginning. He encouraged me to grow, and when it was not possible, he brought me a stool so I could always reach higher. With your help I became a researcher, and everything I learnt will be with me forever. Even though sometimes it was challenging to come to an agreement, this represents the core essence of research and science, and I truly appreciate it. Challenges always inspire us, make us learn, keep us active and take away our fear of creative solutions. And it is always better to have plenty of these.

I also want to express my sincere thanks to Kim Boutilier. She opened her door to me on the first day, and still holds it open. Not only have I learnt new things from the best experts during my stay in her lab, but I got to see a different world, found a second home, sowed and harvested an amazingly huge amount of true and real expat love, and realised that it is the tiniest things (e.g., a tiny pink bike) which fill our lives with happiness.

Family, friends, friends who became family, you all played a role in the part of me that is recorded in this book. The ones who have always walked by my side, the ones who came one day and have never left, the ones who have just arrived but it feels like you have been here forever, the ones who warmly welcome me every time I come back home, wherever home is, even those who only stayed with me for a short while... All of you helped to shape the person who I am now. There is not a moment I regret, a kiss I wish I had never given, a word I would have preferred not to hear. It has been a long road, but this is only the start. Waves keep on hitting the beach, cells keep on growing in our culture dishes, and life, as the show, must go on. The only important thing is being happy. Always looking forward, aiming higher, no retreat, no surrender (Corey Yuen, 1986).

No retreat, no surrender (1986). Film directed by Corey Yuen. USA & Hong Kong.

Abstract

Androgenesis induction is an experimental procedure by which microspores are diverted from their original gametophytic pathway towards embryogenesis by applying specific stresses *in vitro*. It allows for the production of doubled haploid (DH) pure lines through anther culture or isolated microspore culture followed by chromosome doubling. DH technology is interesting for both basic research and plant breeding. In this Thesis, we studied microspore embryogenesis with two parallel approaches: (I) an applied study directed to the development of the first eggplant (*Solanum melongena*) highly embryogenic line and the improvement of the efficiency of eggplant microspore cultures; and (II) a fundamental research study focused on the relationship between microspore embryogenesis ability, intracellular Ca^{2+} levels and the dynamics of callose and cellulose deposition for cell wall formation in microspore-derived structures, using rapeseed (*Brassica napus*) as a model species.

As an applied research, we developed and evaluated an eggplant DH population from a commercial hybrid, and identified and characterized the first eggplant highly androgenic DH line (DH36), which may be used to facilitate the study of eggplant androgenesis and for both basic and applied research. In addition, we evaluated different factors involved in microspore embryogenesis induction efficiency in eggplant and optimized the regeneration protocol for DH production via microspore culture. Together, the applied research on eggplant microspore embryogenesis made in this Thesis resulted in the most efficient protocol existing to date for DH production in eggplant.

As a fundamental research, we studied the dynamics of Ca^{2+} during *in vivo* microsporogenesis and microgametogenesis, as well as during the first stages of *in vitro*-induced microspore embryogenesis, establishing a link between microspore embryogenesis and changes in Ca^{2+} levels and subcellular distribution. In addition, we studied the deposition of callose and cellulose during the first stages of microspore embryogenesis and demonstrated that the abnormally increased callose deposition and the inhibition of cellulose deposition observed in embryogenic microspores is most likely caused by a transient increase in the

intracellular Ca^{2+} levels that occurs right after microspore induction. We also found that this particular dynamics of callose and cellulose deposition is related to microspore embryogenesis ability and is essential for proper progression and success of microspore embryogenesis.

In summary, the research made in this Thesis helps to further understand the basis underlying microspore embryogenesis and cell totipotency, and to apply the powerful DH technology to an economically important crop such as eggplant.

Resumen

La inducción de androgénesis es un procedimiento experimental en el cual las microsporas se desvían de su vía gametofítica original hacia embriogénesis, mediante la aplicación de estreses específicos *in vitro*. Este fenómeno permite la producción de líneas puras dobles haploides (DH) mediante cultivo de anteras o cultivo de microsporas aisladas seguidos de duplicación cromosómica. La tecnología DH es interesante tanto para la investigación básica como para su aplicación a la mejora genética vegetal. En esta Tesis se estudia la embriogénesis de microsporas y la obtención de DHs con dos enfoques paralelos: (I) un estudio aplicado dirigido al desarrollo de la primera línea de berenjena (*Solanum melongena*) con alta respuesta androgénica y a la mejora de la eficiencia de los cultivos de microsporas de berenjena; y (II) un estudio de investigación básica centrado en la relación entre la habilidad para la embriogénesis de microsporas, los niveles intracelulares de Ca^{2+} y la dinámica de la deposición de calosa y celulosa para la formación de paredes celulares en estructuras derivadas de microsporas, utilizando como especie modelo la colza (*Brassica napus*).

Como investigación aplicada, se desarrolló y evaluó una población DH de berenjena a partir de un híbrido comercial, y se identificó y caracterizó la primera línea DH altamente androgénica de berenjena (DH36), que puede usarse para facilitar el estudio de la androgénesis en berenjena y para otros estudios aplicados o básicos. Además, se evaluaron diferentes factores implicados en la eficiencia de la inducción de embriogénesis de microsporas en berenjena, y se optimizó el protocolo de regeneración para la producción de DH mediante cultivo de microsporas. En conjunto, la investigación aplicada sobre la embriogénesis de microsporas realizada en esta Tesis proporciona el protocolo más eficiente existente hasta la fecha para la producción de DH en berenjena.

Como investigación fundamental, se estudió la dinámica del Ca^{2+} durante la microsporogénesis y la microgametogénesis *in vivo*, así como durante las primeras etapas de la embriogénesis de microsporas inducida *in vitro*, y se estableció un vínculo entre la embriogénesis de microsporas y los cambios en el nivel y distribución intracelular de Ca^{2+}. Además, se estudió la deposición de calosa y

celulosa durante las primeras etapas de la embriogénesis de microsporas y se demostró que la excesiva deposición de calosa y la inhibición de la deposición de celulosa, exclusivas de las microsporas embriogénicas, están causadas por el aumento transitorio de Ca^{2+} intracelular que se produce justo tras la inducción. Hemos demostrado que esta particular dinámica de la deposición de calosa y celulosa está relacionada con la capacidad androgénica, y que es fundamental para la correcta progresión y éxito de la embriogénesis de microsporas.

En resumen, la investigación realizada en esta Tesis ayuda a comprender mejor la base de la embriogénesis de microsporas y de la totipotencia celular, y a aplicar la potente tecnología DH a un cultivo económicamente importante como es la berenjena.

Resum

La inducció d'androgènesi és un procediment experimental en el qual les microspores es desvien de la seua via gametofítica original cap a un nou destí embriogènic, mitjançant l'aplicació d'estressos específics in vitro. Aquest fenomen permet la producció de línies pures dobles haploides (DH) mitjançant cultiu d'anteres o cultiu de microsporas aïllades seguits de duplicació cromosòmica. La tecnologia DH és interessant tant per a la recerca bàsica com per a la seua aplicació a la millora genètica vegetal. En aquesta Tesi s'estudia l'embriogènesi de microspores i l'obtenció de DHs amb dos enfocaments paral·lels: (I) un estudi aplicat dirigit al desenvolupament de la primera línia d'albergina (*Solanum melongena*) amb alta resposta androgènica i a la millora de l'eficiència dels cultius de microspores d'albergina; i (II) un estudi de recerca bàsica centrat en la relació entre la capacitat per a l'embriogènesi de microspores, els nivells intracel·lulars de Ca^{2+} i la dinàmica de la deposició de cal·losa i cel·lulosa per a la formació de parets cel·lulars en estructures derivades de microsporas, utilitzant com a espècie model la colza (*Brassica napus*).

Com a recerca aplicada, es va desenvolupar i avaluar una població DH d'albergina a partir d'un híbrid comercial, i es va identificar i caracteritzar la primera línia DH altament androgènica d'albergina (DH36), que pot usar-se per a facilitar l'estudi de l'androgènesi en albergina i per a altres estudis aplicats o bàsics. A més, es van avaluar diferents factors implicats en l'eficiència de la inducció d'embriogènesi de microspores en albergina, i es va optimitzar el protocol de regeneració per a la producció de DH mitjançant cultiu de microspores. En conjunt, la recerca aplicada sobre l'embriogènesi de microspores realitzada en aquesta Tesi proporciona el protocol més eficient existent fins avui per a la producció de DH en albergina.

Com a recerca fonamental, es va estudiar la dinàmica del Ca^{2+} durant la microsporogènesi i la microgametogènesi *in vivo*, així com durant les primeres etapes de l'embriogènesi de microspores induïda *in vitro*, i es va establir un vincle entre l'embriogènesi de microspores i els canvis en el nivell i distribució intracel·lular de Ca^{2+}. A més, es va estudiar la deposició de cal·losa i cel·lulosa

durant les primeres etapes de l'embriogènesi de microspores i es va demostrar que l'excessiva deposició de cal·losa i la inhibició de la deposició de cel·lulosa, exclusives de les microspores embriogèniques, estan causades per l'increment transitori del Ca^{2+} intracel·lular que es produeix just després de la inducció. Hem demostrat que aquesta particular dinàmica de la deposició de cal·losa i cel·lulosa està relacionada amb la capacitat androgènica, i que és fonamental per a la correcta progressió i èxit de l'embriogènesi de microspores.

En resum, la recerca realitzada en aquesta Tesi ajuda a comprendre millor la base de l'embriogènesi de microspores i de la totipotència cel·lular, i a aplicar la potent tecnologia DH a un cultiu econòmicament important com és l'albergina.

Index

Introduction

1. Microspore embryogenesis

Microspore embryogenesis is an experimental androgenic process by which the plant male gamete precursors are deviated from the gametophytic pathway to a new sporophytic fate, and induced to form a new haploid individual without fertilization (Seguí-Simarro 2010). Spontaneously or artificially, the genome of these individuals can be duplicated, giving rise to fully homozygous plants known as doubled haploids (DH). DH technology is interesting both for breeding purposes and for fundamental research. Although other ways of obtaining DHs exist, such as gynogenesis and wide hybridization, the present Thesis will be focused on microspore embryogenesis, the most deeply studied and effective androgenic alternative in most of the species studied (Wedzony *et al*. 2009). Occurrence of spontaneous haploid individuals was first reported in different species long ago (Blakeslee *et al*. 1922; Gaines and Aase 1926), but it was only in 1964 when the first protocol for *in vitro* haploid production, through anther culture, was developed (Guha and Maheshwari 1964). Nowadays, microspore embryogenesis can be induced in several angiosperm species. Up to 2003, DH production protocols were described for more than 250 species (Maluszynski *et al*. 2003) and DH cultivars are routinely used in agricultural production. The first DH crop plant released was the rapeseed (*Brassica napus*) cultivar "Maris Haplona" (Thompson 1972), followed by the "Mingo" barley cultivar (Ho and Jones 1980). However, in many crops of high agronomic interest, the efficiency of DH production is still very low, as is the case in the Solanaceae family. DH technology is only efficiently developed for tobacco, while the other major solanaceous crops (tomato, pepper, eggplant and potato) are still considered recalcitrant species at different degrees (Seguí-Simarro 2016; Seguí-Simarro *et al*. 2011b).

2. Methods for microspore embryogenesis

Most methods for microspore embryogenesis comprise two main *in vitro* steps: the induction of the androgenic process, and the regeneration of haploid or DH plants. The induction phase may be preceded by pretreatments of plants, flowers or anthers and usually consists in a stress treatment. In some species, embryos are directly obtained from the culture, and they can be transferred to a germinating medium and acclimated *ex vitro*. In other species embryogenic microspores develop into calli, and haploids or DHs can be obtained from them through an organogenesis procedure that usually includes several steps such as induction, elongation and rooting. These different developmental pathways often coexist in the same culture and their proportion depends on the genotype and culture conditions (Wedzony *et al*. 2009). From some haploid microspores, spontaneous duplication through nuclear fusion leads to the production of DHs (Seguí-Simarro and Nuez 2008b). When the percentage of spontaneous DHs is not enough for practical purposes, genome duplication treatments including colchicine, among others, must be applied in order to obtain DH individuals.

There are two main experimental *in vitro* methods to induce microspore embryogenesis: anther culture and microspore culture. Anther culture consists in the culture of whole anthers on a semisolid, agar-based medium. Microspores remain enclosed inside the anther locule and the surrounding anther tissues provide a favorable environment for microspore development (Seguí-Simarro and Nuez 2008a). Embryogenic microspores grow inside the anther until microspore-derived embryos (MDEs) are large enough to emerge from it. Anther culture is the most universal method for microspore embryogenesis and for DH production in general, since it is easy to perform and has lower technical requirements. However, the efficiency of the process is generally low, and most of the individuals obtained are haploid, which makes necessary the application of genome duplication treatments (Forster *et al*. 2007).

Microspore culture consists in isolating microspores from anther tissues and culturing them in liquid medium. This is a higher technically demanding procedure that implies more *in vitro* manipulation of the tissues. Since anther tissues are removed, the culture media and conditions are more complex but also more controlled (Kim *et al*. 2008). The fact that microspores are isolated also provides the certainty of completely avoiding the possibility of diploid somatic regenerants. Protocols for DH production via microspore culture are developed for a limited number of species, but in those where it is optimized, the efficiency is usually much higher than with anther culture and spontaneous chromosome duplication occurs more frequently (Forster *et al*. 2007; Seguí-Simarro 2010). A third method for microspore embryogenesis, called shed microspore culture, is also available but less used. In this method, a biphasic liquid-semisolid medium is created. Intact anthers are cultured floating in liquid medium. When anthers dehisce and open, microspores are spontaneously released, sinking and accumulating in the liquid-semisolid interface. The advantage of this method is that microspores are not mechanically released, they are cultured in liquid medium, but count with the presence of the remaining anther tissues, which is usually helpful (Supena *et al*. 2006; Wedzony *et al*. 2009).

3. Main factors involved in microspore embryogenesis

Donor plants

The physiological stage of the donor plants, the growth conditions, the plant age and the part of the plant from where the anthers are harvested have been found to be factors influencing the efficiency of androgenic induction. Unfortunately, there is not a gold rule to be applied to all species or genotypes, since interactions between genotype and growing conditions play also a role. For example, Burnett *et al.* (1992) found that embryo production increased with the age of *Brassica rapa* donor plants, but after two months the plants were too old

and feeble to produce enough buds. They also found that microspores from the primary inflorescences were unable to form any embryo, while anthers from primary tillers in most cereals are more responsive than those from lateral tillers (Dwivedi *et al*. 2015). Since the season has a strong effect over androgenic ability, growth under controlled conditions of light and temperature can often give better results both in terms of embryo production and in terms of regeneration. It has been reported that donor plants of barley grown in growing chambers produced less albino DH regenerants than donor plants grown in the greenhouse (Datta 2005).

The factors above mentioned are important, but less than the genotype. Indeed, the genotype of the donor plant determines the efficiency of *in vitro* (Datta 2005) and *in vivo* (Prigge *et al*. 2012) haploid production. This was evident since the very beginning of the development of microspore embryogenesis methods (Nitsch 1972). The observed interspecific and intraspecific variation is usually so pronounced that some lines or varieties of a species can be highly responsive while others present extremely poor response or are completely nonresponsive (Ferrie *et al*. 1995; Miah *et al*. 1985; Salas *et al*. 2011). Therefore, the identification of genotypes with high androgenic ability is an important step for making androgenic DH production a useful technology. This has been the goal of many studies in different species (reviewed in Ferrie and Caswell, 2011). For example, *Brassica napus* cv. Topas line DH4079 (Ferrie and Keller 1995), *Brassica rapa* cv. CV-2 (Ferrie *et al*. 1995), barley cv. Igri (Hoekstra *et al*. 1992), or wheat cv. Chris, cv. Pavon 70 and cv. Bob White (Kasha *et al*. 2003) are some of the highly embryogenic genotypes widely used both for basic and applied research, as well as for the development of new cultivars. However, they have only been developed in a very limited number of species where, in general, microspore embryogenesis protocols are already well developed. The identification of highly responding genotypes in other economically important crop species is a desirable event that would lead to a better understanding of the process and a more universal application. This is the case, for example, of eggplant, where despite its importance in global agriculture, a high response DH line has not been developed so far.

Developmental stage of the microspore

The stage of microspore development at the initiation of the culture is one of the most important factors influencing the ability of microspores to become totipotent and change their developmental fate. Microspores can only be switched to a sporophytic pathway when they are not yet committed to develop into pollen grains, but when they are sufficiently developed to be able to undergo a change in developmental fate towards embryogenic growth (Zaki and Dickinson 1991). For most species, the moment when microspores are susceptible to androgenic induction is around the first pollen mitosis, which comprises late, vacuolate microspores and early bicellular pollen. It has been suggested that this time point may be especially responsive to external stimuli because it is also a critical changing period during pollen development, the transition between microsporogenesis to microgametogenesis (Reynolds 1997). In most crops, microspore development inside the anthers is asynchronous, so the development of methods to determine markers for the selection of buds with the maximum proportion of potentially responsive microspores is essential for an efficient microspore embryogenesis induction (Bhowmik *et al*. 2011; Parra-Vega *et al*. 2013; Salas *et al*. 2012).

Stress treatment

To induce embryogenesis in microspores, a triggering stress is a common need in all the studied species. The most widely used stresses applied to induce microspore embryogenesis are cold or heat shock and starvation of carbon, nitrogen or both (Shariatpanahi *et al*. 2006). The way how stress treatments lead to changes in the metabolic and developmental pathways of microspores will be discussed later. Heat shock usually consists in incubation of anthers or isolated microspores at sublethal temperatures between 31 and 37 ºC for a variable time ranging from some hours to some days. Cold treatment is carried out at 4-10 ºC from some days to some weeks, and it can be done over isolated microspores or anthers, but also over buds, inflorescences, or even whole donor plants prior to

isolation (Islam and Tuteja 2012; Shariatpanahi *et al*. 2006). Besides the triggering stress, cold shock slows down degradation processes and increases the survival rate of microspores (Shariatpanahi *et al*. 2006). Sugar starvation can be achieved by culturing the microspores in media containing non-metabolizable carbon sources such as mannitol (Touraev *et al*. 2001), or by directly culturing microspores in distilled water as it is the case of eggplant (Miyoshi 1996). One of the main effects of starvation on microspore cultures is the re-entry of the vegetative nucleus of young pollen grains into cell cycle (Zarsky *et al*. 1992), which is normally arrested in G1 during gametophytic development shortly after the pollen first mitosis. This event leads to new vegetative cell divisions. A high number of other stresses have also been reported to induce microspore embryogenesis in some species. These stresses include reduced atmospheric pressure, colchicine, high pH, gamma irradiation, hypertonic shock, application of abscisic acid, auxins, feminizing agents, heavy metals, carrageenan oligosaccharides, mannitol, alcohols such as ethanol, benzyl alcohol or n-butanol, and different inducer chemicals (reviewed in Maraschin *et al*. 2005b; Shariatpanahi *et al*. 2006).

Medium and culture conditions

The constituents of the basal medium and combinations of growth regulators play an important role in DH induction. The reprogramming of microspores from gametophytic to sporophytic pathway depends upon the type and concentration of carbohydrates and plant growth regulators. The requirement of culture medium is species and genotype specific, and there is not a single culture medium suitable for microspore embryogenesis in all crops. However, there are some commonly used recipes for micro and macronutrients, such as potato-2, W-14, NLN, AT3 or A2 (reviewed in Murovec and Bohanec 2012), and they are often used for diverse species. For example, NLN medium initially developed for *Brassica napus* is also useful for *Apiaceae* (Ferrie *et al*. 2011) and *Solanaceae* (Kim *et al*. 2013; Miyoshi 1996). The type and concentration of carbohydrates is also essential. The most

common used carbon source is sucrose, often at concentrations as high as 10% (Kim *et al*. 2008) or 13% (Custers 2003), but the concentration has to be optimized for each species or genotype (Ismaili and Mohammadi 2016; Kim *et al*. 2008). The substitution of sucrose for maltose is sometimes beneficial for embryogenic induction and plantlet regeneration (Gémes Juhász *et al*. 2009; Nageli *et al*. 1999). Auxins and cytokinins control cell division and morphogenesis (Żur *et al*. 2015), hence an appropriate combination of growth regulators is essential for microspore embryogenesis induction and regeneration. However, the addition of exogenous growth regulators is not always needed. In fact, it is not needed in most of the species with high embryogenic response, such as wheat (Touraev *et al*. 1996b), tobacco (Touraev *et al*. 1996a), maize (Gaillard *et al*. 1991) or *B. napus* (Custers 2003), which suggests that these species have an endogenous concentration of growth regulators suitable for microspore embryogenesis. Conversely, exogenous application is needed for induction in species or genotypes with higher recalcitrance, or to help proper MDE development (Żur *et al*. 2015).

Conversion to plantlets

Once the embryogenic pathway has been induced, microspores can take a direct or indirect route to develop into a new individual, and both pathways often occur at the same time. In the direct route, embryos develop directly and proceed through the normal globular, heart-shaped, torpedo and cotyledonary embryo stages, in a way similar to that of zygotic embryos. Once MDEs have been formed, they are usually transferred to a solid germination media to promote their conversion into plants. In the indirect route, random, asynchronous and irregular divisions of the microspore result in the formation of a callus, from where it is possible to obtain haploid or DH individuals through a process of embryogenesis or organogenesis, which usually involves different steps for induction, elongation and rooting. Regeneration of embryos or calli into normal plants can be very difficult in some species, but little effort has been made in the study of regeneration requirements, since it is believed that the induction phase

determines the quality of androgenic embryos or calli, and that is the main factor influencing the regeneration capacity. However, great improvements could be done optimizing regeneration media and protocols because in many studies, the regeneration rate is low and a high quantity of promising induced structures are lost (Wedzony *et al*. 2009). Genotype, developmental stage of the embryo or physiological stage of the callus are important factors to take into consideration, but many different culture conditions such as medium composition, temperature, osmotic pressure, glutathione redox status, and concentration of growth regulators, microelements or vitamins can be modified in order to enhance normal development during the regeneration phase, improving the conversion into plants (Belmonte *et al*. 2006; Huang *et al*. 1991; Tian *et al*. 2004).

Chromosome duplication

Haploid individuals are sterile, and usually present smaller size, less vigor and higher susceptibility to stress and diseases. Therefore, for most practical uses it is desired to obtain DHs. Chromosome doubling occurs spontaneously in a variable percentage of regenerants after microspore embryogenesis, depending on the genotype (Barnabas *et al*. 1999; Henry 1998) and the *in vitro* culture conditions (Kasha *et al*. 2001; Shim *et al*. 2006). Different naturally occurring mechanisms can lead to chromosome doubling in cell plants: endoreduplication (DNA duplication without mitosis), nuclear fusion (mixing of two nuclei already separated) and endomitosis (failed mitosis in the absence of both mitotic spindle and nuclear envelope disassembly) (Seguí-Simarro and Nuez 2008b). Endoreduplication seems to be the most frequent mechanism *in vivo* (d'Amato 1984), but in the case of microspore embryogenesis, numerous studies point to nuclear fusion as the mechanism responsible for chromosome doubling during MDE development (reviewed in Seguí-Simarro and Nuez 2008b).

The ploidy level of the regenerated plants can be determined using flow cytometry (Ochatt 2008), chromosome counting (Maluszynska 2003), and molecular markers, especially SSR (Malik *et al*. 2011) and enzymatic mismatch

cleavage (Hofinger *et al*. 2013). Other less reliable methods such as plant morphology, size of stomata or chloroplast counting are also available (reviewed in Dwivedi *et al*. 2015). When the frequency of spontaneous DHs is not high enough, chemical treatments such as colchicine, trifluralin, oryzalin or amiprophos methyl can be applied to induce chromosome doubling. Among them, colchicine is by far the most frequently agent used. It binds to tubulin units, blocking polymerization and promoting depolymerization of microtubules, which leads to failure in the formation of the mitotic spindle (Seguí-Simarro and Nuez 2008b). The effect of colchicine results in a blockage of mitosis after DNA duplication, and therefore in genome doubling. In addition, the interference with microtubule formation may also affect other cytoskeletal structures, such as the phragmoplast, and facilitate nuclear fusion due to defective cell wall formation (Kasha *et al*. 2006; Parra-Vega *et al*. 2015b).

4. Other methods for DH production

Other androgenic methods

Haploid or DH callus formation can be induced from meiocytes instead of microspores. From these calli, plants are regenerated through organogenesis or indirect embryogenesis (Seguí-Simarro and Nuez 2007). This method is mostly studied in tomato, where it was possible to obtain complete DH plants in some cases (Seguí-Simarro and Nuez 2005; Zagorska *et al*. 2004), and it was reported possible in some other species such as Arabidopsis (Gresshoff and Doy 1972), *Vitis vinifera* (Gresshoff and Doy 1974) or *Digitalis purpurea* (Corduan and Spix 1975). However, this method has no practical use due to its low efficiency and the high rate of mixoploidy in the regenerant material. In some rare *in vivo* cases, a haploid embryo can be formed from a fertilized egg cell where the female nucleus has been inactivated or eliminated. The haploid zygote containing only chromosomes coming from the male parent develops into a haploid or DH embryo. This natural

phenomenon was described by Kostoff (1929), and it has been studied in more detail in maize (Chase 1969), but its extremely low frequency of occurrence makes it useless for practical haploidy induction.

Gynogenesis

In gynogenesis, the unfertilized egg cell (female gamete) is induced to start a sporophytic development. For that, flower buds or isolated ovaries or ovules are cultured in solid media (Fayos *et al*. 2015). This DH production method presents the advantage that reprogramming of the developmental pathway is not required, since the egg cell is already prepared for embryo developing. However, the embryogenic pathway has to be switched on without fertilization. In general, and although regenerants obtained through gynogenesis show higher genetic stability and a lower rate of albino plants (Dwivedi *et al*. 2015), this method is only selected as an alternative when androgenic methods are not effective, mostly because plants produce very fewer egg cells than pollen grains. In addition, gynogenesis has usually a low efficiency. Currently, gynogenesis is the method of choice for breeding species of the genera *Allium* and *Beta*, but a number of other species are responsive, such as barley, wheat, maize, mulberry, coconut, saffron and different *Cucurbitaceae* (Chen *et al*. 2011; Dong *et al*. 2016).

Wide hybridization

Wide hybridization or wide crossing is an *in vivo* method for haploid and DH production, which exploits haploidy from the female gamete and consists in pollination with pollen of a genetically distant species. Fertilization usually takes place, but chromosomes coming from the male parent are eliminated during the early stages of embryo development. For this reason, the endosperm fails to develop properly, which makes it necessary the use of embryo rescue techniques in order to obtain haploid regenerants. This *in vivo* method for haploid or DH induction was first used in barley, using *Hordeum bulbosum* as pollen donor, hence

the term *"bulbosum method"* to refer to this technique (Kasha and Kao 1970). It is the most frequently used method for production of haploids in maize (Prigge and Melchinger 2012) and a common technique in potato breeding (Rokka 2009). Successful attempts were also reported in several other species and crops such as fruit trees, tobacco, different cereals and *Brassica* spp. (reviewed in Dwivedi *et al*. 2015).

Irradiated pollen

Another alternative to produce DHs from unfertilized egg cells is the use of pollen irradiated to inactivate the generative cell and therefore make it unfertile. This has been used in some fruit trees, vegetables crops and ornamental plants (Murovec and Bohanec 2012). Usually, pollen grains germinate on the stigma and pollen tubes grow inside the style, which stimulates embryo development from the egg cell without fertilization. This technique has low efficiency and, as wide crosses, it requires embryo rescue due to the failure in endosperm formation.

Centromere-mediated chromosome elimination

In 2010, a new procedure for haploid production was developed. It is based in genetic modification of the histone CENH3, found in centromeric nucleosomes instead of the regular histone H3. CENH3 controls kinetochore assembly and recruits spindle microtubule attachment factors, which are essential for proper chromosome segregation during mitosis (Black and Bassett 2008). CENH3 is a variable histone, and when different alleles are confronted in a hybrid zygote, chromosomes with weaker alleles may lag behind those with stronger alleles, eventually leading to the elimination of the genome with weaker alleles. This mechanism could explain chromosome elimination phenomena in interspecific crosses. Intentional genetic modification of CENH3 can be used to produce mutated or truncated forms of CENH3 for selective uniparental genome elimination leading to haploid embryo formation (Ravi and Chan 2010). The

defective CENH3, when matched with a wild-type genome in a hybrid zygote, results in failure of chromosome segregation and the progressive loss of the chromosomes coming from the defective parent. The centromere-mediated genome elimination method for haploid production was initially developed in Arabidopsis (Ravi and Chan 2010), but several projects are currently running to apply it to other species including barley, cotton, sugarbeet, banana, tobacco or rice (reviewed in Tek *et al*. 2015).

5. Applications of DHs and DH technology

DHs in plant breeding

Pure lines or true breeding lines are highly homozygous plants traditionally obtained with conventional breeding methods that perform several generations of selfing and selection. The use of DH technology reduces the time and costs required to produce homozygous plants, as it allows a single-step development of complete homozygous lines from heterozygous donor plants (Germanà 2011a). The time saved for the development of a new cultivar using microspore embryogenesis can be up to 50% of the time needed with conventional breeding methods (Germanà 2011b). Once obtained, DH lines may be directly used in cultivar development or subjected to combinatorial ability tests to find the best lines to be used as parentals to produce hybrids. The commercial exploitation of heterosis and homogeneity in F1 hybrids makes the production of DHs an important tool for plant breeding and seed production (Dunwell 2010), that can even be used in woody species characterized by long generations and often self-incompatibility, an additional difficulty for breeding (Chiancone *et al*. 2015). To date, more than 300 DH-derived cultivars have been reported globally. From them, more than 100 cultivars correspond to barley and rice and more than 50 are rapeseed cultivars (Dwivedi *et al*. 2015). In Europe, half of the barley cultivars are produced from pure lines obtained using DH technology (Dunwell 2010). DHs

allow for a fast fixation of desirable traits in new lines or varieties, and it has been demonstrated that, when developed from a F1 hybrid, they can keep or even increase the advantage of heterosis achieved in the hybrid (El-Hennawy *et al*. 2011; Sanguineti *et al*. 1990). Moreover, the size reduction usually observed in haploid individuals may be of horticultural interest in some cases, such as ornamental plants or dwarfing rootstocks for fruit crops (Germanà 2011a). The major limitations for a more universal exploitation of DH technology and specially microspore embryogenesis are the cost of producing DH lines in sufficient quantities for a breeding program, and the recalcitrance of many species and genotypes, hence the importance of continued research input for a better understanding of the mechanistic underlying this process which would allow for its broader application.

DHs as an assisting tool for plant breeding

DH technology is very effective for speeding up plant breeding, as it represents a powerful tool for uncovering recessive traits, establishing marker-trait associations, mapping populations, genetic transformation and for selection and evaluation of lines, among other multiple applications. Most of these applications of DH technology, and especially of microspore embryogenesis, take advantage of the haploid nature of microspores, that can later become a totally homozygous diploid individual, and of the fact that they are a large population of single cells easy to manipulate.

The use of DH populations simplifies breeding and selection for recessive traits, since they are revealed in homozygous recessive individuals (Pauls 1996). DH populations are segregating populations of fully homozygous plants, where dominance effects are avoided and phenotype and genotype are directly linked, which makes easier marker-traits associations. The fact that they can be propagated without further segregation allows for the precise measurement of quantitative traits by repeated trials and for a reduction in the environmental component of the total phenotypic variance (Lu *et al*. 1996). In addition, the

simplified assembly of a haploid genome is the basis for several sequencing and map developing programs (Dunwell 2010; Sato *et al*. 2009). During a backcross process, DH individuals can be obtained at different generations, together with marker-assisted selection, to help fix the introgressed character (Belicuas *et al*. 2007; Toojinda *et al*. 1998). In somatic interspecific hybrids between crop species and their wild relatives, where often sterile and tetraploids individuals are obtained, anther culture can be used to bring back the ploidy level to the diploid status (Rotino *et al*. 2005).

Cultured microspores provide a large population of cells resulting from recombination and therefore showing gametoclonal variation, a genetic variability that could be exploited for breeding. In addition, they constitute an easy platform to target haploid cells for mutation treatment, and then capture the mutation in a homozygous line (Beversdorf and Kott 1987; Castillo *et al*. 2001). Regarding genetic engineering, the haploid nature of microspores and the possibility of culturing them as a population of single cells makes them a perfect target for genetic transformation at the haploid level, avoiding hemizygous transformants and allowing the elimination of detrimental random DNA integration in coding regions (Eudes *et al*. 2014; Kumlehn 2009). In these three cases, selection of specific traits can be made among *in vitro* cultured embryos, considerably reducing the number of plants to be evaluated in the field.

Microspore embryogenesis as a research platform

Plants are characterized by a high level of developmental plasticity, including the ability to form embryos from cells other than the zygote, in a phenomenon known as totipotency (Soriano *et al*. 2013). It may be induced *in vitro* using tissue culture procedures, and the relative ease with which microspores can be induced to reset to the earlier stages of development makes microspore embryogenesis an ideal system to study cell totipotency (Li *et al*. 2014; Pauls *et al*. 2006). Moreover, due to the inaccessibility of the egg cell and the zygote in angiosperm seeds, *in vitro* embryogenesis is a valuable tool to study important developmental events

that take place during early stages of embryogenesis. Although *in vitro* fertilization of egg cells is possible (Kranz and Lorz 1993; Kumlehn *et al*. 1999), it is a highly labor demanding procedure. Somatic embryogenesis may also be a good approach to study embryogenesis *in vitro,* but it presents several difficulties, especially for identifying and tracking single cells committed to embryogenesis inside a tissue (Zimmerman 1993). The fact that microspore derived embryos develop and grow in a suspension culture makes this system a perfect model to study several other plant processes related to embryo development, which are extremely challenging to perform in zygotic embryos. Microspore cultures have been used as platform for studies related to cell cycle regulation, cell division and proliferation (Daghma *et al*. 2014), cell-to-cell communication, cell identity and tissue patterning (Soriano *et al*. 2014) or autophagy (Corral-Martínez *et al*. 2013). These fundamental studies, which address very important biological processes, have been carried out using model species for microspore embryogenesis, such as barley and *B. napus*, since a high response and a well stablished protocol are required in order to make this system useful for basic and applied experimental research. However, a better understanding of the mechanisms underlying microspore embryogenesis, as well as cell totipotency and other developmental processes, are also needed in crop species.

6. The basis of microspore embryogenesis

The microspore is a haploid cell initially programmed to become a pollen grain. The asymmetric first pollen mitosis gives raise to two distinct, totally differentiated and highly specialized cells: the vegetative and the generative cell. The latter will eventually give rise to the two male gametes, the sperm cells. For androgenesis to occur, the microspore is induced to start a sporophytic growth. This fate change requires the complete reprogramming of the developmental plan, from pollen grain formation to a zygotic-like route. The embryogenic process that leads to MDEs can be divided in three overlapping phases (Maraschin *et al*. 2005b):

the induction or acquisition of embryogenic potential, the first cell divisions that form multicellular structures inside pollen exine, and the tissue patterning of the embryo once released from the exine.

Role of stress in embryogenic induction

During the first phase, a stress is needed to trigger the repression of the gametophytic pathway and the dedifferentiation of the microspore. The induction of the sporophytic pathway is only possible at early developmental stages when the microspore is still able to dedifferentiate and become totipotent (Touraev *et al*. 2001). It has been suggested that, in stress conditions, microspores change their developmental pathway as a part of a survival strategy (Shariatpanahi *et al*. 2006).

Asymmetric division, such as the first pollen mitosis, is the primary mechanism to generate cells with different fates (ten Hove and Heidstra 2008). In zygotic development, the first division is also asymmetrical and it establishes the apical-basal axis of the embryo (Friml *et al*. 2003). In conventional microspore embryogenesis systems, when microspores commit to *in vitro* embryogenesis, the occurrence of a symmetric cell division seems to be a general event to start the sporophytic pathway (Indrianto *et al*. 2001; Maraschin *et al*. 2005b; Seguí-Simarro *et al*. 2003; Zaki and Dickinson 1991). In these cases, embryo polarity is usually not established until the globular stage (Hause *et al*. 1994). Stress treatments applied for microspore embryogenesis induction modify many cell processes, and one of the most important for microspore embryogenesis is the occurrence of this symmetrical cell division, instead of the asymmetrical cell division that leads to the formation of the pollen grain. For example, heat stress causes a reorganization of the cytoskeleton that allows for the formation of a preprophase band and thus for symmetrical division (Simmonds and Keller 1999), cold pretreatment alters the actin filament network involved in the reorganization of the cytoplasm that contributes to microspore embryogenesis induction (Fábián *et al*. 2015) and the microtubule depolymerization caused by colchicine repositions the nuclei of vacuolate microspores in the center of the cell, which in turn leads to symmetric

division (Zaki and Dickinson 1991). However, another microspore culture system has been described in *B. napus*, which highly mimics zygotic embryo development (Supena *et al*. 2008). In this system, a milder and shorter heat stress (Joosen *et al*. 2007) or a continuous low-temperature stress (Prem *et al*. 2012) leads to an asymmetrical first cell division, which allows for the differentiation of an apical and a basal cell and to the formation of embryos with well developed suspensors. This milder stress induces cytoskeletal rearrangements in microspores that cause them to re-enter the cell cycle and to undergo a predictable sequence of cell divisions (Dubas *et al*. 2011). It has been demonstrated that, in this route, cell polarity in embryogenic microspores is induced and controlled by early dehiscence of the exine wall (Tang *et al*. 2013), accompanied by intracellular auxin polarity (Dubas *et al*. 2014; Soriano *et al*. 2014).

The wide variety of stress factors able to trigger the developmental switch in microspores may indicate that androgenesis induction is regulated by converging signaling pathways, and that different stress signals may activate the same downstream regulation events (Maraschin *et al*. 2005b). An analogous situation is found in the induction of somatic embryogenesis, regulated by different hormones and stresses, especially auxin (Filonova *et al*. 2000). Although stress is not directly related to zygotic embryogenesis, the level of endogenous auxin also increases after fertilization (Ribnicky *et al*. 2002). It has been suggested that downstream regulatory proteins, such as mitogen-activated protein kinase (MAPK) cascades may link auxin signaling to cell cycle regulation and cell fate determination in different types of cells committed to embryogenesis, since they are activated by auxin and stress-related signaling (Hirt 2000; Maraschin *et al*. 2005b; Seguí-Simarro *et al*. 2005).

Role of calcium in microspore embryogenesis

Heat stress, cold stress, application of alcohols, and possibly other stresses used to induce embryogenesis cause alterations in the cell membrane fluidity (Horvath *et al*. 1998; Vigh *et al*. 1985), and a transient increase in cytoplasmic Ca^{2+}

levels (Liu *et al*. 2005). Moreover, Saidi *et al*. (2009) confirmed the existence of a Ca^{2+}-permeable channel in the plasma membrane which is transiently activated both by mild heating and by chemical perturbation of membrane fluidity. Calcium can be stored within plant cells in different organelles and compartments. The most important Ca^{2+} stores are the vacuole and the cell wall, where it is tightly bound to pectins, and it plays a key role in plant cell wall physiology (Demarty *et al*. 1984). Recently, it was reported that cell wall AGPs act as calcium capacitors to supply Ca^{2+} to the cytosol on demand (Lamport and Varnai 2013). In the vacuole (Rudd and Franklin-Tong 2001), Ca^{2+} acts as a counter-cation for different inorganic and organic anions (White and Broadley 2003). Ca^{2+} stored in these compartments is mobilized to produce changes in intracellular concentration, which plays key signaling and regulating roles in multiple physiological and developmental processes. First of all, the heat-induced Ca^{2+} influx initiates a signaling cascade that induces many components of the heat shock response (HSR), but the function of Ca^{2+} as intracellular messenger is involved in most cell processes. As reviewed in White and Broadley (2003), gradients of Ca^{2+} are essential to control cell division and growth, determine the polarity and the location of pollen pores and the growing pollen tubes (Tirlapur and Willemse 1992) and play an important signaling role for the establishment of embryo polarity and seed germination (Hause *et al*. 1994). In the experimental procedure of microspore embryogenesis, Ca^{2+} has been suggested to be either necessary or helpful for embryogenesis induction in several plant species (Cho and Kasha 1995; Pauls *et al*. 2006; Reynolds 2000), where it seems to activate different signaling cascades, such as MAPK cascades and other phosphatase/kinase cascades (Pearce and Humphrey 2001). After embryogenic induction, calcium has also been reported to play an important role in germination of MDEs and their conversion into plants (Hause *et al*. 1994; Tian *et al*. 2004).

In addition, callose deposition is known to be Ca^{2+}-dependent in general (Verma and Hong 2001). A rapid increase in cytoplasmic Ca^{2+} levels is a common response in plant cells to several stresses, after a pathogen attack (Blume *et al*. 2000) and wounding (León *et al*. 2001), as well as a way to control stomatal

closure, all processes related to callose deposition. Recently, we demonstrated that the presence of callose in the newly formed cell walls of *B. napus* embryogenic microspores is abnormally extended over time and gives rise to transient callose-rich and cellulose-defective cell walls (Parra-Vega *et al*. 2015b), together with the formation of a callose-rich layer below the intine, which is considered an early marker of embryogenic commitment. It has been suggested that Ca^{2+} levels may play a regulating and controlling role in the altered dynamics of polysaccharide deposition observed in embryogenic microspores, but the specific way this regulation is performed and how it is related to the success of microspore embryogenesis still have to be elucidated.

Structural markers related to embryogenic induction

In order to be able to study and control the process, a number of markers for the acquisition of embryogenic potential and the initiation of cell division have been identified, most of them related to the degree of cytoplasmic dedifferentiation. Specific changes in the cell ultrastructure include organelle-free regions, decrease in the number and size of starch granules and lipid bodies, modification in the density of ribosomes, changes in the thickness of cell wall layers or appearance of new ones, cytoskeletal rearrangements, morphologic changes in mitochondria and migration of the nucleus to the center of the cell (Fábián *et al*. 2015; Maraschin *et al*. 2005a; Parra-Vega *et al*. 2015b; Rashid *et al*. 1982; Telmer *et al*. 1995). All of these changes lead to a cytoplasmic remodeling that allows for a symmetric division and the initiation of sporophytic development. Cytoplasmic remodeling occurs both via ubiquitin-26S proteasomal selective degradation of molecules (Maraschin *et al*. 2006) and via autophagy (Corral-Martínez *et al*. 2013), the main intracellular mechanism to degrade and recycle organelles. Both pathways have been showed to be activated under stress conditions (Bassham *et al*. 2006; Smalle and Vierstra 2004).

Tissue patterning and embryo development

After the initial induction and once the developmental pathway has changed, a series of cell divisions lead to the formation of multicellular haploid structures derived from the microspores. Two ways of embryo patterning have been described using *B. napus* microspore cultures as model. As described in Telmer *et al*. (1995), in the conventional microspore embryogenesis system (described in most species), the first divisions are often disorganized, and the first sign of pattern formation is the differentiation of the protoderm through periclinal divisions of the cells that surround the multicellular structure. Later on, embryo symmetry changes from radial to bilateral when cotyledon primordia show up at both sides of the apical meristem at the early heart-shaped stage. In the novel microspore culture system described by (Joosen *et al*. 2007) and (Supena *et al*. 2008), the embryogenic microspore undergoes an asymmetrical first cell division which gives raise to a basal and an apical embryo cell. The basal cell divides transversely to form a filamentous suspensor-like structure, and the apical cell develops into the embryo proper, following the same ordered pattern of cell division and early histodifferentiation as in zygotic embryos.

Embryo patterning mechanisms are complex, and multiple triggering and control mechanisms have been identified. In *Brassica napus*, when a MDE with suspensor is formed, the patterning of the embryo requires polar auxin transport, while when in absence of suspensor, the patterning is auxin-independent at first, but triggered by exine rupture accompanied by auxin gradient polarization (Dubas *et al*. 2014; Soriano *et al*. 2014; Tang *et al*. 2013). Arabinogalactan proteins have also been demonstrated to be involved in early pattern formation and polarity establishment in MDEs (Tang *et al*. 2006). At a later stage, a proper balance of auxins is needed to allow the switch from radial to bilateral symmetry (Ramesar-Fortner and Yeung 2006). The progression in the process of histodifferentiation gives raise to embryos containing all the tissues and organs found in zygotic embryos produced *in vivo* (Yeung *et al*. 1996). However, the majority of the sporophytic structures initially formed arrest and die after a few divisions. In

addition, not all the structures that start dividing randomly are able to eventually adopt an embryo tissue pattern, and the differentiation process can fail at different time points. It may even not start (Fan *et al*. 1988), or it may start and then rapidly switch to a callus-like undifferentiated growth (Corral-Martínez and Seguí-Simarro 2012).

7. Limitations of microspore embryogenesis

Although microspore embryogenesis is undoubtedly a powerful technology holding high interest in different research and development areas, there are still some limitations that difficult its universal application. Some specific technical limitations of the method are segregation distortion, the formation of unwanted somatic diploid regenerants from anther tissues and the high frequency of albinism in cereals. When DH individuals are obtained through duplication of a haploid gamete genome, the possibility of obtaining heterozygous descendants is eliminated, and the classical mendelian segregation is changed to a 1:1 ratio. The expression of deleterious recessive alleles may also distort segregation of specific genes. However, the most important factor affecting segregation distortion in DH populations are the genomic regions controlling the androgenic response itself, where the favorable alleles are usually overrepresented (Cloutier *et al*. 1995; Yamagishi *et al*. 1996). In anther culture, somatic and therefore diploid cells of the anther wall may proliferate and form unwanted somatic diploid callus or, to a lower extent, embryos. These somatic regenerants may be identified using molecular markers, but it implies an extra economic and labor effort. However, this is a minor limitation, and it can easily be overcome by using isolated microspore culture methods, when available. Finally, in the specific case of cereals, a variable rate of albino regenerants is obtained. In these plants, proplastids are unable to develop into functional chloroplasts, and the lack of chlorophyll makes them unable to perform photosynthesis, therefore their development is arrested at early stages. The biological reason for the high frequency of albinism in cereal MDEs is

being studied but has not been elucidated yet (Kumari *et al*. 2009; Makowska and Oleszczuk 2014).

Besides these technical inconveniences of the method, other main obstacles have to be faced in order to achieve a wider exploitation of this powerful technique. One of the main problems, already mentioned above, is the high genotype dependence and the recalcitrance of many important crops, such as solanaceous species, and research model species, such as *Arabidopsis thaliana*. Hundreds of protocols for different species are already available, but the real efficiency of most of them is still very low, and one of the most disregarded parts of the process is the regeneration phase. One of the motivations of the applied part of this Thesis is to improve the protocol for DH production in eggplant, a worldwide important crop still considered recalcitrant to microspore culture because of the formation of calli and the very low rate of conversion into plants.

Another important issue to be addressed is that, in general, basic research about microspore embryogenesis is mainly limited to species and genotypes with high embryogenic response, and fundamental research studies in important crop species are scarce. Since a good embryogenic response facilitates the use of DH technology as a research platform, the identification of genotypes with high androgenic ability is of key importance, not only in model species but also in crop species. To date they have only been identified in highly responding species such as barley, wheat or Brassica sp. One of the motivations of this Thesis is to provide further understanding of the basis underlying recalcitrance, by studying some fundamental aspects related to microspore embryogenesis, such as calcium and callose dynamics, and comparing the highly embryogenic *Brassica napus* to the still recalcitrant eggplant. In addition, we identified the first highly embryogenic eggplant DH line, that hopefully will help to achieve a better knowledge of the processes involved in microspore embryogenesis and cell totipotency, and to the development of efficient protocols, which is needed not only in model species but also in crop species.

Objectives

The main objective of this Thesis is the study of microspore embryogenesis, with two parallel approaches: (I) an applied study directed to the development of the first eggplant (*Solanum melongena*) highly embryogenic line and the improvement of the efficiency of eggplant microspore cultures; and (II) a fundamental research study focused on the relationship between microspore embryogenesis ability, intracellular Ca^{2+} levels and the dynamics of callose and cellulose deposition for cell wall formation in microspore-derived structures. These two approaches were organized in two main blocks, which in turn were divided into different chapters.

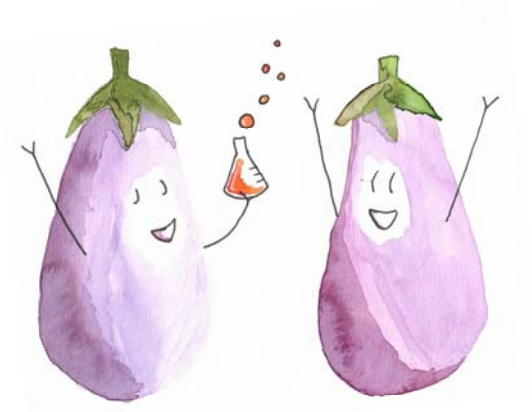
In the **First block**, we optimized the protocol for eggplant DH production via microspore culture, and provided new opportunities for the broader use of this species in the study of microspore embryogenesis.

- In **Chapter 1**, we developed and evaluated an eggplant DH population from a commercial hybrid, and identified the first eggplant highly androgenic DH line (DH36), which may be used to facilitate the study of eggplant androgenesis and embryogenesis for both basic and applied research.
- In **Chapter 2**, we made a further characterization of the *in vitro* performance of DH36 in microspore culture when exposed to different culture conditions, comparing it to some eggplant commercial hybrids. In this work we also provided more precise knowledge about the microspore embryogenesis induction in eggplant.
- In **Chapter 3**, we addressed the optimization of the process of regeneration of eggplant DH plants from microspore-derived calli, one of the main bottlenecks in eggplant DH production. We developed a regeneration protocol that substantially improves the efficiency of DH production published to date in eggplant.

In the **Second block**, the dynamics of Ca^{2+}, callose and cellulose deposition, their role in microspore embryogenesis and their relationship with embryogenic response were studied.

- In **Chapter 4,** we tracked calcium dynamics during *in vivo* microsporogenesis and microgametogenesis, as well as during the first stages of *in vitro*-induced microspore embryogenesis, establishing a link between microspore embryogenesis and changes in Ca^{2+} level and subcellular distribution.
- In **Chapter 5**, we studied the deposition of callose and cellulose during the first stages of microspore embryogenesis, and how it is modulated by different Ca^{2+} levels revealing a link between altered deposition of these polysaccharides and the androgenic response by comparing different genotypes.

First block



Common eggplant (*Solanum melongena*) is one of the most important vegetable crops worldwide. Within vegetable crops, it was ranked in the sixth position in 2014, with more than 50 million of tons produced in 2014, in the ninth position in terms of area harvested, with almost 2 million of hectares (FAOSTAT 2017). China being the main producer country, the first eggplant haploids were obtained through anther culture by a Chinese group of haploid breeding (1978) and soon after by Isouard *et al*. (1979). However, the work of Dumas de Vaulx and Chambonnet (1982) established the first reproducible anther culture protocol for the production of DH plants in eggplant. In this protocol, haploid or DH embryos are directly obtained from cultured anthers and can be germinated and transformed into plants. Adaptations of this method are commonly used to successfully obtain DHs useful for breeding programs (Seguí-Simarro 2016). DH eggplant pure lines obtained through anther culture have already been developed and used to produce hybrids with high yield (Sanguineti *et al*. 1990) or resistant to diseases (Rizza *et al*. 2002). The variability observed among androgenic DH obtained in anther culture, both in quantitative and qualitative traits, makes them useful for selection of lines with interesting characters for breeding (Rotino 1996). They are also used as a tool to assist research, for example to assess gene exchanges (Toppino *et al*. 2008) and to reduce the ploidy of tetraploid somatic hybrids between common eggplant and wild relatives (Rizza *et al*. 2002; Rotino *et al*. 2005). Anther culture, however, has several limitations, including the possible occurrence of somatic regenerants derived from anther walls, the uncontrolled contribution of tapetal cells to culture conditions, and the general low efficiency of the technique (Seguí-Simarro 2015; Seguí-Simarro 2016; Seguí-Simarro *et al*. 2011b). To avoid these problems, it is possible to perform isolated microspore culture, which is usually much more efficient.

Although the first report on eggplant microspore culture was published by Gu (in Chinese) in 1979, it was Miyoshi in 1996 who developed the first well documented protocol for eggplant microspore culture. In this study, the basis of the induction conditions, culture medium composition, calli production and regeneration of DH were established, but microspore development produced calli,

and regeneration of plants was achieved with a low frequency via indirect organogenesis. In 2009, Bal *et al*. developed an alternative method of androgenesis induction from eggplant microspores which gave rise to microspore divisions, but they failed to get calli or embryos. Recently, the application of several external factors proved to be useful to improve the induction efficiency (Corral-Martínez and Seguí-Simarro 2012; 2014). However, eggplant is still considered recalcitrant to microspore culture because the procedure presents two main bottlenecks: the production of calli instead of embryos and the low rate of plant regeneration from these androgenic calli. Although microspore-derived embryos are initially formed, they are unable to switch from radial to bilateral symmetry, and therefore they dedifferentiate to calli before transitioning to heart-shaped embryo (Corral-Martínez and Seguí-Simarro 2012). This fact implies the need to regenerate DH plants through organogenesis, but the studies performed to date achieved a very low rate (Miyoshi 1996) or only focused in the induction of organogenesis, without considering further steps of elongation and rooting (Corral-Martínez and Seguí-Simarro 2012). Overcoming these two main obstacles of eggplant microspore cultures would allow for a wider use of eggplant microspore embryogenesis both in applied plant breeding and in basic research.

Chapter 1

Development and characterization of an eggplant (*Solanum melongena*) doubled haploid population and a doubled haploid line with high androgenic response

Alba Rivas-Sendra, Manuel Campos-Vega, Antonio Calabuig-Serna and Jose M. Seguí-Simarro

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

Keywords

Androgenesis, anther culture, DH36, microspore culture, microspore embryogenesis.

Euphytica (2017) 213:89
DOI 10.1007/s10681-017-1879-3

Abstract

We developed an eggplant doubled haploid (DH) population from a commercial hybrid through androgenesis in microspore culture. Morphological variation, reproductive ability and androgenic responsiveness were evaluated. The DH population showed segregation in vegetative traits related to leaf, flower and fruit, and in reproductive traits such as fruit and seed setting or germination rate. The DH population and subsequent generations also presented variation in the androgenic response, with null, low and high response lines. From this population, we were able to identify the first eggplant highly androgenic DH line (DH36), remarkably similar to the donor hybrid in terms of morphology and reproductive ability, but stably producing four times more calli than the hybrid. The segregating DH population is potentially useful for genetic studies and mapping of several traits, whereas the highly androgenic line DH36 may be used as a model line to facilitate the study of eggplant androgenesis and embryogenesis for both basic and applied research.

Introduction

DH technology is of core importance in breeding programs, since it allows for the production of true breeding (pure) lines, homozygous for all traits, in a way faster and cheaper than classical breeding procedures. This reduces considerably the number of generations needed to produce a pure line, thus decreasing the costs of breeding programs (Germanà 2011b). In addition, the fully homozygous condition of DHs makes them a useful tool to facilitate the establishment of chromosome maps, mapping of genetic markers, marker-trait association and identification of recessive mutations (Forster *et al*. 2007).

One of the androgenic alternatives to obtain DH individuals is microspore embryogenesis, an experimental pathway by which microspores are redirected from their original gametophytic fate towards a new embryogenic program, giving rise to haploid individuals that are converted to DHs either spontaneously or with genome duplication techniques (Seguí-Simarro and Nuez 2008). Microspore embryogenesis is induced *in vitro* by applying specific stresses to microspores either still enclosed inside the anther (anther culture), or isolated and inoculated in liquid culture (isolated microspore culture). Anther culture is the most widely applied method for DH production due to its simplicity (Germanà 2011a). However, isolated microspore culture, although more technically demanding, presents additional advantages including a higher efficiency and the possibility to avoid the occurrence of regenerated somatic plants from anther tissue. Therefore, it constitutes a better way to investigate the biological processes involved in microspore embryogenesis. Microspore cultures hold high interest for research purposes, as they provide a large population of haploid cells in suspension. Any modification made at the haploid level will be carried by the DH plant regenerated, being especially useful for mutagenesis and genetic transformation (Eudes *et al*. 2014). In addition, isolated microspore cultures provide a unique opportunity to study diverse topics related to embryo development, such as cell identity and tissue patterning (Soriano *et al*. 2014), change of cell fate, cell proliferation

(Daghma *et al*. 2014), autophagy (Corral-Martínez *et al*. 2013), totipotency (Li *et al*. 2014), cell-to-cell communication and diverse genetic and genomic studies (Ferrie and Möllers 2011).

The efficiency of microspore embryogenesis varies greatly among species, even among genotypes within the same species (Ferrie *et al*. 1995). The determinant importance of the genotype in the efficiency of *in vitro* production of haploids is known since the first attempts of inducing it (Nitsch 1972). A poor embryogenic response limits the utility of DH technology in breeding programs and in both basic and applied experimental research, so the identification of highly embryogenic genotypes is a fundamental step for progressing in the practical application of this technology. Therefore, countless studies have screened for high androgenic response (reviewed in Ferrie and Caswell 2011). Highly embryogenic genotypes have been identified or developed in some species, and they are widely used for doubled haploidy research. For example, but not only, *Brassica napus* cv. Topas line DH4079 (Ferrie and Keller 1995), barley cv. Igri (Hoekstra *et al*. 1992), or wheat cv. Chris, cv. Pavon 70 and cv. Bob White (Kasha *et al*. 2003).

Common eggplant (*Solanum melongena*) is one of the most important vegetable crops worldwide and important breeding efforts have been made to improve its traits and performance. In terms of production, it was ranked in the sixth position in 2013, with almost 50 million of tons produced (FAOSTAT 2016). Eggplant DH embryos and plants can successfully be obtained through anther culture, and adaptations of the method developed by Dumas de Vaulx and Chambonnet (1982) are commonly used to obtain DHs useful for breeding programs (Seguí-Simarro 2016). They are also used as a tool to assist research, for example to assess gene exchanges (Toppino *et al*. 2008) and to reduce the ploidy of tetraploid somatic hybrids between common eggplant and wild relatives (Rizza *et al*. 2002; Rotino *et al*. 2005). However, eggplant is still considered recalcitrant to microspore culture because, although microspore-derived embryos are initially formed, they do not complete embryogenesis and transform into calli (Corral-Martínez and Seguí-Simarro 2012), making it mandatory additional steps to

regenerate DH plants from these calli through organogenesis (Rivas-Sendra *et al*. 2015). This is why the very scarce studies published about eggplant microspore culture are focused on the improvement of not only induction, but also regeneration rates (Corral-Martínez and Seguí-Simarro 2012; 2014; Miyoshi 1996; Rivas-Sendra *et al*. 2015), and no basic research has been done yet using eggplant microspore cultures as platform.

In the present work we developed an eggplant DH segregating population, characterized its phenotypic variation, and selected lines with high embryogenic response and heritable and stable performance, which hopefully will pave the way for a more intensive utilization of eggplant microspore cultures both in basic and applied research.

Materials and methods

Plant material

In this study, we used eggplant donor plants of cv. Bandera, a commercial F1 hybrid (Seminis Vegetable Seeds Ibérica, S.A., Spain) that showed good androgenic response in previous studies (Corral-Martínez and Seguí-Simarro 2012; Salas *et al*. 2011). Bandera was used as a control reference at each stage of the experiments. We also used a population of *in vitro* obtained DHs derived from Bandera, and a second generation of Bandera-derived DH plants (DHS1) obtained after selfing of the first DH population. Plants were grown in 30 cm pots under natural light at the greenhouses of Universitat Politècnica de València (Spain). Experiments were performed during five consecutive years. Twenty plants of Bandera were grown from March to July of the first year. One plant of each genotype of the DH population was grown from September of the second year to August of the third year. Their androgenic capacity was evaluated through anther culture from January to August of the third year. Twenty plants of each selected selfed DH genotype (S1 lines) were grown from March to August of the fourth year and from

February to May of the fifth year. Their androgenic capacity was evaluated through microspore culture during July and August of the fourth year and during April and May of the fifth year.

Generation of the DH population

Isolated microspore culture was performed according to Corral-Martínez and Seguí-Simarro (2014). Anthers containing mostly vacuolate microspores (Salas *et al*. 2012) were dissected from the bud, surface sterilized with 70% ethanol for 30 s and with 4 g/l sodium hypochlorite for 5 min, and rinsed three times in sterile distilled water. Anthers were crushed in a small beaker with sterile distilled water using a syringe piston, and the microspores contained in the locules were isolated by filtration through a 41 µm nylon mesh (Millipore), followed by three centrifugation steps at 100 *g* for 4 min each. Microspores were suspended in sterile distilled water at a final cell density of 500,000 microspores/ml, plated and incubated at 35ºC in darkness for 3 days to induce embryogenesis. After induction treatment, microspores were collected by centrifugation, resuspended at the same density in NLN medium (Nitsch and Nitsch 1967) supplemented with 20 g/l sucrose, 0.5 mg/l 1-naphthaleneacetic acid and 0.5 mg/l 6-benzilaminopurine, and incubated at 25ºC in darkness.

After 30 days of culture, new individuals were regenerated according to Rivas-Sendra *et al*. (2015). Calli larger than 1 mm were placed in MS medium supplemented with 20 g/l sucrose, 8 g/l plant-agar, 0.2 mg/l indole-3-acetic acid and 4 mg/l zeatin (Miyoshi 1996). Shoots showing a visible meristem surrounded by leaf primordia were transferred to MS medium with 8 g/l plant-agar. Shoots that did not develop roots before the stem elongated were cut and subcultured in fresh basal MS medium with 8 g/l plant-agar to induce rooting. Rooted shoots were finally transferred to pots with substrate and acclimated in a growing chamber. The ploidy of all the new individuals was checked by flow cytometry. DH individuals were transferred to the greenhouse and constituted the DH population used in this study.

Flow cytometry

Small pieces of young leaves taken from *in vitro*-produced plantlets at the moment of acclimatization (when at least 4 true leaves were formed) were chopped with a razor blade in 0.5 ml of nuclei extraction buffer from CyStain UV Precise P kit (Partec). Extracted nuclei was filtered through 30 µm CellTricks filters (Partec), 1.5 ml of DAPI-based staining buffer from CyStain UV Precise P kit (Partec) was added and samples were incubated for 2 min. Samples were immediately analyzed using a Partec CyFlow Ploidy Analyzer flow cytometer.

Anther culture of DH plants

Anther culture was performed according to Salas *et al*. (2011). Flower buds were surface sterilized with 70% ethanol for 30 s and with 4 g/l sodium hypochlorite for 5 min, and rinsed three times in sterile distilled water. Microspore stage from one anther of each bud was assessed under a light microscope and only anthers containing young and mid microspores were used for anther culture. As demonstrated by Salas *et al*. (2012), the unusual thickness of eggplant anthers makes that the best way to ensure that microspores are at the right stages (vacuolate microspores and young bicellular pollen) when medium components reach the anther locule, is to select anthers at previous stages (with young and mid microspores). According to Dumas de Vaulx *et al*. (1981), anthers were plated in C medium supplemented with 120 g/l sucrose, 8 g/l Bacto-agar, 5 mg/l kinetin and 5 mg/l 2,4-D, and incubated at 35ºC in darkness for 8 days, followed by 4 more days at 25ºC in 12/12 photoperiod. Then, they were transferred to R medium supplemented with 30 g/l sucrose, 8% Bacto-agar and 0.1 mg/l kinetin, and incubated in the same conditions. When embryos emerging from anthers were visible, the total number of embryos was recorded for each donor genotype.

Morphological characterization of the DH population

Morphological traits were recorded from individual plants of the original DH population and the parental hybrid Bandera using 10 primary descriptors developed by EGGNET (van der Weerden and Barendse 2007). These descriptors included leaf, flower and fruit characteristics. Leaf prickles, leaf surface shape, corolla color, fruit predominant color, fruit additional color, fruit additional color distribution, fruit cross section, fruit color intensity under calix and fruit calix prickles were measured in a scale with predetermined values corresponding to the EGGNET descriptors. Since this was a characterization of individual DH plants, no replicates could be made. The number of flowers per inflorescence was counted in five inflorescences per plant and expressed as a quantitative trait. Besides these primary descriptors, fruit production, seed production, and germination rate of the DHS1 were also recorded (Table 1). To assess the germination rate, 20 seeds of each line obtained after selfing were sterilized in 4 g/l sodium hypochlorite solution for 5 min, rinsed three times for 5 min each in sterile distilled water and plated in MS medium supplemented with 1.5% sucrose and 0.7% plant agar. Dishes with seeds were incubated at 25ºC with a 12/12 photoperiod and germination rate was recorded after 2 weeks.

Table 1. Morphological and reproductive traits evaluated and their description

Trait	Code	Units/description
Leaf prickles	L-Prickles	0 = none 1 = very few (1-2) 3 = few (3-5) 5 = intermediate (6-10) 7 = many (11-20) 9 = very many (>20)
Leaf surface shape	L-Surface	1 = flat 5 = intermediate 9 = very convex or bullate
Number of flowers per inflorescence	Fl-Number	Quantitative trait
Corolla color	Fl-Color	1 = greenish white 3 = white 5 = pale violet 7 = light violet 9 = bluish violet

Fruit production	Fr-Prod	Yes/No
Fruit predominant color	Fr-MainC	1 = milk white 2 = yellowish 3 = green 4 = unknown 5 =reddish 6 = lilac grey 7 = purple 8 = purple black 9 = black
Fruit additional color	Fr-AddC	1 = milk white 2 = yellowish 3 = green 4 = unknown 5 =reddish 6 = lilac grey 7 = purple 8 = purple black 9 = black
Fruit additional color distribution	Fr-CDistr	1 = uniform 3= mottled 5 = netted 7 = striped 9 = other
Fruit cross section	Fr-Section	1=circular, no grooves 3 = elliptic 5 = few grooves (~4) 7 = many grooves (~8) 9 = very irregular
Fruit color intensity under calix	Fr-UnderC	0 = none 1 = very weak 3 = weak 5 = medium 7 = strong 9 = very strong
Fruit calix prickles	Fr-Prickles	0 = none 1 = very few (<3) 3 = few (~5) 5 = intermediate (~10) 7 = many (~20) 9 = very many (>30)
Seed production	S-Prod	Yes/No
Germination rate	S-Germ	%

Data collection and statistical analysis

In order to evaluate the androgenic ability of the DH genotypes in anther culture, between 15 and 90 anthers of each genotype were cultured, except for the Bandera hybrid, where more plants were available and 135 anthers could be used. The number of embryos produced after 3 months was recorded and the efficiency was expressed as number of embryos produced/100 anthers. In order to evaluate the androgenic ability of the DH genotypes in isolated microspore culture, between 15 and 60 buds of each genotype were used. The number of calli produced was recorded after 30 days of culture and the efficiency was expressed as number of calli/ml of culture. Anther and microspore culture assays were repeated thrice. An ANOVA test ($p \leq 0.05$) was performed to assess global significant differences, and then a Fisher's least significant difference (LSD) test for multiple comparisons was performed in order to group the different genotypes in groups of homogeneity, considering significant differences when p-value was <0.05.

Results and discussion

A population of 80 DH individuals was developed from Bandera microspore cultures. Plantlets were regenerated through indirect organogenesis, acclimatized and hardened in growth chambers, and then grown to flowering in the greenhouse. Their ploidy was checked by flow cytometry, confirming their doubled haploidy. To ensure that each individual of the DH population had a different genotype, only one DH plantlet from each callus was selected and regenerated for this study. Their characterization in terms of morphological traits, reproductive fitness and androgenic competence is described and discussed next.

The eggplant DH population presented moderate morphological variability

Our population of regenerated DH plants showed variability in morphology and performance, as revealed by the different values of the descriptors used. Table 2 shows the values of Bandera, of a chosen DH line (DH36), and the average and ranges of the values of the entire DH population.

Table 2. Results of the evaluation of morphological and reproductive traits of the donor Bandera hybrid, the DH population and a DH line (DH36).

	Bandera Value	DH population Mean [range]	DH36 Value
L-Prickles	0	0.1 [0-7]	0
L-Surface	1	1.5 [1-3]	1
Fl-Number	3-5	3.9 [2-7]	4-5
Fl-Color	7	6.9 [4-8]	7
Fr-Prod	Yes	85% Yes – 15% No	Yes
Fr-MainC	7	4.8 [1-9]	1
Fr-AddC	1	3.5 [1-7]	7
Fr-CDistr	7	6.9 [1-7]	7
Fr-Section	1	1.0 [1-1]	1
Fr-UnderC	9	9.0 [9-9]	9
Fr-Prickles	7	5.2 [1-9]	9
S-Prod	Yes	83% Yes - 17% No	Yes
S-Germ	100%	84.9 [0-100]	95%

Data for Bandera and DH36 genotypes are values of each parameter. Data for the DH population are expressed as the value average and range (bracketed) of each parameter.

Two of the descriptors measured for the fruit, Fr-Section and Fr-UnderC, showed no variability among the DH individuals or compared with the parental hybrid. Fruits always presented a circular section and no grooves (value 1), and the intensity of color under the calix was always the same as in the rest of the fruit (value 9). For the descriptors that presented variability (Figure 1), the most frequent group always corresponded to the value of cv. Bandera, except in the case of Fr-Prickles. This character was highly variable, ranging from less than 3 (value 1) to more than 30 (value 9), but as a group, the DH individuals presented notably fewer prickles in the fruit calix than the parental hybrid.

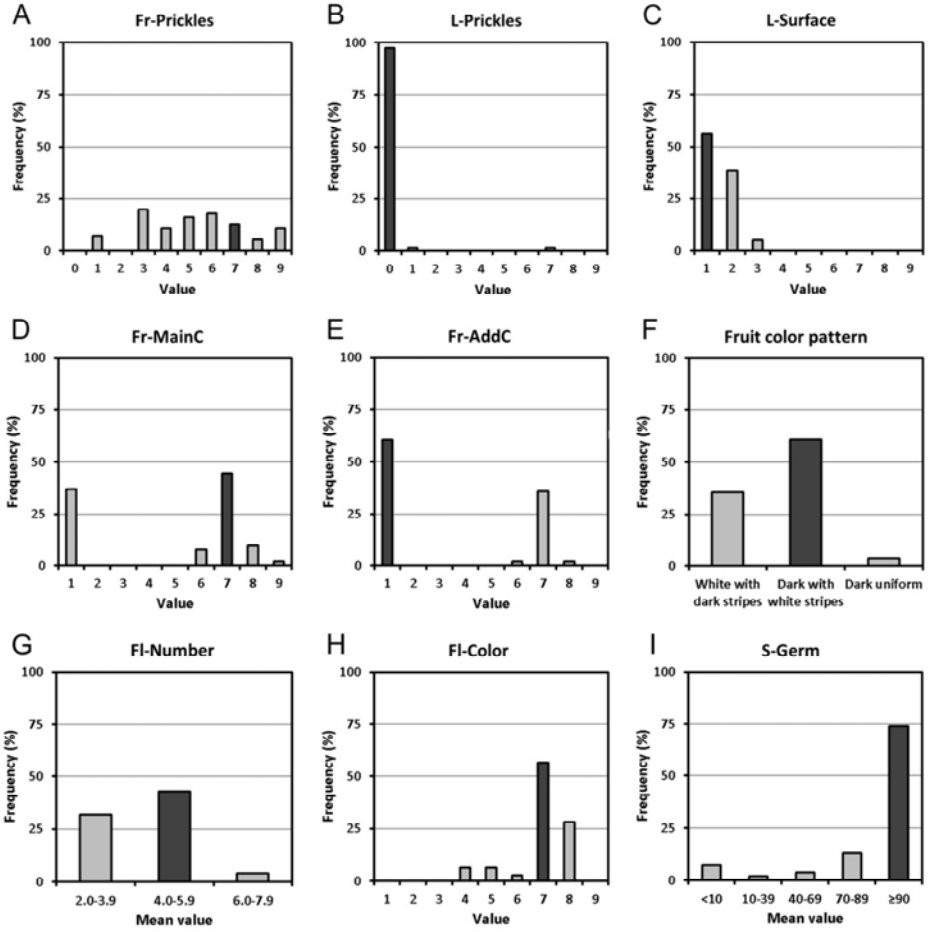
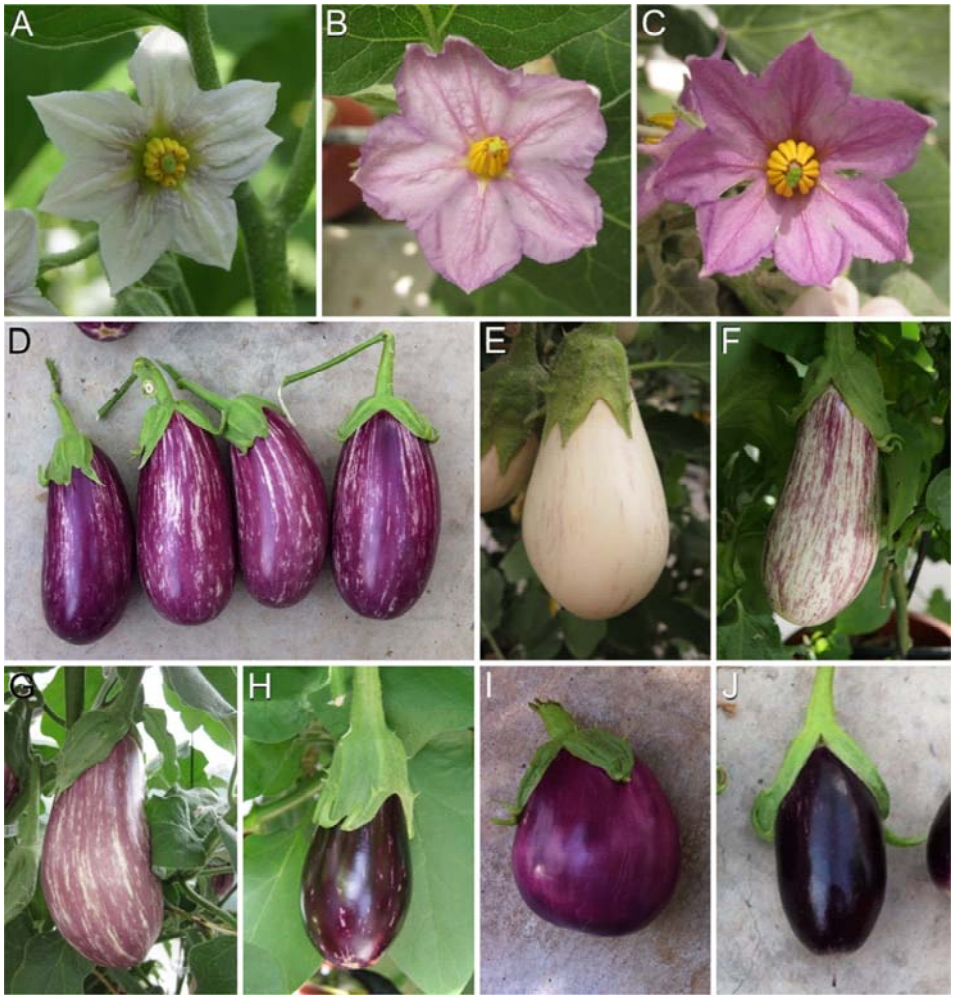


Figure 1. Graphical representation of the variability shown by the DH population. Each chart corresponds to each of the morphological descriptors used. The light grey columns correspond to the values of DH lines, whereas the dark grey columns correspond to the values of cv. Bandera which, with the exception of Fr-Prickles, were always the most frequent values in the DH population.

Leaf related characters presented variability in a short range of values. Most of the genotypes did not have prickles on the leaf, and the bullate of the leaf surface was slightly variable. Flower color (Figures 2A-C) presented variability from almost white (value 4) to strong pinkish-violet (value 8). The number of flowers per inflorescence was highly variable among DH genotypes, ranging from 2 to 7. At commercial ripeness, skin color of Bandera fruit (Figure 2D), which belongs to the

varietal type '*Listada*' (Nuez and Llácer 2001), was characterized as purple with white stripes (Table 2). In our derived DH population, primary and secondary fruit colors presented an interesting variability, being either white or with different purple shades from lilac grey (value 6) to black (value 9). Color distribution was mostly stripped, but 4% of the genotypes presented uniformly colored fruits. For an easier comparison, fruit color patterns were grouped in 3 categories: white with dark stripes (Figures 2E, F), dark with white stripes (Figures 2G, H) and uniformly dark (Figures 2I, J).



◄ **Figure 2.** Morphological variability of the DH population. A-C: Flowers with different petal color including white (A), pinkish (B) and purple (C). These flowers present unusual piece numbers (6-7) and different levels of cohesion in some of their petals. D: Bandera fruits showing, as expected, a remarkable phenotypical homogeneity. E, F: DH fruits with white background and different levels of dark stripes. G, H: DH fruits with different levels of dark background and white stripes. I, J: DH fruits with different levels of uniformly dark background.

All the variability above described was restricted within the ranges of predetermined values of the primary morphological descriptors used. However, we also found some characters not included in these descriptors or values. While the standard number of petals in eggplant flowers is usually five (Frary *et al*. 2007), DHs frequently presented a higher number of whorl pieces, with anther and petal numbers ranging from six to eight and with different degrees of cohesion, as shown in Figures 2A-C. This feature is commonly found in eggplant varieties with globose and round fruit types (Frary *et al*. 2007). Abnormalities were also observed on the leaf surface. While the parental hybrid Bandera had no prickles on the leaf surface (Figure 3A), 2.6% of the individuals showed prickles on the midrib and secondary leaf veins (Figure 3B). However, the most striking abnormality was the presence, in several DH individuals, of small leaf blades growing on the midrib, perpendicular to the main leaf blade (Figures 3C, D). These ectopic leaf-like structures were only observed in young plants cultured in growing chambers, being absent in adult plants, once transferred to the greenhouse. The transient nature of this trait suggests that it might be related to the different environmental conditions of growth chambers and greenhouses, being light intensity and duration the most likely influencing parameters. Indeed, eggplant is known to be highly dependent on light conditions (Uzun 2007). Abnormal morphologic traits were also found in other studies dealing with DH populations. For example, Malik *et al*. (2008) found that some highly responding *B. napus* DH lines presented epinastic leaves, non-abscised petals and pale flower color. Nevertheless, these unusual traits appear irrelevant as long as they do not compromise biological performance or agronomical usefulness.

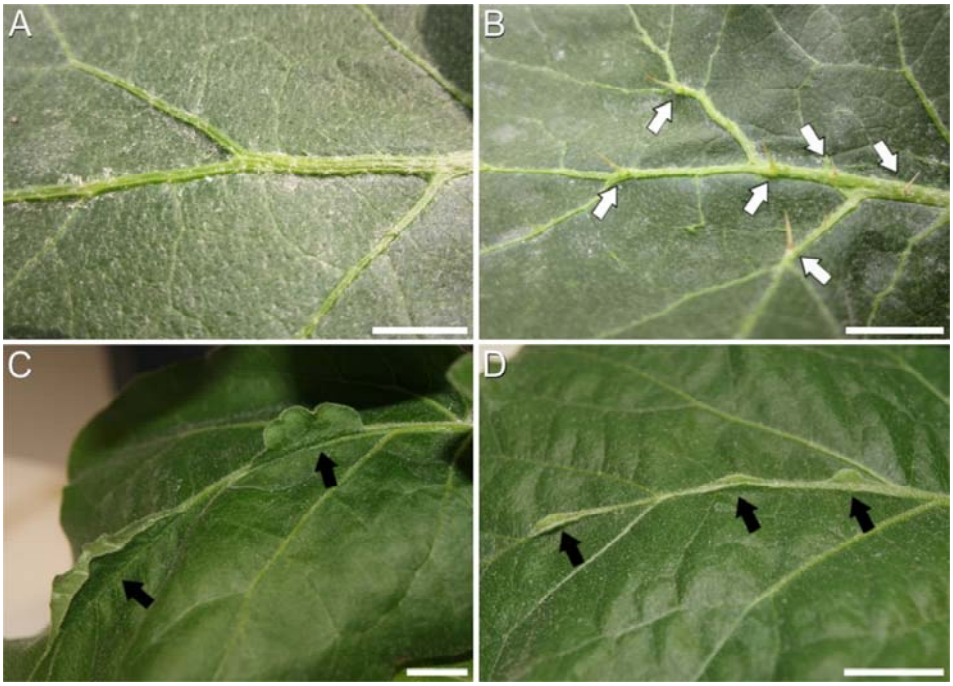


Figure 3. Examples of leaf surfaces of the Bandera hybrid (A) with no prickles, and of some DH individuals with prickles (white arrows) on the midrib and secondary leaf veins (B), and with large (C) and small (D) leaf blades (black arrows) on the midrib. Bars: 1 cm.

This variability generated in gametic cells cultured *in vitro* is referred to as gametoclonal variation (Veilleux 1998) and may be caused by meiotic recombination, by spontaneous mutations or by the process of *in vitro* culture itself (Kaeppler *et al*. 2000; Malik *et al*. 2008). One advantage of doubled haploidy is the possibility to unmask, fixing them in homozygosity, many of these events that might otherwise remain silent due to dominance effects in heterozygous individuals. Indeed, previous studies in eggplant demonstrated the convenience of DH lines as a powerful tool to reveal variability in diverse important agronomical traits, including fruit number and yield (Rotino *et al*. 1991), even in cases when donor plants had a high level of homozygosity (Sanguineti *et al*. 1990). It is known that the impact of gametoclonal variation differs among species from null to high (Snape *et al*. 1988). Our study, together with others (Rotino *et al*. 1991), showed

that in the particular case of eggplant, gametoclonal variation exists and may be used for breeding purposes, but it is not dramatic.

The eggplant DH population presented a slightly reduced reproductive ability

All individuals of the DH population were selfed to produce a second DH generation (DHS1) through which we evaluated the reproductive ability of the DH population. As seen in Table 2, 85% of the DHs were able to set fruits. From them, 83% produced seeds. The germination rate was higher than 70% in 87% of the lines, and only 7% produced seeds unable to germinate in our conditions (two weeks at 25ºC and 12/12 photoperiod). In summary, 63% of the individuals of the total DH population could be reproduced by seed. These results indicate that the reproductive traits of our DH population are slightly affected. This is not surprising, since DH lines fully express deleterious recessive genes that may be masked by dominance in the heterozygous parental. The strong selection pressure during the *in vitro* regeneration eliminates genotypes with major lethal genes, but this selection is only effective on genes related to vegetative growth, while no selection pressure is exerted on reproductive traits (Bohanec 2002). Other possible causes of the reduced reproductive ability include increased seed dormancy, low pollen fertility due to high frequency of meiotic irregularities such as formation of univalents, trivalents, or unequal chromosome segregation, known to occur as a consequence of *in vitro* culture (Doğramacı-Altuntepe *et al*. 2001; Immonen and Robinson 2000), or due to the particularities of the species used (Oleszczuk *et al*. 2011). Alternatively, inbreeding depression may also be a source of reduction in reproductive ability, which makes reasonable to expect the moderate signs of reduced reproductive ability we found. However, eggplant is generally considered a self-pollinating species (Frary *et al*. 2007), so it is expected to be able to bear high or complete homozygosis without important detrimental effects.

The DH population showed a wide range of androgenic competence

The androgenic competence of the 66 surviving DHs (the rest up to 80 aged or died before this assay started) and the donor hybrid Bandera was compared by culturing anthers of each single plant. Although microspore culture is the method of choice due to its higher efficiency, this method requires a minimum number of buds at the right stage for microspore isolation. This is possible when working with homogeneous populations (e.g. hybrids or DH lines), but when analyzing individual plants, the amount of available buds at a given time is insufficient. This is why we opted for anther culture. Bandera yielded 146.5 embryos/100 anthers, a value that was set as the reference. The response of DHs was variable among lines, ranging from 0 to 237.5 embryos/100 anthers (Table 3). 29 DHs did not produce any embryo (omitted in Table 3). A Fisher's least significant difference (LSD) test for multiple comparisons grouped the embryogenic yield of these DH lines in 5 categories of homogeneity. The individuals with highest yield, belonging to the higher group (e in Table 3), included DH36, DH40, DH15, DH39, DH72, DH41 and DH34, together with the parental hybrid Bandera. Five of them produced more embryos than Bandera (up to 1.6x). However, these increased yields were not significantly different from Bandera. The DH individual with the highest yield, DH36, only appeared in the highest category, being absent from the others.

DH plants were selfed and DHS1 seed was collected separately from each line. To further assess their androgenic competence, our goal was to evaluate to what extent androgenic competence was inherited in the next seed generation. We aimed to use DHS1 plants from the seven genotypes with the highest yield as donor plants for isolated microspore cultures, and then compare their efficiency with the parental hybrid. However, DH39 did not produce any fruit and DH34 and DH72 produced very few seeds with a low germination rate (less than 60%), so they were discarded.

Table 3. Androgenic competence in anther culture of DH individuals and the donor hybrid (Bandera). Fisher's least significant difference (LSD) test found statistically significant differences among the androgenic competence of the DHs, as well as compared to Bandera.

Genotype	Anthers plated	Responding anthers	Embryos/100 anthers (mean ± SD)	LSD				
				G1	G2	G3	G4	G5
DH47	86	1	1,19 ±1.68	a				
DH68	48	1	2.63 ±3.72	a	b			
DH50	29	1	3.33 ±4.71	a	b			
DH52	46	2	4.76 ±6.73	a	b			
DH76	32	1	7.14 ±10.10	a	b			
DH13	71	4	7.69 ±10.88	a	b			
DH46	87	4	8.54 ±12.07	a	b			
DH33	28	1	8.82 ±12.48	a	b			
DH22	37	1	9.09 ±12.86	a	b			
DH14	28	1	10.00 ±14.14	a	b			
DH20	23	1	10.00 ±14.14	a	b			
DH75	38	1	10.00 ±14.14	a	b			
DH64	19	1	10.71 ±15.15	a	b			
DH21	23	1	11.76 ±16.64	a	b			
DH66	24	2	12.22 ±1.57	a	b			
DH60	36	2	14.29 ±20.20	a	b			
DH63	32	1	14.29 ±20.20	a	b			
DH19	37	2	14.58 ±20.62	a	b			
DH53	27	2	14.71 ±20.80	a	b			
DH70	40	3	14.79 ±6.03	a	b			
DH37	62	2	20.45 ±28.93	a	b	c		
DH43	33	2	22.22 ±31.43	a	b	c		
DH42	23	2	22.35 ±19.82	a	b	c		
DH31	43	1	23.08 ±32.64	a	b	c		
DH29	60	2	27.47 ±11.91	a	b	c		
DH65	29	2	30.28 ±35.75	a	b	c		
DH30	85	1	37.21 ±52.62	a	b	c		
DH16	30	3	38.24 ±54.07	a	b	c		
DH17	35	2	47.37 ±66.99	a	b	c		
DH74	33	5	68.33 ±73.07	a	b	c		
DH34	47	2	72.22 ±102.14	a	b	c	d	e
DH41	54	5	74.24 ±104.99	a	b	c	d	e
Bandera	135	24	146.46 ±137.45		b	c	d	e
DH72	18	3	157.69 ±201.25	a	b	c	d	e
DH39	29	6	162.50 ±229.81	a	b	c	d	e
DH15	31	3	185.00 ±261.63			c	d	e
DH40	23	5	231.82 ±327.84				d	e
DH36	22	1	237.50 ±335.88					e

Means are placed in five homogeneity groups (a-e). Note that one DH individual can belong to more than one homogeneity group, and means followed with at least a common letter are not statistically different at $p<0.05$. All the non-responsive genotypes (not shown in this table) belonged to group a.

The androgenic ability in isolated microspore culture of the remaining four DHS1 lines was higher than Bandera, which yielded 65.1 calli/ml (Table 4). The differences in yield between the DH lines and 'Bandera' were higher than for anther culture, ranging from 1.2 to 4.1-fold. DH36 was also in this case the line with the highest callus yield (compare Figures 4A and B).

Table 4. Androgenic competence in isolated microspore culture of DHS1 lines derived from DH individuals and the donor hybrid (Bandera).

Genotype	Calli/ml (mean ± SD)	LSD[a]
Bandera	65.08 ±54.11	a
DH15	76.85 ±68.56	a
DH41	92.00 ±27.65	b
DH40	149.11 ±79.49	c
DH36	267.36 ±33.96	d

[a] Fisher's least significant difference (LSD) test found statistically significant differences among the androgenic competence of DHS1 lines and Bandera. Means are placed in 4 homogeneity groups (a-d).

When evaluated in anther culture, the DH lines showed a high variability in their androgenic response, as shown by the high values of SD in Table 3. This variability was notably reduced when androgenic response was evaluated in microspore culture, as shown in Table 4. A high number of factors, difficult to control, influence the efficiency of microspore embryogenesis, including growing conditions of donor plants, seasonal effects, and *in vitro* culture conditions (Rotino 1996). Among the *in vitro* conditions, anther tissues have a prominent role in the excretion of different substances that may promote or inhibit embryo development (Seguí-Simarro *et al*. 2011), making the exact composition of the culture medium more unpredictable than in microspore cultures. This is why it is not surprising to find higher variability in the response of microspores to anther culture than to microspore culture. Microspore culture is a more technically demanding procedure, but once optimized it allows for a higher control of culture conditions,

which in turn provides more stable efficiency (Forster *et al*. 2007), as it was the case in our experiments.

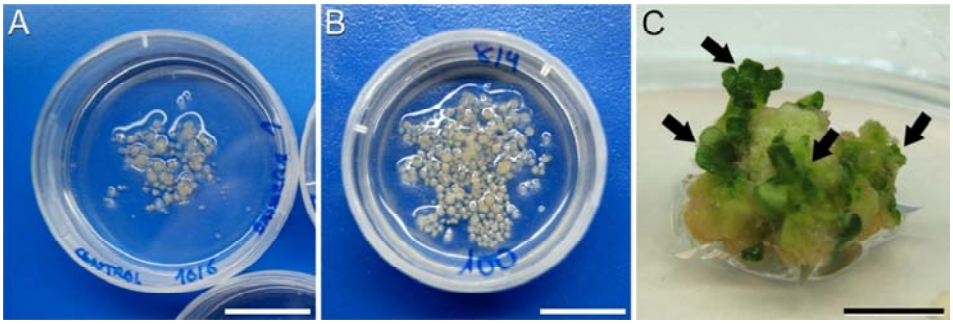


Figure 4. Isolated microspore culture dishes of Bandera (A) and DH36 (B). Note the difference between genotypes in terms of androgenic response. C shows a microspore-derived callus with shoot organogenic nodules (arrows) developing on its surface. Bars: 1 cm.

Our results clearly showed that androgenic response is highly variable in the segregating DH population. Since plants were cultured together under the same conditions, such variability suggests that androgenic competence is a genetically controlled trait, and not all DH regenerants in our population carry the most favorable allele combinations. Genetic control of androgenic responsiveness has already been studied in several species, including rice (Miah *et al*. 1985; Quimio and Zapata 1990; Yamagishi *et al*. 1996), *Brassica napus* (Zhang and Takahata 2001) and even eggplant (Salas *et al*. 2011). In short, the conclusion of these studies was that this is a recessive trait controlled by a few recessive nuclear genes, with strong additive effects. Due to this, efforts have been made to breed for improved androgenic response. In maize, Petolino *et al*. (1988) showed it possible, and suggested that anther culture *per se* allows for the selection of genes favoring an increased response. In their experiments, a single cycle of selection resulted in more than a six-fold increase. Similarly, Malik *et al*. (2008) obtained dramatic increases from initially recalcitrant genotypes in *B. napus*. The four-fold increase in microspore culture after a single *in vitro* DH generation adds to these evidences, including eggplant in the list of species where breeding for improved androgenic response is possible.

DH36 is a DH line with stably high embryogenic competence

Our DH36 line had an average yield of 237.5 embryos per 100 anthers cultured, and 267.36 calli/ml in isolated microspore culture. Previous reports on eggplant androgenesis with different genotypes found results far behind the yield of DH36. In anther culture experiments, the response of the commercial hybrids Ecavi (Salas *et al*. 2011) and Cristal (Salas *et al*. 2012) was reported to be 60.9 and 53 embryos/100 anthers, respectively. Other studies reported maximal responses of 14.2 embryos (Başay *et al*. 2011) and 3.67 embryos/100 anthers with the most responsive of the genotypes used (Alpsoy and Seniz 2007). In microspore culture, Corral-Martínez and Seguí-Simarro (2012) reported 5 calli/ml for Ecavi and 2 calli/ml for Cristal. The yield of the DH36 line obtained in our study greatly surpassed all other genotypes previously tested, including Bandera donors. In addition, DH36 calli showed a good organogenic and regenerative performance (Figure 4C). In general, it is thought that genotypes with highly androgenic response use to be recalcitrant to organogenesis and regeneration, which makes them difficult to use for genetic transformation (Malik *et al*. 2008). Our observations showed that this seems not the case for DH36. In addition, as seen in Table 2, the values of morphological descriptors for DH36 were identical to Bandera, with the exception of some fruit traits including predominant (1 vs 7) and additional color (7 vs 1) and the presence of prickles (9 vs 7). In terms of reproductive ability, the only difference (minor) was a seed germination rate of 95% vs 100% in Bandera. In conclusion, DH36 could be considered a high response line, phenotypically and agronomically similar to the donor hybrid (Bandera), genetically stable and self-perpetuating, and therefore useful for basic and applied research in eggplant microspore embryogenesis.

Concluding remarks

We developed here an eggplant DH population from a commercial hybrid

which showed variability in morphological and reproductive traits, as well as in androgenic competence. This population can be perpetuated by seed without further segregation, which makes it useful for genetic analysis and mapping of segregating characters. It may also be used to facilitate the study of the genetic control of androgenic competence in eggplant. We also developed the, to the best of our knowledge, first eggplant DH line with high androgenic response. The use of this line will provide material to be used both for basic research about morphogenesis, and for applied research of *in vitro* DH production. It could even be used to explore the possibility of using crosses to transfer its androgenic competence to recalcitrant genotypes of agronomic interest. Hopefully, this high response line will encourage the use of eggplant as a research platform for the study of this fascinating experimental phenomenon.

Acknowledgements

We would like to thank the reviewers of this manuscript for their critical and helpful comments. This work was supported by grant AGL2014-55177-R to JMSS from Spanish Ministerio de Economía y Competitividad (MINECO) jointly funded by FEDER. ARS is supported by a predoctoral fellowship from the FPI Program of Universitat Politècnica de València.

References

Alpsoy HC, Seniz V (2007) Researches on the *in vitro* androgenesis and obtaining haploid plants in some eggplant genotypes. Acta Hort 729: 137-141

Başay S, Şeniz V, Ellialtioğlu Ş (2011) Obtaining dihaploid lines by using anther culture in the different eggplant cultivars. J Food Agric Environ 9: 188-190

Bohanec B (2002) Doubled-haploid onions. In: Rabinowitch HD, Currah L (eds) Allium Crop Science: Recent Advances. CABI Publishing, pp 145-157

Corral-Martínez P, Parra-Vega V, Seguí-Simarro JM (2013) Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. J Exp Bot 64: 3061-3075

Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica 187: 47-61

Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382

Daghma DES, Hensel G, Rutten T, Melzer M, Kumlehn J (2014) Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging. Front Plant Sci 5: 675

Doğramacı-Altuntepe M, Peterson TS, Jauhar PP (2001) Anther Culture-Derived Regenerants of Durum Wheat and Their Cytological Characterization. J Hered 92: 56-64

Dumas de Vaulx R, Chambonnet D (1982) Culture *in vitro* d'anthères d'aubergine (*Solanum melongena* L.): stimulation de la production de plantes au moyen de traitements à 35ºC associés à de faibles teneurs en substances de croissance. Agronomie 2: 983-988

Dumas de Vaulx R, Chambonnet D, Pochard E (1981) Culture *in vitro* d'anthères de piment (*Capsicum annuum* L.): amèlioration des taux d'obtenction de plantes chez différents génotypes par des traitments à +35ºC. Agronomie 1: 859-864

Eudes F, Shim Y-S, Jiang F (2014) Engineering the haploid genome of microspores. Biocatalysis and Agricultural Biotechnology 3: 20-23

FAOSTAT (2016) http://faostat.fao.org [last checked December 2016].

Ferrie A, Caswell K (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104: 301-309

Ferrie A, Möllers C (2011) Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tissue Organ Cult 104: 375-386

Ferrie AMR, Keller WA (1995) Microspore Culture for Haploid Plant Production. In: Gamborg OL, Phillips GC (eds) Plant Cell, Tissue and Organ Culture: Fundamental Methods. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 155-164

Ferrie AMR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpe TA (ed) *In vitro* embryogenesis in plants. Kluwer Academic Publishers, Dordretch, pp 309-344

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12: 368-375

Frary A, Doganlar S, Daunay MC (2007) Eggplant. In: Kole C (ed) Vegetables. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 287-313

Germanà MA (2011a) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104: 283-300

Germanà MA (2011b) Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep 30: 839-857

Hoekstra S, Vanzijderveld MH, Louwerse JD, Heidekamp F, Vandermark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv Igri. Plant Sci 86: 89-96

Immonen S, Robinson J (2000) Stress treatments and ficoll for improving green plant regeneration in triticale anther culture. Plant Sci 150: 77-84

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43: 179-188

Kasha KJ, Simion E, Miner M, Letarte J, Hu TC (2003) Haploid wheat isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 77-82

Li H, Soriano M, Cordewener J, Muiño JM, Riksen T, Fukuoka H, Angenent GC, Boutilier K (2014) The histone deacetylase inhibitor Trichostatin A promotes totipotency in the male gametophyte. Plant Cell 26: 195-209

Malik MR, Wang F, Dirpaul J, Zhou N, Hammerlindl J, Keller W, Abrams SR, Ferrie AMR, Krochko JE (2008) Isolation of an embryogenic line from non-embryogenic *Brassica napus* cv. Westar through microspore embryogenesis. J Exp Bot 59: 2857-2873

Miah MAA, Earle ED, Khush GS (1985) Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor Appl Genet 70: 113-116

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Nitsch C, Nitsch JP (1967) Induction of flowering *in vitro* in stem segments of *Plumbago indica* L. I Production of vegetative buds. Planta 72: 355-&

Nitsch JP (1972) Haploid plants from pollen. Z Pflanzenzüchtg 67: 3-18

Nuez F, Llácer G (2001) La horticultura española, Ediciones de Horticultura, S.L., Reus, Spain

Oleszczuk S, Rabiza-Swider J, Zimny J, Lukaszewski AJ (2011) Aneuploidy among androgenic progeny of hexaploid triticale (XTriticosecale Wittmack). Plant Cell Rep 30: 575-586

Petolino JF, Jones AM, Thompson SA (1988) Selection for increased anther culture response in maize. Theor Appl Genet 76: 157-159

Quimio CA, Zapata FJ (1990) Diallel Analysis of Callus Induction and Green-Plant Regeneration in Rice Anther Culture. Crop Sci 30: 188-192

Rivas-Sendra A, Corral-Martínez P, Camacho-Fernández C, Seguí-Simarro JM (2015) Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis. Plant Cell Tissue Organ Cult 122: 759-765

Rizza F, Mennella G, Collonnier C, Shiachakr D, Kashyap V, Rajam MV, Prestera M, Rotino GL (2002) Androgenic dihaploids from somatic hybrids between *Solanum melongena* and *S. aethiopicum* group *Gilo* as a source of resistance to *Fusarium oxysporum* f. sp. *melongenae*. Plant Cell Rep 20: 1022-1032

Rotino GL (1996) Haploidy in eggplant. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 115-141

Rotino GL, Restaino F, Gjomarkaj M, Massimo M, Falavigna A, Schiavi M, Vicini E (1991) Evaluation of genetic variability in embryogenetic and androgenetic lines of eggplant. Acta Hort 300: 357-362

Rotino GL, Sihachakr D, Rizza F, Vale G, Tacconi MG, Alberti P, Mennella G, Sabatini E, Toppino L, D'Alessandro A, Acciarri N (2005) Current status in production and utilization of dihaploids from somatic hybrids between eggplant (*Solanum melongena* L.) and its wild relatives. Acta Physiol Plant 27: 723-733

Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther culture in common eggplant and related species. Euphytica 182: 261–274

Salas P, Rivas-Sendra A, Prohens J, Seguí-Simarro JM (2012) Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther cultures. Euphytica 184: 235-250

Sanguineti MC, Tuberosa R, Conti S (1990) Field evaluation of androgenetic lines of eggplant. Acta Hort 280: 177-182

Seguí-Simarro JM (2016) Androgenesis in solanaceae. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis. Springer Science + Business Media, New York, pp 209-244

Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011) Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778

Seguí-Simarro JM, Nuez F (2008) Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet Genome Res 120: 358-369

Snape JW, Sitch LA, Simpson E, Parker BB (1988) Tests for the presence of gametoclonal variation in barley and wheat doubled haploids produced using the *miah*system. Theor Appl Genet 75: 509-513

Soriano M, Li H, Jacquard C, Angenent GC, Krochko J, Offringa R, Boutilier K (2014) Plasticity in Cell Division Patterns and Auxin Transport Dependency during *in vitro* Embryogenesis in *Brassica napus*. Plant Cell 26: 2568-2581

Toppino L, Mennella G, Rizza F, D'Alessandro A, Sihachakr D, Rotino GL (2008) ISSR and isozyme characterization of androgenetic dihaploids reveals tetrasomic inheritance in tetraploid somatic hybrids between *Solanum melongena* and *Solanum aethiopicum* group Gilo. J Hered 99: 304-315

Uzun S (2007) Effect of light and temperature on the phenology and maturation of the fruit of eggplant (*Solanum melongena*) grown in greenhouses. New Zeal J Crop Hort Sci 35: 51-59

van der Weerden GM, Barendse GWM (2007) A web-based searchable database developed for the EGGNET project and applied to the Radboud University Solanaceae database. Acta Hort 745: 503-506

Veilleux RE (1998) Gametoclonal variation in crop plants. In: Jain SM, Brar DS, Ahloowalia BS (eds) Somaclonal variation and induced mutations in crop improvement. Springer Netherlands, Dordrecht, pp 123-133

Yamagishi M, Yano M, Fukuta Y, Fukui K, Otani M, Shimada T (1996) Distorted segregation of RFLP markers in regenerated plants derived from anther culture of an F1 hybrid of rice. Genes Genet Syst 71: 37-41

Zhang FL, Takahata Y (2001) Inheritance of microspore embryogenic ability in Brassica crops. Theor Appl Genet 103: 254-258

Chapter 2

Evaluation of the *in vitro* performance of microspores from DH36, a highly embryogenic eggplant doubled haploid line

Alba Rivas-Sendra, Carolina Camacho-Fernández, Patricia Corral-Martínez, Edgar García-Fortea and Jose M. Seguí-Simarro

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

Keywords

Androgenesis, hormones, iron, microspore culture, microspore embryogenesis, *Solanum melongena*.

Abstract

Despite of the importance of eggplant in global horticulture, this species is still considered recalcitrant for doubled haploid production, which precludes the rapid generation of pure lines for breeding programs on a routine basis. We recently developed an eggplant doubled haploid population from which a highly embryogenic line (DH36) was selected. In this work, we present a characterization of the *in vitro* performance of DH36 microspores when exposed to different culture conditions, including modifications in concentrations of salts and vitamins, sucrose, growth regulators and iron, and also modifications in the microspore density used. We compared it with some eggplant commercial hybrids. Our work builds up on the already existing studies about eggplant microspore culture by providing more precise knowledge about the optimization of the different parameters evaluated. But most importantly, our results lead us to think that DH36 approaches the features of the genotypes considered as models for the study of microspore embryogenesis, at least in terms of induction rates.

Introduction

Microspore embryogenesis is an androgenic pathway whereby immature pollen grains (microspores) are able to form new haploid or doubled haploid (DH) individuals in absence of egg fecundation. By the application of specific stresses *in vitro*, microspores are deviated from their original gametophytic pathway and induced to start dividing sporophytically. DH technology is highly interesting both for plant breeding purposes, because it allows for a fast production of totally homozygous plants useful in breeding programs, and for fundamental research, since it provides an haploid platform for genetic studies and genetic transformation, and an *in vitro* model system for embryogenesis. From the different methods available to produce DHs, microspore culture is preferred in the species where it is possible, since it is the method where conditions are most controlled and provides a high efficiency.

The efficiency of microspore culture is hard to predict, because many factors are involved. Perhaps, the genotype is the most important one. Its role in the androgenic response is very well known (Dwivedi *et al*. 2015; Ferrie *et al*. 1995), and the fact that different genotypes from the same species greatly vary in their capacity to produce DHs indicates that the androgenic response is genetically controlled. Studies in different species, including rice (Yamagishi *et al*. 1996), *B. napus* (Zhang and Takahata 2001) and eggplant (Salas *et al*. 2011), pointed to a genetic control by nuclear recessive genes with additive effects. On the other hand, for most species there is a narrow window during anther development where microspores are responsive to induction treatments. This window revolves around the first pollen mitosis (Seguí-Simarro *et al*. 2011). Finally, stress treatments and culture conditions such as the culture medium composition and the plating density are very important factors that regulate the efficiency of the process (Seguí-Simarro *et al*. 2011; Shariatpanahi *et al*. 2006).

In eggplant, the most widely used method to obtain DH is still anther culture. Advances have been made in microspore cultures, but this technique has still a

short history (reviewed in Seguí-Simarro 2016). Miyoshi (1996) developed the first well documented protocol for embryogenesis induction, calli production and regeneration of DH from isolated eggplant microspores, establishing the basis of the induction conditions and culture medium composition. Since then, several medium modifications have been tested in order to improve the efficiency of the procedure and to stimulate embryo progression, with different results. For example, it was found that the addition of polyethylene glycol improved calli induction and proliferation (Corral-Martínez and Seguí-Simarro 2012), while the addition of epibrassinolide improved calli induction but had a negative effect on calli proliferation (Corral-Martínez and Seguí-Simarro 2014). The addition of abscisic acid prevented disorganization at the beginning of the culture, but could not stop the dedifferentiation process that the initially formed microspore-derived embryos (MDEs) undergo after the globular stage (Corral-Martínez and Seguí-Simarro 2014). Gum arabic, a mix of arabinogalactans and arabinogalactan proteins, was the only compound able to promote embryo development beyond the globular stage, but the MDEs formed presented an aberrant morphology and were defective in shoot apices (Corral-Martínez and Seguí-Simarro 2014).

Microspore embryogenesis is a powerful methodology that can provide a large quantity of material useful both for breeding and for research purposes, since a single anther contains thousands of potentially embryogenic microspores. However, a low embryogenic response limits the use of DH technology and only high responding species and genotypes can be successfully exploited. Recently, a new highly androgenic eggplant DH line has been developed (Rivas-Sendra *et al*. 2017), with a high and stable response both in anther and in microspore culture. The morphology, reproductive traits and agronomical behavior have already been described as very similar to the parental line. In the present work, we aim to a further characterization of the *in vitro* performance of the highly androgenic eggplant DH36 line, and to study its response to culture conditions modifications, in comparison with some eggplant commercial hybrids.

Materials and methods

Plant material

Two eggplant F1 hybrids and one DH line were used in this study as donor plants. The hybrids were provided by Vilmorin & Cie (France) and for this study, they were named G1 and G3, since their identity is protected under a confidentiality agreement. The DH line DH36 is a highly androgenic line developed in our group (Rivas-Sendra *et al*. 2017), derived from the F1 hybrid cv. Bandera originally commercialized by Seminis (Spain). Twenty plants of each line were grown in 30 cm pots at the greenhouse under controlled temperature (25ºC) and natural light.

Isolated microspore culture

Prior to performing isolated microspore cultures, the length of the anther containing mostly vacuolate microspores and young bicellular pollen was determined for each genotype. Isolated microspore culture was performed according to Corral-Martínez and Seguí-Simarro (2014). Anthers containing mostly vacuolate microspores were dissected from the bud, surface sterilized with 70% ethanol for 30 seconds and with 4g/l sodium hypochlorite for 5 minutes, and rinsed three times in sterile distilled water. Anthers were crushed in a small beaker with sterile distilled water using a syringe piston, and their locular content was isolated by filtration through a 41 µm nylon mesh (Millipore), and by three centrifugation steps at 100 g for 4 min each. Microspores were suspended in sterile distilled water at a final cell density of 500,000 microspores/ml (if not specified otherwise in Results), plated and incubated at 35ºC in darkness for 3 days to induce embryogenesis. After induction treatment, microspores were collected by centrifugation, resuspended at the same density in the culture medium and incubated at 25ºC in darkness for the progression of culture. The basal culture medium used for eggplant microspore culture was NLN salts and vitamins (Nitsch

and Nitsch 1967) supplemented with 2% sucrose, 0.5 mg/l 1-naphthaleneacetic acid and 0.5 mg/l 6-benzylaminopurine. Different modifications of this culture medium were tested, as explained in Results.

Data collection and statistical analysis

In order to evaluate the effect of different culture conditions on the androgenic performance of the different genotypes used, embryo-like and callus-like structures were observed and counted under a light microscope after 7 and 14 days of culture, and were expressed as number of structures per ml of culture. After 30 days of culture, two more parameters were recorded: the total number of calli as an estimation of induction efficiency, and the number of calli larger than 1 mm (calli>1mm) as an estimation of callus growth. Both parameters were expressed per ml of culture medium. Microspore cultures performed in different culture conditions were always repeated at least three times. An ANOVA test ($p \leq 0.05$) was performed to assess global significant differences, and then a Fisher's least significant difference (LSD) test for multiple comparisons was performed in order to group the effect of the treatments in groups of homogeneity, considering significant differences when p-value was <0.05.

Results

In this work, we characterized the androgenic performance of DH36, comparing it with that of some eggplant F1 hybrids, and evaluating its embryogenic response in different *in vitro* conditions. The conditions included the modification of experimental parameters such as the concentrations of NLN salts and vitamins, sucrose, growth regulators (GR), chelated iron and the plating microspore density as factors influencing the yield of cultures. Results are presented next.

Response to changes in GR, salts, vitamins and sucrose concentrations

The first assay performed was to increase the medium concentration (NLN salts, vitamins and sucrose) combined with the reduction of the concentration of GR. Two levels of medium concentration (1x NLN + 2% sucrose and 2x NLN + 4% sucrose) and GR (100% and 20%) were tested. After 30 days of culture, the total number of calli and the number of calli>1mm was recorded. Increased NLN and sucrose concentration did not have any significantly positive effect on total callus production (Figure 1A) nor on the number of calli>1mm (Figure 1B) in G1 microspore cultures with respect to controls (1x NLN + 2% sucrose + 100% GR). Reducing the concentration of GR to 20% increased 3.4-fold the number of total calli and 3.7-fold the number of calli>1mm compared to controls. The combination of increased NLN and sucrose concentration and reduced GR concentration interacted positively, increasing the number of total calli 4.6-fold respect to the control conditions. However, it did not have any positive effect in the number of calli>1mm (Figure 1B). For G3, the response to the modifications in GR concentration was weaker than in G1, but the combination of increased NLN and sucrose concentration and a reduced GR concentration had a strong positive effect too (Figure 1C), although the number of calli>1mm remained unaffected (Figure 1D). Thus, it seemed clear that in F1 hybrids, an increase in NLN salts, vitamins and sucrose concentration combined with a reduction of GR concentration (20%) were beneficial in terms of increasing the efficiency of embryogenesis induction.

In DH36, as expected, callus production in control microspore cultures was much higher than that of hybrid genotypes (11.4-fold more than G1 and 10.2-fold more than G3). Interestingly, the response of the DH line to medium modifications was also different from hybrids in qualitative terms (Figures 1E and 1F). Higher NLN salts, vitamins and sucrose concentrations had a positive effect on the number of total calli and multiplied the production by a factor of 2.1, but it was negative for the number of calli>1mm. A reduced GR concentration did not have any positive effect on the number of total calli, but slightly increased 1.4-fold the number of calli>1mm. In addition, increased concentration of NLN and sucrose combined

with reduced GR concentration had a negative effect on the number of calli>1mm. This particular behavior of the DH line was confirmed in experiments excluding GR from medium. In G1 and G3 hybrids, no embryos or calli were induced at all, whereas in DH36, up to 4 calli/ml were formed. These calli presented a characteristic external appearance, different from those of control conditions. Regular calli were usually white and disorganized, with a dense core and a fluffy appearance in the outer layers (Figure 2A), whereas calli formed in GR-free medium (Figure 2B) showed lobulation, a creamy coloration and a smoother surface, similar to that of eggplant zygotic embryos and MDEs (Seguí-Simarro *et al*. 2011). Although they lacked the typical embryo pattern, they appeared to have a higher tissue organization. Together, these results showed that hybrid genotypes had, in general terms, a similar response to medium modifications, whereas the DH line response was markedly different.

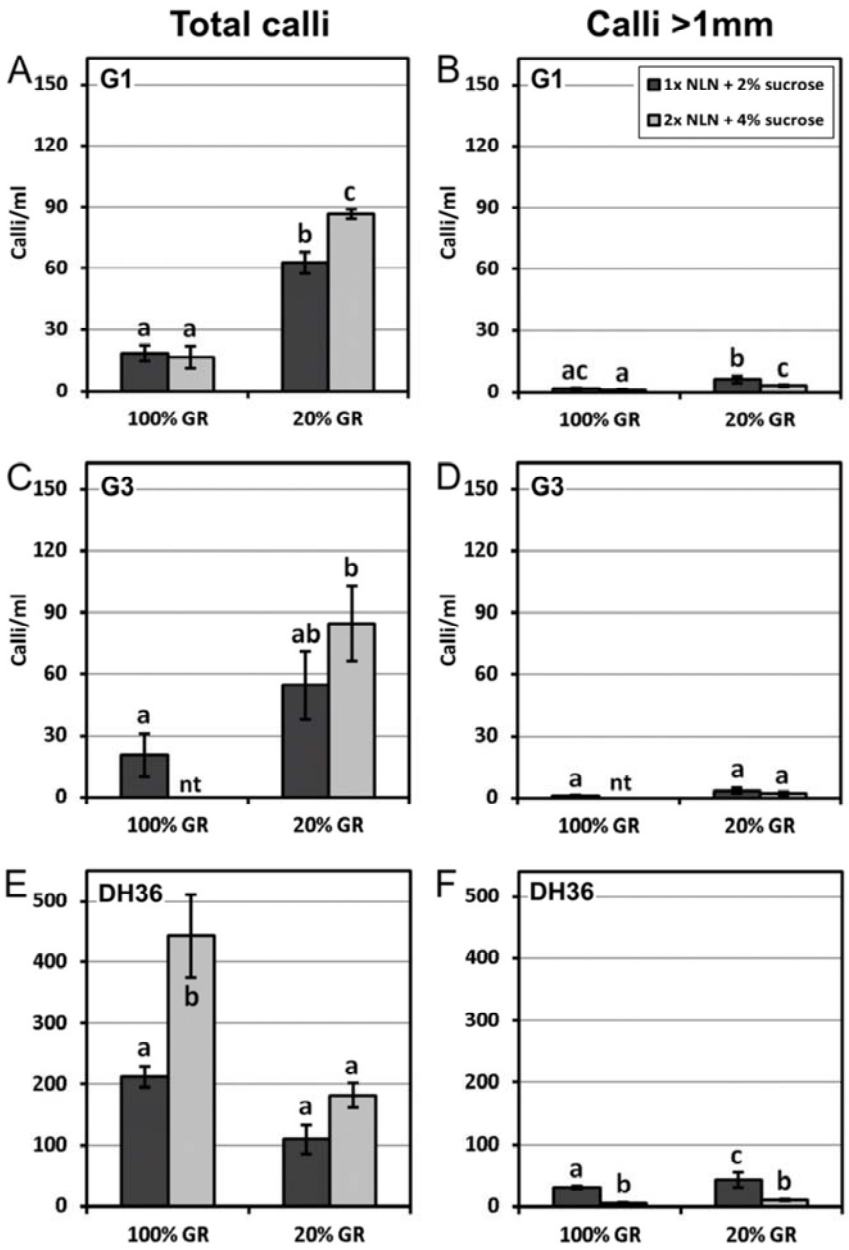


Figure 1. Effect of increased medium (sucrose and NLN salts and vitamins) concentration and reduced GR concentration on the three assayed genotypes. **A, C** and **E** represent the total number of calli formed after 30 days of culture. **B, D** and **F** represent the number of calli>1mm formed after 30 days of culture. Note that the scale of DH36 charts (**E** and **F**) is different. The first column in each chart corresponds to the control conditions. Different letters indicate statistically significant differences found by a Fisher's least significant difference (LSD) test. **nt:** not tested.

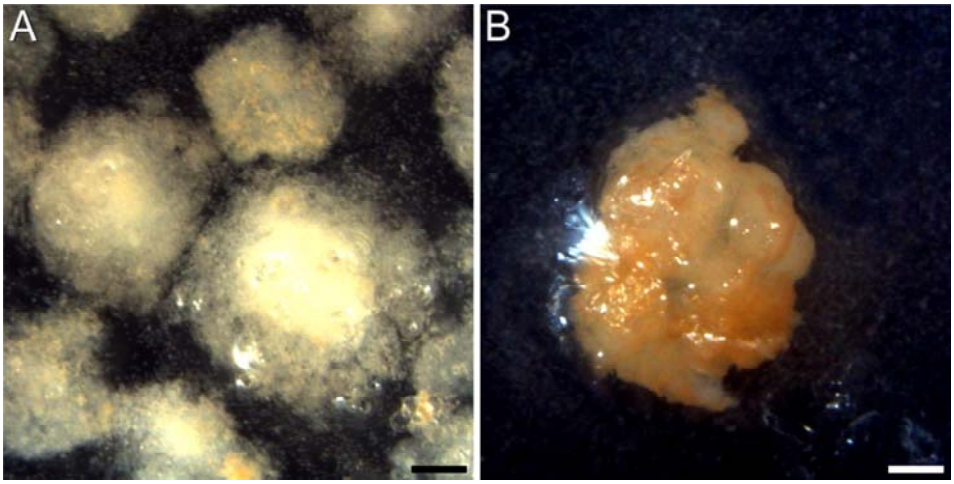


Figure 2. Calli belonging to DH36 formed after 30 days of culture in control conditions (**A**) and in absence of GR (**B**). Bars: 5 mm.

Next, we wanted to find out whether the above described effects were due to sucrose alone, NLN (salts + vitamins), or both. Two levels of sucrose concentration (2% and 5%) were tested in the three genotypes, keeping all other parameters unchanged. As seen in Figure 3A, 5% sucrose greatly increased the number of total calli in G1 and G3, improving callus production by 5.4 and 66.6-fold respectively, but not in DH36. As to the number of calli>1mm, it was unaffected for G1 and G3, but reduced for DH36 (Figure 3B). Additional experiments showed that a sucrose concentration of 10% inhibited induction in all cases (data not shown). Thus, the increase of sucrose alone up to 5% was clearly beneficial for hybrids, but not for DH36, which suggested that the beneficial effects previously observed in DH36 would be due to the increase in NLN salts + vitamins concentration. To test this, we checked in DH36 the effect of increasing only NLN salts and vitamins under two levels of GR concentration, keeping fixed the rest of conditions. The trend was similar to that observed when NLN and sucrose concentration were increased at the same time (compare Figures 1E and 4A), although differences were not significant in this case. Among all the combinations, the highest yields were achieved with 2x NLN, but 100% GR was needed for highest total callus yield (Figure 4A), and 20% GR for highest calli>1mm yield (Figure 4B), which was the

only statistically significant result. However, the principal finding drawn from these experiments was that the response of DH36 to changes in the culture medium is markedly different from that of G1 and G3 hybrids.

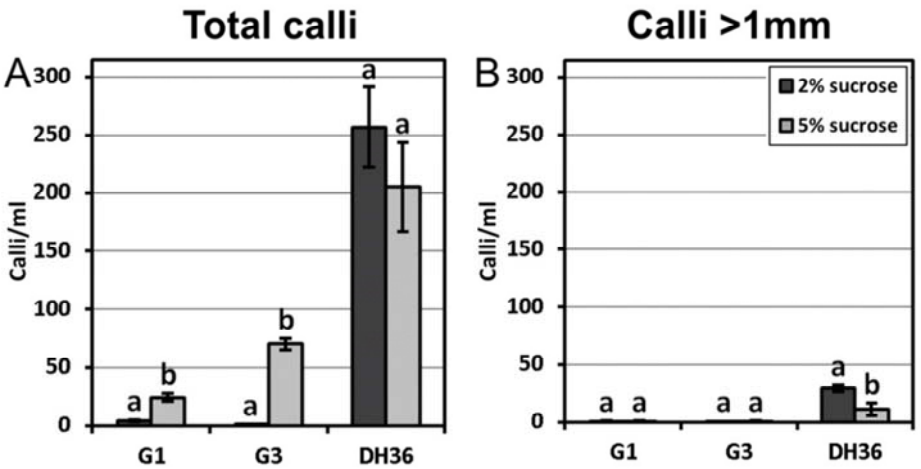


Figure 3. Effect of increased sucrose concentration on the three genotypes tested. **A** represents the total number of calli formed after 30 days of culture. **B** represents the number of calli>1mm formed after 30 days of culture. The first column of each genotype corresponds to the control conditions. Different letters indicate statistically significant differences found by a Fisher's least significant difference (LSD) test.

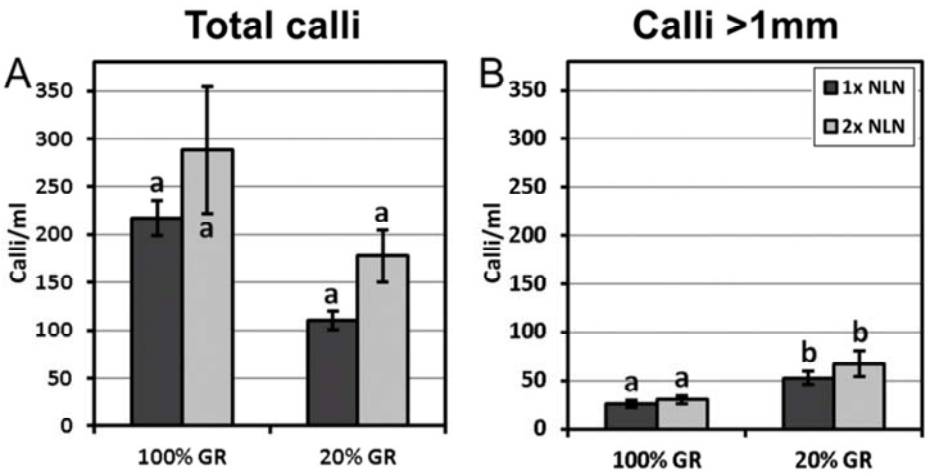


Figure 4. Effect of increased NLN salts and vitamins concentration on DH36. **A** represents the total number of calli formed after 30 days of culture. **B** represents the number of calli>1mm formed after 30 days of culture. The first column of each genotype corresponds to the control conditions. Different letters indicate statistically significant differences found by a Fisher's least significant difference (LSD) test.

Response to different concentrations of chelated iron

We tested the effect of increasing the concentration of chelated iron in the androgenic response of DH36. Embryo-like and callus-like structures were recorded at different time points. After 7 days of culture, multicellular embryogenic structures were observed. They presented spherical shape, internal cell walls and several nuclei still enclosed within the exine coat (Figure 5A). Higher concentrations of chelated iron in the culture medium increased the number of embryogenic structures during the first days of culture. A clear dose-response curve could be observed (Figure 6A), with an optimal concentration at 800 μM, which produced 3.8-fold more embryogenic structures than control concentration. After 14 days of culture, larger globular MDEs were observed, with dense cells organized in layers and a clear, differentiated protoderm (Figure 5B). In addition, callus-like structures began to be observed together with globular MDEs. In these calli, tissue organization in layers was disrupted, and the cells were larger and more translucent (Figure 5C). As reported by Corral-Martínez and Seguí-Simarro (2012), these callus-like structures are globular MDEs that entered a dedifferentiation process. Increasing concentrations of chelated iron gave rise to different rates of MDE dedifferentiation (Figure 6B). 54.6% of the structures formed in control conditions (100 μM) were calli, while concentrations of 800 and 1600 μM produced, respectively, 33.9% and 42.0%, percentages significantly lower than control. 1600 μM also produced a total number of structures slightly higher (1.3x) than control. Increasing the concentration to 3200 μM produced the highest number of total structures (1.9-fold more than control), but the rate of dedifferentiation was comparable to control conditions. After 30 days of culture, all the multicellular structures transformed into calli (Figure 5D). All the concentrations produced more calli than control (Figure 6C), being 800 and 3200 μM the concentrations with the highest differences (1.4 and 1.5-fold, respectively). It is interesting to note that the response curve observed at day 7 (Figure 6A) gradually disappeared as the culture progressed (Figures 6B and 6C). In summary, increasing the concentration of chelated iron in the culture medium greatly increased the formation of embryogenic structures during the first culture stages,

and delayed the embryo dedifferentiation process. It seemed, thus, that higher iron levels were beneficial for the initial culture stages. However, the number of calli obtained with the different treatments was almost leveled up after one month of culture.

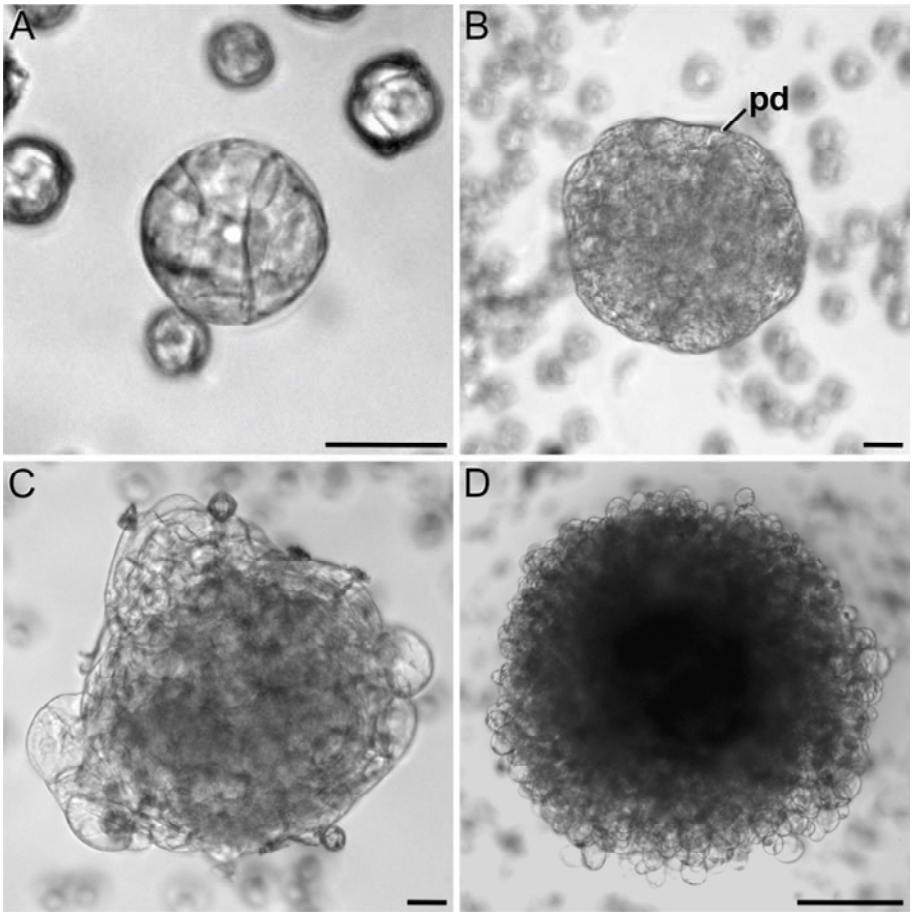


Figure 5. Structures found at different time points in cultures with increased chelated iron concentration. **A**: embryo-like structure at 7 days. **B**: globular embryo at 14 days. Note the differentiated protoderm (**pd**) surrounding the structure. **C**: callus-like structure at 14 days. **D**: calli at 30 days. Bars: 20 µm in **A-C**, 200 µm in **D**.

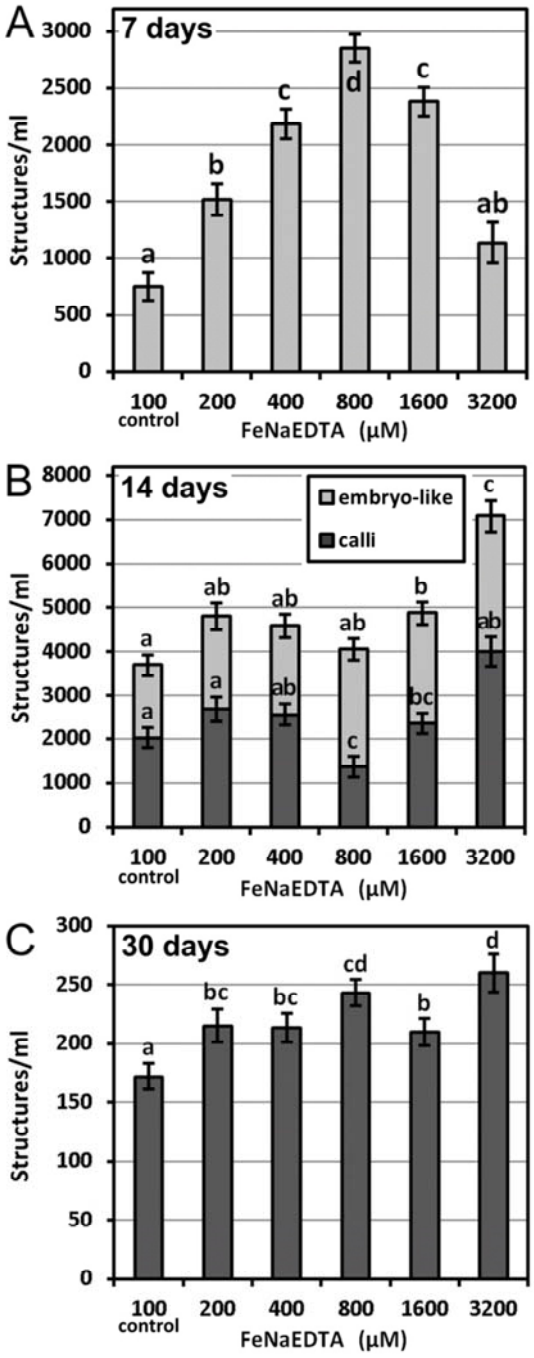


Figure 6. Effect of increased chelated iron concentration on DH36. **A** represents the number of embryo-like structures formed after seven days of culture. Different letters indicate statistically significant differences. **B** represents the total number of structures formed after 14 days of culture, divided in embryo-like (light grey) and callus-like (dark grey). Different letters over upper bars indicate statistically significant differences between the total number of structures formed, different letters over lower bars indicate statistically significant differences between the percentage of calli (rate of dedifferentiation). **C** represents the total number of calli formed after 30 days of culture. Different letters indicate statistically significant differences found by a Fisher's least significant difference (LSD) test.

Response to different plating densities

To test whether cell density has an effect on DH36 callus production, we tested a range of different initial plating densities from 50,000 to 2,000,000 microspores per ml. After 30 days of culture, the total number of calli and the number of calli>1mm was recorded. In order to exclude the effect of the different densities used, numbers of calli were expressed using a normalized parameter, the number of calli per 500,000 microspores, which is the standard density used so far in eggplant microspore cultures (Corral-Martínez and Seguí-Simarro 2014; Miyoshi 1996; Rivas-Sendra *et al*. 2015) and in this work as the control to compare with.

As seen in Figure 7A, a slight reduction of the plating density below the standard density had in general a positive effect on the number of total calli and the number of calli>1mm. When 200,000 microspores/ml where plated, the total number of calli reached a maximum (1.4-fold higher than the control). This density increased the number of calli>1mm too, although the highest increase (1.6-fold higher than control) was observed for 300,000 microspores/ml (Figure 7B). A reduction to 100,000 microspores/ml reduced dramatically the total number of calli (7.7-fold lower than control), and dishes with a density of 50,000 microspores/ml were unable to produce calli at all (Figure 7A). On the other side, an increase to 1,000,000 microspores/ml showed no change versus control, and dishes with 2,000,000 microspores/ml were unable to produce any calli, because bacterial contamination appeared systematically in all the culture dishes (data not shown). These results showed that DH36 responds differently to different microspore plating densities. In general, the response is higher with densities below the standard generally established for eggplant hybrids, but there is a lower threshold below which, response is inhibited.

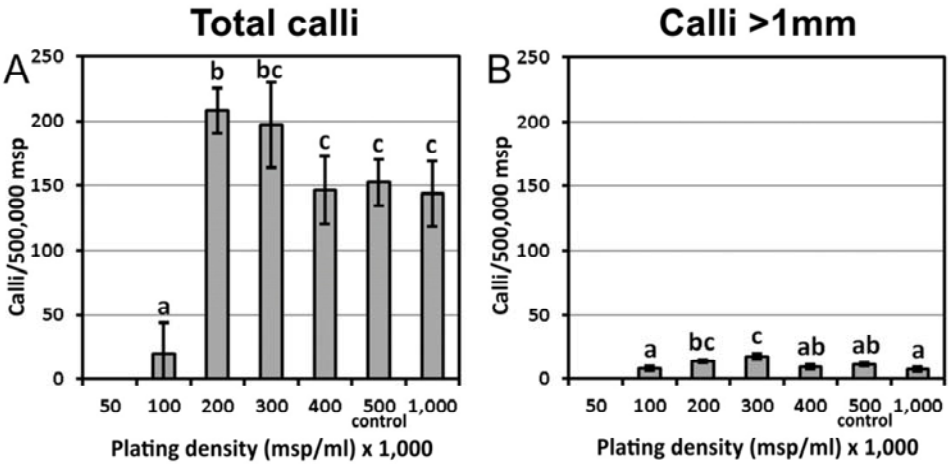


Figure 7. Effect of different plating densities on DH36 microspore cultures. **A** represents the number of total calli formed after 30 days of culture, B represents the number of calli>1mm after 30 days of culture. Note that the efficiency has been expressed in calli per 500,000 microspores in order to allow an easy comparison between different conditions. Different letters indicate statistically significant differences found by a Fisher's least significant difference (LSD) test.

Discussion

DH36 does not respond to changes in culture medium conditions as hybrid genotypes do

Carbon source is a key factor for *in vitro* culture. Several studies have been conducted to find the optimum type and concentration to induce microspore embryogenesis in different species (Ismaili and Mohammadi 2016; Kim *et al*. 2008). In model species, or in species where high rates of MDEs are usually obtained, sucrose concentration is generally high. For example, 13% is used for *Brassica napus* (Custers 2003), 8.6% for tobacco (Touraev *et al*. 1996a), 5.1% for maize (Gaillard *et al*. 1991) or 10% for hot pepper (Kim *et al*. 2008). In eggplant anther culture, a sucrose concentration of 12% is used during the initial culture stage (Chambonnet 1988), whereas for microspore culture, the standard culture medium contains 2% (Corral-Martínez and Seguí-Simarro 2012; 2014; Miyoshi 1996). These data made us think that higher sucrose concentrations could perhaps

have a beneficial effect in callus production. However, results were not uniform. Combined with 2x NLN concentration and 20% GR, sucrose gave rise to more calli in G1 and G3, but not in DH36, where higher yields were only obtained when high sucrose was combined with 2x NLN. In order to elucidate the source of such effect, high sucrose alone was tested, evidencing a strongly positive effect in G1 and G3, but not in DH36. Thus, it seems that medium concentration (NLN salts and vitamins) does not influence callus production or size in hybrid genotypes, but is positive for DH36. In contrast, sucrose concentration alone has a great impact in callus production in both hybrids, but not in DH36.

With respect to GRs, Miyoshi (1996) obtained the best results with 0.5 mg/l NAA and 0.5 mg/l BAP. Later studies indicated that eggplant microspores seem to be more sensitive to absolute concentrations of GRs than to their relative ratios (Corral-Martínez and Seguí-Simarro 2014), reporting a higher production of calli from eggplant hybrids when using reduced concentrations of NAA and BAP. In line with this, we showed that a reduced concentration had a clear and strong positive effect on G1 and G3 hybrids. However, this was not the case of DH36. Altogether, these results evidence that the response of DH36 to modifications in the medium composition is in general opposite to that of the hybrid genotypes, responding negatively or not responding to these modifications. It seems that this line reached a maximum of performance, difficult to increase by medium modifications.

Increased iron boosts embryo induction but does not prevent dedifferentiation

Iron is an essential micronutrient for metabolic and developmental processes, including photosynthesis (Briat *et al*. 2007) redox reactions (Briat *et al*. 1995) and induction of somatic embryogenesis (Loh and Lim 1992). However, the key role of iron in embryogenesis seems to be related to embryo progression. The concentration of chelated iron in NLN medium was determined by Nitsch (1972). Working with tobacco anther cultures, they found that when iron concentration was too low, MDE development arrested at the globular stage. Recent studies

suggested that, in the absence of surrounding anther tissues, the endogenous iron level of cells would be enough to sustain just a limited number of divisions during the very first stages of *B. napus* embryogenesis induction, and an exogenous addition of chelated iron is necessary for further development of MDEs (Leroux *et al*. 2016). Likewise, an optimal concentration of iron is needed for somatic embryo progression through advanced stages in *Pinus taeda* (Pullman *et al*. 2003) and cherry (Gutierrez and Rugini 2004). More specifically, Saha *et al*. (2010) analyzed in *Plantago ovata* how the concentration of several metallic ions in embryonic tissues changed during the different stages, and suggested that providing the culture medium with the ion concentration optimal for each developmental stage is key for a successful *in vitro* embryogenesis. In addition, an iron transporter protein (ITP) has been found to be present in high concentrations during late stages of embryogenesis in many species, suggesting that iron is an element commonly needed for the development and progression of embryos (Kruger *et al*. 2002). These observations made us think that higher iron concentrations could help MDE progression, overcoming the developmental arrest observed between the globular and heart-shaped stages, which makes them become calli (Corral-Martínez and Seguí-Simarro 2012). Our results showed that increased iron concentration boosted embryogenesis induction. It was also able to slow down the dedifferentiation of MDEs into calli, reducing the callus rate after 14 days of culture and allowing the globular MDEs to grow for a longer period before transformation into calli. Such a positive effect had an optimum (800 μM FeNaEDTA) beyond which the effect decreased. However, increased iron concentration was unable to stop dedifferentiation, and calli were eventually obtained in any case. Thus, it seems that, as opposed to its role in other systems, iron has a role in the initial stages where microspores become embryogenic, but it is not the key factor needed by DH36 MDEs for a successful development.

Lower plating density reduces competence and allows for a higher efficiency

In most *in vitro* culture systems, determining the optimal plating density is crucial for success. This may influence not only the number of calli or MDEs produced, but also the quality and the speed of development (Castillo *et al*. 2000; Kim *et al*. 2008).In the case of microspore cultures, a different plating density is used in each species, from as low as 40,000 microspores/ml in *B. napus* (Robert *et al*. 2015) and maize (Aulinger 2002), 50,000 in tobacco (Touraev and Heberle-Bors 2003), 100,000 in pepper (Kim *et al*. 2013) or wheat (Sinha and Eudes 2015), to the very high 1,000,000 in barley (Esteves *et al*. 2014) or 1,400,000 in apple (Höfer 2004). In order to check whether cell density affected the efficiency of the process in DH36, we tested a wide range of plating densities. Our results showed that lower densities (200,000-300,000 microspores/ml) allowed for higher rates of total callus production, but also of callus>1mm, confirming that high densities negatively affect MDE induction. This might be due to excessive accumulation of compounds, excreted by cells, that may be toxic and/or inhibitory for embryogenesis induction, or simply due to the competence of more cells for a limited amount of space and resources available. All our other experiments point to the second option, since they showed a similar inverse relationship between the total number of calli and the number of calli>1mm in G1 and G3 hybrids, but especially in DH36, where induction efficiency is higher. It is interesting to note that the experiment where more dramatic increases in number of calli were observed in DH36 consisted in the addition of twice the normal amount of NLN salts, vitamins and sucrose (Figure 1E). Doubling the amount of available nutrients led to twice the regular amount of calli. These were already induced structures, not affected by the putative presence of inhibitory substances. Thus, it seems that when the embryogenesis rate is boosted, more MDEs are forced to share and therefore to compete for a limited (fixed) amount of space and *in vitro* resources, reducing the chances of reaching older stages.

Our results also showed that a minimum cell density is needed for embryogenesis to be induced, since very few calli were obtained at 100,000 microspores/ml and no one at 50,000 microspores/ml. A similar observation was reported by Kim *et al*. (2008) in hot pepper microspore cultures. Embryogenic microspores release different substances to the culture medium, including GRs and arabinogalactan proteins among others, that have a positive effect in other microspores, promoting their switch to embryogenesis (Borderies *et al*. 2004; Paire *et al*. 2003; Żur *et al*. 2015). If cell density is too low, these substances are too diluted to be effective. Other interesting observation was that bacterial contamination systematically appeared at high densities (10x our optimal density). The most likely explanation for this is the presence of endogenous bacteria inside eggplant anthers, since during isolation, it is only possible to surface-sterilize them before microspore extraction. Lower densities would contribute a bacterial inoculum insufficient to proliferate, but there is a threshold beyond which contamination is manifested. These observations are not exceptional, since endogenous bacterial contamination has been repeatedly reported in pepper microspore cultures, making the use of antibiotics mandatory (Lantos *et al*. 2012; Parra-Vega and Seguí-Simarro 2013; Supena *et al*. 2006).

DH36 embryogenic response approaches that of the model species

In this study we used two hybrid genotypes and one highly androgenic DH line. Callus production of DH36 was in all our tests enormously higher than that of hybrids, ranging from 10 to 85 times higher. A 4-fold increase in callus production was reported when compared with the parental hybrid from which DH36 was developed (Rivas-Sendra *et al*. 2017). Under the best experimental conditions, we showed that DH36 may yield up to ~450 calli/ml, obviously coming from ~450 MDEs/ml. These numbers are comparable to those of the best performing genotypes of model species. Our experiments also made clear that androgenic efficiency of DH36 was difficult to increase with medium modifications. Most of the treatments applied also had a negative effect on the size of the calli produced, and

in general had an effect on DH36 opposite to that of the hybrid genotypes. This would suggest that the homozygous nature of alleles present in DH36, already highly favorable for embryogenesis induction, could approach the efficiency of this line close to a maximum, being therefore non-responsive to minor medium modifications. Indeed, the efficiency of its parental hybrid line ('Bandera') could still be notably enhanced by altering medium composition (Corral-Martínez and Seguí-Simarro 2012; 2014).

Most of the model species with high embryogenic response in microspore cultures do not need the addition of GRs to the culture medium. This is the case, for example, of wheat (Touraev *et al*. 1996b), tobacco (Touraev *et al*. 1996a), maize (Gaillard *et al*. 1991) and *B. napus* (Custers 2003). In these species, embryos are produced instead of calli with none or very low GR addition needed for proper embryo induction, which suggests that their endogenous GR production suffices their needs. However, this is not the case of recalcitrant species, where exogenous application of auxins and cytokinins is needed to control cell division and morphogenesis. This GR unbalance may be negatively affecting eggplant MDE progression and causing the transformation of globular MDEs into calli, the main bottleneck in eggplant microspore culture. The fact that DH36 did not respond positively to changes in GR concentration might indicate that this genotype presents an endogenous GR balance better adapted for high embryogenesis induction rates under the standard GR regime. However, it also seems able to respond under other, lower GR regimes. Indeed DH36 was able to respond (though at low frequency) even in absence of GRs, producing calli with an external appearance closer to MDEs than to regular calli. These facts indirectly support the notion that DH36 is sensitive but less dependent on exogenous GR application than hybrid genotypes. The only step left to consider DH36 a model line would be to overcome the blockage of globular embryos, in order to consistently achieve successful embryogenesis.

Acknowledgements

This work was supported by grants AGL2010-17895 and AGL2014-55177 to JMSS from Spanish MICINN and MINECO, respectively, jointly funded by FEDER. ARS was supported by a predoctoral fellowship from the FPI Program of Universitat Politècnica de València.

References

Aulinger IE (2002) Combination of *in vitro* androgenesis and biolistic transformation: an approach for breeding transgenic maize (*Zea mays* L.) lines. Swiss Federal Institute of Technology, Zurich, p 115

Borderies G, le Bechec M, Rossignol M, Lafitte C, Le Deunff E, Beckert M, Dumas C, Matthys-Rochon E (2004) Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur J Cell Biol 83: 205-212

Briat J-F, Curie C, Gaymard F (2007) Iron utilization and metabolism in plants. Curr Opin Plant Biol 10: 276-282

Briat JF, Fobis-Loisy I, Grignon N, Lobréaux S, Pascal N, Savino G, Thoiron S, von Wirén N, Van Wuytswinkel O (1995) Cellular and molecular aspects of iron metabolism in plants. Biol Cell 84: 69-81

Castillo AM, Valles MP, Cistue L (2000) Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. Euphytica 113: 1-8

Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica 187: 47-61

Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382

Custers J (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 185-193

Chambonnet D (1988) Production of haploid eggplant plants. Bulletin interne de la Station d'Amelioration des Plantes Maraicheres d'Avignon-Montfavet, France: 1-10

Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: Constraints and opportunities in plant breeding. Biotechnol Adv 33: 812-829

Esteves P, Clermont I, Marchand S, Belzile F (2014) Improving the efficiency of isolated microspore culture in six-row spring barley: II-exploring novel growth regulators to maximize embryogenesis and reduce albinism. Plant Cell Rep: In press

Ferrie AMR, Palmer CE, Keller WA (1995) Haploid embryogenesis. In: Thorpe TA (ed) *In vitro* embryogenesis in plants. Kluwer Academic Publishers, Dordretch, pp 309-344

Gaillard A, Vergne P, Beckerte M (1991) Optimization of maize microspore isolation and culture conditions for reliable plant regeneration. Plant Cell Rep 10: 55-58

Gutierrez P, Rugini E (2004) Influence of plant growth regulators, carbon sources and iron on the cyclic secondary somatic embryogenesis and plant regeneration of transgenic cherry rootstock 'Colt' (Prunus avium · P. pseudocerasus). Plant Cell, Tissue and Organ Culture (PCTOC) 79: 223-232

Höfer M (2004) *In vitro* androgenesis in apple—improvement of the induction phase. Plant Cell Rep 22: 365-370

Ismaili A, Mohammadi PP (2016) Effect of genotype, induction medium, carbohydrate source, and polyethylene glycol on embryogenesis in maize (Zea mays L.) anther culture. Acta Physiol Plant 38

Kim M, Jang I-C, Kim J-A, Park E-J, Yoon M, Lee Y (2008) Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. Plant Cell Rep 27: 425-434

Kim M, Park E-J, An D, Lee Y (2013) High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). Plant Cell, Tissue and Organ Culture (PCTOC) 112: 191-201

Kruger C, Berkowitz O, Stephan U, Hell R (2002) A Metal-binding Member of the Late Embryogenesis Abundant Protein Family Transports Iron in the Phloem of *Ricinus communis L*. The journal of biological chemestry 277: 25062-25069

Lantos C, Juhasz AG, Vagi P, Mihaly R, Kristof Z, Pauk J (2012) Androgenesis induction in microspore culture of sweet pepper (*Capsicum annuum* L.). Plant Biotechnol Rep 6: 123-132

Leroux BJG, Potin P, Larher FR, Bodin M (2016) Enhancement of embryo yield from isolated microspores of *Brassica napus* by early iron starvation. Plant Biotechnol Rep 10: 483-486

Loh C, Lim G (1992) The influence of medium components on secondary embryogenesis of winter oil seed Rape, *Brassica napus* L. spp oleifera New Phytol 121: 425-430

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Nitsch C, Nitsch JP (1967) Induction of flowering *in vitro* in stem segments of *Plumbago indica* L. I Production of vegetative buds. Planta 72: 355-&

Nitsch JP (1972) Haploid plants from pollen. Z Pflanzenzüchtg 67: 3-18

Paire A, Devaux P, Lafitte C, Dumas C, Matthys-Rochon E (2003) Proteins produced by barley microspores and their derived androgenic structures promote *in vitro* zygotic maize embryo formation. Plant Cell Tissue Organ Cult 73: 167-176

Parra-Vega V, Seguí-Simarro JM (2013) Improvement of an isolated microspore culture protocol for Spanish sweet pepper (*Capsicum annuum* L.). In: Lanteri S, Rotino GL (eds) Breakthroughs in the genetics and breeding of *Capsicum* and *Eggplant*. Universita degli Studi di Torino, Torino, Italy, pp 161-168

Pullman G, Montello P, Cairney J, Xu N, Feng X (2003) Loblolly pine (*Pinus taeda L.*) somatic embryogenesis: maturation improvements by metal analyses of zygotic and somatic embryos. Plant Sci 164: 955-969

Rivas-Sendra A, Campos-Vega M, Calabuig-Serna A, Seguí-Simarro JM (2017) Development and characterization of an eggplant (*Solanum melongena*) doubled haploid population and a doubled haploid line with high androgenic response. Euphytica 213: 89

Rivas-Sendra A, Corral-Martínez P, Camacho-Fernández C, Seguí-Simarro JM (2015) Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis. Plant Cell Tissue Organ Cult 122: 759-765

Robert HS, Grunewald W, Sauer M, Cannoot B, Soriano M, Swarup R, Weijers D, Bennett M, Boutilier K, Friml J (2015) Plant embryogenesis requires AUX/LAX-mediated auxin influx. Development 142: 702-711

Saha P, Raychaudhuri S, Sudarshan M, Chakraborty A (2010) Analysis of Trace Elements During Different Developmental Stages of Somatic Embryogenesis in *Plantago ovata* Forssk Using Energy Dispersive X-ray Fluorescence. Biol Trace Elem Res 135: 283-294

Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther culture in common eggplant and related species. Euphytica 182: 261–274

Seguí-Simarro JM (2016) Androgenesis in solanaceae. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis. Springer Science + Business Media, New York, pp 209-244

Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011) Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiol Plant 127: 519-534

Sinha R, Eudes F (2015) Dimethyl tyrosine conjugated peptide prevents oxidative damage and death of triticale and wheat microspores. Plant Cell, Tissue and Organ Culture (PCTOC): 1-11

Supena EDJ, Suharsono S, Jacobsen E, Custers JBM (2006) Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). Plant Cell Rep 25: 1-10

Touraev A, Heberle-Bors E (2003) Anther and microspore culture in tobacco. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 223-228

Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep 15: 561-565

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996b) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperatures. Sex Plant Reprod 9: 209-215

Yamagishi M, Yano M, Fukuta Y, Fukui K, Otani M, Shimada T (1996) Distorted segregation of RFLP markers in regenerated plants derived from anther culture of an F1 hybrid of rice. Genes Genet Syst 71: 37-41

Zhang FL, Takahata Y (2001) Inheritance of microspore embryogenic ability in Brassica crops. Theor Appl Genet 103: 254-258

Żur I, Dubas E, Krzewska M, Janowiak F (2015) Current insights into hormonal regulation of microspore embryogenesis. Front Plant Sci 6: 424

Chapter 3

Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis

Alba Rivas-Sendra, Patricia Corral-Martínez, Carolina Camacho-Fernández and Jose M. Seguí-Simarro

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

Keywords

Androgenesis, microspore culture, microspore embryogenesis, *Solanum melongena*

Plant Cell Tissue and Organ Culture (2015) 122:759–765
DOI 10.1007/s11240-015-0791-6

Abstract

Doubled haploid (DH) technology allows for the production of pure lines, useful for plant breeding, through a one-generation procedure that reduces considerably the time and resources needed to produce them. Despite the advantages of microspore culture to obtain DHs, this technique is still insufficiently developed in eggplant, where DHs are produced from microspore-derived calli through organogenesis. At present, very little is known on the best *in vitro* conditions to promote this process. This is why in this work we addressed the optimization of the process of regeneration of eggplant DH plants from microspore-derived calli. We evaluated the effect of different media compositions in the induction of organogenesis, in the promotion of shoot growth and elongation, and in root growth. According to our results, we propose the repeated subculture of the calli in MS medium with 0.2 mg/l IAA and 4 mg/l zeatin to produce shoots, and then the repeated subculture of the excised shoots in basal MS medium to promote their conversion into entire plantlets. This procedure yielded 7.6 plants per 100 cultured calli, which represents a ~4x increase with respect to previous reports. We also evaluated by flow cytometry and SSR molecular markers the effect of these *in vitro* culture conditions in the rate of DH plant production, finding that ~70% of the regenerated plants were true DHs. These results substantially improve the efficiencies of DH recovery published to date in eggplant, and may be useful to those working in the field of eggplant doubled haploidy and breeding.

Production of DHs reduces the time and costs required to produce homozygous, pure lines, essential for hybrid seed production. This is why there is an increasing effort to extend this technology to new species and to optimize the protocols already existing for agronomically interesting crops (Asif *et al*. 2014; Castillo *et al*. 2014; Eshaghi *et al*. 2015; Kim *et al*. 2013; Parra-Vega *et al*. 2013). In eggplant, doubled haploids (DHs) are typically produced through anther culture. This approach, however, has several limitations, including the possible occurrence of somatic regenerants derived from anther walls, the uncontrolled contribution of tapetal cells to culture conditions, and the general low efficiency of the technique (Seguí-Simarro 2016; Seguí-Simarro *et al*. 2011). To avoid these problems, it is possible to perform isolated microspore culture, which is much more efficient (Corral-Martínez and Seguí-Simarro 2012, 2014). Unfortunately, microspore culture in eggplant has a bottleneck still to be solved, which is the arrest of the globular-to-heart-shaped embryo transition, producing undifferentiated calli (Corral-Martínez and Seguí-Simarro 2012). This implies that DHs must be obtained through organogenesis from the microspore-derived calli. Although the first part of the process (induction of microspore embryogenesis) is highly efficient, little progress has been made in the second part (organogenesis from calli). In literature, different authors working with different eggplant explants proposed different protocols to regenerate plantlets (Franklin *et al*. 2004; Gisbert *et al*. 2006; Borgato *et al*. 2007; Xing *et al*. 2010; Kaur *et al*. 2011). Only two studies addressed the particular case of regeneration from microspore-derived callus (Miyoshi 1996; Corral-Martínez and Seguí-Simarro 2012), and their results were not conclusive. Therefore, the aim of this work is to optimize the process of regeneration of DH plants from androgenic calli obtained through isolated microspore culture.

We isolated and cultured microspores from 'Bandera' (a F1 hybrid from Seminis Vegetable Seeds Ibérica, S.A., Spain) according to the protocol described in Corral-Martínez and Seguí-Simarro (2012, 2014). 'Bandera' was selected because in previous studies, it showed the highest response among different eggplant genotypes (Salas *et al*. 2011; Corral-Martínez and Seguí-Simarro 2012). To promote organogenesis, calli exceeding 1 mm after 30 days of culture were

isolated and transferred to two modified MS media (pH 5.8) previously described as useful to induce organogenesis in eggplant calli, explants or protoplasts. One of them (M1) was supplemented with 2% sucrose, 0.8% plant-agar, 0.2 mg/l IAA and 4 mg/l zeatin (Miyoshi 1996), and the other (M4) was supplemented with 3% sucrose, 0.4% Phytagel, 0.1 mg/l indole acetic acid (IAA) and 2 mg/l zeatin (Borgato *et al*. 2007; Xing *et al*. 2010). In our previous studies using 8 different media, M1 and M4 produced the best results in terms of organogenesis induction (Corral-Martínez and Seguí-Simarro 2012). Cultures were kept at 25ºC under a 12/12 photoperiod and subcultured every 5 weeks. Callus growth could be observed 4-5 days after callus isolation. After 2 weeks of callus culture, the first organogenic buds were formed on the callus surface (Figure 1A). After 5 weeks, we were able to discriminate between two different types of calli. The first type appeared as compact and hard cell masses, non friable, and sometimes greenish (Figure 1B). This kind of callus is, in general, non organogenic (Seguí-Simarro and Nuez 2006; Corral-Martínez and Seguí-Simarro 2012). We could confirm this since no further development was observed in their surface. They eventually necrosed and died. The second type consisted of white, spongy and friable masses. We frequently observed the formation of shoot primordia in these organogenic calli, normally surrounded by two or more leaf primordia (Figure 1C). After 4 months of culture, calli in M1 produced 441 shoot primordia whereas calli in M4 produced 26 shoot primordia. 157 out of the 1,392 calli cultured in M1 (11.3%) produced at least one shoot primordium, while this rate was reduced to 16 out of 1,311 calli (1.2%) in M4. Most of the primordia produced were hyperhydrated and did not develop further (data not shown). Differentiated shoots with an elongated stem, a normal appearance, fully formed leaves, and no hyperhydration (i.e. ready for rooting; Figure 1D) could only be observed in 51 out of the 2,703 calli (M1+M4). M1 was also better than M4 in producing this type of shoot (37 vs 14 shoots, respectively). All these results considered, M1 clearly appeared as better than M4 for organogenesis induction, but none of the media tested was appropriate to promote shoot growth and elongation efficiently. It is interesting to note that, as opposed to our results, Corral-Martínez and Seguí-Simarro (2012) chose M4

instead of M1. This apparent discrepancy can be explained by the time point used for the analysis. In the previous work, shoots were studied after 5 weeks of culture (not after 4 months as hereby). Although M1 and M4 produced callus growth and shoot induction at a similar rate, M4 promoted rooting at a higher rate. Nevertheless, the final percentage of entire DH plants was very low, justifying the need for the improved regeneration protocol we hereby present.

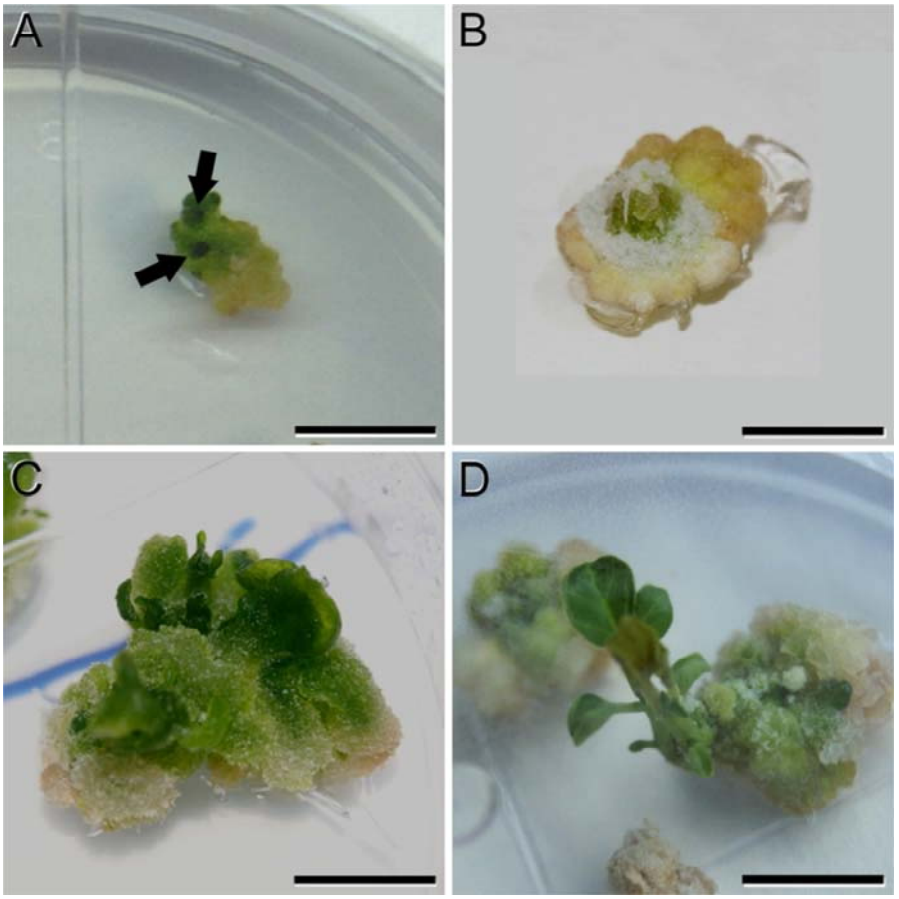


Figure 1. Evolution of calli in shoot induction media. A: Organogenic callus after two weeks in shoot induction media. Note the formation of shoot primordia (arrows) on the callus surface. B: Non-organogenic callus after 5 weeks in shoot induction media. C: organogenic callus after 5 weeks in shoot induction media. D: Shoot emerging from a callus. Bars: 1 cm.

To increase the rate of normal-appearing, elongated shoots, we tested the effect of different media on shoot growth and elongation (Table 1) as a previous step before rooting.

Table 1. Performance of the M11, M12, M13 and M14 media after 6 months expressed as the number of shoots presenting spontaneous rooting, ready for transference to rooting medium (RM), acclimated from RM, and total acclimated (spontaneous rooting + acclimated from RM). Numbers between brackets express percentages.

Medium	Shoots	Calli	Spontaneous rooting	Transferred to RM	Acclimated from RM	Total acclimated	Yield (plants/100 calli)
M11	150	501	19 (12.7)	27 (18%)	19 (70.4)	38 (25.3)	**7.6%**
M12	128	428	1 (0.8)	14 (10.9)	10 (71.4)	11 (8.6)	**2.6%**
M13	153	511	2 (1.3)	40 (26.1)	29 (72.5)	31 (20.3)	**6.1%**
M14	124	415	2 (1.6)	19 (15.3)	13 (68.4)	15 (12.1)	**3.6%**
Total	555	1855	24	100	71	95	

We produced a new batch of 1,855 calli cultured in M1 medium. From them, we excised 555 shoots with leaf primordia surrounding a visible meristem, and transferred the shoots to four different media: M11, M12, M13 and M14. All of them included MS medium (pH 5.8), 0.8% plant-agar and different growth regulators, as follows. M11 included no regulators, M12 was supplemented with 0.3 g/l 6-benzyladenine (BA) according to Kaur *et al*. (2011), M13 was supplemented with 1.5 mg/l giberellic acid (GA_3; Shivaraj and Rao 2011) and M14 was supplemented with 1.5 mg/l GA3 and 8 mg/l AgNO3 (Xing *et al*. 2010). A minimum of 124 shoots were cultured in each of the four media. First, we evaluated shoot elongation after 30 days of culture, classifying them in five discrete types: (0) dead shoots; (1) no growth, or formation of swollen, hyperhydrated organs with no stem growth (Figure 2A); (2) formation of new, no hyperhydrated organs, but no stem elongation (Figure 2B); (3) formation of new, no hyperhydrated organs and stem elongation (Figure 2C); (4) root formation in addition to all the features of type 3 (Figure 2D).

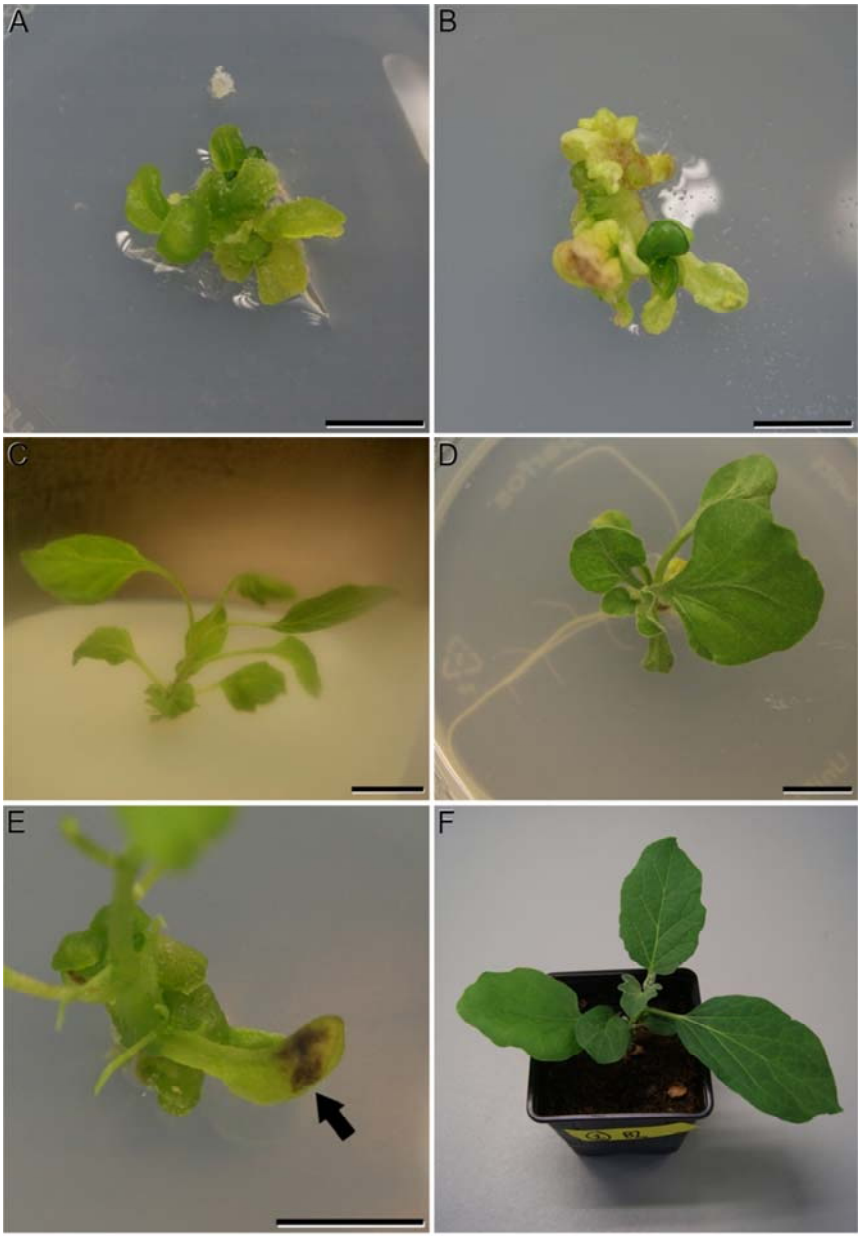


Figure 2. Different types of shoots formed after 30 days in elongation media. A: type 1, no shoot growth and formation of swollen, hyperhydrated organs with no stem growth; B: type 2, formation of new, no hyperhydrated organs, but no stem elongation; C: type 3, formation of new, no hyperhydrated organs and stem elongation; D: type 4, root formation in addition to all the features of type 3. E: presence of necrotic lesions in shoots cultured in $AgNO_3$-containing M14 medium. F: Fully regenerated and acclimated, microspore-derived eggplant DH plant. Bars: 1 cm.

Shoot development was found to be dependent on the elongation medium used according to a $\chi 2$ test ($p \leq 0.05$). As seen in Figure 3, 61% of the shoots cultured in M11 formed new, normal-appearing organs (type 2, 3 and 4). From them, elongation was limited to 11% of the shoots, and formation of roots was observed in some cases (3%). In contrast, in GA3-containing media (M13 and M14) the production of new organs was lower than in M11 (55 and 53%, respectively), although elongation was induced in a percentage of shoots similar to M11 (11%). In the particular case of M14, shoots elongated quickly, but many of the newly formed organs presented necrotic spots (Figure 2E). Overall, M12 was the worst medium in terms of promotion of shoot growth and elongation.

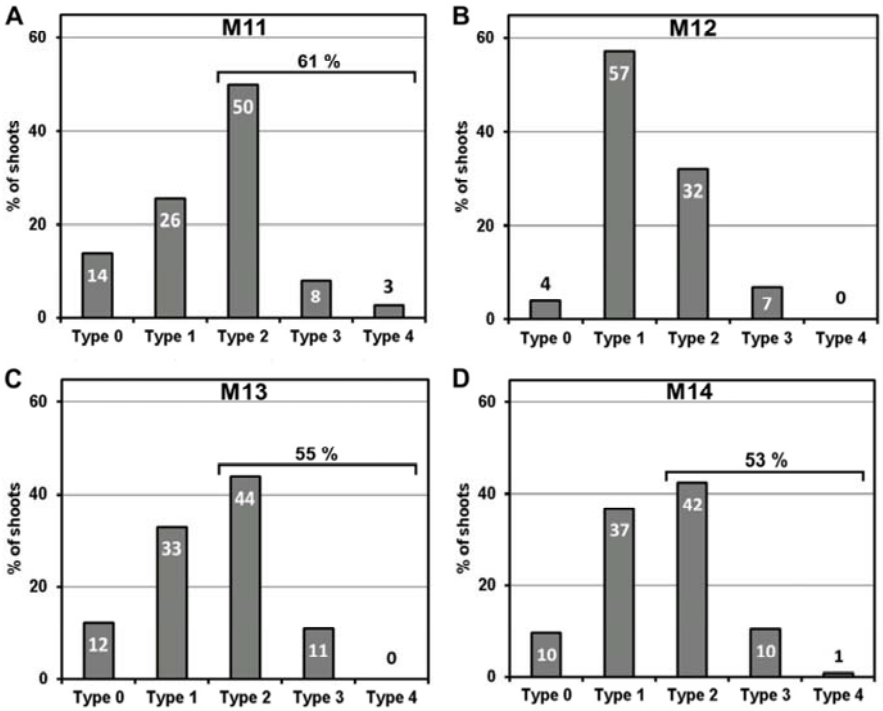


Figure 3. Percentages of the different shoot types observed after 30 days in four elongation media: M11 (A), M12 (B), M13 (C) and M14 (D). For each elongation medium the shoots produced are classified in five types: type 0 (dead shoots), type 1 (no growth, or formation of swollen, hyperhydrated organs with no stem growth), type 2 (formation of new, no hyperhydrated organs, but no stem elongation), type 3 (formation of new, no hyperhydrated organs and stem elongation) and type 4 (root formation in addition to all type 3 features. The percentages above the brackets correspond to the sum of the shoot types forming new, normal-appearing organs (types 2+3+4).

Non-rooting shoots were kept in their elongation media beyond the 30-day checkpoint, and individually transferred to rooting medium once they reached a minimal length of 2 cm. During the 6-month period considered, M13 was the medium that induced elongation in more shoots (Figure 4A; Table 1). Most of the non-rooted shoots developed within the first 3 months (Figure 4B). In particular, shoots in M14 reached the stage suitable for rooting faster than shoots from other media, including M13. Both media have 1.5 mg/l GA3, known to be involved in many developmental responses, including the promotion of stem elongation (Moshkov *et al*. 2008). This would explain the higher shoot elongation in these media. M14 included also AgNO3, an ethylene inhibitor known to promote shoot growth and elongation in several species (Mohinuddin *et al*. 1997; Sgamma *et al*. 2015; Zhang *et al*. 2001) including eggplant (Xing *et al*. 2010). However, the results of M14 after 6 months were clearly below those of M13. It is likely that the Ag+ cations present in M14 had a toxic effect (Veen and van de Geijn 1978) that accounted for the necrosed areas observed in these explants (Figure 2E), and prevented further development.

On the other hand, some shoots were able to form roots while they were cultured in elongation medium, before the stem was 2 cm long, and therefore, before being transferred to rooting medium. M11 induced rooting in 12.7% of the shoots, while in the other media the frequency was much lower (Figure 4A; Table 1). These results pointed to M11 (basal MS medium) as the best medium to induce the development of new normal-appearing organs, including roots, and therefore the formation of entire plantlets. Previous studies also pointed to this medium as the best to induce rooting in different explants and calli of eggplant and wild relatives (Miyoshi 1996; Franklin *et al*. 2004; Gisbert *et al*. 2006; Xing *et al*. 2010). However, the frequency of rooting in M11 during the elongation period was relatively low (12.7%), possibly due to the fact that most these shoots were not differentiated enough to be able to root spontaneously. This is why we considered the addition of a rooting step (with M11) to the protocol. Around 71% of the elongated shoots produced in M11, M12, M13 and M14 developed roots when transferred to M11, forming entire *in vitro* plantlets. As seen in Table 1, the rooting

percentages were remarkably similar, ruling out a different effect of the elongation medium used in the rooting capacity of the shoots after transference to rooting (basal) medium. Taken together, our short-term results (1 month) and mid-term results (6 months) showed that M11 and M13 were the best performing media, and while M13 produced more elongated shoots after 6 months, M11 produced more entire plantlets. Besides, it appeared that the addition of an elongation step after shoot initiation was beneficial not only to produce normal-appearing, elongated shoots, but also to increase their rooting ability.

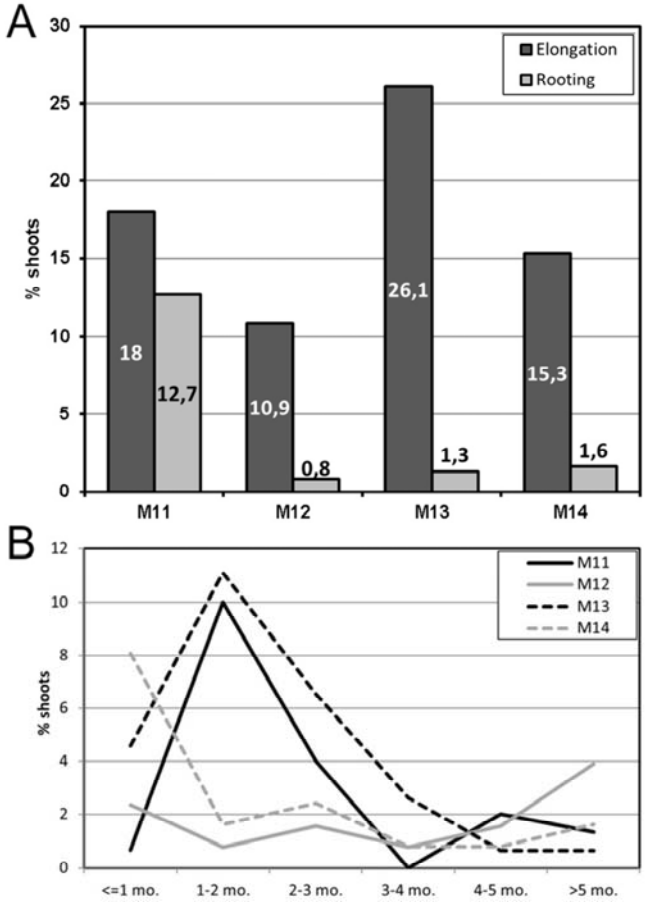


Figure 4. A: Frequencies of the elongated and rooted shoots observed after 6 months in the elongation media used. B: Time needed by shoots cultured in different elongation media to be ready for transference to rooting medium. See text for further details.

Rooted plants were transferred to pots with substrate and kept in a growth chamber at 25ºC and a 16/8 photoperiod (Figure 2F). For acclimatization and hardening, a transparent plastic cup was placed covering the whole plant, and humidity was progressively reduced by drilling the cup. Once plants were acclimated, their ploidy was checked by flow cytometry. Small pieces of young leaves were chopped, processed basically as described in Corral-Martínez and Seguí-Simarro (2012), and analyzed using a Partec PA-I Ploidy Analyzer. Ploidy analysis of 88 microspore-derived plants revealed that 5 (5.7%) were haploid, while 61 (69.3%) were diploid and 18 (20.5%) showed higher ploidies. The histograms of 4 plants presented multiple peaks or excessive background noise that precluded an unambiguous determination of their ploidy. Diploid plants were analyzed with microsatellite (SSR) molecular markers using the procedure and markers described in Corral-Martínez and Seguí-Simarro (2012). These markers were proven heterozygous for the 'Bandera' donor plants. The 61 diploid plants analyzed were consistently found homozygous for all the SSR makers used, as expected since they came from isolated microspore cultures where anther wall tissue is filtered out. These results are in agreement with our previous studies with these SSR markers in 'Bandera', where we repeatedly proved a gametophytic (initially haploid) origin for all the regenerants obtained (Corral-Martínez and Seguí-Simarro 2012). Genetic variation, from gross ploidy differences to subtle genetic changes, is a well-known phenomenon inherent to all *in vitro* culture processes (reviewed in Dunwell 2010). However, the consistency of the SSR analyses reported hereby and previously published make us think that in this particular case, genetic variation should not have a great impact. In addition, ~70% of DHs is a percentage better than previous results from microspore cultures in this and other eggplant cultivars, which revolved around 60% (Miyoshi 1996; Corral-Martínez and Seguí-Simarro 2012). This percentage is much better than those obtained through anther culture (46.4% of DHs in Salas *et al*. 2011, 25.6% in Rotino 1996, 23.5% in Tuberosa *et al*. 1987 and 15.4% in Dumas de Vaulx and Chambonnet 1982). Thus, it appeared that the prolonged *in vitro* culture time

did not affect the rate of occurrence of higher ploidies, while it seemed beneficial in terms of reducing the rate of haploids.

In this work we addressed the main limiting step of DH production in eggplant through microspore culture: the regeneration of entire DH plants from microspore-derived calli. After testing several media previously proposed in the literature to regenerate eggplant plantlets from explants of different origins, the results presented hereby clearly point to the use of M1 (Miyoshi 1996) to obtain the highest rate of shoot-producing organogenic calli. However, this medium alone is not sufficient to promote shoot growth. According to our results, we propose the repeated subculture (approximately on a monthly basis) of the microspore-derived calli in M1, and then the repeated subculture of the excised shoots in basal MS medium (M11) to promote the development of new normal-appearing organs and the rooting of shoots, and therefore the formation of entire plantlets. As shown in Table 1, this procedure allowed us to obtain 7.6 plants every 100 cultivated calli, which represents a remarkable improvement (~4x) compared with the 2% obtained in the only previous reference of entire plant regeneration from eggplant microspore-derived calli (Miyoshi 1996). We also showed that media supplemented with GA3, and in particular M13, produced more elongated shoots, ready for rooting, than any other media. However, the inability of this medium to promote root growth, and the increased time and expenses associated to the use of an additional step with M13, make us to discourage its use. Finally, we obtained a high frequency of DH individuals (69.3%), which is also higher than those previously reported for eggplant DHs coming from both anther and microspore culture. To increase this frequency, the genome of the 5.7% haploids might be duplicated with colchicine or oryzalin (Dhooghe *et al*. 2011). However, ~70% of DHs seems a percentage good enough for most breeding programs, and makes us propose to discard the addition of a step for genome doubling of the haploid individuals, which can just be disposed of.

Acknowledgements

We acknowledge Dr. Rosa Peiró for her statistical advice, and the staff of the COMAV greenhouses for their valuable help. This work was supported by the AGL2014-55177-R grant from Spanish MINECO to JMSS.

References

Asif M, Eudes F, Randhawa H, Amundsen E, Spaner D (2014) Phytosulfokine alpha enhances microspore embryogenesis in both triticale and wheat. Plant Cell Tissue Organ Cult 116: 125-130

Borgato L, Conicella C, Pisani F, Furini A (2007) Production and characterization of arboreous and fertile *Solanum melongena* plus *Solanum marginatum* somatic hybrid plants. Planta 226: 961-969

Castillo AM, Nielsen NH, Jensen A, Vallés MP (2014) Effects of n-butanol on barley microspore embryogenesis. Plant Cell Tissue Organ Cult 117: 411-418

Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica 187: 47-61

Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382

Dhooghe E, Van Laere K, Eeckhaut T, Leus L, Van Huylenbroeck J (2011) Mitotic chromosome doubling of plant tissues *in vitro*. Plant Cell Tissue Organ Cult 104: 359-373

Dumas de Vaulx R, Chambonnet D (1982) Culture *in vitro* d'anthères d'aubergine (*Solanum melongena* L.): stimulation de la production de plantes au moyen de traitements à 35ºC associés à de faibles teneurs en substances de croissance. Agronomie 2: 983-988

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8: 377-424

Eshaghi ZC, Abdollahi MR, Moosavi SS, Deljou A, Seguí-Simarro JM (2015) Induction of androgenesis and production of haploid embryos in anther

cultures of borage (*Borago officinalis* L.). Plant Cell Tissue Organ Cult: 1-9. doi: 10.1007/s11240-015-0768-5

Franklin G, Sheeba CJ, Sita GL (2004) Regeneration of eggplant (*Solanum melongena* L.) from root explants. *In vitro* Cellular & Developmental Biology-Plant 40: 188-191

Gisbert C, Prohens J, Nuez F (2006) Efficient regeneration in two potential new crops for subtropical climates, the scarlet (*Solanum aethiopicum*) and gboma (*S. macrocarpon*) eggplants. New Zeal J Crop Hort Sci 34: 55-62

Kaur M, Dhatt AS, Sandhu JS, Gosal SS (2011) *In vitro* plant regeneration in brinjal from cultured seedling explants. Indian Journal of Horticulture 68: 61-65

Kim M, Park E-J, An D, Lee Y (2013) High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112: 191-201

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Mohinuddin AKM, Chowdhury MKU, Abdullah Zaliha C, Napis S (1997) Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. Plant Cell Tissue Organ Cult 51: 75-78

Moshkov IE, Novikova GV, Hall MA, George EF (2008) Plant Growth Regulators III: Gibberellins, Ethylene, Abscisic Acid, their Analogues and Inhibitors; Miscellaneous Compounds. In EF George, MA Hall, GJ De Klerk, eds), Plant Propagation by Tissue Culture, Ed 3 Vol 1. Springer, Dordrecht, The Netherlands

Parra-Vega V, Renau-Morata B, Sifres A, Seguí-Simarro JM (2013) Stress treatments and *in vitro* culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (*Capsicum annuum* L.). Plant Cell Tissue Organ Cult 112: 353-360

Rotino GL (1996) Haploidy in eggplant. In SM Jain, SK Sopory, RE Veilleux, eds), *In vitro* haploid production in higher plants, Vol 3. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 115-141

Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther culture in common eggplant and related species. Euphytica 182: 261–274

Seguí-Simarro JM (2015) Androgenesis in solanaceae. In MA Germanà, M Lambardi, eds), *In vitro* embryogenesis. Springer Science + Business Media, The Netherlands, p In Press

Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011) Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778

Seguí-Simarro JM, Nuez F (2006) Androgenesis induction from tomato anther cultures: callus characterization. Acta Hort 725: 855-861

Sgamma T, Thomas B, Muleo R (2015) Ethylene inhibitor silver nitrate enhances regeneration and genetic transformation of *Prunus avium* (L.) cv Stella. Plant Cell Tissue Organ Cult 120: 79-88

Shivaraj G, Rao S (2011) Rapid and efficient plant regeneration of eggplant (*Solanum melongena* L.) from cotyledonary leaf explants. Indian J Biotechnol 10: 125-129

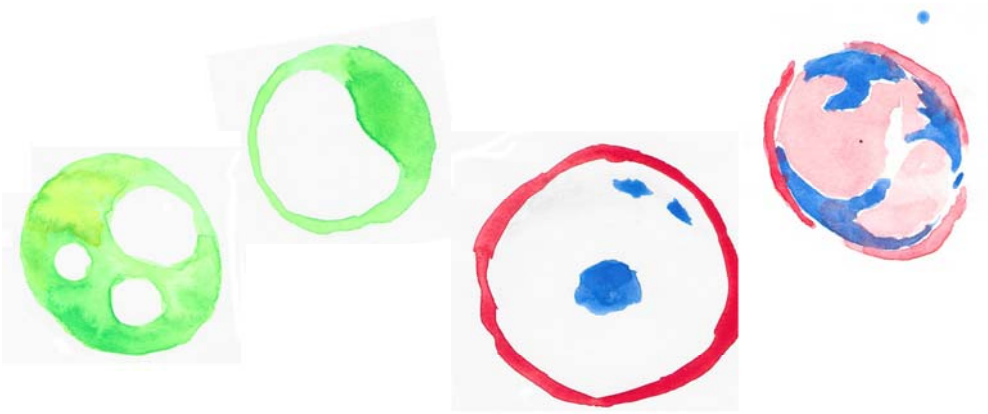
Tuberosa R, Sanghineti MC, Conti S (1987) Anther culture of eggplant *Solanum melongena* L. lines and hybrids. Genética Agrária 41: 267-274

Veen H, van de Geijn S (1978) Mobility and Ionic Form of Silver as Related to Longevity of Cut Carnations. Planta 140: 93-96

Xing Y, Yu Y, Luo X, Zhang JN, Zhao B, Guo YD (2010) High efficiency organogenesis and analysis of genetic stability of the regenerants in *Solanum melongena*. Biol Plant 54: 231-236

Zhang P, Phansiri S, Puonti-Kaerlas J (2001) Improvement of cassava shoot organogenesis by the use of silver nitrate *in vitro*. Plant Cell Tissue Organ Cult 67: 47-54

Second block



Rapeseed (*Brassica napus*) is an oilseed rape, also referred as canola, which belongs to the Brassicaceae family. Brassica species are widely used in the cuisine of many cultures, and different species and types of Brassicas are significant oilseed crops, vegetables or forage crops, and are used in the production of condiments, such as mustard. Within oilcrops, *B. napus* was ranked in the fourth position in 2014 in terms of production, with almost 74 million of tons produced, and in the second position in terms or area harvested, with more than 36 million of hectares, Canada being the main producer (FAOSTAT 2017).

The first protocol for androgenic DH production in *B. napus* was published in 1975 for anther culture (Thomas and Wenzel 1975). Later, protocols for shed microspore culture (Lichter 1981) and for isolated microspore culture (Lichter 1982) were also developed. Microspore culture showed to be much more efficient than anther culture (Lichter *et al*. 1988; Siebel and Pauls 1989), and nowadays it is a routine procedure in this species, but improvements of the protocol are continuously being published (Ahmadi and Shariatpanahi 2015; Custers 2003; Ferrie and Keller 2007; Leroux *et al*. 2016; Li *et al*. 2014; Möllers *et al*. 1994; Telmer *et al*. 1992). *B napus* DHs have been used in breeding (Dormann *et al*. 1998; Henderson and Pauls 1992; Zhang *et al*. 2006), and *B. napus* DH populations are being used for mapping quantitative traits and establishing marker-traits association (Fredua-Agyeman and Rahman 2016; Fu *et al*. 2017). Aside of the applied use of *B. napus* DHs, a major interest of *B. napus* resides in its use as a model species for microspore embryogenesis. *B. napus* microspore cultures present several positive characteristics that make them an ideal system, including a high rate of embryogenic cells, a quick embryo induction process, and no need for exogenous addition of growth regulators. In addition, it belongs to the same family as the model species *Arabidopsis thaliana*, where DH technology is not developed yet due to its extreme recalcitrance. Homologues of genes identified in *Brassica* are likely to be found in Arabidopsis genome and databases, so combined studies for genetic and genomic analysis are possible (Custers *et al*. 2001). Indeed, during the last 20 years, several genetic and genomic studies have been published focusing on the genetic control of microspore embryogenesis ability (Foisset *et al*.

1997; Zhang *et al*. 2003; Zhang and Takahata 2001) and on the identification of genes expressed during early embryogenesis, potentially useful as markers for embryo development (Elhiti *et al*. 2010; Joosen *et al*. 2007; Malik *et al*. 2007; Tsuwamoto *et al*. 2007).

In parallel, many other works using *B. napus* microspore cultures focused on the study of the events associated to embryogenesis induction, in an attempt to find cellular markers of the embryogenic switch. These events include structural and cellular changes during the first divisions (Dubas *et al*. 2011; Hause *et al*. 1993; Satpute *et al*. 2005; Seguí-Simarro *et al*. 2006; Telmer *et al*. 1995; Zaki and Dickinson 1990), changes in DNA synthesis and gene expression (Binarova *et al*. 1993; Boutilier *et al*. 1994; Hays *et al*. 1999; Seguí-Simarro *et al*. 2011a), the role of epigenetic modifications (Li *et al*. 2014; Rodríguez-Sanz *et al*. 2014; Solís *et al*. 2012), regulatory cascades (Chan and Pauls 2007; Seguí-Simarro *et al*. 2005), autophagy and cytoplasmic cleaning (Corral-Martínez *et al*. 2013; Parra-Vega *et al*. 2015a), altered cytokinesis (Parra-Vega *et al*. 2015b), and changes in protein synthesis (Cordewener *et al*. 2000; Cordewener *et al*. 1994; Pechan *et al*. 1991), with special focus on heat-shock proteins expression (Cordewener *et al*. 1995; Seguí-Simarro *et al*. 2003; Testillano *et al*. 2000; Zhao *et al*. 2003). Comparative studies between MDEs and zygotic embryos (Ilic-Grubor *et al*. 1998; Yeung *et al*. 1996) have shown that MDEs may hold a high morphologic similarity to zygotic embryos, and they can be used as a model to study general embryogenesis characteristics, as well as the role of different plant hormones during embryogenesis initiation and embryo development (Belmonte *et al*. 2010; Hays *et al*. 2001; Hays *et al*. 2000; Hays *et al*. 2002; Robert *et al*. 2015).

In 2015, using also *B. napus* microspore cultures as a model system, we reported the formation of a callose-rich layer below the intine which was considered an early marker of embryogenic commitment, and an abnormally prolonged presence of callose in the newly formed cell walls (Parra-Vega *et al*. 2015b). This specific dynamics of callose gives rise to transient callose-rich and cellulose-defective cell walls, which often leads to irregular and discontinuous cell

walls, and eventually to nuclear fusions between coalescing nuclei. Indirect evidence suggested that Ca^{2+} levels might regulate the observed polysaccharide deposition. In the following chapters we present a detailed study about the relationship between Ca^{2+} dynamics, polysaccharide deposition and success in microspore embryogenesis.

Chapter 4

Dynamics of calcium during *in vivo* microspore development and *in vitro* microspore embryogenesis in *Brassica napus*

Alba Rivas-Sendra and Jose M. Seguí-Simarro

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

Keywords

Androgenesis, FluoForte, *in vitro* culture, microgametogenesis, microspore culture, microsporogenesis, rapeseed.

Abstract

Calcium is widely known to have a role as a signaling molecule in many different processes, including stress response and activation of the embryogenic program. However, there are no direct clues about the role of calcium in microspore embryogenesis, an experimental process that combines a developmental switch towards embryogenesis and the simultaneous application of different stressing factors. In this work, we used FluoForte, a calcium-specific fluorescent vital dye, to track, by means of confocal microscopy, the changes in levels and subcellular distribution of calcium in living microspores and pollen grains during *in vivo* development, as well as during the first stages of *in vitro*-induced microspore embryogenesis. During *in vivo* development, a clear peak of cytosolic Ca^{2+} was observed in vacuolate microspores and young pollen grains, the stages more suitable for embryogenesis induction. Just after *in vitro* induction, Ca^{2+} levels increased specifically in embryogenic microspores at levels dramatically higher than during *in vivo* development. The increase was observed in the cytosol, but predominantly in the vacuoles. Non-embryogenic forms such as callus-like and pollen-like structures presented remarkably different calcium patterns. After the heat shock-based inductive treatment, Ca^{2+} levels progressively decreased in all cases. Together, our results reveal a unique calcium dynamics in cells reprogrammed towards embryogenesis, establishing a link between changes in Ca^{2+} level and subcellular distribution, and microspore embryogenesis.

Introduction

Microspores are the precursors of pollen grains, haploid gametophytes that will give rise to the male gamete in plants. By applying specific stresses and conditions *in vitro*, microspores can be deviated from the original gametophytic pathway towards a new, experimentally induced embryogenic fate. These already differentiated cells are then reprogrammed to become totipotent and start dividing sporophytically to produce a new haploid microspore-derived embryo (MDE). Either spontaneously or using genome duplication treatments, haploid MDEs may become doubled haploid (DH) and therefore 100% homozygous. This experimental process is highly interesting for both basic research and applied plant breeding. At the basic research level, the main interest of microspore embryogenesis resides on being an exceptional example of the fascinating developmental plasticity of plant cells. The change in cell fate undergone by microspores constitutes an excellent system to understand the cellular and molecular mechanisms underlying cell totipotency. Once the particular characteristics of this system are well understood, it may serve as a model for the study of the very early stages of zygotic embryogenesis itself, overcoming the technical difficulties imposed by the maternal tissues surrounding the zygotic embryo.

In *Brassica napus* microspore cultures, embryogenic development starts from late uninucleated microspores and young bicellular pollen. A series of sporophytic divisions make the multicellular MDE grow inside the pollen exine, stretching and thinning it until the increasing size causes its rupture (Hause *et al*. 1994). MDEs that emerge from the exine follow the typical morphogenic pattern of zygotic embryos through globular, heart-shaped, torpedo and cotyledonary stages, while microspores in culture either arrest and die (Seguí-Simarro and Nuez 2008), or continue a gametophytic-like development to become pollen-like structures which, after 5-6 days in culture, usually burst and therefore die (Soriano *et al*. 2013).

To be induced to embryogenesis, microspores of most species, including those of *B. napus*, are submitted to a heat stress treatment. The first perception of heat stress occurs at the level of the plasma membrane via changes in its fluidity (Horvath *et al*. 1998; Vigh *et al*. 1985), which, together with the activation of stress-specific Ca^{2+}-permeable channels, causes a transient increase in cytosolic Ca^{2+} levels (Liu *et al*. 2005). Elevation of cytosolic calcium levels is thought to be a primitive and universal response to stress (White and Broadley 2003). As a response to specific Ca^{2+} perturbations, cells activate specific combinations of Ca^{2+} sensors (Ca^{2+}-binding proteins). Binding to Ca^{2+} change their properties, which in turn modify the way they interact with target proteins, thereby altering many different aspects of cell physiology which, altogether, may result in stress tolerance and/or a developmental switch (White and Broadley 2003). However, the roles and locations of calcium go far beyond being an intracellular messenger in the cytoplasm. Plant cells store calcium in different compartments, including the endoplasmic reticulum, nucleus, cell wall and vacuoles. At first, it was thought that the major source of stored calcium was the cell wall, where it is tightly bound to pectins and plays a key role in cell wall physiology (Demarty *et al*. 1984). Recently, it was proposed that cell wall AGPs act as calcium capacitors to supply Ca^{2+} to the cytosol on demand (Lamport and Varnai 2013). However, other studies pointed out that, regardless of the roles of other compartments as calcium stores, the major calcium store in plants appeared to be the vacuole (Rudd and Franklin-Tong 2001), where it acts as a counter-cation for different inorganic and organic anions (White and Broadley 2003). From these compartments, calcium is mobilized to produce fluctuations in cytoplasmic Ca^{2+} levels that play key signaling and regulating roles in multiple physiological and developmental processes, including cell division and growth, stomatal closure, and response to several stresses, including pathogen attack and wounding (reviewed in (White and Broadley 2003). Using the potassium pyroantimonate technique, calcium distribution and levels have been assessed during anther development in different species. From these and other studies, it was deduced that calcium has a direct role in pollen development. Indeed, altered calcium distribution in anther walls and pollen in tobacco, wheat

and rice leads to pollen abortion (Li *et al*. 2001; Meng *et al*. 2000; Tian *et al*. 1998), and gradients of Ca^{2+} are important to determine the polarity and location of pollen pores and growing pollen tubes (Tirlapur and Willemse 1992). Later, during post-zygotic development, gradients of Ca^{2+} are important for the establishment of embryo polarity and seed germination (Hause *et al*. 1994).

Even in the context of the experimental induction of microspore embryogenesis, Ca^{2+} has been suggested to be either necessary or helpful for microspore induction as well as for MDE germination and conversion into plants. Experimenting with extracellular calcium concentrations and calcium signaling modulators, a relationship between calcium modulation and embryogenesis induction was proposed in *B. napus* (Pauls *et al*. 2006), barley (Cho and Kasha 1995) and bread wheat (Reynolds 2000). Usually, stresses mobilize calcium stores, eliciting an increase in cytosolic free Ca^{2+} levels which, in turn, activates mitogen-activated protein kinase (MAPK) cascades, among other protein phosphatase/kinase cascades (Pearce and Humphrey). Interestingly, different components of MAPK cascades have been found differentially regulated during stress-mediated induction of microspore embryogenesis in *B. napus* and tobacco (Coronado *et al*. 2002; Seguí-Simarro *et al*. 2005). Hause *et al*. (1994) studied the distribution of calmodulin and free cytosolic Ca^{2+} in globular and elongated MDEs, finding some degree of polarization in the latter. Calcium was also found helpful for conversion of *B. napus* into plantlets (Tian *et al*. 2004). However, although its role in embryogenesis induction is suspected from indirect evidences and deductions, the knowledge about the precise role of calcium in this process is still very scarce and poorly understood. Perhaps due to the technical difficulties of detecting calcium in a complex *in vitro* system like this, to our knowledge nobody has shown how calcium distributes and accumulates during microspore embryogenesis. In this work we study the changes in levels and subcellular distribution of calcium during microspore/pollen *in vivo* development and during the first stages of *in vitro*-induced microspore embryogenesis, establishing a link between calcium increases and microspore embryogenesis.

Materials and methods

Plant material

Donor plants of *Brassica napus* L. cv. Topas line DH4079 were grown in growing chambers of the COMAV Institute (Universitat Politècnica de València, Valencia, Spain). Plants were grown in 20 cm pots at 60% humidity and 16/8 photoperiod, kept at 20ºC until flowering and then transferred to 15ºC.

Brassica napus microspore culture

Isolation, induction treatment and microspore culture were performed according to Custers (2003). Flower buds containing mostly late uninucleated microspores and young bicellular pollen were dissected from the plant, surface sterilized with 2% sodium hypochlorite for 10 min, and washed three times in sterile distilled water for a total of 15 min. Buds were crushed with a sterile syringe piston in NLN-13 medium. NLN13 medium consist of NLN medium as described by (Nitsch and Nitsch 1967) supplemented with 13 % sucrose and sterilized by filtration though 0.22 µm filter. Microspores were isolated from the suspension by filtration through 40 µm nylon mesh (Millipore) followed by three rounds of centrifugation at 100 g for 4 min each. Microspore density was calculated with a hemocytometer and adjusted to 4×10^4 microspores per ml. The cellular suspension was plated, incubated in darkness for 3 days at 32.5 ºC to induce embryogenesis, and then at 25ºC in darkness for embryogenesis progression.

Callose and calcium staining and detection

B. napus microspores and pollen at different stages during microsporogenesis and microgametogenesis were stained with FluoForte (FF; Enzo Life Sciences, ENZ-52015) for calcium detection and with aniline blue (Fluka, 96290) for callose

detection. Microspores and pollen were isolated from flower buds at different stages. To minimize the stress, the extraction process was done as quickly as possible and the solutions and plant material used were kept at 4ºC. Excised buds were crushed with a syringe piston in phosphate-buffered saline (PBS), filtered through a 40 μm nylon mesh and centrifuged at 100 g for 4 min. The supernatant was discarded and the concentrated cell suspension was mixed with the same volume of 0.2 g/l FF in PBS and incubated in darkness during 30 min. Then, cells were washed with 1 ml PBS and centrifuged in an Eppendorf centrifuge at 200 g for 2 min. The supernatant was discarded and pelleted cells were mounted in a microscope slide and immediately observed. Microspore cultures were prepared as described above, collected at different culture times and stained following the same protocol, with two modifications: samples and solutions were kept at room temperature and 0.2% aniline blue was also added to the staining mix. Stained cells were mounted in a microscope slide and immediately observed. In all cases, observations were carried out with a Zeiss LSM 780 confocal laser scanning microscope.

Image analysis of fluorescence

Digital images were processed with Leica Application Suite Advanced Fluorescence (LAS AF) and FIJI software. Spectral imaging of mature pollen grains stained with FF was carried out in the confocal laser scanning microscope with laser excitation at 488 nm. A set of images was obtained, with each image being acquired with a separate narrow bandwidth (8.9 nm), representing the complete spectral distribution of the fluorescence emission signals for every point of the image. The spectral analysis of defined areas and the visualization of the images in coded colors depending on the emission spectrum was performed using the advanced linear unmixing function (LAS software), which separates mixed signals pixel by pixel using the entire emission spectrum of each defined fluorescent compound in the sample. Autofluorescence and FF signal were differentiated by comparison with the reference emission spectrum of FF provided by the

manufacturer (www.enzolifesciences.com). Using Fiji software, different cell regions were selected (vacuoles, cytosol+nucleus, and exine) and their mean fluorescence intensity was measured.

Results

Dynamics of FF during observation in living cells

FF has been successfully used in animal cells (Blaauw *et al*. 2012; Kim *et al*. 2013) but, to the best of our knowledge, has not yet been used to detect calcium in plant cells. Conventional calcium-sensitive fluorescent dyes are known to undergo compartmentalization after some time of incubation (Fricker *et al*. 2001). Therefore, we performed some preliminary tests to adjust the best conditions to study calcium levels and distribution in *in vivo* and *in vitro* cultured microspores. Previous experiments with *B. napus* root tips (data not shown) helped us to adjust the best working concentration of FF to 0.1 g/l. Then, we studied the dynamics of FF staining in living, freshly isolated microspores and pollen grains at different time points. Approximately 15 min after mounting samples in the slides (Fig. 1A), both vacuolate microspores (Figs. 2A, A') and pollen grains (Figs. 2B, B') clearly showed an intense nuclear-cytosolic FF signal, vacuoles exhibiting a very low signal, barely eye-detectable. Prolonged observation of these cells revealed a progressive decrease of the nuclear-cytosolic signal and a parallel increase of the vacuolar signal. Thereby, 60 min after mounting (Fig. 1B), the FF signal was almost excluded from the nucleus-cytosol and principally localized in the vacuoles. Such a transition from cytosolic to vacuolar signal was observed in both vacuolate microspores (Figs. 2C, C') and pollen grains (Figs. 2D, D'). After one hour, no further changes were observed. Quantitatively, the nucleo-cytosol/vacuole ratio averaged for the three stages shown in Fig. 1 was 4.2 (4-fold more signal in the nucleus-cytosol than in vacuole) when observed within 15 min, and 0.03 (30-fold less signal in nucleus-cytosol than in vacuole) when observed after 60 min. Thus,

we concluded that prolonged incubation times caused a progressive loss of signal together with a compartmentalization of the FF signal into vacuoles. However, observation of cells around 15 min after incubation and mounting consistently showed a clear nucleo-cytosolic signal in these cells. Thus, we assumed this time point as the optimal to reliably detect calcium with FF in our cells.

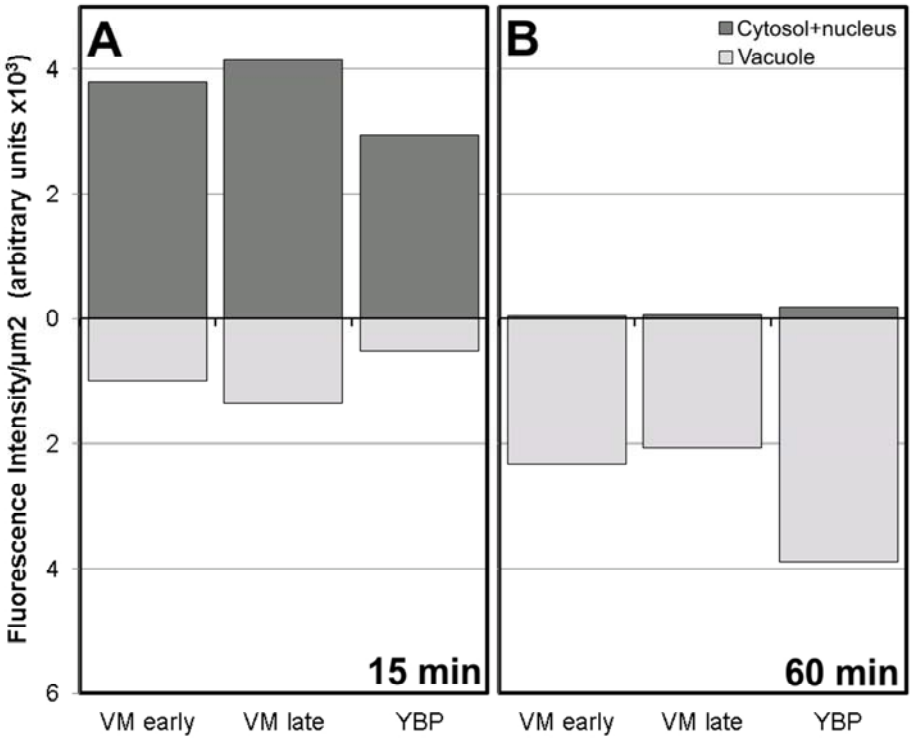


Figure 1. Fluorescence intensity of FF staining in the cytosol+nucleus region and in vacuoles of vacuolated microspores and young bicellular pollen, 15 min (A) and 60 min (B) after mounting the samples. VM: vacuolate microspore, YBP: young bicellular pollen.

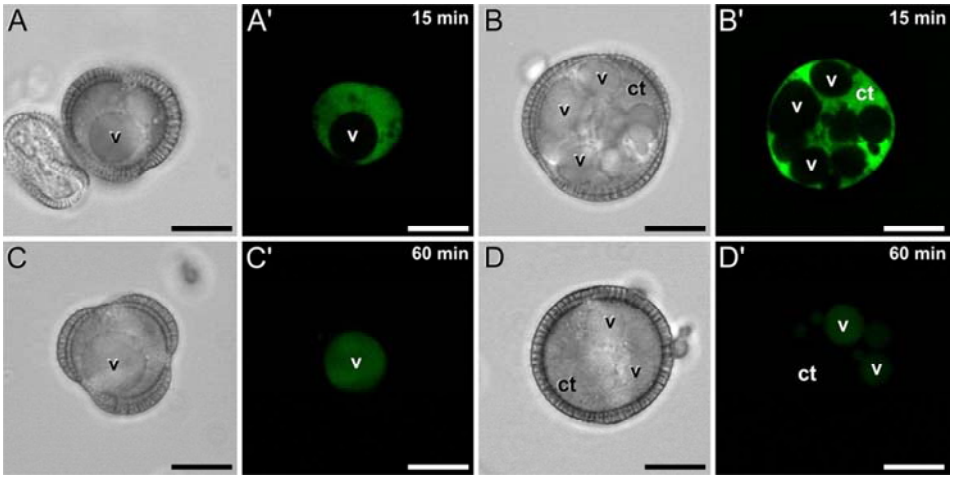


Figure 2. FF staining of microspores and pollen after 15 and 60 minutes of observation. Bright field and FF staining pairs of pictures are shown. Vacuolate microspore (A, A') and early binucleated pollen (B, B') after 15 min of observation. Vacuolated microspore (C, C') and early binucleated pollen (D, D') after 60 min of observation. Note the change of FF staining localization from nuclear-cytosolic to vacuolar in both cell types. ct: cytosol, v: vacuole. Bars: 10 µm.

Calcium distribution during microspore and pollen development

We carefully examined a minimum of 20 cells at each of the stages studied. Observations were remarkably consistent for each stage. A representative example of the calcium distribution at each stage during microsporogenesis and microgametogenesis is shown in Fig. 3. Tetrads (Figs. 3A, A') and young microspores just released from the tetrad (Figs 3B, B') presented a very low calcium signal, principally located in few, small cytosolic foci, likely corresponding to cytoplasmic organelles. Mid microspores showed no detectable calcium signal (Figs 3C, C'). At the onset of microspore vacuolation, calcium signal accumulated in the cytosol and nucleus, while no detectable signal was found in the vacuole (Figs. 2A, A'). Late unicellular, vacuolated microspores (Figs. 3D, D') also showed a nucleo-cytosolic signal, but brighter than in the previous stage. As in the previous stage, the large, central vacuole was devoid of fluorescence, but in this stage, the nuclear region appeared slightly brighter than the cytosol. The highest signal intensity was observed after the first pollen mitosis, in early binucleated pollen

(Figs. 3E, E'; Supplementary Movie S1). In this stage, an intense fluorescence was observed in the cytosol and even more intense in the nuclei, but not in the mid-sized vacuoles that resulted from fragmentation of the large vacuole. In the mid pollen grain (Figs. 3F, F') calcium staining was in general less intense, but it was observed again in the cytosol and the centrally positioned nuclei. The small and numerous vacuoles and the starch granules typical from this pollen stage showed no detectable signal. In order to confirm our qualitative observations, we calculated for all the cells studied at each stage, the average FF fluorescence intensity/μm2 in all the cell area and in each of the identifiable cell regions. As seen in Table 1 and in Fig. 4A, this analysis confirmed that at the stages of microspore vacuolation and first pollen division, calcium signal strongly increases in the nuclear-cytosolic regions, staying very low in vacuoles. In mature pollen grains (Figs. 5A, A'), a faint, barely detectable signal was observed inside the pollen grain, in line with the progressive decrease observed after the first pollen mitosis. However, numerous bright foci were detected in the exine, outside the cell. To exclude exine autofluorescence, a known and well documented phenomenon (Roshchina 2012), we analyzed both emission spectra. Spectral imaging as described in Materials and Methods was carried out in preparations containing both microspores and mature pollen grains and exposed to the same excitation conditions. Small regions were selected in the pollen and microspore exine (Fig. 5C, green and pink arrowheads, respectively), and the fluorescence emission spectra of the selected areas were analyzed. As seen in Fig. 5D, the pollen exine spectrum (green curve) presented the characteristic emission spectrum of FF (one neat peak with a maximum in 525 nm), whereas the microspore exine spectrum (pink curve) showed multiple peaks at different wavelengths, typical of autofluorescence. Indeed, when the linear unmixing procedure provided by the LAS AF software was used to assign different color to areas with different emission spectrum, both signals perfectly overlaid (Fig. 5E). Thus, the exine signal observed in mature pollen grains was not due to autofluorescence.

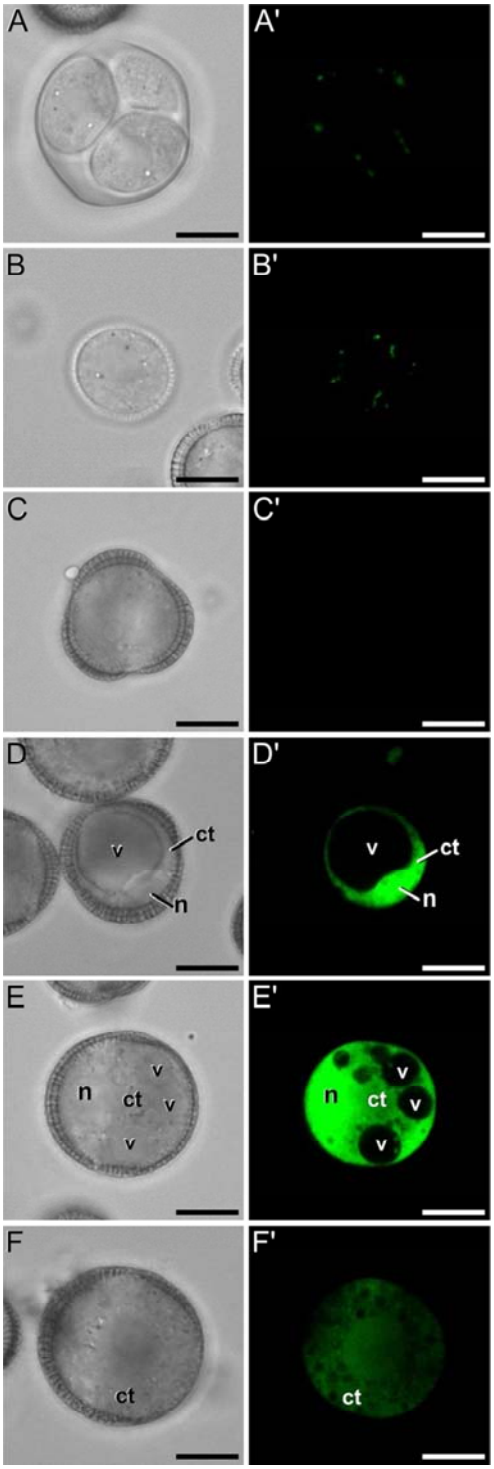


Figure 3. FF staining during *in vivo* microspore/pollen development. Bright field and FF staining pairs of pictures of a tetrad (A, A'), young microspore (B, B'), mid microspore (C, C'), vacuolated microspore (D, D'), young bicellular pollen (E, E') and mid pollen grain (F, F') are shown. ct: cytosol, v: vacuole, n: nucleus. Bars: 10 µm.

Table 1. Average intensities of FF fluorescent staining for different regions of microspores/pollen during *in vivo* development. Fluorescence intensity is given in arbitrary units ×10^3/µm^2.

Stage	Vacuoles (mean ± SD)	Cytosol + nucleus (mean ± SD)	Exine (mean ± SD)
Mid microspore	0.01±0.01	0.01±0.01	n.a.[a]
Early vacuolated microspore	1.00±1.11	3.80±1.75	n.a.[a]
Late vacuolated microspore	0.28±0.39	2.45±1.65	n.a.[a]
Young bicellular pollen	0.52±0.29	2.94±2.27	n.a.[a]
Mature pollen	0.03±0.02	0.03±0.02	4.04±1.63

[a] not available

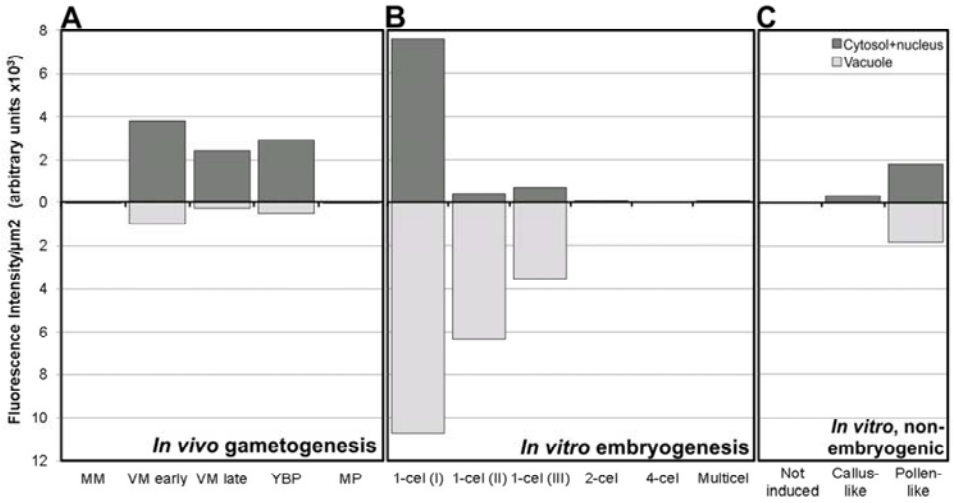


Figure 4. Fluorescence intensity of FF staining in cytoplasm and vacuoles of cells at different stages during *in vivo* gametogenesis (A), *in vitro* embryogenesis (B) and non-embryogenic *in vitro* development (C). MM: mid microspore, VM: vacuolate microspore, YBP: young bicellular pollen, MP: mature pollen, cel: cell/cells.

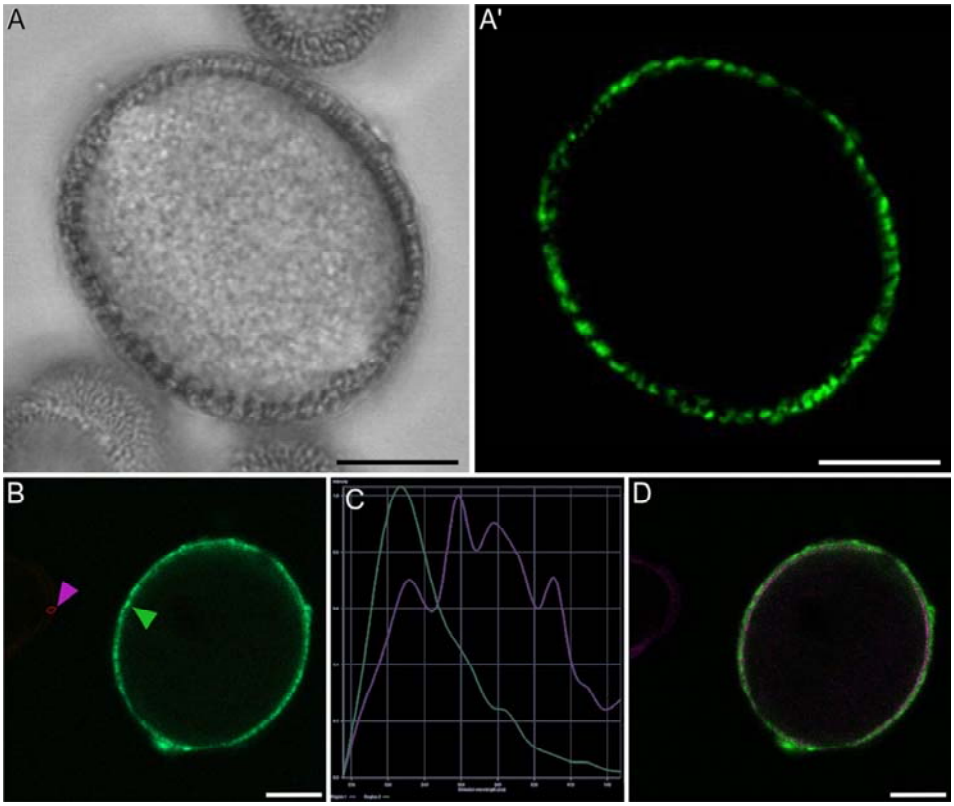


Figure 5. Mature pollen grain observed under bright field (A) and stained with FF (A'). B-D show the analysis of fluorescence emission spectra of exine autofluorescence and FF. B: Small areas were selected from mature pollen exine (green arrowhead) and microspore exine (pink arrowhead), C: Fluorescence emission spectrum of pollen exine (green) and microspore exine (pink), D: Areas with different emission spectra are represented in coded colors, allowing the identification of FF staining (green) and exine autofluorescence (pink). Note that in the pollen wall, FF staining and exine autofluorescence are overlaid. Bars: 10 µm.

In conclusion, we showed that during *in vivo* development, calcium levels increased progressively in the cytosol and nucleus (but not in vacuoles) from the tetrad stage to the late vacuolate microspore stage, first as discrete foci and then as a dispersed signal that reached a maximum at the young pollen stage. From then on, calcium levels progressively decreased to become barely detectable in the cytosol of mature pollen. However, an intense calcium signal was observed at this stage in the pollen exine.

Calcium distribution during induction of microspore embryogenesis

Next, we studied the intracellular levels and distribution of calcium signal in microspores isolated and *in vitro* induced to embryogenesis. To identify them we used different morphological markers (size, shape, cytoplasmic appearance, etc.), amongst which the presence of internal cell walls was the most evident one. In order to identify microspores committed to embryogenesis but still not divided, we stained cells in parallel with aniline blue to identify the development of the callose-rich subintinal layer, described as an early marker of embryogenic commitment (Parra-Vega *et al*. 2015b). One day after induction (Figs. 6A, A'), we found many cells where the intracellular calcium signal increased dramatically with respect to that found in *in vivo* isolated microspores. These cells also presented aniline blue staining at discrete peripheral regions, indicative of the onset of subintinal layer formation. The calculation of the average FF fluorescence intensity/µm2 showed that in these cells (1-cel (I) in Table 2 and Fig. 4B), in addition to a ~2.5x increase in the nuclear-cytosolic signal, these microspores accumulated signal in vacuoles at levels considerably higher (~19x) than during *in vivo* development. Indeed, the most striking difference between *in vivo* and *in vitro* development was the massive internalization of calcium signal to vacuoles. In one day-old cultures we also observed microspores with larger peripheral regions stained with aniline blue, indicating a later stage in subintinal layer formation. Interestingly, these cells (1-cel (II) in Table 2 and Fig. 4B) showed a decrease in the nuclear-cytosolic calcium signal, with nearly all the signal concentrated in vacuoles, now located at the cell periphery (Figs. 6B, B'; Supplementary Movie S2). A third type of unicellular microspores (1-cel (III) in Table 2 and Fig. 4B) was fully surrounded by aniline blue-positive subintinal layer and showed a decrease in vacuolar signal (Figs. 6C, C'), suggesting that as the cell progresses in embryogenesis, calcium signal decreases. This notion was confirmed in embryogenic structures with clearly visible cell divisions (Figs. 6D, D'), where vacuoles showed no detectable signal, and the nuclear-cytosolic signal dropped down to very low levels. Four-celled (Figs. 6E, E') and multicellular embryogenic microspores (Figs. 6F, F') from 6-day old cultures, where the subintinal layer is

already dismantled (Parra-Vega *et al*. 2015b), followed this trend, with almost no detectable calcium signal neither in the vacuoles nor in the rest of the cell.

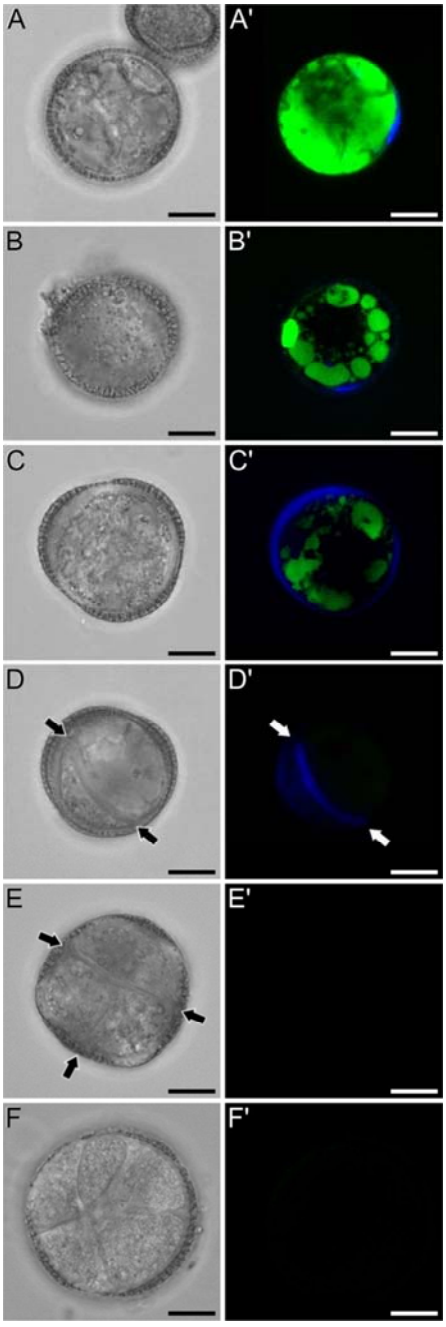


Figure 6. FF and aniline blue staining during *in vitro* microspore embryogenesis. A, A': Induced microspore showing high FF green signal, and few aniline blue stained regions below the intine. B, B': Microspore showing FF staining only in vacuoles, and aniline blue signal in the subintinal layer. C, C': Embryogenic microspore with decreased vacuolar FF signal and fully surrounded by aniline blue-positive subintinal layer. D, D': Embryogenic microspore with a clear aniline blue-stained cell division (arrows) and very low level of FF staining. E, E': Four-celled embryogenic structure (arrows indicate inner cell walls), without detectable fluorescent signal. F, F': Multicellular embryogenic structure without detectable fluorescent signal. Bars: 10 µm.

Table 2. Average intensities of FF fluorescent staining for different regions of *in vitro*- cultured structures. Fluorescence intensity is given in arbitrary units $\times 10^3/\mu m^2$

Stage	Vacuole	Cytosol + nucleus
1-cel (I)	10.74±1.92	7.58±2.55
1-cel (II)	6.36±2.54	0.40±0.24
1-cel (III)	3.54±2.20	0.69±0.87
2-cel	0.04±0.08	0.04±0.08
4-cel	n.d	n.d
Multicellularl	0.01±0.01	0.01±0.01
Not induced	0.01±0.01	0.01±0.01
Callus-like	0.05±0.08	0.31±0.20
Pollen-like	1.84±2.12	1.84±2.12

In light of these results, we concluded that the first signs of embryogenic commitment are accompanied by an increase in intracellular calcium levels. In particular, calcium accumulated in vacuoles, disappearing from the nucleus and cytosol. However, later embryogenic stages, when microspores start the successive division rounds, are characterized by a progressive decrease in calcium levels, reaching undetectable levels not only in the cytosol, but also in vacuoles.

Calcium distribution in non-induced, in vitro cultured cells

In isolated microspore cultures, microspores induced to embryogenesis coexist with other forms, non-sensitive to the inductive treatment and therefore, not induced to embryogenesis. For instance, there are cells arrested or dead at different culture stages (Fig. 7A), microspores induced to divide and proliferate but in a non-embryogenic manner, giving rise to disorganized, callus-like structures (Figs. 7B, C), and microspores that follow a gametophytic-like pathway, becoming pollen-like grains (Figs. 7D, E) with many of the features typical from pollen grains, including enlarged size, vegetative and generative nuclei, and starch granules,

among others. Microspores apparently arrested or dead, not showing any sign of development, showed no detectable signal in any case (Figs. 7A, A'). Dividing cells that followed a callus-like pathway showed a pattern of FF staining remarkably different from embryogenic microspores. These structures presented a faint but clearly detectable signal in the nucleo-cytosolic region (Figs. 7B, B'), combining a dispersed pattern with the presence of some discrete foci as those observed in *in vivo* microspores. Interestingly, we detected this pattern of calcium signal in these structures even in three day-old cultures, a stage when calcium could not be detected in embryogenic, dividing structures. In all cases, signal was not present in vacuoles, at least at detectable levels. After 6 days of culture (Figs. 7C, C'), however, calcium signal in callus-like structures became eventually non detectable in our conditions. Pollen-like structures presented a dual pattern of calcium distribution. Some of them presented very scarce signal, concentrated in few small, peripheral foci (Figs. 7D, D'). Others presented abundant cytosolic signal, principally in peripheral regions (Figs. 7E, E'). Interestingly, some of them showed a broken exine and part of the cytoplasm emerging out of the grain, resembling germinating pollen (Figs. 7F, F'). In any case we could find in pollen-like structures a calcium distribution pattern similar to that found in embryogenic microspores.

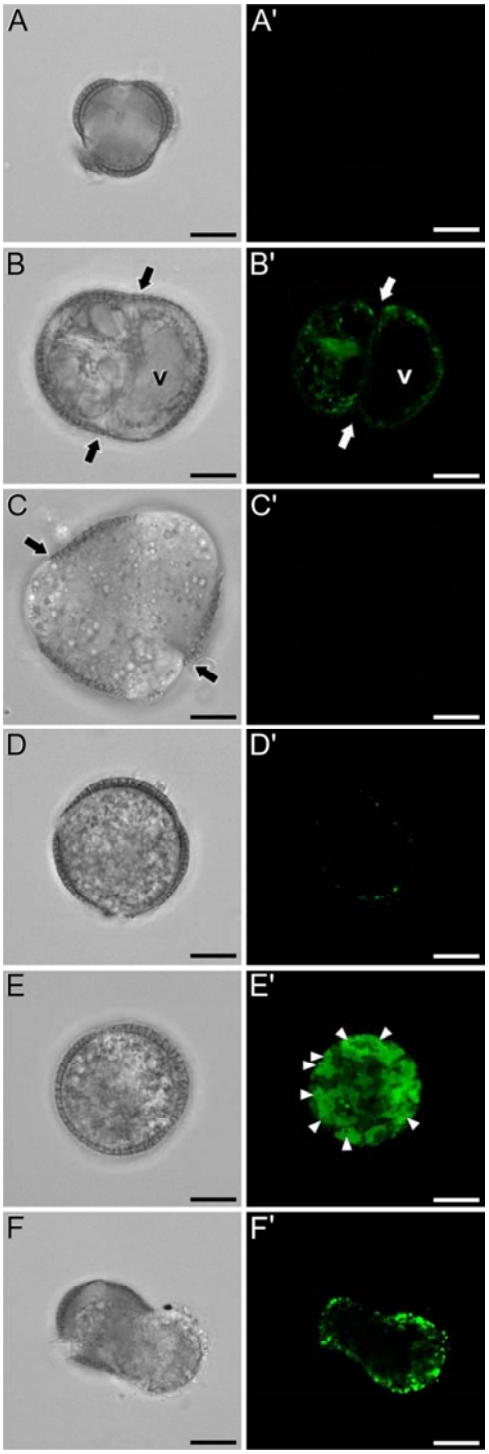


Figure 7. FF and aniline blue staining during non-embryogenic *in vitro* development. A, A': Non-induced, arrested or dead microspore. B, B': Callus-like structure with non-embryogenic cell divisions (arrows). C, C': Six day-old multicellular callus-like structure (arrows indicate inner cell walls). D, D' and E, E': Pollen-like structures with different levels of FF signal, from low and concentrated in discrete peripheral foci (D, D') to intense and distributed throughout the cytosol. F, F': Pollen-like structure with broken exine resembling germinating pollen, showing FF signal in the peripheral cytosolic area. Bars: 10 µm.

Discussion

FluoForte staining is a convenient way to detect intracellular Ca^{2+} in living in vivo and in vitro cultured microspores

Despite the interest of the long pursued study of the changes in intracellular calcium levels and distribution in plant cells, this study has not been always easy. Among the different technical alternatives, the most used have been Ca^{2+}-sensitive fluorescent dyes. Different dyes are commercially available, but they generally do not diffuse well into cells because at physiological pH, they are negatively charged (Grynkiewicz *et al*. 1985). The alternative, uncharged acetoxymethyl (AM) esters of the dyes are able to passively enter the cytosol, where they interact with intracellular esterases that switch them to the negatively charged, Ca^{2+}-sensitive form (Tsien 1981). In plants, this approach may be limited by the presence of esterases (pectin methyl-esterases, PMEs) in the cell wall (Micheli 2001), which hydrolyze AM-esters in the extracellular space prior to access the cytosol. However, Solis *et al*. (2016) found that the expression of PMEs in *B. napus* vacuolated microspores, pollen grains and embryogenic microspores was very low. Thus, it seems that the presence of cell wall PMEs should not be a problem in our case. Our results confirmed it, since we were able to detect an intense signal within microspores and pollen grains. As to the presence of FF signal in the exine of mature pollen, it could be due to the presence of other, mature pollen-specific PMEs, or to the contribution of the senescing inner anther wall layers which, together with the release of calcium (Ge *et al*. 2007b; Kuang and Liao 2015; Qiu *et al*. 2009) and of partially and non methyl-esterified pectins to the locular fluid (Corral-Martínez *et al*. 2016), could be delivering pectin-associated PMEs.

Another problem previously documented with the use of fluorescent calcium dyes in plant cells is intracellular compartmentalization after loading (Fricker *et al*. 2001). However, this event strongly depends on the cell type and the dye used. For example, Bush and Jones (1987) used two AM-ester dyes, Indo-1 and Fura-2, to image Ca^{2+} in barley aleurone protoplasts, finding that while Indo-1 was not well

hydrolyzed, Fura-2 compartmentalized in the vacuole. Similarly, in rhizoid cells of *Fucus serratus*, Fura-2 was found sequestered into vacuoles and vesicles (Brownlee and Pulsford 1988). Using FF in *B. napus* microspores, we also found some degree of dye compartmentalization in the vacuole, but one hour was needed to observe it. When observed within 15 min after mounting, calcium signal was most predominantly cytosolic. Other studies, using absolutely different approaches and species (Ge *et al*. 2007b; Kong and JIA 2004; Kuang and Liao 2015; Qiu *et al*. 2009; Wei *et al*. 2015), consistently showed a clearly cytoplasmic signal, which counts in favor of this notion, too. Thus, we can assume that in *in vivo* microspores, the signal we observed corresponded to cytosolic Ca^{2+}. Similarly, we can also assume that when we see, under identical preparative conditions, cells with mostly vacuolar signal (as in embryogenic microspores), we are detecting Ca^{2+} initially located in vacuoles, and not compartmentalized. It must be noted, though, that compartmentalization prevented us from using live imaging of Ca^{2+} changes in the same microspores during the first embryogenic stages. This was the reason to take different samples for different time points, instead of observing the same cells over time. Since we kept our *in vivo* samples at 4ºC to avoid the putative effect of heat on calcium levels, it could also be argued that cold might also have an effect. However, calcium oscillations due to cold shock are brief (seconds, according to Knight 1999; White and Broadley 2003), which implies that after a 15 min incubation, we should not observe them. All this considered, we can rely on the use of FF to detect calcium in *in vivo* and *in vitro* cultured *B. napus* microspores.

B. napus shows a particular calcium dynamics during in vivo development

From the tetrad stage on, cytosolic Ca^{2+} progressively increased from a very low level in tetrads up to a maximum in late microspores and young pollen grains. From that stage on, Ca^{2+} progressively disappeared, reaching in mature pollen cytosolic levels as low as those of tetrads. The pattern of Ca^{2+} dynamics during microsporogenesis and microgametogenesis seems to be species-specific. For example, using the potassium pyroantimonate cytochemistry for electron

microscopy, in *Larix principis-rupprechtii*, calcium was almost undetectable during microspore development, whereas in pollen grains, it was only clearly observed in the pollen coat (Kong and JIA 2004). In lettuce and oil tea, a progressive increase during microsporogenesis, a decrease during vacuolation, a second increase in young pollen grains, and a final decrease in mature pollen was described (Qiu *et al*. 2009). In *Uncaria hirsuta*, calcium precipitates increased as microsporogenesis proceeded, with a peak in bicellular pollen and a decrease in mature pollen (Kuang and Liao 2015). In tobacco, calcium was detected in the cytoplasm and nucleus of vacuolated microspores and bicellular pollen, decreasing at late pollen stages (Ge *et al*. 2007b). As seen, there are species where the level of calcium in vacuolated microspores is low, and others where it is remarkably high, accumulating preferentially in the nuclei. The observed sharp calcium peak at the microspore-pollen transition seems to be not exclusive of *B. napus*, but also occurring, for example, in tobacco. Interestingly, these two species are considered model species in terms of response to induction of microspore embryogenesis. Perhaps, this particularly high Ca^{2+} level just at the stages more suitable for embryogenesis induction is related to their ability to undergo embryogenesis.

The unique calcium pattern of embryogenic microspores would reflect the simultaneous occurrence of multiple stresses

In microspores induced to embryogenesis, Ca^{2+} was found in the cytosol and nucleus at levels remarkably higher than in *in vivo* vacuolated microspores and young pollen grains. This is not surprising, since cultured microspores are suspended in a calcium-rich medium (500 mg/l Ca(NO3)2), and it is known that one of the consequences of heat shock exposure is fluidization of the plasma membrane, which makes it more permeable to cations such as Ca^{2+}, among others. Thus, it seems reasonable to deduce that the dramatic increase in embryogenic microspores is due to the entry of Ca^{2+} from the culture medium. According to White and Broadley (2003), the magnitude and duration of a stress-associated calcium increase depends on the severity of the stress and the number of different

stresses acting together. *B. napus* isolated microspore culture is a system where different stress sources are simultaneously applied to the same population. These stresses include, at least, a mechanical stress from isolation procedures, an osmotic stress from culture in a medium with high sucrose levels (130 g/l), and a 24 h-long heat stress at 32.5ºC. In turn, they induce the production of reactive oxygen species which generate additional oxidative stress. It is known that some stresses, such as mechanical stress or cold shock induce immediate, transient Ca^{2+} short pulses, whereas heat shock, hyper-osmotic stress, and exposure to oxidative stress first elicit an immediate, short Ca^{2+} pulse, but also a second, prolonged elevation that may last even hours (reviewed in White and Broadley 2003). Our experimental conditions precluded us from detecting the first short pulse, but the extremely elevated Ca^{2+} levels observed after 24 h of *in vitro* culture would reflect the second, prolonged elevation of such biphasic calcium signature caused not only by exposition to heat stress, but also to hyper-osmotic and oxidative stress. Thus, just induced embryogenic microspores combine, simultaneously, high initial Ca^{2+} levels and a series of stress-inducing factors characterized by prolonged Ca^{2+} elevations. Such unique combination would be the cause of their disparate Ca^{2+} levels.

The unique calcium pattern of embryogenic microspores might be involved in the developmental switch

In this cellular scenario, it is tempting to speculate with the consequences of this unique Ca^{2+} perturbation. First, it might be related to autophagy. We demonstrated that induction of embryogenesis in B. napus is tightly associated with massive autophagy and excretion processes involving the formation of autophagosomes and plastolysomes (Corral-Martínez *et al*. 2013; Parra-Vega *et al*. 2015a). On the other hand, a clear link between calcium signaling and autophagy induction through regulation of PPP3/calcineurin (a calcium-dependent phosphatase) was recently demonstrated in mammal cells (Medina *et al*. 2015). Although possible, this hypothesis is still weak, since a similar link between calcium and autophagy is still to be demonstrated in plants. However, the links

between Ca^{2+} perturbations and plant embryogenesis induction are significantly stronger and well founded. It is widely accepted that during zygotic embryogenesis, calcium is needed for egg cell activation. In both plants and animals, the initial step of egg activation involves dramatic Ca^{2+} increase and oscillations. In mouse oocytes, it was demonstrated that the promotion of calcium uptake with the A23187 ionophore was sufficient to activate nearly 50% of the treated oocytes (Nakasaka *et al*. 2000). In plants, there are multiple evidences of Ca^{2+} increases upon fertilization that point to a key role of calcium in egg cell activation (reviewed in Ge *et al*. 2007a). The most notable example comes from maize, where the first events of the embryogenic program can be induced just by triggering Ca^{2+} influx (Antoine *et al*. 2001). In other embryo-forming processes, the involvement of calcium is similar. Experimentally elevated Ca^{2+} levels were found to stimulate somatic embryogenesis in *Coffea canephora* (Ramakrishna *et al*. 2012) and carrot (Takeda *et al*. 2003), where initiation of somatic embryogenesis was found to coincide with a rise in the level of cytosolic Ca^{2+} (Timmers *et al*. 1996). Although there is no evidence for an androgenesis-specific calcium signature, the calcium pattern we hereby describe is the most detailed description of calcium dynamics during the first stages of MDE induction, and presents remarkable similarities with calcium dynamics in other embryo-forming processes. Since it is widely accepted that explicit calcium perturbations produce specific signatures which trigger defined physiological responses (reviewed in White and Broadley 2003), we speculate that the unique combination of elevated initial Ca^{2+} levels and additional stresses in embryogenic microspores would be somehow mimicking the specific calcium perturbations that appear to initiate embryogenesis in egg and somatic cells under defined circumstances.

Interestingly, calcium increase is not the only common link with other embryogenic processes. The formation of a callose-rich layer surrounding the cell as soon as it acquires embryogenic identity is a common feature of embryogenic microspores (Parra-Vega *et al*. 2015b), somatic embryos (Dubois *et al*. 1991; Maheswaran and Williams 1985; You *et al*. 2006) and zygotic embryos as well (Jensen 1968; Williams *et al*. 1984). The similarities between calcium patterns

would add to the growing body of evidences that relate the different embryogenic pathways not only ant the genetic level, but also at the cellular and physiological levels.

Vacuolar calcium internalization could help cells prevent toxicity of calcium excess

An additional difference with *in vivo* microspores was the massive internalization of calcium in vacuoles during the inductive treatment. It is known that there is a maximal concentration and duration beyond which, prolonged increases in cytosolic Ca^{2+} become toxic and even lethal for cells (White and Broadley 2003). Indeed, sustained high Ca^{2+} levels were shown involved in programmed cell death during both normal development and abnormal situations such as hypersensitive responses to pathogens (Levine *et al*. 1996). Thus, vacuolar storage of calcium excess in embryogenic microspores may be a mechanism to keep calcium homeostasis under control and therefore avoid calcium toxicity or death induction. It is interesting to note that the presence of markers of embryogenic commitment such as the callose-rich subintinal layer (Parra-Vega *et al*. 2015b) was inversely related to the presence of Ca^{2+} in vacuoles. A callose-rich, impermeable wall may constitute an effective barrier against Ca^{2+} influx across a plasma membrane permeabilized during heat shock. Therefore, as the subintinal layer grows and covers progressively more plasma membrane, it will be less necessary to store Ca^{2+} excess in vacuoles. In line with this, once cells were transferred to 25ºC, no Ca^{2+} was detected in vacuoles, reaching a situation similar to that of young microspores or mature pollen. In conclusion, the storage of calcium in vacuoles during heat shock exposure seems to be a cellular response to reduce excessive concentration to safe cytosolic levels.

Stress-induced calcium perturbations are not the only players involved

A remarkable feature of microspore cultures is that all microspores are initially exposed to the same *in vitro* conditions, but not all adopt the same developmental pathway nor present the same calcium patterns, as we demonstrated hereby. Embryogenic microspores showed a dramatic rise during the first stages, accumulating most of the Ca^{2+} in vacuoles, whereas callus-like structures presented almost no calcium increase, being always cytosolic, not vacuolar. In turn, pollen-like structures showed either very scarce signal, which might indicate pollen latency, or abundant peripheral signal associated in some cases to morphological evidences of pollen germination. *In vivo*, this calcium distribution pattern has been associated to germinating pollen (Ge *et al*. 2007a), which confirms the pollen-like behavior of these *in vitro* induced structures. In summary, we showed three defined calcium patterns associated to three developmental fates, all triggered in adjacent cells exposed to identical stress conditions. Thus, the question arises as to why there are different responses to identical stimuli? According to Gilroy and Trewavas (2001), this phenotypic plasticity may be explained because calcium levels are not the only players involved on these responses. The levels and activity of Ca^{2+} sensors and target proteins, among other elements, are also important, and they may not be the same in all cells, probably due to subtle developmental differences (even being at the same microspore stage) which lead to minute differences in transcript, protein and/or enzyme profiles. This is why identical stimuli may give rise to different Ca^{2+} perturbations which, in turn, may lead to different developmental fates in two adjacent microspores.

Acknowledgements

We want to express our thanks to Marisol Gascón, from the IBMCP Microscopy Service, for her excellent technical help. This work was supported by

grant AGL2014-55177-R to JMSS from Spanish Ministerio de Economía y Competitividad (MINECO) jointly funded by FEDER. ARS was supported by a predoctoral fellowship from the FPI Program of Universitat Politècnica de València.

References

Antoine A-F, Faure J-E, Dumas C, Feijo JA (2001) Differential contribution of cytoplasmic Ca^{2+} and Ca^{2+} influx to gamete fusion and egg activation in maize. Nat Cell Biol 3: 1120-1123

Blaauw B, del Piccolo P, Rodriguez L, Hernandez Gonzalez V-H, Agatea L, Solagna F, Mammano F, Pozzan T, Schiaffino S (2012) No evidence for inositol 1,4,5-trisphosphate–dependent $Ca(^{2+})$ release in isolated fibers of adult mouse skeletal muscle. J Gen Physiol 140: 235-241

Brownlee C, Pulsford AL (1988) Visualization of the cytoplasmic Ca^{2+} gradient in *Fucus serratus* rhizoids: correlation with cell ultrastructure and polarity. J Cell Sci 91: 249-256

Bush DS, Jones RL (1987) Measurement of cytoplasmic calcium in aleurone protoplasts using indo-1 and fura-2. Cell Calcium 8: 455-472

Coronado MJ, Gonzalez-Melendi P, Seguí-Simarro JM, Ramirez C, Barany I, Testillano PS, Risueno MC (2002) MAPKs entry into the nucleus at specific interchromatin domains in plant differentiation and proliferation processes. J Struct Biol 140: 200-213

Corral-Martínez P, García-Fortea E, Bernard S, Driouich A, Seguí-Simarro JM (2016) Ultrastructural Immunolocalization of Arabinogalactan Protein, Pectin and Hemicellulose Epitopes Through Anther Development in *Brassica napus*. Plant Cell Physiol 57: 2161-2174

Corral-Martínez P, Parra-Vega V, Seguí-Simarro JM (2013) Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. J Exp Bot 64: 3061-3075

Custers J (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 185-193

Cho UH, Kasha KJ (1995) The effect of calcium on ethylene production and microspore-derived embryogenesis in barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.) anther cultures. J Plant Physiol 146: 677-680

Demarty M, Morvan C, Thellier M (1984) Calcium and the cell wall. Plant, Cell & Environment 7: 441-448

Dubois T, Guedira M, Dubois J, Vasseur J (1991) Direct somatic embryogenesis in leaves of *Cichorium*: a histological and SEM study of early stages. Protoplasma 162: 120-127

Fricker M, Parsons A, Tialka M, Blancaflor E, Gilroy S, Meyer A, Plieth C (2001) Fluorescent probes for living plant cells. In: Hawes C, Satiat-Jeunemaitre B (eds) Plant Cell Biology. Oxford University Press, Oxford, UK, pp 35-84

Ge LL, Tian HQ, Russell SD (2007a) Calcium function and distribution during fertilization in angiosperms. Am J Bot 94: 1046-1060

Ge LL, Xie CT, Wei DM, Qiu YL, H.Q. T (2007b) Calcium distribution during the anther development of tobacco. Int J Plant Dev Biol 1: 287–293

Gilroy S, Trewavas A (2001) Signal processing and transduction in plant cells: the end of the beginning? Nat Rev Mol Cell Biol 2: 307-314

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440-3450

Hause B, van Veenendaal WLH, Hause G, van Lammeren AAM (1994) Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L cv. Topas. Botanica Acta 107: 407-415

Horvath I, Glatz A, Varvasovszki V, Torok Z, Pali T, Balogh G, Kovacs E, Nadasdi L, Benko S, Joo F, Vigh L (1998) Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: Identification of hsp17 as a "fluidity gene". Proc Natl Acad Sci USA 95: 3513-3518

Jensen WA (1968) Cotton embryogenesis: The zygote. Planta 79: 346-366

Kim J-H, Xu EY, Sacks DB, Lee J, Shu L, Xia B, Kong A-NT (2013) Identification and Functional Studies of a New Nrf2 Partner IQGAP1: A Critical Role in the Stability and Transactivation of Nrf2. Antioxid Redox Signal 19: 89-101

Knight H (1999) Calcium Signaling during Abiotic Stress in Plants. Int Rev Cytol Volume 195: 269-324

Kong H-Y, JIA G-X (2004) Calcium Distribution During Pollen Development of *Larix principis-rupprechtii*. Acta Bot Sin 46: 69—76

Kuang Y-F, Liao J-P (2015) Calcium distribution during pollen development of *Uncaria hirsuta* Havil. (Rubiaceae), with special reference to the function of the protruding oncus. Grana 54: 12-25

Lamport DT, Varnai P (2013) Periplasmic arabinogalactan glycoproteins act as a calcium capacitor that regulates plant growth and development. New Phytol 197: 58-64

Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr Biol 6: 427-437

Li R, Zhu Y, Meng X, Wang J (2001) The distribution of calcium in the pollen and connective tissue of Honglian-Yuetai cytoplasmic male sterile rice. Zuo Wu Xue Bao 27: 230-233

Liu HT, Sun DY, Zhou RG (2005) Ca^{2+} and AtCaM3 are involved in the expression of heat shock protein gene in Arabidopsis. Plant Cell Environ 28: 1276-1284

Maheswaran G, Williams EG (1985) Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann Bot 56: 619-630

Medina DL, Di Paola S, Peluso I, Armani A, De Stefani D, Venditti R, Montefusco S, Scotto-Rosato A, Prezioso C, Forrester A, Settembre C, Wang W, Gao Q, Xu H, Sandri M, Rizzuto R, De Matteis MA, Ballabio A (2015) Lysosomal calcium signalling regulates autophagy through calcineurin and TFEB. Nat Cell Biol 17: 288-299

Meng X-H, Wang J-B, LI R-Q (2000) Effect of photoperiod on calcium distribution in photoperiod-sensitive cytoplasmic male-sterile wheat during anther development. Acta Bot Sin 42: 15-22

Micheli F (2001) Pectin methylesterases: cell wall enzymes with important roles in plant physiology. Trends Plant Sci 6: 414-419

Nakasaka H, Yamano S, Hinokio K, Nakagawa K, Yoshizawa M, Aono T (2000) Effective activation method with A23187 and puromycin to produce haploid parthenogenones from freshly ovulated mouse oocytes. Zygote (Cambridge, England) 8: 203-208

Nitsch C, Nitsch JP (1967) Induction of flowering *in vitro* in stem segments of *Plumbago indica* L. I Production of vegetative buds. Planta 72: 355-&

Parra-Vega V, Corral-Martínez P, Rivas-Sendra A, Seguí-Simarro JM (2015a) Formation and excretion of autophagic plastids (plastolysomes) in *Brassica napus* embryogenic microspores. Front Plant Sci 6 (94)

Parra-Vega V, Corral-Martínez P, Rivas-Sendra A, Seguí-Simarro JM (2015b) Induction of embryogenesis in *Brassica napus* microspores produces a callosic subintinal layer and abnormal cell walls with altered levels of callose and cellulose. Front Plant Sci 6: 1018

Pauls KP, Chan J, Woronuk G, Schulze D, Brazolot J (2006) When microspores decide to become embryos - cellular and molecular changes. Can J Bot 84: 668-678

Pearce AK, Humphrey TC (2001) Integrating stress-response and cell-cycle checkpoint pathways. Trends Cell Biol 11: 426-433

Qiu Y-L, Liu R-S, Wei D-M, Tian H-Q (2009) Calcium distribution in developing anthers of lettuce (*Lactuca sativa*). Ann Bot Fenn 46: 101-106

Ramakrishna A, Giridhar P, Jobin M, Paulose CS, Ravishankar GA (2012) Indoleamines and calcium enhance somatic embryogenesis in *Coffea canephora* P ex Fr. Plant Cell, Tissue and Organ Culture (PCTOC) 108: 267-278

Reynolds TL (2000) Effects of calcium on embryogenic induction and the accumulation of abscisic acid, and an early cysteine-labeled metallothionein gene in androgenic microspores of *Triticum aestivum*. Plant Sci 150: 201-207

Roshchina VV (2012) Vital autofluorescence: application to the study of plant living cells. Int J Spectrosc 2012: 14

Rudd JJ, Franklin-Tong VE (2001) Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. New Phytol 151: 7-33

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134: 1-12

Seguí-Simarro JM, Testillano PS, Risueño MC (2005) MAP kinases are developmentally regulated during stress-induced microspore embryogenesis in *Brassica napus* L. Histochem Cell Biol 123: 541-551

Solis MT, Berenguer E, Risueno MC, Testillano PS (2016) BnPME is progressively induced after microspore reprogramming to embryogenesis, correlating with pectin de-esterification and cell differentiation in *Brassica napus*. BMC Plant Biol 16: 176

Soriano M, Li H, Boutilier K (2013) Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod 26: 181-196

Takeda T, Inose H, Matsuoka H (2003) Stimulation of somatic embryogenesis in carrot cells by the addition of calcium. Biochem Eng J 14: 143-148

Tian H, Yao CY, Sun MX (2004) High frequency conversion of microspore-derived embryos of *Brassica napus* cv. Topas by supplemental calcium and vitamins. Plant Cell Tissue Organ Cult 76: 159-165

Tian HQ, Kuang A, Musgrave ME, Russell SD (1998) Calcium distribution in fertile and sterile anthers of a photoperiod-sensitive genic male-sterile rice. Planta 204: 183-192

Timmers ACJ, Reiss H-D, Bohsung J, Traxel K, Schel JHN (1996) Localization of calcium during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma 190: 107-118

Tirlapur UK, Willemse MTM (1992) Changes in calcium and calmodulin levels during microsporogenesis, pollen development and germination in Gasteria verrucosa (Mill.) H. Duval. Sex Plant Reprod 5: 214-223

Tsien RY (1981) A non-disruptive technique for loading calcium buffers and indicators into cells. Nature 290: 527-528

Vigh L, Gombos Z, Joo F (1985) Selective modification of cytoplasmic membrane fluidity by catalytic-hydrogenation provides evidence on its primary role in chilling susceptibility of the blue-green alga, Anacystis nidulans. Febs Letters 191: 200-204

Wei D, Gao C, Yuan D (2015) Calcium distribution during anther development in oil tea (*Camellia oleifera* Abel.). J Am Soc Hortic Sci 140: 88-93

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92: 487-511

Williams EG, Knox RB, Kaul V, Rouse JL (1984) Post-pollination callose development in ovules of *Rhododendron* and *Ledum* (Ericaceae): zygote special wall. J Cell Sci 69: 127-135

You XL, Yi JS, Choi YE (2006) Cellular change and callose accumulation in zygotic embryos of *Eleutherococcus senticosus* caused by plasmolyzing pretreatment result in high frequency of single-cell-derived somatic embryogenesis. Protoplasma 227: 105-112

Chapter 5

Callose deposition in the cell wall and subintinal layer during induction of microspore embryogenesis are independent processes, differently modulated by calcium and variable among genotypes

Alba Rivas-Sendra, Patricia Corral-Martínez and Jose M. Seguí-Simarro

Cell biology Group - COMAV Institute, Universitat Politècnica de València, Valencia, Spain

Keywords

Androgenesis, Ca^{2+}, cellulose, doubled haploid, eggplant, rapeseed

Abstract

Induction of microspore embryogenesis in *Brassica napus* is associated to dramatic alterations of intracellular Ca^{2+} concentrations, and to the formation of a callose-rich subintinal layer exclusive for embryogenic microspores. In this work, we studied the deposition of callose and cellulose during the first stages of microspore embryogenesis in a highly responding (DH4079) *B. napus* line, and in an eggplant DH line (DH36). Our results revealed that the formation of a callose-rich subintinal layer is a common feature of microspore embryogenesis in different species, but the amount of callose in this layer is related to the androgenic response, being higher in the highly responding genotype of *B. napus*. In this genotype, we studied the relationship between Ca^{2+} and callose and cellulose deposition by modulating intracellular Ca^{2+} levels with caffeine, digitonin, chitosan and endosidin 7. Abnormal callose deposition in the inner cell walls was found Ca^{2+}-dependent, whereas deposition in the subintinal layer was also modulated by Ca^{2+}, but to a lower extent. Callose deposition was inversely related to cellulose deposition, indicating that these are incompatible processes. In turn, callose deposition in the cell wall and the subintinal layer are independent processes, likely regulated by different callose synthases. The significance and implications of these findings are discussed.

Introduction

Microspore embryogenesis is an experimental, *in vitro*-induced system whereby embryogenic development can be induced in cells initially programmed to become male gametophytes, i.e., male gamete precursors. Through the application of specific abiotic stresses, haploid microspores are pushed to exit their original gametophytic program and proliferate first, and then to differentiate into haploid or doubled haploid (DH) microspore-derived embryos (MDEs). In microspore cultures, different developmental fates coexist. Some microspores arrest and die, while others continue a gametophytic-like development to become pollen-like structures which, after 5-6 days in culture, usually burst and therefore die (Soriano *et al*. 2013). Among the dividing individuals, some adopt a proliferative, callus-like pathway that sooner or later will end up in cell arrest and death, and only a few of them are effectively induced to embryogenesis (Seguí-Simarro and Nuez 2008). In them, a series of symmetrical division are triggered, following a pattern and mechanism more similar to cytokinesis of somatic cells than to the typical asymmetric division of the first pollen mitosis (Parra-Vega *et al*. 2015; Zaki and Dickinson 1991). In conventional, somatic-type cytokinesis, the first polysaccharides that form the new inner cell walls are pectins and hemicelluloses transported to the nascent cell plate via Golgi-derived vesicles, whereas callose is synthesized *in situ* and deposited in the cell plate lumen (reviewed in Drakakaki 2015). By the widening force of callose accumulation in the cell plate lumen, the initial tubulo-vesicular membranous network develops into a wide tubular network and finally into a fenestrated-sheet cell plate (Seguí-Simarro *et al*. 2004). At this stage, callose walls are discontinuous, flexible and delicate. Transient callose walls serve as a stabilizing scaffold to expand the cell plate prior to deposition of cellulose fibrils and callose removal by β-1,3-glucanases (reviewed in Seguí-Simarro *et al*. 2008). At the end of the process, the primary cell wall is formed by a cellulose-hemicellulose network where pectins arrange in the middle lamella, and callose is only present around plasmodesmata (Levy *et al*. 2007).

As opposed to this pattern, embryogenic microspores produce cell walls with altered polysaccharide composition. Induction of embryogenesis was associated to a particular composition in terms of pectins and xyloglucans in *Capsicum annuum* (Barany *et al*. 2010) and of arabinogalactan proteins in *B. napus* (El-Tantawy *et al*. 2013). Cellulose was found to be absent from the first, newly formed cell walls of *B. napus* embryogenic microspores of up to 6 days in culture (Dubas *et al*. 2013). With respect to callose, its presence seems to be extended over time. It was recently demonstrated that the cell walls of few-celled *B. napus* MDEs accumulate callose in cell plate stages beyond the fenestrated-sheet, even in mature cell walls where cellulose should already have replaced it, giving rise to callose-rich and cellulose-defective cell walls (Parra-Vega *et al*. 2015). This characteristic feature of cytokinesis in embryogenic microspores appeared to be transient, associated to the first stages of the process, since no abnormal callose presence was detected in advanced MDEs. At the same time, Parra-Vega *et al*. (2015) described a callose-rich subintinal layer (SL) formed between the intine and the plasma membrane in *B. napus* embryogenic microspores, even before the first embryogenic division is observed. The SL was variable in thickness, but fully surrounded the cells of the structure during the first stages of embryogenic commitment. The SL was found to be exclusive from induced microspores, being absent in pollen-like structures exposed to the same induction and culture conditions. All these features led to the notion that the presence of a callose-rich subintinal layer could be an early marker of embryogenic commitment (Parra-Vega *et al*. 2015).

The stress used to induce embryogenesis in most species, including *B. napus,* is heat. The way cells perceive heat involves transient increases in cytoplasmic Ca^{2+} levels mediated by changes in plasma membrane fluidity and activation of Ca^{2+}-permeable channels (Horvath *et al*. 1998; Liu *et al*. 2005; Saidi *et al*. 2009). The signaling cascade initiated by heat-induced Ca^{2+} influx acts as an inducer of many components of the heat shock response (HSR), but the effects of Ca^{2+} as intracellular messenger go far beyond. As reviewed in White and Broadley (2003), cytosolic Ca^{2+} levels play a key role in the regulation of many different processes, including those occurring during microspore embryogenesis: induction of

embryogenesis and response to several stresses. In this context, calcium was traditionally suspected to have a role, principally during the first stages of the process (Pauls *et al*. 2006). However, its precise role has not been elucidated yet. Recently, we demonstrated that, as a consequence of induction, embryogenic microspores accumulate Ca^{2+} in the cytosol, but principally in vacuoles, at levels dramatically higher than during *in vivo* development (Rivas-Sendra and Seguí-Simarro 2017). The increase was specifically associated to the change in developmental fate, since non-embryogenic callus-like and pollen-like structures exhibited remarkably different calcium patterns. Plant cells store Ca^{2+} in different compartments such as the endoplasmic reticulum, the vacuole or the cell wall, where it is tightly bound to pectins. It plays a key role in cell wall formation and in the regulation of specific callose synthase (CalS) complexes, either interacting with Ca^{2+}-sensitive proteins such as annexins or directly with the Ca^{2+}-binding domain of CalS, when present (Verma and Hong 2001). Twelve different callose synthase genes have been described in *Arabidopsis thaliana* (Hong *et al*. 2001), named *CalS* (from callose synthase) or *GLS* (from *GLUCAN SYNTHASE-LIKE*) indistinctly. From them, five CalS have been found in microspores and pollen, *CalS5* (*GSL2*) being the CalS with higher level of expression (Dong *et al*. 2008). CalS11 and CalS12 (GSL1 and GSL5, respectively) are present at all pollen developmental stages, but their role is the synthesis of callose walls during post-meiotic cytokinesis (Enns *et al*. 2005). CalS5 is responsible for the formation of a thin callose layer in the cell wall of young microspores, needed as a scaffold for proper exine deposition and patterning (Dong *et al*. 2005). CalS9 and CalS10 (GSL10 and GSL8, respectively) produce the callose wall separating the generative and vegetative cell after the first pollen mitosis (Töller *et al*. 2008). Finally, callose deposition in walls and plugs of pollen tubes is brought about by CalS5 (Nishikawa *et al*. 2005) and CalS11 (Doblin *et al*. 2001).

Considering the link between heat stress, alteration of membrane fluidity and Ca^{2+} increase, together with the role of Ca^{2+} in pollen development, microspore embryogenesis and callose synthesis, it was tempting to speculate that Ca^{2+} regulation is involved in the formation of the abnormal, callose-rich inner cell walls

and the SL that define the first stages of microspore embryogenesis (Parra-Vega *et al*. 2015). To shed light on this hypothesis, in this work we characterized the dynamics of cell wall formation, including callose and cellulose deposition during normal *B. napus* microspore embryogenesis, and in cultured microspores exposed to several compounds known to interfere with intracellular Ca^{2+} levels, with plasma membrane fluidity and/or with callose synthesis and deposition. These compounds include caffeine, chitosan, digitonin, and endosidin 7 (ES7). Caffeine and related methylxanthines are known for their ability to inhibit cell plate formation during plant cytokinesis (Becerra and López-Sáez 1978; López-Saez *et al*. 1966). By altering the intracellular Ca^{2+} levels, and specially by reducing membrane-associated Ca^{2+} (Paul and Goff 1973), caffeine causes phragmoplast microtubule depolymerization (Yasuhara 2005), inhibition of callose synthesis (Samuels and Staehelin 1996) and blockage of the last steps of vesicle fusion (Rasmussen and Tenenhouse 1968), therefore preventing the conversion of the tubulo-vesicular network into a more stable tubular network cell plate (Samuels and Staehelin 1996). Chitosan is a naturally occurring polymer of β-1,4-glucosamine derived from chitin and found as a structural component of fungal walls. In turn, chitin is the main protein component of the insect exoskeleton. These facts make chitosan to act as a strong elicitor of plant anti-pathogen response, triggering a huge variety of events. As a polycation (positive polyelectrolyte), it is able to bind to all negatively charged places, thus causing many biochemical and molecular changes in plant cells (Hadwiger 2013) including the displacement of membrane-bound cations such as Ca^{2+} from anionic centers (Siegel and Daly 1966), disturbance of plasma membrane stability and increase of its fluidity (Young *et al*. 1982). Together with digitonin, a detergent well known to increase membrane permeability (Wu and Wasserman 1993), both compounds were found to increase membrane fluidity, Ca^{2+} uptake and callose synthesis in *in vitro*-cultured cells (Hirano *et al*. 2012; Waldmann *et al*. 1988; Zuppini *et al*. 2003). ES7 was found by Drakakaki *et al*. (2011) and further characterized by Park *et al*. (2014) to inhibit callose deposition specifically during somatic-type cytokinesis and during pollen tube growth, but not in other processes such as the pathogen

response in physically wounded tissues and the formation of the sieve plate in sieve elements. We used all these compounds to interfere with normal Ca^{2+} levels, membrane fluidity and callose deposition in order to know to what extent these processes are related. To find out whether these abnormal callose deposition events are specific of embryogenic microspores of the highly responding *B. napus* cv. Topas DH4079 or it is a more general feature of embryogenesis induction, we also studied the dynamics of callose deposition in a recalcitrant species, eggplant (*Solanum melongena*). Together, our results shed light on the relationship between Ca^{2+} levels, membrane fluidity and callose deposition, establishing a link between them and induction of microspore embryogenesis.

Materials and methods

Plant materials

Donor plants of *Brassica napus* L. cv. Topas line DH4079 were grown in growing chambers of the COMAV Institute (Universitat Politècnica de València, Valencia, Spain) and the Plant Research International (Wageningen, The Netherlands). Plants were grown in 20 cm pots at 60% humidity and 16/8 photoperiod, kept at 20ºC until flowering and then transferred to 10ºC. Donor plants of *Solanum melongena* cv. Bandera line DH36 (Rivas-Sendra *et al*. 2017) were grown in 30 cm pots under natural light in the greenhouse of the COMAV Institute.

B. napus microspore culture

Isolation, induction treatment and microspore culture were performed according to Custers (2003). Flower buds containing mostly late uninucleated microspores and early binucleated pollen were dissected from the plant, surface

sterilized with 2% sodium hypochlorite for 10 min, and washed three times in sterile distilled water for a total of 15 min. Buds were crushed with a sterile syringe piston in NLN-13 medium. NLN-13 medium consist of NLN medium as described by (Nitsch and Nitsch 1967) supplemented with 13% sucrose and sterilized by filtration though 0.22 µm filter. Microspores were isolated from the suspension by filtration through 30 µm nylon mesh (Millipore) followed by three rounds of centrifugation at 100 *g*, 4 min each. Microspore density was calculated with a hemacytometer and adjusted to 4×10^4 microspores per ml. The cellular suspension was plated, incubated in darkness for 24 h at 32.5ºC to induce embryogenesis, and then at 25ºC in darkness for embryogenesis progression.

***S. melongena* microspore culture**

Isolated microspore culture of eggplant was performed according to (Rivas-Sendra *et al*. 2017). Anthers containing mostly vacuolate microspores were dissected from the bud, surface sterilized with 70% ethanol for 30 seconds and with 4g/l sodium hypochlorite for 5 minutes, and rinsed three times in sterile distilled water. Anthers were crushed in sterile distilled water using a syringe piston, and their locular content was isolated by filtration through a 41 µm nylon mesh and successive retention in a 11 µm nylon mesh (both from Millipore), and three centrifugation steps at 100 *g*, 4 min each. Microspores were resuspended in sterile distilled water at a final cell density of $5x10^5$ microspores per ml, plated and incubated at 35ºC in darkness for 3 days to induce embryogenesis. After the induction treatment, microspores were collected by centrifugation in the same conditions, resuspended at the same density in NLN medium (Nitsch and Nitsch 1967) supplemented with 2% sucrose, 0.5 mg/l 1-naphthaleneacetic acid and 0.5 mg/l 6-benzilaminopurine, and incubated at 25ºC in darkness for embryogenesis progression.

Chemicals

For caffeine experiments, caffeine at the working concentration was added directly to the culture medium prior to sterilization. The rest of chemicals were diluted to working concentration from stock solutions previously prepared. Stock solution of 1 g/l digitonin was prepared in distilled water. Stock solution of 1 g/l of chitosan was prepared in acetic acid 1%; the solution was stirred overnight at 4ºC and pH was adjusted to 5.8 with sodium hydroxide. Stock solutions of 5 and 20 mM ES7 were prepared in DMSO. All chemicals were purchased from Sigma-Aldrich with the exception of ES7 (Hit2Lead). All stock solutions were sterilized by filtration through 0.22 µm filters and stored at -20ºC. The corresponding volumes of each stock solution were added to microspore cultures to reach the desired final concentration in cultures, as explained in Results.

Callose and cellulose staining and detection

Samples of *B. napus* microspore cultures were collected at days 3 and 6 after isolation, and fixed overnight at 4ºC with 4% paraformaldehyde in phosphate saline buffer (PBS) with pH 7.4. After three washings with PBS, samples were stored at 4ºC in 0.1% paraformaldehyde in PBS until use. Before staining, samples were immobilized by mixing 10 µl of microspore pellet with the same volume of 1.8% agarose, letting it to solidify on a microscope slide. Staining and detection of callose and cellulose in microspores was performed according to Parra-Vega *et al*. (2015). For callose detection, aniline blue (AB; Fluka) was used (Evans *et al*. 1984), together with propidium iodide (PI; Fluka) to counterstain. Samples were incubated with 10 µg/ml PI in PBS for 10 min, washed three times with PBS, stained with 0.1% AB in PBS for 20 min, washed three times with the same buffer and mounted with Mowiol antifading mounting solution. Mowiol solution was prepared with 17% Mowiol 4-88 (Sigma-Aldrich) and 33% glicerol (v/v) in PBS. For cellulose detection, Pontamine Fast Scarlet (S4B) was used for cellulose staining and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) for nuclei staining. Samples were stained with 0.01% S4B in 0.1 M PBS for

30 min (Anderson *et al*. 2010), washed three times with PBS, mounted with a 1:1 mix of Mowiol solution and 2.5 μg/ml DAPI prepared as described in Custers (2003), and incubated for at least 15 minutes. All incubations were performed in darkness. Samples were observed with a Leica CTR 5500 and a Zeiss LSM 780 confocal laser scanning microscopes. Digital images were processed with Leica Application Suite Advanced Fluorescence (LAS AF) and FIJI software.

Processing and inmunogold labelling of B. napus and eggplant microspore cultures for transmission electron microscopy

For immunogold labelling of callose, we used OsO_4-treated, epoxy-embedded samples. Although these procedures are not generally used for immunolabeling because they alter the conformation of protein epitopes, the fact that our epitope was a carbohydrate allowed us to use this procedure which, in addition to provide an excellent ultrastructural preservation, does not interfere with carbohydrate epitopes, as shown in Corral-Martínez *et al*. (2016) and Parra-Vega *et al*. (2015). Samples of *B. napus* and eggplant microspore cultures were collected at day 3 and day 9 after isolation, respectively. These time points correspond to equivalent development stages. Microspores were concentrated by centrifugation, cryoprotected with 20% dextran, transferred to aluminum sample holders and high pressure frozen in a Leica HPM100 high-pressure freezer (Leica Microsystems, Vienna, Austria). The samples were then freeze substituted in 2% OsO_4 in anhydrous acetone at -80ºC for 4 days, followed by slow warming to room temperature over a period of 24 h. After rinsing in several acetone washes, they were removed from the holders and infiltrated with increasing concentrations of Spurr resin (Ted Pella, Redding, CA) in acetone according to the following schedule: 4 h in 2% and 5% resin, 12 h in 10%, 25%, 50% and 75% resin, and 40 h in 100% resin. Polymerization was performed at 70ºC for 30 h. Using a Leica UC6 ultramicrotome, thin sections (1 μm) were obtained for light microscopy observation, and ultrathin sections (~80 nm) were obtained for electron microscopy.

Ultrathin sections were mounted on Formvar and carbon-coated, 150 mesh nickel grids and were used for inmunodetection of callose with an anti-callose monoclonal antibody (mouse IgG) crossreacting with linear β-1,3-oligosaccharide segments in β-1,3-glucans (Meikle *et al*. 1991). Sections were hydrated with distilled water for 1 min, with PBS for 1 min, and blocked with 5% BSA in PBS for 5 min. Then, sections were incubated for 1 h at 25ºC with anti-callose antibody, diluted 1:5,000 in 1% BSA. Next, sections were washed three times with PBS and incubated for 45 min at 25ºC with a goat anti-mouse secondary antibody conjugated with 10 nm colloidal gold (BBI Solutions, UK), diluted 1:25 in 1% BSA. Then, sections were washed three times with PBS, incubated 10 min with 1% formaldehyde in PBS, and washed again three times with PBS and three times with distilled water. Finally, sections were counterstained with uranyl acetate and lead citrate and observed in a Jeol JEM 1010 transmission electron microscope.

Results

In *B. napus* microspore cultures, freshly isolated microspores (Fig. 1A) are isolated and inoculated into *in vitro* culture dishes. Some microspores are not sensitive to the heat-based inductive treatment, and follow a gametophytic-like program to become pollen-like structures (Fig. 1B). Many other microspores or pollen-like structures do not longer develop and eventually arrest and die (Fig. 1C). However, those microspores sensitive to the inductive treatment are reprogrammed towards embryogenesis, reabsorbing the large vacuole and dividing symmetrically. 2-4-celled embryogenic structures could be observed just three days after inoculation (Fig. 1D). In the highly embryogenic DH4079 line, many of these cells presented AB-stained (callosic) walls and SL, being thick in general but discontinuous (Fig. 1D'). In some instances, we also observed embryogenic structures with thin, continuous, but not always complete S4B-positive (cellulosic) inner cell walls, suggesting the onset of callose replacement by

cellulose. However, we never observed a similar S4B-positive signal in the SL in these structures (Fig. 1D''). In parallel, pollen-like grains in culture increased considerably in volume and, in some instances, underwent the second pollen mitosis (Fig. 1E). Pollen-like grains showed no detectable callose staining (Fig. 1E'), whereas S4B staining revealed a thick and intensely stained cellulose layer beneath the exine, likely corresponding to the intine (Fig. 1E''). Multicellular structures (around 8 cells) can be found in 6-day old cultures (Fig. 1F). These structures showed no callose in inner cell walls, and thin SL residuals were occasionally observed, in general only around apertures (Fig. 1F'). At this stage, we observed continuous but irregularly thick cellulose walls surrounding all cells (Fig. 1F''), which indicated that in 6-day old embryogenic structures, the SL was almost dismantled and callose of the inner cell walls was replaced by cellulose. In summary, callose was considerably more abundant than cellulose at the beginning of the development of embryogenic structures, but in the transition from day 3 to day 6, inner cell walls had callose progressively replaced by cellulose and in parallel, the callose-rich SL was dismantled. These observations indicated that inner cell walls and SL have a different timing for their formation and development during the early stages of microspore embryogenesis.

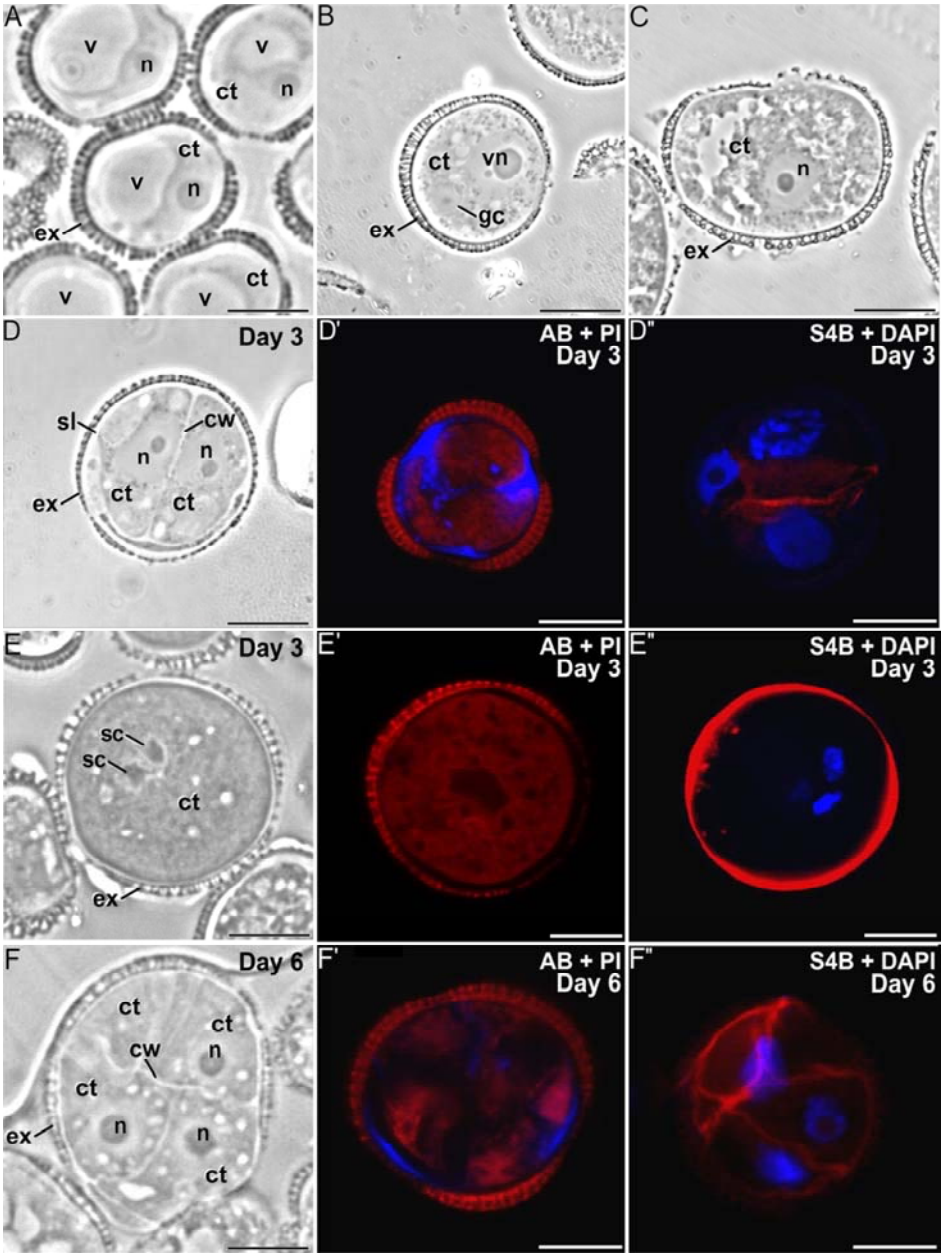


Figure 1. Development of *B. napus* untreated microspores. A: isolated microspores before induction. B: Pollen-like development in culture. C: Dead structure in culture. D-D": 3-day old embryogenic microspore. E-E": pollen-like development in culture. F-F": 6-day old multicellular embryogenic structure. Bright field (A, B, C, D, E, F), callose staining (blue) with aniline blue and propidium iodide (D', E', F'), and cellulose staining (red) with S4B and DAPI (F, F', F") sets of pictures are shown. Bars: 10 µm.

Caffeine reduced callose and cellulose deposition and prevented cell wall formation

To test whether the formation of cell walls and SL is affected by changes in intracellular Ca^{2+}, microspore cultures were performed in the presence of 1 and 10 mM caffeine. 3-day old microspores treated with 1 mM caffeine (Fig. 2A) presented thinner, spottier and more discontinuous callose walls than untreated (control) microspores. The overall appearance of the SL was similar to untreated cultures. S4B staining showed a thin but continuous cellulose wall around the different cells of the structure (Fig. 2A'), remarkably similar to that produced in conventional somatic-type cytokinesis. At the 6-day stage, the presence of callose (Fig. 2B) and cellulose (Fig. 2B') showed no remarkable differences with the untreated cultures, except for some ectopic wall fragments positive for AB staining (arrowheads in Fig. 2B). When cultures were treated with 10 mM caffeine, embryogenic structures were unable to develop beyond the first round of division. At the 3-day stage, binucleated cells without inner cell walls were often observed, with the two nuclei closely apposed (Fig. 2C) or even fused (Fig. 2C'). When present, cell walls were very thin, discontinuous, fragmented and faintly stained for both callose (Fig. 2D) and cellulose (Fig. 2D'). Treatment with 10 mM caffeine did not have an equivalent effect in the formation of the SL. Callose was present in a discontinuous and irregular layer, even in cells where inner cell walls were absent (Fig. 2C), and cellulose staining was negative (Fig. 2C'). Six days after isolation all cells were dead. Together, these results showed a dose-dependent effect of caffeine in callose deposition. At low doses, a slight reduction in callose synthesis was accompanied by a slight increase for cellulose. At higher doses, caffeine prevented the formation of inner cell walls, while SL was considerably less affected.

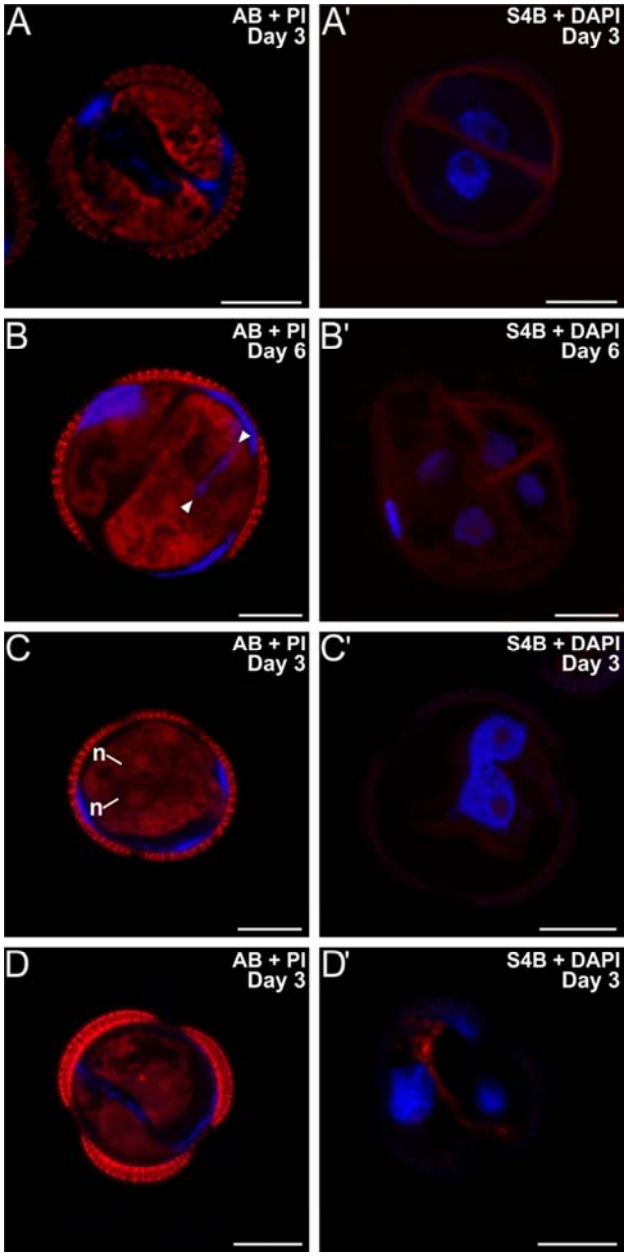


Figure 2. *B. napus* microspores treated with caffeine. A-A': 3-day old embryogenic microspores treated with 1 mM caffeine. B-B': 6-day old embryogenic microspores treated with 1 mM caffeine. C-C': 3-day old embryogenic microspores treated with 10mM caffeine. D-D': 6-day old embryogenic microspores treated with 10 mM caffeine. Callose staining (blue) with aniline blue and propidium iodide, and cellulose staining (red) with S4B and DAPI pairs of pictures are shown. Bars: 10 µm.

Digitonin caused an increase of callose and reduction of cellulose

We used digitonin to increase membrane fluidity and therefore Ca^{2+} uptake, in order to check out whether such alteration affects callose deposition. Microspores were cultured with 1, 5, 10 and 100 mg/l digitonin. When digitonin was added at 5, 10 and 100 mg/l, cells were all dead after 3 days of culture. However, microspores treated with 1 mg/l digitonin survived, and at the 3-day stage (Fig. 3A) presented callose walls more continuous and uniform than untreated, control cells. Cellulose deposition pattern, as revealed by S4B staining, was abnormal, with multiple ectopic, cytoplasmic wall fragments (Fig. 3A'). At the 6-day stage, callose was observed in inner cell walls and in the SL, forming thin, discontinuous and fragmented deposits (Fig. 3B). Most of the multicellular structures observed presented diffuse and weak S4B staining in the SL and in inner cell walls, revealing thin and incomplete cellulose deposits (Fig. 3B'). These results indicated that low concentrations of digitonin increased callose deposition and extended callose presence over time, while cellulose deposition was reduced and severely altered.

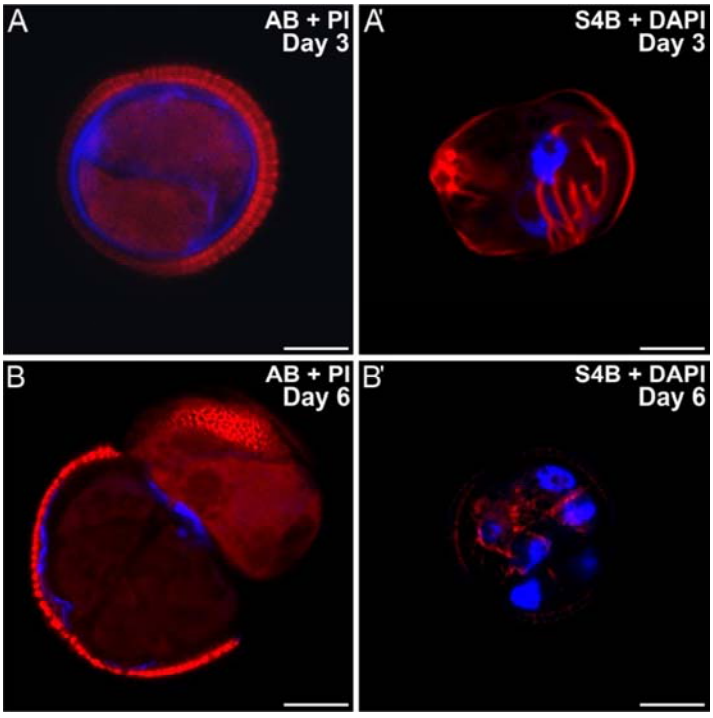


Figure 3. *B. napus* microspores treated with 1 mg/l digitonin. A-A': 3-day old embryogenic microspores. B-B': 6-day old embryogenic microspores. Callose staining (blue) with aniline blue and propidium iodide, and cellulose staining (red) with S4B and DAPI are shown. Bars: 10 µm.

Chitosan induced callose plugs and abnormal cellulose deposition

As with digitonin, chitosan was used in an attempt to increase membrane fluidity and therefore intracellular Ca^{2+} levels. To test whether this had any effect in the formation of cell walls and the SL, 1, 10 and 100 μg/l chitosan was added to microspore cultures. No changes with respect to control cultures were observed when 1 μg/l chitosan was used, whereas cells exposed to 100 μg/l were unable to develop (data not shown). Thus, we focused on the study of the effects of 10 μg/l, which in general reduced the embryogenic response and favoured the development of callus-like structures. These structures showed a combination of sporophytic and gametophytic features, with cell divisions and a characteristic granulated cytoplasm resembling that of pollen-like structures (Fig. 4A). At the 3-day stage, callose was scarce, and only present in some fragments of the SL and some small cytoplasmic deposits, being never observed in inner cell walls (Fig. 4A). In general, cellulose was abundant in all inner and outer walls, completely surrounding all cells of the structure, as well as in small cytoplasmic spots (Fig 4A'). Chitosan treatment also produced binucleated cells, with no callose and a severely altered pattern of cellulose deposition, showing fragmented and wrinkled wall stubs, but no clear cell walls (Fig. 4B). Pollen-like structures seemed also affected, as cytoplasmic cellulose dots and ectopic wall stubs were formed beneath the pollen wall (Fig. 4C). At the 6-day stage, AB staining revealed unusual, compact and well delimited cytoplasmic plugs of callose, especially in non embryogenic cells and burst pollen (Fig. 4D). S4B staining revealed a thick and intensely stained wall of cellulose entirely surrounding each individual cell, together with small cytoplasmic dots (Fig. 4D'). In summary, chitosan presence clearly reduced callose synthesis and increased cellulose synthesis both in cell walls and the SL. However, the most remarkable effect was the induction of abnormal patterns of ectopic deposition for both polysaccharides.

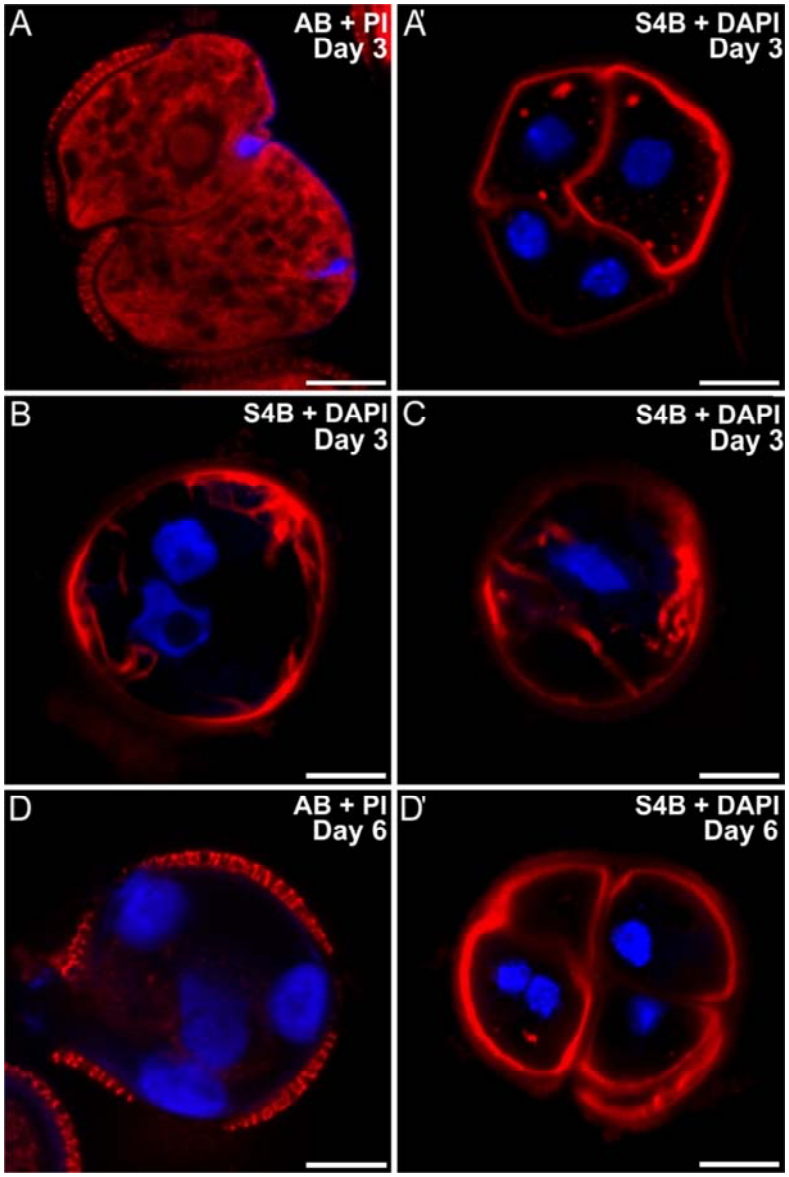


Figure 4. Structures formed in *B. napus* microspore cultures treated with chitosan 10 µg/l. A-A': 3-day old callus-like structures. Callose staining (blue) with aniline blue and propidium iodide (A), and cellulose staining (red) with S4B and DAPI (B) are shown. B: cellulose staining of a 3-day old binucleated cell. C: Cellulose staining of a 3-day old pollen-like cell. D-D': 6-day old structures stained for callose (D) and cellulose (D'). Bars: 10 µm.

ES7 prevents callose deposition in the cell walls but not in the SL and other callose-containing structures

We used ES7 to shed light on the mechanism responsible for callose deposition in cell walls and the SL of embryogenic microspores. Microspores were cultured in the presence of 10 μM and 25 μM ES7. As ES7 was dissolved in DMSO, a control with DMSO alone was also considered. DMSO at the used concentration slightly reduced the efficiency of embryogenic induction, but it did not have a noticeable effect in polysaccharide deposition (data not shown). With 10 μM ES7, in 3-day old embryogenic microspores, callose was found in the SL and beneath the apertures, but it was rarely or not detected at all in inner cell walls (Fig. 5A). Cellulose was found in some inner cell walls, but never in the SL (Fig. 5A'). At the 6-day stage, very small callose residuals, if any, were found beneath the intine, but never in inner cell walls (Fig. 5B). Cellulose deposition was unaffected, as it was found in the thin inner cell walls and surrounding each cell (Fig. 5B'). Treatment with 25 μM ES7 had an effect even stronger than 10 μM. Binucleated cells were frequently observed in day 3 cultures, occasionally showing fusing nuclei (Supplementary Fig. S1A). Structures with more than 3 nuclei were never observed. At this stage, callose was present in the SL (Supplementary Fig. S1B), with an amount and pattern similar to control and 10 μM ES7 cultures. However, no callose was found in inner cell walls. Cellulose was faintly detected in the scarce, thin and fragmented cell walls and SL present in these structures (Supplementary Fig. S1C). At the 6-day stage most cell walls were broken and the cytoplasm, shrunk and fragmented, was poured out of the cell. The only callose observed was in the form of large cytoplasmic plugs (Supplementary Fig. S1D), and no cellulose walls were observed (data not shown).

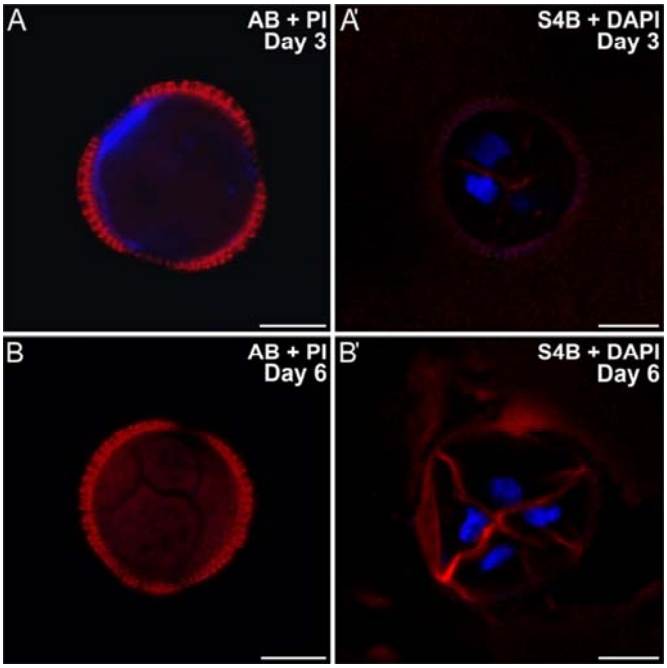


Figure 5. *B. napus* microspores treated with ES7 10 µM. A-A': 3-days old embryogenic microspores. B-B': 6-day old embryogenic microspores. Callose staining (blue) with aniline blue and propidium iodide, and cellulose staining (red) with S4B and DAPI are shown. Bars: 10 µm.

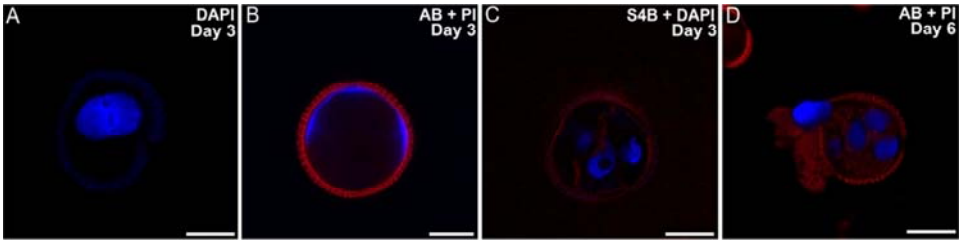


Figure S1. *B. napus* microspores treated with ES7 25 µM. A: DAPI stained 3-day old microspore showing two fusing nuclei. B: 3 day-old embryogenic microspore stained for callose (blue) with aniline blue and propidium iodide. C: 3-day old embryogenic microspore stained for cellulose (red) with S4B and DAPI. D: 6-day old burst cell showing callose plugs (blue). Bars: 10 µm.

Interestingly, the effect of ES7 (either at 10 µM and 25 µM) in pollen-like structures, following a gametophytic-like pathway, was remarkably different from that observed in dividing microspores. We found a clear callose wall beneath the exine, thickened just below the apertures (Fig. 6A), as opposed to that of control (untreated) pollen grains, devoid of callose (Fig. 1E') and with cellulose (Fig. 1E''). In those cases where the pollen-like structure germinated *in vitro* and initiated pollen tube emission, the tube wall was intensely stained with AB, indicating the presence of callose (Fig. 6B). In addition, unusual callose depositions, forming large plugs in the cytoplasm, were also observed in 6 day-old pollen-like grains (Fig. 6C).

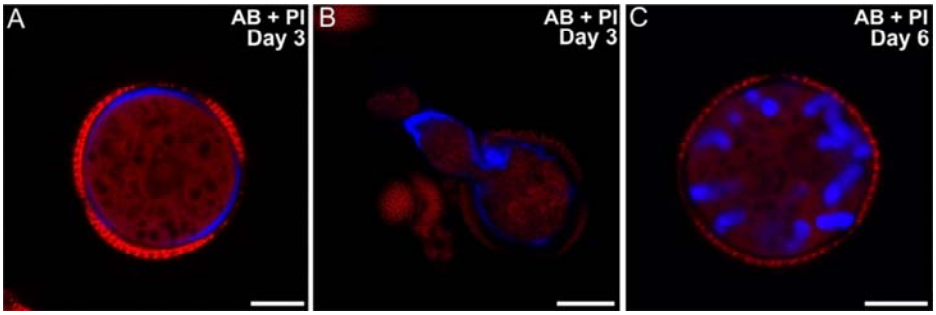


Figure 6. Effect of 10 µM ES7 treatment in *B. napus* pollen-like structures. A: 3-day old pollen grain in culture. B: pollen grain forming a pollen-tube structure. C: 6-day old pollen-like cell. Callose staining (blue) with aniline blue and propidium iodide is shown. Bars: 10 µm.

From the experiments shown above, we deduced that the application of ES7 affected the cells in a dose-dependent manner. At a low concentration, ES7 strongly reduced callose deposition while cellulose deposition was slightly increased. At a higher concentration, it clearly blocked callose deposition in the cell plate and therefore, cells failed to divide. However, the SL remained unaffected and developed normally. Most importantly, pollen-like grains underwent an unusual accumulation of callose beneath the exine, in the pollen tube wall and in the form of large cytoplasmic plugs. These results suggested that while somatic-type cytokinesis is severely affected by ES7, callose deposition associated to gametophytic-like development was not negatively affected, or even promoted, at least for callose related to pollen tube growth.

Eggplant embryogenic microspores develop thinner SL and callose cell walls

In order to test whether the development of callosic rich SL and inner cell walls is exclusive for *B. napus* embryogenic microspores, we studied the ultrastructure of the cell walls of embryogenic microspores from a recalcitrant species (eggplant), and compared it with that of *B. napus* embryogenic microspores. The callosic SL of *B. napus* embryogenic microspores presented an electron light appearance and an irregular thickness (Fig. 7A). The SL was continuous with the inner cell walls, which presented the same electron light appearance when observed under the TEM, except in the places where excreted cytoplasmic material (asterisk in Fig. 7B) was deposited (Corral-Martínez *et al*. 2013). As expected, anti-callose immunogold labelling revealed the callosic composition of both the SL and the inner cell walls (Fig. 7B). In eggplant embryogenic microspores, an electron light layer between the plasma membrane and the intine, distinct from the intine, irregular in thickness and continuous with the inner cell walls, was also found (Fig. 7C). However, this layer was thinner than that of *B. napus*. The layer was identical in appearance to the callosic SL of *B. napus* embryogenic microspores, and immunogold labelling with anti-callose antibodies revealed the presence of this carbohydrate in its composition (Fig. 7D). Callose was also detected in the inner cell walls of eggplant embryogenic microspores (Fig. 7E), but labelling was in general reduced to few, isolated gold particles, much scarcer than in *B. napus* inner walls. Particle clusters were only found around plasmodesmata (Fig. 7F), which is not surprising since callose is known to be present in plasmodesmata as part of the regulatory mechanism of symplastic transport (Vatén *et al*. 2011). All these observations suggested that the development of a callose rich SL observed in embryogenic microspores is not a unique feature of the model species *B. napus*, but it is also extensible to other, recalcitrant species such as eggplant. However, in eggplant it is not as evident as in *B. napus*. In line with this, the presence of callose in inner cell walls was also different, being almost negligible in eggplant.

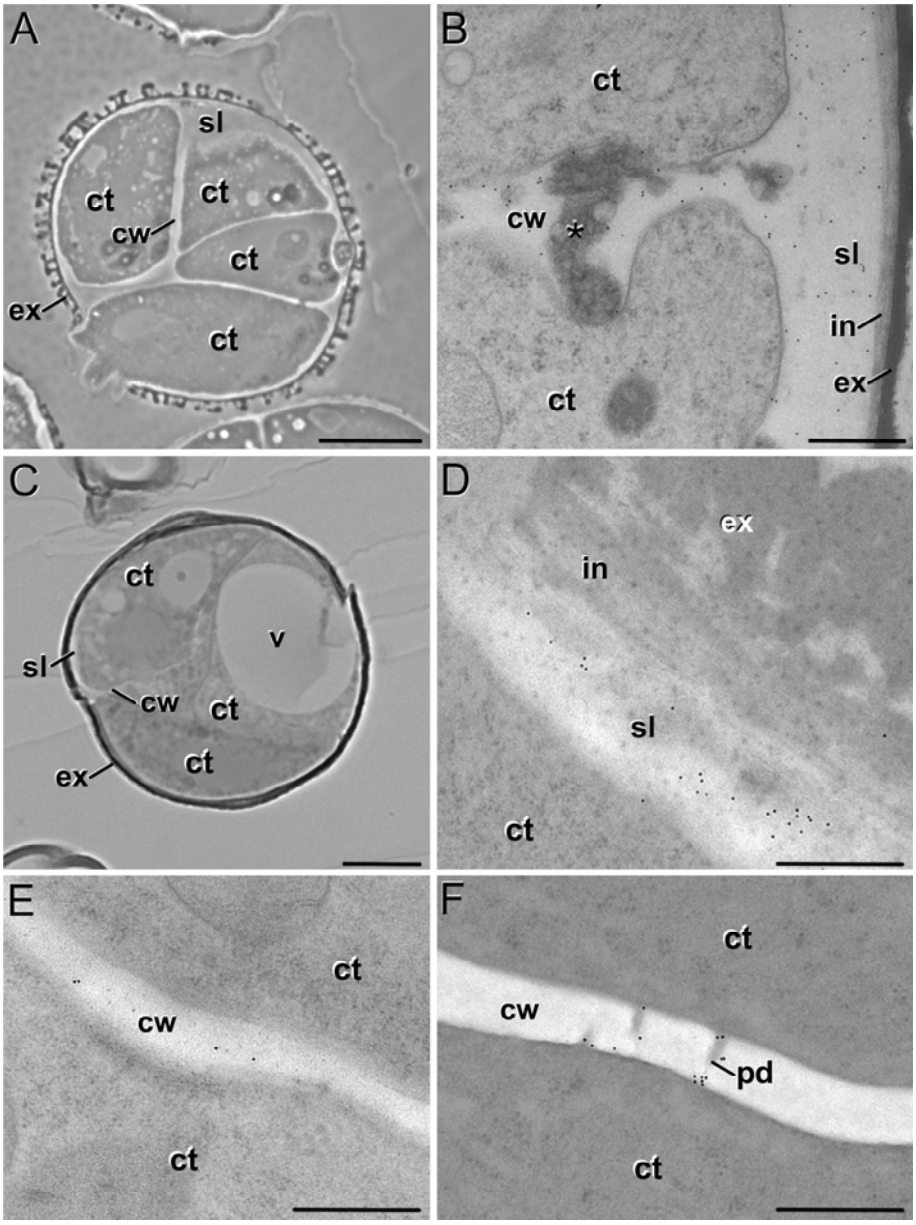


Figure 7. Anti-callose immunogold labeling of *B. napus* and eggplant embryogenic microspores. A: *B. napus* embryogenic microspore. B: Detail of the cell wall (cw) and subintinal layer (sl) showing abundant gold particles. Asterisk indicates deposits of excreted material. C: Eggplant embryogenic microspore. D: Detail of the subintinal layer. ct: cytoplasm; cw: cell wall; ex: exine; in: intine; v: vacuole. Bars: A, C: 10 µm. B, D: 500 nm.

Discussion

In this work, we studied the relationship between intracellular Ca^{2+} levels and the dynamics of callose and cellulose deposition for cell wall formation during microspore embryogenesis. For this, we used a series of chemicals to (1) decrease Ca^{2+} cytoplasmic levels (caffeine), (2) increase Ca^{2+} levels through alteration of plasma membrane fluidity (chitosan and digitonin), and (3) selectively inhibit some callose synthases (ES7). The regulatory mechanisms, the putative enzymes involved and the implications of callose walls in microspore embryogenesis are discussed next.

The formation of a callosic SL is a common feature of microspore embryogenesis in different species

Our results confirmed that two types of callose-rich walls are formed during the first stages of *B. napus* microspore embryogenesis. As previously reported (Parra-Vega *et al*. 2015), induction of microspore embryogenesis in *B. napus* is associated to the *de novo* formation of a SL mostly made of callose, and with the presence of abnormal cell walls where callose persists and cellulose is absent. In the present study, we compared *B. napus* and eggplant embryogenic microspores and found that eggplant embryogenic microspores also develop a new layer beneath the intine. This SL is clearly distinct from the intine and its electron light appearance, remarkably similar to that of *B. napus* embryogenic microspores, suggests that they may have the same nature. Indeed, we confirmed the presence of callose in both SLs. Therefore, we demonstrate that the presence of callosic SLs is an inherent characteristic of embryogenic induction, not only in the highly embryogenic *B. napus* line DH4079, but also in other, less responsive species such as eggplant.

Callose layers have been described at the onset of other natural and *in vitro* developmental processes such as male and female meiosis (Abramova *et al*. 2003), male gamete formation (Töller *et al*. 2008), *in vitro*-induced organogenesis (Fortes *et al*. 2002), zygotic embryogenesis (Jensen 1968; Williams *et al*. 1984) and somatic embryogenesis (Maheswaran and Williams 1985). In the latter, Dubois *et al*. (1991) found that callose presence in the cell surface initiated exactly when mesophyll cells showed the first signs of dedifferentiation, even before the onset of the first division. Interestingly, this is what we showed to happen in *B. napus* microspores. In this case, callose deposition was considered as the first sign of the entrance of cells into a new morphogenic program. Callose seems to form a protective layer that isolates specific cells from the influence of surrounding cells, which indicates that limiting exchange and/or communication with the surroundings is essential for cells to be able to commit to a change of their developmental fate. Callose deposition stands therefore as a marker for cell fate change and embryogenic commitment not only in *B. napus* microspores. It may serve to identify embryogenic microspores even before the first visual evidence of division.

Callose deposition associated to induction of microspore embryogenesis is modulated by calcium

The relationship of Ca^{2+} with microspore embryogenesis has been long suspected, as reviewed in Pauls *et al*. (2006). Working with a microspore embryogenesis model in wheat, Reynolds (2000) found that the expression of an early cysteine-labeled metallothionein (EcMt) transcript was a marker of embryogenic commitment, since it was expressed only during the first moments of the developmental switch. He also deduced that external Ca^{2+} is required for such a switch, since reduced Ca^{2+} concentrations in the medium suppressed both EcMt transcript accumulation and embryogenesis induction. The calmodulin antagonist W-7 suppressed embryogenesis induction and EcMt levels. These results led him to propose that Ca^{2+} might play a role in signal transduction during microspore

embryogenesis. Similarly, Ca^{2+} was associated to enhanced induction frequency and improved MDE structure in *Solanum carolinense* (Reynolds 1990), *Hordeum vulgare* (Hoekstra *et al*. 1997) or *Triticum aestivum* (Cho and Kasha 1995). Recently, we demonstrated that just after induction, intracellular Ca^{2+} transiently increased in embryogenic microspores at levels dramatically higher than before induction, whereas non-embryogenic forms such as callus-like and pollen-like structures presented remarkably different calcium patterns (Rivas-Sendra and Seguí-Simarro 2017).

In an attempt to reproduce in *B. napus* the positive results of Ca^{2+} increase, we used digitonin and chitosan, known to rise intracellular Ca^{2+} levels and induce callose synthesis stronger and more specifically than the ionophore A23187 (Pfeiffer *et al*. 1974; Waldmann *et al*. 1988). Our results with digitonin resulted, as expected, in an increased initial deposition of callose in the inner cell walls, and in the persistence of callose in time. These results confirmed the link between increased Ca^{2+} internalization and callose deposition during the initial stages of the embryogenic switch in *B. napus* isolated microspore culture. Moreover, the reduction of intracellular Ca^{2+} induced by caffeine treatment clearly blocked callose deposition in the cell plate and prevented cell division. Microspores cultured in the presence of 10 mM caffeine were induced to start their embryogenic development, but the lack of a properly formed cell wall precluded further nuclei division without cytokinesis, and eventually caused cell arrest and death. These results indicate that Ca^{2+} plays a regulatory role over callose deposition in the cell plate, essential for proper microspore embryogenesis progression and success.

Callose and cellulose deposition are incompatible processes

In somatic-type cytokinesis, proper callose deposition is needed for subsequent deposition of cellulose. However, the high level of Ca^{2+} needed for callose synthesis prevents the premature cellulose synthesis (Verma 2001). When Ca^{2+} level decreases, callose synthesis is reduced and cellulose synthesis starts. Our

experiments with digitonin showed that a moderate increase in callose deposition is accompanied by a moderate reduction in cellulose deposition. In parallel, experiments with chitosan and with low concentration of caffeine showed that limiting the level of Ca^{2+} gave rise to a reduction of callose deposition, and lead to a premature and increased cellulose deposition. Nevertheless, the results with higher doses of caffeine and with ES7 showed that when callose deposition in the cell plate is completely inhibited, cellulose is unable to deposit. This indicates that, as in conventional somatic-type cytokinesis (Verma 2001), the presence of a preliminary callose layer is needed as a scaffold to stabilize the cell plate and for proper cellulose deposition. However, callose must be removed to allow for cellulose deposition. Therefore, callose and cellulose synthesis would be related but incompatible processes.

Callose deposition in the cell wall and the SL are independent processes

The different timing and pattern of callose deposition in different locations and in response to different stimuli, even when occurring at the same time, indicate a local stimulation of callose synthases present, although possibly inactive, in microspores. Our results consistently showed that during the first stages of microspore embryogenesis, the dynamics of carbohydrate deposition is different in the newly formed inner cell walls and the SL. While callose accumulated in the SL of *B. napus* and eggplant microspores, it was significantly present only in inner cell walls of *B. napus*, but not eggplant microspores. In *B. napus*, the replacement of callose by cellulose seemed to be slower in the SL. Our experiments with caffeine showed that a reduction in intracellular Ca^{2+} concentration led to reduced callose deposition at the nascent cell plate, but the callose content of the SL was unaffected, irrespective of the caffeine dose used. In parallel, cytokinesis in microspores was affected by ES7, while the development of the SL proceeded normally. These evidences indicate that the formation of inner cell walls and the SL are two independent processes with different timing, responding to different stimuli and therefore subjected to different regulatory mechanisms. Considering

that some callose synthases are activated by Ca^{2+} whereas others are Ca^{2+}-insensitive, and that ES7 inhibits callose synthesis specifically associated to somatic-type cytokinesis and pollen tube germination, but not to other processes (Park *et al*. 2014), it is reasonable to deduce that different sets of callose synthases are involved in the formation of the SL and inner cell walls.

CalS9 and CalS10 could be involved in callose deposition in the first inner walls

In *Brassica napus*, more than 50 genes orthologous to arabidopsis CalS genes, including several duplicates, have been found. They have been divided in 11 groups and named after their counterparts in arabidopsis, with the exception of *CalS4* (*GSL9*) (Wang *et al*. 2014). Considering the short phylogenetic distance between *B. napus* and arabidopsis, we assume that *B. napus* CalS functions and characteristics would be related to the equivalent and highly homologous genes of arabidopsis. Vacuolate microspores are programmed to undergo the first pollen mitosis to produce the generative and vegetative cells. This division is asymmetrical and the wall formed around the generative cell is a transient, hemispherical wall made of callose (Park and Twell 2001). The enzymes responsible for the synthesis of this wall, CalS9 and CalS10, would already be present or ready for delivery to the cell plate when a microspore changes its fate, so they are the most reasonable candidates for callose deposition in the first walls formed in embryogenic microspores. Moreover, CalS10 is also involved in somatic-type cytokinesis (Chen *et al*. 2009; Thiele *et al*. 2009), being functionally redundant with the cell plate-specific CalS1 (GSL6) enzyme (Hong *et al*. 2001; Verma and Hong 2001). This implies that CalS10 must be Ca^{2+}-dependent and inhibited by ES7 when interfering with cytokinesis, which is consistent with our results. Although CalS1 is believed to be the most important CalS operating in somatic-type cytokinesis, knockout mutants did not display cytokinesis-defective phenotypes, suggesting the involvement of more than one CalS (Hong and Verma 2007). In addition, CalS1 was found in tissues all over the plant except for petals, pollen and

embryos (Dong *et al*. 2008), which supports the hypothesis of this enzyme not being involved in at least the first cytokinesis rounds of embryogenic microspores.

CalS5 and CalS12 could be involved in the formation of the subintinal layer

The pattern of callose deposition in the SL resembles that of the callose deposited between the plasma membrane and the cell wall as a defense barrier against external conditions or pathogen attack. Callose is rapidly deposited forming plugs called papillae, but it also forms layers beneath the cell wall. By aniline blue staining, Jacobs *et al*. (2003) showed how a continuous layer of callose was deposited by CalS12 in the periphery of epidermal cells in response to the penetration of fungal haustoria. On the other hand, the location of the SL could also be related to the callose layer deposited by CalS5 surrounding young microspores to serve as a scaffold for exine formation. Our experiments repeatedly showed that changes in intracellular Ca^{2+} altered callose deposition at the inner cell walls, but the SL was much less (or not) affected, being always a basal level of callose deposition. This suggests that in callose deposition at the SL, more than one CalS would be involved, and one would be sensitive to Ca^{2+} changes while the other would not. Our hypothesis is that these two enzymes could well be CalS12 and CalS5. CalS12 was suggested to be Ca^{2+}–sensitive (Jacobs *et al*. 2003). CalS5 presents a Ca^{2+}-binding domain in the central cytoplasmic hydrophilic loop and is involved in callose deposition during pollen tube growth (Dong *et al*. 2005). However, it was shown that such a deposition does not need Ca^{2+}, which was interpreted as CalS5 being Ca^{2+}–independent, at least in the context of pollen tube growth (Schlüpmann *et al*. 1993). This is not surprising, since multiple CalS genes are known to be expressed in the same tissues or cell types, being functionally redundant but responding to different regulatory cues (Dong *et al*. 2008). In our system, the Ca^{2+}-independent but sensitive to ES7 CalS5 and the Ca^{2+}-dependent but insensitive to ES7 CalS12 would perfectly complement each other and make

callose deposition at the SL a robust event, relatively insensitive to Ca^{2+} levels modification and independent from cytokinesis.

Chitosan appears to elicit callose synthesis related to pathogen attack response

Our results with chitosan were somehow unexpected, since a beneficial effect was previously reported (Ahmadi and Shariatpanahi 2015). We applied a long treatment, so the observed effects are probably due to the toxicity of the compound. Indeed, shorter treatments with higher doses showed a similar negative effect, causing high callus formation (Ahmadi and Shariatpanahi 2015). Anyway, the most striking result at the cell wall level was the pattern of callose deposition, remarkably different from that of other callose-inducing compounds. The level of callose deposition in cell plates was reduced, while large cytoplasmic plugs were formed. Previous works reported similar structures described as '*big plugs*' and '*cap-like*' areas, heavily stained by aniline blue (Köhle *et al*. 1985; Waldmann *et al*. 1988). Chitosan is a derivative of chitin, a naturally occurring polymer present in fungal cell walls and in the exoskeleton of insects, which elicits a pathogenesis-related (PR) response upon pathogen attack (Hadwiger 2013). As a part of this response, chitosan produces rapid membrane transient depolarization (Amborabé *et al*. 2008), which causes influx of H^+ and Ca^{2+} which, in turn, elicits callose synthesis and lignification (Pearce and Ride 1982) as a physical barrier against pathogen penetration. PR response also includes the synthesis of chitinases to fight against the pathogen. Interestingly, a series of enzymes involved in PR response were strongly upregulated upon stress treatment in barley anther cultures (Jacquard *et al*. 2009). Some of these enzymes were chitinases. Two chitinase isoforms were found to be excreted by developing maize MDEs (Borderies *et al*. 2004). As seen, there are interesting coincidences between PR response and microspore embryogenesis. It seems that *in vitro* cultured microspores perceive heat and chitosan as stressors and react by triggering general plant defense mechanisms which would include chitinase and callose

synthesis. CalS12, the main CalS related to pathogen defense, is present in *B. napus* microspores and a good candidate to be activated by the heat shock-related Ca^{2+} influx to produce the SL and, in a stronger and more specific way, by chitosan.

Concluding remarks

When entering the embryogenic pathway, a transient additional wall may be needed to isolate the cell and to protect it from the *in vitro* environment. But why callose? There are several reasons to choose callose for this. First, because the SL must be transient. As opposed to cellulose, callose can be easily degraded into its glucose building blocks by specific cell wall enzymes (Verma and Hong 2001). Interestingly, among the different proteins secreted to the culture medium by maize embryogenic microspores, one β-1,3-glucanase and two isoforms of thaumatin, a PR protein with 1,3 glucan-hydrolyzing activity, were found (Borderies *et al*. 2004). In addition, the gel-like properties of callose may confer embryogenic microspore cells the high wall plasticity needed during the first stages of rapid growth while still encaged within the exine coat. However, the main reason to use callose may be related to viability of these delicate cells in an artificial, osmotically unbalanced *in vitro* environment. Callose walls are known to be formed as a response to osmotic stress in cells cultured *in vitro* in hypertonic media, which in turn allows them to enter embryogenesis (You *et al*. 2006). In these conditions, BY-2 cells that developed a thick callose wall produced by altered expression of CalS5, were more tolerant to osmotic stress, preventing them from plasmolysis (Xie *et al*. 2012). On the other hand, these callosic walls made cells more prone to cytolysis, due to the above mentioned plasticity of callose. This means that these cells are more likely to burst when cytoplasmic turgor increases. This may well be the situation in our *B. napus* DH4079 microspores with callose walls, exposed to a hypertonic medium with, among others, 130 g/l sucrose. Thick callose walls may provide them the impermeability needed to prevent water loss and plasmolysis. This would be beneficial in terms of prevention of plasmolysis,

but in parallel, it would prevent proper cellulose deposition, which would produce the abnormal walls we observed, for example, with digitonin treatments. The plasticity of the walls formed would also allow to premature exine rupture, which is also a phenomenon frequently observed in microspore cultures of different species, and determinant for MDE fate (Tang *et al*. 2013). Indeed, we have repeatedly observed in different species that when exine is ruptured prematurely, microspores usually develop as loose callus-like structures (our unpublished observations). This hypothesis also explains why less responsive species such as eggplant show thinner callosic SLs and they are less protected against plasmolysis.

Our present and previous (Parra-Vega *et al*. 2015; Rivas-Sendra and Seguí-Simarro 2017) results establish a clear link between Ca^{2+} influx, callose deposition, abnormal cellulose synthesis and induction of microspore embryogenesis. It is not clear whether the former are a cause or a consequence of the latter. We showed that two different species, with markedly different androgenic response, produce different amounts of callose, clearly related to their response. Our data point to a sequence of events starting with the application of the stress treatment. This stress would be perceived differently in different genotypes, generating a different embryogenic response. In highly responsive genotypes, stress response would generate an increased Ca^{2+} influx and in turn an increased and excessive callose synthesis, preventing cellulose synthesis and producing abnormal callose-rich inner walls and the callosic SL which would isolate microspores from their environments and would therefore allow for proper initial MDE development. In less responsive genotypes, callose synthesis is much less induced, producing inner cell walls with little or no callose and thinner SLs with less callose, but leaving microspores more exposed to the environment, and therefore increasing the chances of developmental abortion and collapse. In conclusion, the species-specific differences in embryogenic response might be related to differences in response against abiotic stresses (involving the heat shock response, for example) or even biotic stresses, involving perhaps the PR response genes.

Acknowledgements

We want to express our thanks to the staff of the Electron Microscopy Service of Universitat Politècnica de València and to Marisol Gascón, from the IBMCP Microscopy Service, for their excellent technical help. Thanks are also due to Dr. Kim Boutilier (Plant Research International, Wageningen, The Netherlands) for her help during the stay of ARS at her lab, and to Dr. Samantha Vernhettes (Institut Jean-Pierre Bourgin, INRA Versailles, France) for her kind gift of for Pontamine Fast Scarlet 4B staining. This work was supported by grant AGL2014-55177-R to JMSS from Spanish Ministerio de Economía y Competitividad (MINECO) jointly funded by FEDER. ARS was supported by a predoctoral fellowship from the FPI Program of Universitat Politècnica de València.

References

Abramova LI, Avalkina NA, Golubeva EA, Pyzhenkova ZS, Golubovskaya IN (2003) Synthesis and Deposition of Callose in Anthers and Ovules of Meiotic Mutants of Maize (*Zea mays*). Russian Journal of Plant Physiology 50: 324-329

Ahmadi B, Shariatpanahi M (2015) Proline and chitosan enhanced efficiency of microspore embryogenesis induction and plantlet regeneration in *Brassica napus* L. Plant Cell, Tissue and Organ Culture (PCTOC) 123: 57-65

Amborabé B-E, Bonmort J, Fleurat-Lessard P, Roblin G (2008) Early events induced by chitosan on plant cells. J Exp Bot 59: 2317-2324

Anderson CT, Carroll A, Akhmetova L, Somerville C (2010) Real-Time Imaging of Cellulose Reorientation during Cell Wall Expansion in Arabidopsis Roots. Plant Physiol 152: 787-796

Barany I, Fadon B, Risueno MC, Testillano PS (2010) Cell wall components and pectin esterification levels as markers of proliferation and differentiation events during pollen development and pollen embryogenesis in *Capsicum annuum* L. J Exp Bot 61: 1159-1175

Becerra J, López-Sáez JF (1978) Effects of caffeine, calcium and magnesium on plant cytokinesis. Exp Cell Res 111: 301-308

Borderies G, le Bechec M, Rossignol M, Lafitte C, Le Deunff E, Beckert M, Dumas C, Matthys-Rochon E (2004) Characterization of proteins secreted during maize microspore culture: arabinogalactan proteins (AGPs) stimulate embryo development. Eur J Cell Biol 83: 205-212

Corral-Martínez P, García-Fortea E, Bernard S, Driouich A, Seguí-Simarro JM (2016) Ultrastructural Immunolocalization of Arabinogalactan Protein, Pectin and Hemicellulose Epitopes Through Anther Development in *Brassica napus*. Plant Cell Physiol 57: 2161-2174

Corral-Martínez P, Parra-Vega V, Seguí-Simarro JM (2013) Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. J Exp Bot 64: 3061-3075

Custers J (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 185-193

Chen XY, Liu L, Lee E, Han X, Rim Y, Chu H, Kim SW, Sack F, Kim JY (2009) The Arabidopsis callose synthase gene GSL8 is required for cytokinesis and cell patterning. Plant Physiol 150: 105-113

Cho UH, Kasha KJ (1995) The effect of calcium on ethylene production and microspore-derived embryogenesis in barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.) anther cultures. J Plant Physiol 146: 677-680

Doblin MS, De Melis L, Newbigin E, Bacic A, Read SM (2001) Pollen tubes of Nicotiana alata express two genes from different beta-glucan synthase families. Plant Physiol 125: 2040-2052

Dong X, Hong Z, Sivaramakrishnan M, Mahfouz M, Verma DPS (2005) Callose synthase (CalS5) is required for exine formation during microgametogenesis and for pollen viability in Arabidopsis. The Plant Journal 42: 315-328

Dong XY, Hong ZL, Chatterjee J, Kim SH, Verma DPS (2008) Expression of callose synthase genes and its connection with Npr1 signaling pathway during pathogen infection. Planta 229: 87-98

Drakakaki G (2015) Polysaccharide deposition during cytokinesis: Challenges and future perspectives. Plant Sci 236: 177-184

Drakakaki G, Robert S, Szatmari A-M, Brown MQ, Nagawa S, Van Damme D, Leonard M, Yang Z, Girke T, Schmid SL, Russinova E, Friml J, Raikhel NV, Hicks GR (2011) Clusters of bioactive compounds target dynamic endomembrane networks *in vivo*. Proceedings of the National Academy of Sciences 108: 17850-17855

Dubas E, Custers J, Kieft H, Wedzony M, van Lammeren AA (2013) Characterization of polarity development through 2- and 3-D imaging during the initial phase of microspore embryogenesis in *Brassica napus* L. Protoplasma

Dubois T, Guedira M, Dubois J, Vasseur J (1991) Direct somatic embryogenesis in leaves of *Cichorium*: a histological and SEM study of early stages. Protoplasma 162: 120-127

El-Tantawy A-A, Solís M-T, Costa ML, Coimbra S, Risueño M-C, Testillano PS (2013) Arabinogalactan protein profiles and distribution patterns during microspore embryogenesis and pollen development in *Brassica napus*. Plant Reprod 26: 231-243

Enns LC, Kanaoka MM, Torii KU, Comai L, Okada K, Cleland RE (2005) Two callose synthases, GSL1 and GSL5, play an essential and redundant role in plant and pollen development and in fertility. Plant Mol Biol 58: 333-349

Evans NA, Hoyne PA, Stone BA (1984) Characteristics and specificity of the interaction of a fluorochrome from aniline blue (Sirofluor) with polysaccharides. Carbohydrate Polymers 4: 215-230

Fortes AM, Testillano PS, Risueno MD, Pais MS (2002) Studies on callose and cutin during the expression of competence and determination for organogenic nodule formation from internodes of Humulus lupulus var. Nugget. Physiol Plant 116: 113-120

Hadwiger LA (2013) Multiple effects of chitosan on plant systems: Solid science or hype. Plant Sci: 42-49

Hirano Y, Frey B, Brunner I (2012) Contrasting reactions of roots of two coniferous tree species to aluminum stress. Environ Exp Bot 77: 12-18

Hoekstra S, van Bergen S, van Brouwershaven IR, Schilperoort RA, Wang M (1997) Androgenesis in *Hordeum vulgare* L.: Effects of mannitol, calcium and abscisic acid on anther pretreatment. Plant Sci 126: 211-218

Hong Z, Delauney AJ, Verma DPS (2001) A cell plate specific callose synthase and its interaction with phragmoplastin. Plant Cell 13: 755-768

Hong Z, Verma DPS (2007) Molecular Analysis of the Cell Plate Forming Machinery. In: Verma DPS, Hong Z (eds) Cell Division Control in Plants. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 303-320

Horvath I, Glatz A, Varvasovszki V, Torok Z, Pali T, Balogh G, Kovacs E, Nadasdi L, Benko S, Joo F, Vigh L (1998) Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: Identification of hsp17 as a "fluidity gene". Proc Natl Acad Sci USA 95: 3513-3518

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 15: 2503-2513

Jacquard C, Mazeyrat-Gourbeyre F, Devaux P, Boutilier K, Baillieul F, Clément C (2009) Microspore embryogenesis in barley: anther pre-treatment stimulates plant defence gene expression. Planta 229: 393-402

Jensen WA (1968) Cotton embryogenesis: The zygote. Planta 79: 346-366

Köhle H, Jeblick W, Poten F, Blaschek W, Kauss H (1985) Chitosan-elicited callose synthesis in soybean cells as a Ca^{2+}-dependent process. Plant Physiol 77: 544-551

Levy A, Erlanger M, Rosenthal M, Epel BL (2007) A plasmodesmata-associated beta-1,3-glucanase in Arabidopsis. Plant J 49: 669-682

Liu HT, Sun DY, Zhou RG (2005) Ca^{2+} and AtCaM3 are involved in the expression of heat shock protein gene in Arabidopsis. Plant Cell Environ 28: 1276-1284

López-Saez JF, Risueño MC, Giménez-Martin G (1966) Inhibition of cytokinesis in plant cells. Journal of Ultrastructural Research 14: 85-94

Maheswaran G, Williams EG (1985) Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann Bot 56: 619-630

Meikle PJ, Bonig I, Hoogenraad NJ, Clarke AE, Stone BA (1991) The location of (1--3)-β-glucans in the walls of pollen tubes of *Nicotiana alata* using a (1--3)-β-glucan-specific monoclonal antibody. Planta 185: 1-8

Nishikawa S-i, Zinkl GM, Swanson RJ, Maruyama D, Preuss D (2005) Callose (β-1,3 glucan) is essential for Arabidopsis pollen wall patterning, but not tube growth. BMC Plant Biol 5: 1-9

Nitsch C, Nitsch JP (1967) Induction of flowering *in vitro* in stem segments of *Plumbago indica* L. I Production of vegetative buds. Planta 72: 355-&

Park E, Díaz-Moreno SM, Davis DJ, Wilkop TE, Bulone V, Drakakaki G (2014) Endosidin 7 Specifically Arrests Late Cytokinesis and Inhibits Callose Biosynthesis, Revealing Distinct Trafficking Events during Cell Plate Maturation. Plant Physiol 165: 1019-1034

Park SK, Twell D (2001) Novel patterns of ectopic cell plate growth and lipid body distribution in the Arabidopsis *gemini pollen1* mutant. Plant Physiol 126: 899-909

Parra-Vega V, Corral-Martínez P, Rivas-Sendra A, Seguí-Simarro JM (2015) Induction of embryogenesis in *Brassica napus* microspores produces a callosic subintinal layer and abnormal cell walls with altered levels of callose and cellulose. Front Plant Sci 6: 1018

Paul DC, Goff DV (1973) Comparative effects of caffeine, its analogues and calcium deficiency on cytokinesis. Exp Cell Res 78: 399-413

Pauls KP, Chan J, Woronuk G, Schulze D, Brazolot J (2006) When microspores decide to become embryos - cellular and molecular changes. Can J Bot 84: 668-678

Pearce RB, Ride JP (1982) Chitin and related compounds as elicitors of the lignification response in wounded wheat leaves. Physiological Plant Pathology 20: 119-123

Pfeiffer DR, Reed PW, Lardy HA (1974) Ultraviolet and fluorescent spectral properties of the divalent cation ionophore A23187 and its metal ion complexes. Biochemistry 13

Rasmussen H, Tenenhouse A (1968) Cyclic adenosin monophosphate, Ca++, and membranes. Proceedings of the National Academy of Sciences 59: 1364-1370

Reynolds TL (1990) Interactions between calcium and auxin during pollen androgenesis in anther cultures of *Solanum carolinense* L. Plant Sci 72: 109-114

Reynolds TL (2000) Effects of calcium on embryogenic induction and the accumulation of abscisic acid, and an early cysteine-labeled metallothionein gene in androgenic microspores of *Triticum aestivum*. Plant Sci 150: 201-207

Rivas-Sendra A, Campos-Vega M, Calabuig-Serna A, Seguí-Simarro JM (2017) Development and characterization of an eggplant (*Solanum melongena*) doubled haploid population and a doubled haploid line with high androgenic response. Euphytica 213: 89

Rivas-Sendra A, Seguí-Simarro JM (2017) Dynamics of calcium during *in vivo* microspore development and *in vitro* microspore embryogenesis in *Brassica napus* In preparation

Saidi Y, Finka A, Muriset M, Bromberg Z, Weiss YG, Maathuis FJM, Goloubinoff P (2009) The Heat Shock Response in Moss Plants Is Regulated by Specific Calcium-Permeable Channels in the Plasma Membrane. Plant Cell 21: 2829-2843

Samuels AL, Staehelin LA (1996) Caffeine inhibits cell plate formation by disrupting membrane reorganization just after the vesicle fusion step. Protoplasma 195: 144-155

Schlüpmann H, Bacic A, Read SM (1993) A novel callose synthase from pollen tubes of Nicotiana. Planta 191: 470-481

Seguí-Simarro JM, Austin JR, White EA, Staehelin LA (2004) Electron tomographic analysis of somatic cell plate formation in meristematic cells of arabidopsis preserved by high-pressure freezing. Plant Cell 16: 836-856

Seguí-Simarro JM, Nuez F (2008) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134: 1-12

Seguí-Simarro JM, Otegui MS, Austin JR, Staehelin LA (2008) Plant cytokinesis - Insights gained from electron tomography studies. In: Verma DPS, Hong Z (eds) Cell Division Control in Plants. Springer, Berlin/Heidelberg, pp 251-287

Siegel SM, Daly O (1966) Regulation of betacyanin efflux from beet root by poly-L-lysine, Ca-ion and other substances. Plant Physiol 41: 1429-1434

Soriano M, Li H, Boutilier K (2013) Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod 26: 181-196

Tang X, Liu Y, He Y, Ma L, Sun M-x (2013) Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical–basal axis of the embryo. J Exp Bot

Thiele K, Wanner G, Kindzierski V, Jürgens G, Mayer U, Pachl F, Assaad FF (2009) The timely deposition of callose is essential for cytokinesis in Arabidopsis. The Plant Journal 58: 13-26

Töller A, Brownfield L, Neu C, Twell D, Schulze-Lefert P (2008) Dual function of Arabidopsis glucan synthase-like genes GSL8 and GSL10 in male gametophyte development and plant growth. The Plant Journal 54: 911-923

Vatén A, Dettmer J, Wu S, Stierhof Y-D, Miyashima S, Yadav Shri R, Roberts Christina J, Campilho A, Bulone V, Lichtenberger R, Lehesranta S, Mähönen Ari P, Kim J-Y, Jokitalo E, Sauer N, Scheres B, Nakajima K, Carlsbecker A, Gallagher Kimberly L, Helariutta Y (2011) Callose Biosynthesis Regulates Symplastic Trafficking during Root Development. Dev Cell 21: 1144-1155

Verma DPS (2001) Cytokinesis and building of the cell plate in plants. Annual Review of Plant Physiology and Plant Molecular Biology 52: 751-784

Verma DPS, Hong Z (2001) Plant callose synthase complexes. Plant Mol Biol 47: 693-701

Waldmann T, Jeblick W, Kauss H (1988) Induced net Ca^{2+} uptake and callose biosynthesis in suspension-cultured plant cells. Planta 173: 88-95

Wang H, Wang J, Zhou Y, Guan C, Hua W, Liu S, Fan G, Liu W, Hu Q, Sun F (2014) *Brassica napus* whole genome shotgun (WGS) project, assembly v 1.0, accesion number JMKK01000000 NCBI

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92: 487-511

Williams EG, Knox RB, Kaul V, Rouse JL (1984) Post-pollination callose development in ovules of *Rhododendron* and *Ledum* (Ericaceae): zygote special wall. J Cell Sci 69: 127-135

Wu A, Wasserman BP (1993) Limited proteolysis of (1,3)-β-glucan (callose) synthase from Beta vulgaris L: topology of protease-sensitive sites and polypeptide identification using Pronase E. The Plant Journal 4: 683-695

Xie B, Deng Y, Kanaoka MM, Okada K, Hong Z (2012) Expression of Arabidopsis callose synthase 5 results in callose accumulation and cell wall permeability alteration. Plant Sci 183: 1-8

Yasuhara H (2005) Caffeine inhibits callose deposition in the cell plate and the depolymerization of microtubules in the central region of the phragmoplast. Plant Cell Physiol 46: 1083–1092

You XL, Yi JS, Choi YE (2006) Cellular change and callose accumulation in zygotic embryos of *Eleutherococcus senticosus* caused by plasmolyzing pretreatment result in high frequency of single-cell-derived somatic embryogenesis. Protoplasma 227: 105-112

Young DH, Kohle H, Kauss H (1982) Effect of chitosan on membrane permeability of suspension-cultured *Glycine max* and *Phaseolus vulgaris* cells. Plant Physiol 70: 1449-1454

Zaki MAM, Dickinson HG (1991) Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4: 48-55

Zuppini A, Baldan B, Millioni R, Favaron F, Navazio L, Mariani P (2003) Chitosan induces Ca^{2+}-mediated programmed cell death in soybean cells. New Phytol 161: 557-568

General discussion

Microspore embryogenesis is an experimental procedure by which microspores are diverted from their original gametophytic pathway towards a new embryogenic fate by applying specific stresses *in vitro*. It allows for the production of DH (pure) lines through anther culture or isolated microspore culture followed by chromosome doubling. DH technology is interesting for both basic research and plant breeding. In this Thesis, we studied both applied and fundamental aspects of microspore embryogenesis, with the final goal to know them better and improve the efficiency of the process.

Importance of highly androgenic lines

In **Chapter 1** of this Thesis we developed an eggplant DH population from a commercial hybrid through microspore culture (Rivas-Sendra *et al*. 2017). The DH population showed segregation in vegetative and reproductive traits, and due to its totally homozygous condition, it can be perpetuated by seed without further segregation, which makes it useful for genetic analysis and mapping of segregating traits. From this population, we were able to identify the first highly androgenic DH eggplant line (DH36), which is remarkably similar to the donor hybrid in terms of morphology and reproductive ability, but which stably produces four times more calli than the parental hybrid and has a much higher response than other genotypes. In **Chapter 2** we compared the *in vitro* behavior of this highly responsive DH line to other eggplant commercial hybrids. These studies confirmed that the DH36 response is stable and much higher than responsive commercial hybrids. Two evidences strongly indicate that the genetic combination present in this line produces an endogenous concentration of growth regulators that is highly favorable for embryogenic response. On the one hand, DH36 embryogenic response was almost irresponsive to modifications of the concentration of growth regulators in the culture medium, while reducing this concentration increased the response of the commercial hybrids. On the other hand, in DH36 microspore cultures, embryogenesis could take place, although at a very low rate, in absence of exogenous growth regulators. Highly responsive species, usually considered model

species, do not need exogenous application of growth regulators to undergo microspore embryogenesis, as it is the case of wheat (Touraev *et al*. 1996b), tobacco (Touraev *et al*. 1996a), maize (Gaillard *et al*. 1991) and *B. napus* (Custers 2003), among others. Since the genotype-dependence in androgenic response is one of the main limitations of the DH technology, the identification of genotypes with high androgenic response is necessary not only in model species but also in crop species. To date, highly androgenic lines have been identified only in a limited number of species that usually already present good androgenic ability, such as some Brassicas and cereals (Ferrie *et al*. 1995; Ferrie and Keller 1995; Hoekstra *et al*. 1992; Kasha *et al*. 2003), but less efforts have been made in identifying highly responding genotypes in other economically important crop species. Our results suggest that the eggplant DH36 line could be used as a model line to provide material both for basic research on induced totipotency and embryo morphogenesis, and for applied research on *in vitro* DH production. The availability of a genotype with high and stable response to androgenesis induction, but still presenting some of the problems associated to recalcitrance, such the production of calli instead of embryos, may be very helpful to reach a better understanding of the mechanisms controlling androgenic ability and recalcitrance by comparison with model species, as we made in **Chapter 5** of this Thesis. Hopefully, this high response line will encourage the use of eggplant as a research platform for the study of the fascinating experimental phenomenon of microspore embryogenesis. Obviously, a highly androgenic line belonging to an economically important crop such as eggplant holds great interest for breeding purposes as well. For example, it could be used to transfer its androgenic competence to other recalcitrant genotypes of agronomic interest.

DH production in eggplant

One of the main applied interests of DH technology in general and microspore embryogenesis in particular is the use of DH as pure lines for hybrid production. In certain species such as rice, corn, wheat, barley, tobacco, or different Brassicas, the

protocols for DH production are efficient enough to allow for routine use in breeding programs. However, other species with high agronomic and economic interest, including solanaceous crops such as tomato or eggplant, or species with high scientific interest such as arabidopsis, are to some extent still recalcitrant to microspore embryogenesis.

Production of DHs in eggplant through microspore cultures is possible since 1996 (Miyoshi 1996), but it presents two main bottlenecks: the formation of calli instead of embryos, and the low frequency of plant regeneration from these calli. (Corral-Martínez and Seguí-Simarro 2012) demonstrated that embryos are initially formed in eggplant microspore cultures but they fail to transition from radial to bilateral symmetry after the globular stage. Production of embryos would be desirable since they are more genetically stable than calli and easier to transform into plants. By increasing chelated iron concentration during the microspore cultures, in **Chapter 2** of this Thesis we made a step towards the improvement of the embryo patterning in eggplant MDEs. Although we were not able to overcome the conversion into calli, we were able to slow down the process of dedifferentiation. That is a promising result, since evidence suggest that optimization of the concentration and timing of the treatment could lead to better control of the process. However, the fact that exogenous growth regulators are needed for eggplant microspore embryogenesis might be the cause for the failure in embryo development. Therefore, the most straightforward approach to improve the protocol for eggplant DH production through microspore at present is to obtain a high number of good quality androgenic calli, and to be able to transform them into entire DH plants. In **Chapter 2** we showed that adjusting the basic components of the culture medium, such as NLN salts and vitamins or sucrose, highly increases the efficiency of eggplant microspore cultures in different genotypes, and allows for a reduction of growth regulators in the culture medium without reducing efficiency. On the other hand, we addressed the problem of the low rate of regeneration of eggplant DH plants from microspore-derived calli. In **Chapter 3** we evaluated the effect of different media compositions in the induction of organogenesis, in the promotion of shoot growth and elongation, and in root

growth (Rivas-Sendra *et al*. 2015). The protocol we developed, with 70% of the regenerated plants being true DHs, represents a four-fold increase in plant production with respect to previous reports (Miyoshi 1996). Together, the applied research on eggplant microspore embryogenesis made in this Thesis resulted in the most efficient protocol existing to date for DH production in eggplant.

The role of calcium in microspore embryogenesis induction

The signaling and regulatory role of calcium in many cell processes, including stress response and activation of the embryogenic program, is widely known and studied. Indirect evidence drawn from experimenting with extracellular calcium concentrations and calcium signaling modulators in different species suggested a relationship between calcium regulation and microspore embryogenesis induction (Cho and Kasha 1995; Pauls *et al*. 2006; Reynolds 2000), and exogenous application of calcium has been found to improve microspore induction and MDEs germination and conversion into plants. However, up to date there were no direct clues about the precise role of calcium in microspore embryogenesis.

In **Chapter 4** of this Thesis, we used a Ca^{2+}-specific fluorescent vital dye to track and describe the changes in levels and in subcellular distribution of free cytosolic Ca^{2+} in living microspores and pollen grains during *in vivo* development, as well as during the first stages of *in vitro*-induced microspore embryogenesis. During *in vivo* development, we observed a clear peak of cytosolic Ca^{2+} in vacuolate microspores and young pollen grains, the stages more suitable for embryogenesis induction. The dynamics of Ca^{2+} accumulation during the *in vivo* process of microsporogenesis and microgametogenesis seems to be different for every species (Ge *et al*. 2007b; Kong and JIA 2004; Kuang and Liao 2015; Qiu *et al*. 2009), but the sharp peak in vacuolate microspores and young pollen is not only observed in *B. napus*, but also in other highly responding species such as tobacco (Ge *et al*. 2007b). Our observations suggest that this particularly high Ca^{2+} level just at the

stages more suitable for embryogenesis induction could be related to the microspore embryogenesis ability.

Just after *in vitro* induction of microspore embryogenesis, the first signs of embryogenic commitment are accompanied by a dramatic increase in intracellular Ca^{2+} levels, which becomes much higher than during *in vivo* development. We observed a transition of Ca^{2+} from the cytoplasm to the vacuoles during first stages of induction, probably as a mechanism to avoid calcium toxicity in the cytosol, and a progressive decrease in Ca^{2+} as embryogenesis proceeds. When microspores have undergone successive division rounds, Ca^{2+} concentration reaches undetectable levels both in vacuoles and cytosol. At the same time, non-embryogenic forms such as callus-like and pollen-like structures, although subjected to the same culture conditions, presented very different calcium patterns. For the first time, we described a detailed pattern of Ca^{2+} dynamics during the early stages of MDE induction. Other embryogenesis processes, such as somatic and zygotic embryogenesis, are also characterized by specific intracellular calcium perturbations, specifically transient Ca^{2+} peaks (Antoine *et al*. 2001; Ge *et al*. 2007a; Timmers *et al*. 1996), which suggests that the elevated Ca^{2+} levels we found during the initials of microspore embryogenesis play a key role in developmental switch and embryogenesis initiation. The similarities between calcium patterns would add to the growing body of evidences that relate the different embryogenic pathways not only ant the genetic level, but also at the cellular and physiological levels.

The role of callose in microspore embryogenesis

In previous studies of our group, we used HPF/FS to study *B. napus* embryogenic microspores, and showed for the first time that a dramatic and transient increase in callose in the newly formed cell walls is an inherent characteristic of embryogenic induction (Parra-Vega *et al*. 2015b). Abnormally increased levels of callose in *B. napus* embryogenic microspores were found both in the newly formed inner cell walls and in a new layer beneath the pollen intine.

In **Chapter 4** of this Thesis we showed how the presence of the callose-rich subintinal layer, considered a marker of embryogenic commitment (Parra-Vega *et al*. 2015b), was inversely related to the presence of Ca^{2+} in vacuoles, which is most likely a mechanism to avoid the potentially toxic effect of a high Ca^{2+} concentration in the cytosol. As a callose-rich, impermeable new cell wall is formed and progressively covers more area of the plasma membrane, which was permeabilized during heat shock, the influx of Ca^{2+} decreases, and the protective storage of Ca^{2+} in vacuoles is no longer needed. In **Chapter 5** of this Thesis, we showed that Ca^{2+} increase is not the only common link of microspore embryogenesis with other embryogenic processes. We studied the relationship between intracellular Ca^{2+} levels and the dynamics of callose and cellulose deposition for cell wall formation during microspore embryogenesis in *B. napus*, and demonstrated that the formation of a callose-rich subintinal layer is not an exclusive feature of *B. napus*, but also occurs in different species, such as eggplant (*Solanum melongena*). Our results revealed that the amount of callose in this layer, and in the newly formed inner cell walls, is related to the androgenic response, being higher in the highly responding genotype. The formation of a callose-rich layer surrounding the cell during the very first stages of embryogenic commitment is a common event of embryogenic microspores, somatic embryos (Dubois *et al*. 1991; Maheswaran and Williams 1985; You *et al*. 2006) and also zygotic embryos (Jensen 1968; Williams *et al*. 1984).

In **Chapter 5**, we used different chemical compounds known to interfere with normal Ca^{2+} levels, membrane fluidity and callose synthesis in order to determine to what extent these processes are related. Our experiments proved that the transient intracellular Ca^{2+} increase observed just after microspore induction, previously described in **Chapter 4**, is most likely causing the abnormally increased callose deposition observed in *B. napus* embryogenic microspores and early MDEs. We also demonstrated that this prolonged and increased callose deposition in the cell wall is essential for proper microspore embryogenesis progression and success, since cell walls defective in callose prevented cell division and led to cell arrest and death. The presence of a preliminary callose layer is needed as a scaffold

to stabilize the cell plate and mandatory for proper cellulose deposition. However, callose must be removed to allow for cellulose deposition, since they showed to be consecutive but incompatible processes. In addition, our results clearly indicated that callose deposition in the cell plate and in the subintinal layer are independent and differently regulated, since they follow different rhythms and patterns. While callose deposition in the cell plate showed to be completely Ca^{2+}-dependent, callose deposition in the subintinal layer was much less (or not) affected by Ca^{2+} concentration alteration, and a basal level of callose deposition was always present irrespective to the treatment applied, which demonstrates that different sets of callose synthases must be involved in each process. We proposed that callose synthases which already have a function in pollen grains are most likely the responsible for the unique callose deposition observed in embryogenic microspores. Although initially programmed to perform specific functions during pollen grain development *in vivo*, some CalS may participate in microspore embryogenesis as well, and carry out the synthesis and deposition of callose in different cellular events. Taking together all our results, and the known specific characteristics of the *B. napus* CalSs, which present high homology with those of Arabidopsis, we hypothesized a model where, on the one hand, CalS9 and CalS10, could be the enzymes responsible for callose deposition in the cell walls of embryogenic microspores, and on the other hand, CalS5 and CalS12 may be responsible for the formation of the subintinal layer. Being CalS5 Ca^{2+}-independent and sensitive to ES7, and CalS12 Ca^{2+}-dependent but insensitive to ES7 (Jacobs *et al*. 2003; Park *et al*. 2014; Schlüpmann *et al*. 1993), they would perfectly complement each other and make callose deposition in the subintinal layer a robust event.

Conclusions

In this Thesis:

1. We obtained a hybrid-derived eggplant DH population segregating for vegetative and reproductive traits as well as for androgenic response. This population may be useful for genetic studies and mapping of several traits.

2. We generated the first eggplant highly androgenic DH line, which presents a stable androgenic response that may be comparable to that of the best performing genotypes of model species, although proper embryo progression has still to be achieved. The highly androgenic line DH36 may facilitate the study of eggplant androgenesis and embryogenesis for both basic and applied research.

3. We developed the most efficient protocol existing to date for eggplant DH regeneration from microspore culture, which implies a reduced use of growth regulators while providing a high DH rate.

4. We made a detailed study of Ca^{2+} dynamics during microsporogenesis and microgametogenesis, revealing a sharp peak during the androgenesis-responsive stages that may be related to the ability of these stages to undergo microspore embryogenesis.

5. We studied Ca^{2+} during microspore embryogenesis and found that the first signs of embryogenic commitment are accompanied by a dramatic increase in intracellular Ca^{2+} level, which progressively decreases as embryogenesis proceeds. This increase, similar to that observed in cells committed to embryogenic fates, may be behind such a commitment.

6. We confirmed that this transient intracellular Ca^{2+} increase is causing the abnormally increased callose deposition observed in *B. napus* embryogenic microspores and early MDEs, found to be essential for proper microspore embryogenesis progression and success.

7. We demonstrated that the formation of callose-rich subintinal layer and cell walls is not an exclusive feature of *B. napus* embryogenic microspores, and that it is related to the androgenic ability of the genotype.

8. We proved that callose deposition in the subintinal layer and cell plate are independent processes, and proposed a model where different sets of callose synthases are responsible for these two processes.

References

Ahmadi B, Shariatpanahi M (2015) Proline and chitosan enhanced efficiency of microspore embryogenesis induction and plantlet regeneration in *Brassica napus* L. Plant Cell, Tissue and Organ Culture (PCTOC) 123: 57-65

Antoine A-F, Faure J-E, Dumas C, Feijo JA (2001) Differential contribution of cytoplasmic Ca^{2+} and Ca^{2+} influx to gamete fusion and egg activation in maize. Nat Cell Biol 3: 1120-1123

Bal U, Ellialtioglu S, Abak K (2009) Induction of symmetrical nucleus division and multi-nucleate structures in microspores of eggplant (*Solanum melongena* L.) cultured *in vitro*. Sci Agric 66: 535-539

Barnabas B, Obert B, Kovacs G (1999) Colchicine, an efficient genome-doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Rep 18: 858-862

Bassham DC, Laporte M, Marty F, Moriyasu Y, Ohsumi Y, Olsen LJ, Yoshimoto K (2006) Autophagy in Development and Stress Responses of Plants. Autophagy 2: 2-11

Belicuas PR, Guimaraes CT, Paiva LV, Duarte JM, Maluf WR, Paiva E (2007) Androgenetic haploids and SSR markers as tools for the development of tropical maize hybrids. Euphytica 156: 95-102

Belmonte M, Elhiti M, Waldner B, Stasolla C (2010) Depletion of cellular brassinolide decreases embryo production and disrupts the architecture of the apical meristems in *Brassica napus* microspore-derived embryos. J Exp Bot 61: 2779-2794

Belmonte MF, Ambrose SJ, Ross ARS, Abrams SR, Stasolla C (2006) Improved development of microspore-derived embryo cultures of *Brassica napus* cv Topas following changes in glutathione metabolism. Physiol Plant 127: 690-700

Beversdorf W, Kott L (1987) An *in vitro* mutagenesis/selection system for *Brassica napus*. Iowa State J Res 61: 435-443

Bhowmik P, Dirpaul J, Polowick P, Ferrie A (2011) A high throughput *Brassica napus* microspore culture system: influence of percoll gradient separation and bud selection on embryogenesis. Plant Cell Tissue Organ Cult 106: 359-362

Binarova P, Straatman KR, Hause B, Hause G, van Lammeren AAM (1993) Nuclear DNA synthesis during the induction of embryogenesis in cultured microspores and pollen of *Brassica napus* l. Theor Appl Genet 87: 9-16

Black BE, Bassett EA (2008) The histone variant CENP-A and centromere specification. Curr Opin Cell Biol 20: 91-100

Blakeslee AF, Belling J, Farnham ME, Bergner AD (1922) A haploid mutant in the Jimson weed *Datura stramonium*. Science 55: 646-647

Blume B, Nurnberger T, Nass N, Scheel D (2000) Receptor-mediated increase in cytoplasmic free calcium required for activation of pathogen defense in parsley. Plant Cell 12: 1425-1440

Boutilier KA, Gines MJ, DeMoor JM, Huang B, Baszczynski CL, Iyer VN, Miki BL (1994) Expression of the BnmNAP subfamily of napin genes coincides with the induction of Brassica microspore embryogenesis. Plant Mol Biol 26: 1711-1723

Breeding RGoH (1978) Induction of haploid plants of *Solanum melongena*. Proceedings of the Symposium on Plant Tissue Culture. Science Press, Peking, pp 227-232

Burnett L, Yarrow S, Huang B (1992) Embryogenesis and plant regeneration from isolated microspores of *Brassica rapa* L ssp Oleifera. Plant Cell Rep 11: 215-218

Castillo AM, Cistué L, Vallés MP, Sanz JM, Romagosa I, Molina-Cano JL (2001) Efficient production of androgenic doubled-haploid mutants in barley by the application of sodium azide to anther and microspore cultures. Plant Cell Rep 20: 105-111

Cloutier S, Cappadocia M, Landry BS (1995) Study of microspore-culture responsiveness in oilseed rape (*Brassica napus* L.) by comparative mapping of a F2 population and two microspore-derived populations. Theor Appl Genet 91: 841-847

Cordewener J, Bergervoet J, Liu CM (2000) Changes in protein synthesis and phosphorylation during microspore embryogenesis in *Brassica napus*. J Plant Physiol 156: 156-163

Cordewener JHG, Busink R, Traas J, Custers JBM, Dons HJM, van Lookeren-Campagne MM (1994) Induction of microspore embryogenesis in *Brassica napus* L. is accompanied by specific changes in protein synthesis. Planta 195: 50-56

Cordewener JHG, Hause G, Görgen E, Busink R, Hause B, Dons HJM, van Lammeren AAM, van Lookeren-Campagne MM, Pechan P (1995) Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196: 747-755

Corduan G, Spix C (1975) Haploid callus and regeneration of plants from anthers of *Digitalis purpurea* L. Planta 124: 1-11

Corral-Martínez P, Parra-Vega V, Seguí-Simarro JM (2013) Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning. J Exp Bot 64: 3061-3075

Corral-Martínez P, Seguí-Simarro JM (2012) Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.). Euphytica 187: 47-61

Corral-Martínez P, Seguí-Simarro JM (2014) Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins. Euphytica 195: 369-382

Custers J (2003) Microspore culture in rapeseed (*Brassica napus* L.). In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 185-193

Custers JBM, Cordewener JHG, Fiers MA, Maassen BTH, van Lookeren-Campagne MM, Liu CM (2001) Androgenesis in *Brassica*: a model system to study the initiation of plant embryogenesis. In: Bhojwani SS, Soh WY (eds) Current trends in the embryology of angiosperm. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 451-470

Chan J, Pauls KP (2007) *Brassica napus* Rop GTPases and their expression in microspore cultures. Planta 225: 469-484

Chase SS (1969) Monoploids and monoploid - derivatives of maize (*Zea mays* L.). Botanical Review 35: 117-167

Chen J-F, Cui L, Malik AA, Mbira KG (2011) *In vitro* haploid and dihaploid production via unfertilized ovule culture. Plant Cell, Tissue and Organ Culture (PCTOC) 104: 311-319

Chiancone B, Marli Gniech Karasawa M, Gianguzzi V, Abdelgalel AM, Bárány Iv, Testillano PS, Torello Marinoni D, Botta R, Germanà MA (2015) Early embryo achievement through isolated microspore culture in *Citrus clementina* Hort. ex Tan., cvs. 'Monreal Rosso' and 'Nules'. Front Plant Sci 6

Cho UH, Kasha KJ (1995) The effect of calcium on ethylene production and microspore-derived embryogenesis in barley (Hordeum vulgare L.) and wheat (Triticum aestivum L.) anther cultures. J Plant Physiol 146: 677-680

d'Amato F (1984) Role of polyploidy in reproductive organs and tissues. In: Johri BM (ed) Embryology of angiosperms. Springer-Verlag, New York, pp 519-566

Daghma DES, Hensel G, Rutten T, Melzer M, Kumlehn J (2014) Cellular dynamics during early barley pollen embryogenesis revealed by time-lapse imaging. Front Plant Sci 5: 675

Datta SK (2005) Androgenic haploids: Factors controlling development and its application in crop improvement. Curr Sci 89: 1870-1878

Demarty M, Morvan C, Thellier M (1984) Calcium and the cell wall. Plant, Cell & Environment 7: 441-448

Dong Y-Q, Zhao W-X, Li X-H, Liu X-C, Gao N-N, Huang J-H, Wang W-Y, Xu X-L, Tang Z-H (2016) Androgenesis, gynogenesis, and parthenogenesis haploids in cucurbit species. Plant Cell Rep: 1-29

Dormann M, Datla N, Hayden A, Puttick D, Quandt J (1998) Non-destructive screening of haploid embryos for glufosinate ammonium resistance four weeks after microspore transformation in *Brassica*. Acta Hort 459: 91-197

Dubas E, Custers J, Kieft H, Wędzony M, van Lammeren A (2011) Microtubule configurations and nuclear DNA synthesis during initiation of suspensor-bearing embryos from *Brassica napus* cv. Topas microspores. Plant Cell Rep 30: 2105–2116

Dubas E, Moravčíková J, Libantová J, Matušíková I, Benková E, Żur I, Krzewska M (2014) The influence of heat stress on auxin distribution in transgenic *B. napus* microspores and microspore-derived embryos. Protoplasma: 1-11

Dubois T, Guedira M, Dubois J, Vasseur J (1991) Direct somatic embryogenesis in leaves of *Cichorium*: a histological and SEM study of early stages. Protoplasma 162: 120-127

Dumas de Vaulx R, Chambonnet D (1982) Culture *in vitro* d'anthères d'aubergine (*Solanum melongena* L.): stimulation de la production de plantes au moyen de traitements à 35ºC associés à de faibles teneurs en substances de croissance. Agronomie 2: 983-988

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotechnol J 8: 377-424

Dwivedi SL, Britt AB, Tripathi L, Sharma S, Upadhyaya HD, Ortiz R (2015) Haploids: Constraints and opportunities in plant breeding. Biotechnol Adv 33: 812-829

El-Hennawy MA, Abdalla AF, Shafey SA, Al-Ashkar IM (2011) Production of doubled haploid wheat lines (Triticum aestivum L.) using anther culture technique. Annals of Agricultural Sciences 56: 63-72

Elhiti M, Tahir M, Gulden RH, Khamiss K, Stasolla C (2010) Modulation of embryo-forming capacity in culture through the expression of *Brassica* genes involved in the regulation of the shoot apical meristem. J Exp Bot 61: 4069-4085

Eudes F, Shim Y-S, Jiang F (2014) Engineering the haploid genome of microspores. Biocatalysis and Agricultural Biotechnology 3: 20-23

Fábián A, Földesiné Füredi PK, Ambrus H, Jäger K, Szabó L, Barnabás B (2015) Effect of n-butanol and cold pretreatment on the cytoskeleton and the ultrastructure of maize microspores when cultured *in vitro*. Plant Cell, Tissue and Organ Culture (PCTOC) 123: 257-271

Fan Z, Armstrong KC, Keller WA (1988) Development of microspores *in vivo* and *in vitro* in *Brassica napus* L. Protoplasma 147: 191-199

FAOSTAT (2017) http://faostat.fao.org [last checked March 2017].

Fayos O, Vallés MP, Garcés-Claver A, Mallor C, Castillo AM (2015) Doubled haploid production from Spanish onion (*Allium cepa* L.) germplasm: embryogenesis induction, plant regeneration and chromosome doubling. Front Plant Sci 6

Ferrie A, Caswell K (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104: 301-309

Ferrie AMR, Bethune TD, Mykytyshyn M (2011) Microspore embryogenesis in Apiaceae. Plant Cell, Tissue and Organ Culture (PCTOC) 104: 399-406

Ferrie AMR, Epp DJ, Keller WA (1995) Evaluation of *Brassica rapa* L. genotypes for microspore culture response and identification of a highly embryogenic line. Plant Cell Rep 14: 580-584

Ferrie AMR, Keller WA (1995) Microspore Culture for Haploid Plant Production. In: Gamborg OL, Phillips GC (eds) Plant Cell, Tissue and Organ Culture: Fundamental Methods. Springer Berlin Heidelberg, Berlin, Heidelberg, pp 155-164

Ferrie AMR, Keller WA (2007) Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the Brassicaceae. *In vitro* Cell Dev Biol -Pl 43: 348-355

Filonova LH, Bozhkov PV, Brukhin VB, Daniel G, Zhivotovsky B, von Arnold S (2000) Two waves of programmed cell death occur during formation and development of somatic embryos in the gymnosperm, Norway spruce. J Cell Sci 113: 4399-4411

Foisset N, Delourme R, Lucas MO, Renard M (1997) *In vitro* androgenesis and segregation distortion in *Brassica napus* L: Spontaneous versus colchicine-doubled lines. Plant Cell Rep 16: 464-468

Forster BP, Heberle-Bors E, Kasha KJ, Touraev A (2007) The resurgence of haploids in higher plants. Trends Plant Sci 12: 368-375

Fredua-Agyeman R, Rahman H (2016) Mapping of the clubroot disease resistance in spring *Brassica napus* canola introgressed from European winter canola cv. 'Mendel'. Euphytica 211: 201-213

Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamman T, Offringa R, Juergens G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature 426: 147-153

Fu Y, Zhang DQ, Gleeson M, Zhang YF, Lin BG, Hua SJ, Ding HD, Frauen M, Li JN, Qian W, Yu HS (2017) Analysis of QTL for seed oil content in *Brassica napus* by association mapping and QTL mapping. Euphytica 213

Gaillard A, Vergne P, Beckerte M (1991) Optimization of maize microspore isolation and culture conditions for reliable plant regeneration. Plant Cell Rep 10: 55-58

Gaines EF, Aase HC (1926) A haploid wheat plant. Am J Bot 13: 373-385

Ge LL, Tian HQ, Russell SD (2007a) Calcium function and distribution during fertilization in angiosperms. Am J Bot 94: 1046-1060

Ge LL, Xie CT, Wei DM, Qiu YL, H.Q. T (2007b) Calcium distribution during the anther development of tobacco. Int J Plant Dev Biol 1: 287–293

Gémes Juhász A, Kristóf Z, Vági P, Lantos C, Pauk J (2009) *In vitro* anther and isolated microspore culture as tools in sweet and spice pepper breeding. Acta Hort 829: 61-64

Germanà MA (2011a) Anther culture for haploid and doubled haploid production. Plant Cell Tissue Organ Cult 104: 283-300

Germanà MA (2011b) Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep 30: 839-857

Gresshoff PM, Doy CH (1972) Haploid *Arabidopsis thaliana* callus and plants from anther culture. Australian Journal of Biological Sciences 25: 259

Gresshoff PM, Doy CH (1974) Derivation of a haploid cell line from *Vitis vinifera* and importance of stage of meiotic development of anthers for haploid culture of this and other genera. Z Pflanzenphysiol 73: 132-141

Gu S-r (1979) Plantlets from isolated pollen cultures of eggplant (*Solanum melongena* L.). Acta Bot Sin 21: 30-36

Guha S, Maheshwari SC (1964) *In vitro* production of embryos from anthers of *Datura*. Nature 204: 497

Hause B, Hause G, Pechan P, van Lammeren AAM (1993) Cytoskeletal changes and induction of embryogenesis in microspore and pollen cultures of *Brassica napus* L. Cell Biol Int 17: 153-168

Hause B, van Veenendaal WLH, Hause G, van Lammeren AAM (1994) Expression of polarity during early development of microspore-derived and zygotic embryos of *Brassica napus* L cv. Topas. Botanica Acta 107: 407-415

Hays DB, Mandel RM, Pharis RP (2001) Hormones in zygotic and microspore embryos of *Brassica napus*. Plant Growth Regul 35: 47-58

Hays DB, Reid DM, Yeung EC, Pharis RP (2000) Role of ethylene in cotyledon development of microspore-derived embryos of *Brassica napus*. J Exp Bot 51: 1851-1859

Hays DB, Wilen RW, Sheng C, Moloney MM, Pharis RP (1999) Embryo-specific gene expression in microspore-derived embryos of *Brassica napus*. An interaction between abscisic acid and jasmonic acid. Plant Physiol 119: 1065-1072

Hays DB, Yeung EC, Pharis RP (2002) The role of gibberellins in embryo axis development. J Exp Bot 53: 1747-1751

Henderson CAP, Pauls KP (1992) The use of haploidy to develop plants that express several recessive traits using light-seeded canola (*Brassica napus*) as an example. Theor Appl Genet 83: 476-479

Henry Y (1998) Origin of microspore-derived dihaploid and polyhaploid *in vitro* plants. Plant Tissue Culture and Biotechnology 4: 127-135

Hirt H (2000) Connecting oxidative stress, auxin, and cell cycle regulation through a plant mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 97: 2405-2407

Ho KM, Jones GE (1980) Mingo barley. Canadian Journal of Plant Science 60: 279-280

Hoekstra S, Vanzijderveld MH, Louwerse JD, Heidekamp F, Vandermark F (1992) Anther and microspore culture of *Hordeum vulgare* L. cv Igri. Plant Sci 86: 89-96

Hofinger BJ, Huynh OA, Jankowicz-Cieslak J, Müller A, Otto I, Kumlehn J, Till BJ (2013) Validation of doubled haploid plants by enzymatic mismatch cleavage. Plant Methods 9: 43

Horvath I, Glatz A, Varvasovszki V, Torok Z, Pali T, Balogh G, Kovacs E, Nadasdi L, Benko S, Joo F, Vigh L (1998) Membrane physical state controls the signaling mechanism of the heat shock response in Synechocystis PCC 6803: Identification of hsp17 as a "fluidity gene". Proc Natl Acad Sci USA 95: 3513-3518

Huang B, Bird S, Kemble R, Miki B, Keller W (1991) Plant regeneration from microspore-derived embryos of *Brassica napus*: Effect of embryo age, culture temperature, osmotic pressure, and abscisic acid. *In vitro* Cell Dev Biol -Pl 27: 28-31

Ilic-Grubor K, Attree SM, Fowke LC (1998) Comparative morphological study of zygotic and microspore-derived embryos of *Brassica napus* L. as revealed by scanning electron microscopy. Ann Bot 82: 157-165

Indrianto A, Barinova I, Touraev A, Heberle-Bors E (2001) Tracking individual wheat microspores *in vitro*: identification of embryogenic microspores and body axis formation in the embryo. Planta 212: 163-174

Islam SMS, Tuteja N (2012) Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species. Plant Sci 182: 134-144

Ismaili A, Mohammadi PP (2016) Effect of genotype, induction medium, carbohydrate source, and polyethylene glycol on embryogenesis in maize (Zea mays L.) anther culture. Acta Physiol Plant 38

Isouard G, Raquin C, Demarly Y (1979) Obtention de plantes haploides et diploides par culture *in vitro* d'anthères dáubergine (*Solanum melongena* L.). C R Acad Sci Paris 288: 987-989

Jacobs AK, Lipka V, Burton RA, Panstruga R, Strizhov N, Schulze-Lefert P, Fincher GB (2003) An Arabidopsis callose synthase, GSL5, is required for wound and papillary callose formation. Plant Cell 15: 2503-2513

Jensen WA (1968) Cotton embryogenesis: The zygote. Planta 79: 346-366

Joosen R, Cordewener J, Supena EDJ, Vorst O, Lammers M, Maliepaard C, Zeilmaker T, Miki B, America T, Custers J, Boutilier K (2007) Combined transcriptome and proteome analysis identifies pathways and markers associated with the establishment of rapeseed microspore-derived embryo development. Plant Physiol 144: 155-172

Kasha KJ, Hu TC, Oro R, Simion E, Shim YS (2001) Nuclear fusion leads to chromosome doubling during mannitol pretreatment of barley (*Hordeum vulgare* L.) microspores. J Exp Bot 52: 1227-1238

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225: 874-876

Kasha KJ, Shim YS, Simion E, Letarte J (2006) Haploid production and chromosome doubling. In: Fari MG, Holb I, Bisztray GD (eds) Acta Hort. ISHS, Debrecen, Hungary, pp 817-828

Kasha KJ, Simion E, Miner M, Letarte J, Hu TC (2003) Haploid wheat isolated microspore culture protocol. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled haploid production in crop plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 77-82

Kim M, Jang I-C, Kim J-A, Park E-J, Yoon M, Lee Y (2008) Embryogenesis and plant regeneration of hot pepper (*Capsicum annuum* L.) through isolated microspore culture. Plant Cell Rep 27: 425-434

Kim M, Park E-J, An D, Lee Y (2013) High-quality embryo production and plant regeneration using a two-step culture system in isolated microspore cultures of hot pepper (*Capsicum annuum* L.). Plant Cell, Tissue and Organ Culture 112: 191-201

Kong H-Y, JIA G-X (2004) Calcium Distribution During Pollen Development of *Larix principis-rupprechtii*. Acta Bot Sin 46: 69—76

Kostoff D (1929) An androgenic *Nicotiana* haploid. Z Zellforsch 9: 640-642

Kranz E, Lorz H (1993) *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. Plant Cell 5: 739-746

Kuang Y-F, Liao J-P (2015) Calcium distribution during pollen development of *Uncaria hirsuta* Havil. (Rubiaceae), with special reference to the function of the protruding oncus. Grana 54: 12-25

Kumari M, Clarke HJ, Small I, Siddique KH (2009) Albinism in plants: a major bottleneck in wide hybridization, androgenesis and doubled haploid culture. Critical Reviews in Plant Science 28: 393-409

Kumlehn J (2009) Embryogenic pollen culture: a promising target for genetic transformation. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer Science + Business Media The Netherlands, pp 295-305

Kumlehn J, Lorz H, Kranz E (1999) Monitoring individual development of isolated wheat zygotes: a novel approach to study early embryogenesis. Protoplasma 208: 156-162

Lamport DT, Varnai P (2013) Periplasmic arabinogalactan glycoproteins act as a calcium capacitor that regulates plant growth and development. New Phytol 197: 58-64

León J, Rojo E, Sánchez-Serrano JJ (2001) Wound signalling in plants. J Exp Bot 52: 1-9

Leroux BJG, Potin P, Larher FR, Bodin M (2016) Enhancement of embryo yield from isolated microspores of *Brassica napus* by early iron starvation. Plant Biotechnol Rep 10: 483-486

Li H, Soriano M, Cordewener J, Muiño JM, Riksen T, Fukuoka H, Angenent GC, Boutilier K (2014) The histone deacetylase inhibitor Trichostatin A promotes totipotency in the male gametophyte. Plant Cell 26: 195-209

Lichter R (1981) Anther culture of *Brassica napus* in a liquid culture medium. Z Pflanzenphysiol 103: 229-237

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105: 427-434

Lichter R, Degroot E, Fiebig D, Schweiger R, Gland A (1988) Glucosinolates determined by HPLC in the seeds of microspore-derived homozygous lines of rapeseed (*Brassica napus* L). Plant Breed 100: 209-221

Liu HT, Sun DY, Zhou RG (2005) Ca^{2+} and AtCaM3 are involved in the expression of heat shock protein gene in Arabidopsis. Plant Cell Environ 28: 1276-1284

Lu C, Shen L, Tan Z, Xu Y, He P, Chen Y, Zhu L (1996) Comparative mapping of QTLs for agronomic traits of rice across environments using a doubled haploid population. Theor Appl Genet 93: 1211-1217

Maheswaran G, Williams EG (1985) Origin and development of somatic embryoids formed directly on immature embryos of *Trifolium repens in vitro*. Ann Bot 56: 619-630

Makowska K, Oleszczuk S (2014) Albinism in barley androgenesis. Plant Cell Rep 33: 385-392

Malik A, Cui L, Zhang S, Chen J-f (2011) Efficiency of SSR markers for determining the origin of melon plantlets derived through unfertilized ovary culture. Horticultural Science 38: 27-34

Malik MR, Wang F, Dirpaul JM, Zhou N, Polowick PL, Ferrie AMR, Krochko JE (2007) Transcript profiling and identification of molecular markers for early microspore embryogenesis in *Brassica napus*. Plant Physiol 144: 134-154

Maluszynska J (2003) Cytogenetic tests for ploidy level analyses — chromosome counting. In: Maluszynski M, Kasha KJ, Forster BP, Szarejko I (eds) Doubled Haploid Production in Crop Plants: A Manual. Springer Netherlands, Dordrecht, pp 391-395

Maluszynski M, Kasha KJ, Forster BP, Szarejko I (2003) Doubled haploid production in crop plants. A manual., Kluwer Academic Publishers, Dordrecht, The Netherlands

Maraschin SD, Vennik M, Lamers GEM, Spaink HP, Wang M (2005a) Time-lapse tracking of barley androgenesis reveals position-determined cell death within pro-embryos. Planta 220: 531-540

Maraschin SDF, Caspers M, Potokina E, Wulfert F, Graner A, Spaink HP, Wang M (2006) CDNA array analysis of stress-induced gene expression in barley androgenesis. Physiol Plant 127: 535-550

Maraschin SF, de Priester W, Spaink HP, Wang M (2005b) Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective. J Exp Bot 56: 1711-1726

Miah MAA, Earle ED, Khush GS (1985) Inheritance of callus formation ability in anther cultures of rice, *Oryza sativa* L. Theor Appl Genet 70: 113-116

Miyoshi K (1996) Callus induction and plantlet formation through culture of isolated microspores of eggplant (*Solanum melongena* L). Plant Cell Rep 15: 391-395

Möllers C, Iqbal MCM, Robbelen G (1994) Efficient production of doubled haploid *Brassica napus* plants by colchicine treatment of microspores. Euphytica 75: 95-104

Murovec J, Bohanec B (2012) Haploids and doubled haploids in plant breeding. In: Abdurakhmonov I (ed) Plant Breed. InTech, Rijeka, Croatia

Nageli M, Schmid JE, Stamp P, Buter B (1999) Improved formation of regenerable callus in isolated microspore culture of maize: impact of carbohydrates, plating density and time of transfer. Plant Cell Rep 19: 177-184

Nitsch JP (1972) Haploid plants from pollen. Z Pflanzenzüchtg 67: 3-18

Ochatt SJ (2008) Flow cytometry in plant breeding. Cytometry Part A 73A: 581-598

Park E, Díaz-Moreno SM, Davis DJ, Wilkop TE, Bulone V, Drakakaki G (2014) Endosidin 7 Specifically Arrests Late Cytokinesis and Inhibits Callose Biosynthesis, Revealing Distinct Trafficking Events during Cell Plate Maturation. Plant Physiol 165: 1019-1034

Parra-Vega V, Corral-Martínez P, Rivas-Sendra A, Seguí-Simarro JM (2015a) Formation and excretion of autophagic plastids (plastolysomes) in *Brassica napus* embryogenic microspores. Front Plant Sci 6 (94)

Parra-Vega V, Corral-Martínez P, Rivas-Sendra A, Seguí-Simarro JM (2015b) Induction of embryogenesis in *Brassica napus* microspores produces a callosic subintinal layer and abnormal cell walls with altered levels of callose and cellulose. Front Plant Sci 6: 1018

Parra-Vega V, González-García B, Seguí-Simarro JM (2013) Morphological markers to correlate bud and anther development with microsporogenesis and microgametogenesis in pepper (*Capsicum annuum L.*). Acta Physiol Plant 35: 627-633

Pauls KP (1996) The utility of doubled haploid populations for studying the genetic control of traits determinated by recessive alleles. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* Haploid Production in Higher Plants: Volume 1 — Fundamental Aspects and Methods. Springer Netherlands, Dordrecht, pp 125-144

Pauls KP, Chan J, Woronuk G, Schulze D, Brazolot J (2006) When microspores decide to become embryos - cellular and molecular changes. Can J Bot 84: 668-678

Pearce AK, Humphrey TC (2001) Integrating stress-response and cell-cycle checkpoint pathways. Trends Cell Biol 11: 426-433

Pechan PM, Bartels D, Brown DCW, Schell J (1991) Messenger RNA and protein changes associated with induction of *Brassica* microspore embryogenesis. Planta 184: 161-165

Prem D, Solís MT, Bárány I, Rodríguez-Sanz H, Risueño MC, Testillano PS (2012) A new microspore embryogenesis system under low temperature which mimics zygotic embryogenesis initials, expresses auxin and efficiently regenerates doubled-haploid plants in *Brassica napus*. BMC Plant Biol 12

Prigge V, Melchinger AE (2012) Production of haploids and doubled haploids in maize. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant Cell Culture Protocols. Humana Press, pp 161-172

Prigge V, Xu X, Li L, Babu R, Chen S, Atlin GN, Melchinger AE (2012) New Insights into the Genetics of *in vivo* Induction of Maternal Haploids, the Backbone of Doubled Haploid Technology in Maize. Genetics 190: 781-793

Qiu Y-L, Liu R-S, Wei D-M, Tian H-Q (2009) Calcium distribution in developing anthers of lettuce (*Lactuca sativa*). Ann Bot Fenn 46: 101-106

Ramesar-Fortner NS, Yeung EC (2006) Physiological influences in the development and function of the shoot apical meristem of microspore-derived embryos of *Brassica napus* 'Topas'. Can J Bot 84: 371-383

Rashid A, Siddiqui AW, Reinert J (1982) Subcellular aspects of origin and structure of pollen embryos of *Nicotiana*. Protoplasma 113: 202-208

Ravi M, Chan SWL (2010) Haploid plants produced by centromere-mediated genome elimination. Nature 464: 615-618

Reynolds TL (1997) Pollen embryogenesis. Plant Mol Biol 33: 1-10

Reynolds TL (2000) Effects of calcium on embryogenic induction and the accumulation of abscisic acid, and an early cysteine-labeled metallothionein gene in androgenic microspores of *Triticum aestivum*. Plant Sci 150: 201-207

Ribnicky DM, Cohen JD, Hu W-S, Cooke TJ (2002) An auxin surge following fertilization in carrots: a mechanism for regulating plant totipotency. Planta 214: 505-509

Rivas-Sendra A, Campos-Vega M, Calabuig-Serna A, Seguí-Simarro JM (2017) Development and characterization of an eggplant (*Solanum melongena*) doubled haploid population and a doubled haploid line with high androgenic response. Euphytica 213: 89

Rivas-Sendra A, Corral-Martínez P, Camacho-Fernández C, Seguí-Simarro JM (2015) Improved regeneration of eggplant doubled haploids from microspore-derived calli through organogenesis. Plant Cell Tissue Organ Cult 122: 759-765

Rizza F, Mennella G, Collonnier C, Shiachakr D, Kashyap V, Rajam MV, Prestera M, Rotino GL (2002) Androgenic dihaploids from somatic hybrids between *Solanum melongena* and *S. aethiopicum* group *Gilo* as a source of resistance to *Fusarium oxysporum* f. sp. *melongenae*. Plant Cell Rep 20: 1022-1032

Robert HS, Grunewald W, Sauer M, Cannoot B, Soriano M, Swarup R, Weijers D, Bennett M, Boutilier K, Friml J (2015) Plant embryogenesis requires AUX/LAX-mediated auxin influx. Development 142: 702-711

Rodríguez-Sanz H, Moreno-Romero J, Solís MT, Köhler C, Risueño MC, Testillano PS (2014) Changes in Histone Methylation and Acetylation during Microspore Reprogramming to Embryogenesis Occur Concomitantly with Bn*HKMT* and Bn*HAT* Expression and Are Associated with Cell Totipotency, Proliferation, and Differentiation in *Brassica napus*. Cytogenet Genome Res 143: 209-218

Rokka VM (2009) Potato haploids and breeding. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, Dordrecht, The Netherlands, pp 199-208

Rotino GL (1996) Haploidy in eggplant. In: Jain SM, Sopory SK, Veilleux RE (eds) *In vitro* haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 115-141

Rotino GL, Sihachakr D, Rizza F, Vale G, Tacconi MG, Alberti P, Mennella G, Sabatini E, Toppino L, D'Alessandro A, Acciarri N (2005) Current status in production and utilization of dihaploids from somatic hybrids between eggplant (*Solanum melongena* L.) and its wild relatives. Acta Physiol Plant 27: 723-733

Rudd JJ, Franklin-Tong VE (2001) Unravelling response-specificity in Ca^{2+} signalling pathways in plant cells. New Phytol 151: 7-33

Saidi Y, Finka A, Muriset M, Bromberg Z, Weiss YG, Maathuis FJM, Goloubinoff P (2009) The Heat Shock Response in Moss Plants Is Regulated by Specific Calcium-Permeable Channels in the Plasma Membrane. Plant Cell 21: 2829-2843

Salas P, Prohens J, Seguí-Simarro JM (2011) Evaluation of androgenic competence through anther culture in common eggplant and related species. Euphytica 182: 261–274

Salas P, Rivas-Sendra A, Prohens J, Seguí-Simarro JM (2012) Influence of the stage for anther excision and heterostyly in embryogenesis induction from eggplant anther cultures. Euphytica 184: 235-250

Sanguineti MC, Tuberosa R, Conti S (1990) Field evaluation of androgenetic lines of eggplant. Acta Hort 280: 177-182

Sato K, Nankaku N, Takeda K (2009) A high-density transcript linkage map of barley derived from a single population. Heredity 103: 110-117

Satpute G, Long H, Seguí-Simarro JM, Risueño MC, Testillano PS (2005) Cell architecture during gametophytic and embryogenic microspore development in *Brassica napus*. Acta Physiol Plant 27: 665-674

Schlüpmann H, Bacic A, Read SM (1993) A novel callose synthase from pollen tubes of Nicotiana. Planta 191: 470-481

Seguí-Simarro JM (2010) Androgenesis revisited. Bot Rev 76: 377-404

Seguí-Simarro JM (2015) Doubled haploidy in model and recalcitrant species. Front Plant Sci 6: 1175

Seguí-Simarro JM (2016) Androgenesis in solanaceae. In: Germanà MA, Lambardi M (eds) *In vitro* embryogenesis. Springer Science + Business Media, New York, pp 209-244

Seguí-Simarro JM, Bárány I, Suárez R, Fadón B, Testillano PS, Risueño MC (2006) Nuclear bodies domain changes with microspore reprogramming to embryogenesis. Eur J Histochem 50: 35-44

Seguí-Simarro JM, Corral-Martínez P, Corredor E, Raska I, Testillano PS, Risueño MC (2011a) A change of developmental program induces the remodeling of the interchromatin domain during microspore embryogenesis in *Brassica napus* L. J Plant Physiol 168: 746-757

Seguí-Simarro JM, Corral-Martínez P, Parra-Vega V, González-García B (2011b) Androgenesis in recalcitrant solanaceous crops. Plant Cell Rep 30: 765-778

Seguí-Simarro JM, Nuez F (2005) Meiotic metaphase I to telophase II is the most responsive stage of microspore development for induction of androgenesis in tomato (*Solanum lycopersicum*). Acta Physiol Plant 27: 675-685

Seguí-Simarro JM, Nuez F (2007) Embryogenesis induction, callogenesis, and plant regeneration by *in vitro* culture of tomato isolated microspores and whole anthers. J Exp Bot 58: 1119-1132

Seguí-Simarro JM, Nuez F (2008a) How microspores transform into haploid embryos: changes associated with embryogenesis induction and microspore-derived embryogenesis. Physiol Plant 134: 1-12

Seguí-Simarro JM, Nuez F (2008b) Pathways to doubled haploidy: chromosome doubling during androgenesis. Cytogenet Genome Res 120: 358-369

Seguí-Simarro JM, Testillano PS, Risueño MC (2003) Hsp70 and Hsp90 change their expression and subcellular localization after microspore embryogenesis induction in *Brassica napus* L. cv Topas. J Struct Biol 142: 379-391

Seguí-Simarro JM, Testillano PS, Risueño MC (2005) MAP kinases are developmentally regulated during stress-induced microspore embryogenesis in *Brassica napus* L. Histochem Cell Biol 123: 541-551

Shariatpanahi ME, Bal U, Heberle-Bors E, Touraev A (2006) Stresses applied for the re-programming of plant microspores towards *in vitro* embryogenesis. Physiol Plant 127: 519-534

Shim YS, Kasha KJ, Simion E, Letarte J (2006) The relationship between induction of embryogenesis and chromosome doubling in microspore cultures. Protoplasma 228: 79-86

Siebel J, Pauls KP (1989) A comparison of anther and microspore culture as a breeding tool in *Brassica napus*. Theor Appl Genet 78: 473-479

Simmonds DH, Keller WA (1999) Significance of preprophase bands of microtubules in the induction of microspore embryogenesis of *Brassica napus*. Planta 208: 383-391

Smalle J, Vierstra RD (2004) The ubiquitin 26S proteasome proteolytic pathway. Ann Rev Plant Biol 55: 555-590

Solís M-T, Rodríguez-Serrano M, Meijón M, Cañal M-J, Cifuentes A, Risueño MC, Testillano PS (2012) DNA methylation dynamics and MET1a-like gene

expression changes during stress-induced pollen reprogramming to embryogenesis. J Exp Bot 63: 6431-6444

Soriano M, Li H, Boutilier K (2013) Microspore embryogenesis: establishment of embryo identity and pattern in culture. Plant Reprod 26: 181-196

Soriano M, Li H, Jacquard C, Angenent GC, Krochko J, Offringa R, Boutilier K (2014) Plasticity in Cell Division Patterns and Auxin Transport Dependency during *in vitro* Embryogenesis in *Brassica napus*. Plant Cell 26: 2568-2581

Supena EDJ, Suharsono S, Jacobsen E, Custers JBM (2006) Successful development of a shed-microspore culture protocol for doubled haploid production in Indonesian hot pepper (*Capsicum annuum* L.). Plant Cell Rep 25: 1-10

Supena EDJ, Winarto B, Riksen T, Dubas E, van Lammeren A, Offringa R, Boutilier K, Custers J (2008) Regeneration of zygotic-like microspore-derived embryos suggests an important role for the suspensor in early embryo patterning. J Exp Bot 59: 803-814

Tang X, Liu Y, He Y, Ma L, Sun M-x (2013) Exine dehiscing induces rape microspore polarity, which results in different daughter cell fate and fixes the apical–basal axis of the embryo. J Exp Bot

Tang XC, He YQ, Wang Y, Sun MX (2006) The role of arabinogalactan proteins binding to Yariv reagents in the initiation, cell developmental fate, and maintenance of microspore embryogenesis in *Brassica napus* L. cv. Topas. J Exp Bot 57: 2639-2650

Tek AL, Stupar RM, Nagaki K (2015) Modification of centromere structure: a promising approach for haploid line production in plant breeding. Turkish Journal of Agriculture and Forestry 39: 557-562

Telmer CA, Newcomb W, Simmonds DH (1995) Cellular changes during heat shock induction and embryo development of cultured microspores of *Brassica napus* cv. Topas. Protoplasma 185: 106-112

Telmer CA, Simmonds DH, Newcomb W (1992) Determination of developmental stage to obtain high frequencies of embryogenic microspores in *Brassica napus*. Physiol Plant 84: 417-424

ten Hove CA, Heidstra R (2008) Who begets whom? Plant cell fate determination by asymmetric cell division. Curr Opin Plant Biol 11: 34-41

Testillano PS, Coronado MJ, Seguí-Simarro JM, Domenech J, Gonzalez-Melendi P, Raska I, Risueño MC (2000) Defined nuclear changes accompany the reprogramming of the microspore to embryogenesis. J Struct Biol 129: 223-232

Thomas E, Wenzel G (1975) Embryogenesis from microspores of *Brassica napus*. Z Pflanzenzuecht 74: 77-81

Thompson KF (1972) Oil-seed rape. Reports of the Plant Breeding Institute. Cambridge University Press, Cambridge, pp 94–96

Tian H, Yao CY, Sun MX (2004) High frequency conversion of microspore-derived embryos of *Brassica napus* cv. Topas by supplemental calcium and vitamins. Plant Cell Tissue Organ Cult 76: 159-165

Timmers ACJ, Reiss H-D, Bohsung J, Traxel K, Schel JHN (1996) Localization of calcium during somatic embryogenesis of carrot (*Daucus carota* L.). Protoplasma 190: 107-118

Tirlapur UK, Willemse MTM (1992) Changes in calcium and calmodulin levels during microsporogenesis, pollen development and germination in Gasteria verrucosa (Mill.) H. Duval. Sex Plant Reprod 5: 214-223

Toojinda T, Baird E, Booth A, Broers L, Hayes P, Powell W, Thomas W, Vivar H, Young G (1998) Introgression of quantitative trait loci (QTLs) determining stripe rust resistance in barley: an example of marker-assisted line development. Theor Appl Genet 96: 123-131

Toppino L, Mennella G, Rizza F, D'Alessandro A, Sihachakr D, Rotino GL (2008) ISSR and isozyme characterization of androgenetic dihaploids reveals tetrasomic inheritance in tetraploid somatic hybrids between *Solanum melongena* and *Solanum aethiopicum* group Gilo. J Hered 99: 304-315

Touraev A, Ilham A, Vicente O, Heberle-Bors E (1996a) Stress-induced microspore embryogenesis in tobacco: an optimized system for molecular studies. Plant Cell Rep 15: 561-565

Touraev A, Indrianto A, Wratschko I, Vicente O, Heberle-Bors E (1996b) Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperatures. Sex Plant Reprod 9: 209-215

Touraev A, Pfosser M, Heberle-Bors E (2001) The microspore: A haploid multipurpose cell. Adv Bot Res 35: 53-109

Tsuwamoto R, Fukuoka H, Takahata Y (2007) Identification and characterization of genes expressed in early embryogenesis from microspores of *Brassica napus*. Planta 225: 641-652

Verma DPS, Hong Z (2001) Plant callose synthase complexes. Plant Mol Biol 47: 693-701

Vigh L, Gombos Z, Joo F (1985) Selective modification of cytoplasmic membrane fluidity by catalytic-hydrogenation provides evidence on its primary role in chilling susceptibility of the blue-green alga, Anacystis nidulans. Febs Letters 191: 200-204

Wedzony M, Forster BP, Zur I, Golemiec E, Szechynska-Hebda M, Dubas E, Gotebiowska G (2009) Progress in doubled haploid technology in higher plants. In: Touraev A, Forster BP, Jain SM (eds) Advances in haploid production in higher plants. Springer, Dordrecht, Netherlands, pp 1-33

White PJ, Broadley MR (2003) Calcium in plants. Ann Bot 92: 487-511

Williams EG, Knox RB, Kaul V, Rouse JL (1984) Post-pollination callose development in ovules of *Rhododendron* and *Ledum* (Ericaceae): zygote special wall. J Cell Sci 69: 127-135

Yamagishi M, Yano M, Fukuta Y, Fukui K, Otani M, Shimada T (1996) Distorted segregation of RFLP markers in regenerated plants derived from anther culture of an F1 hybrid of rice. Genes Genet Syst 71: 37-41

Yeung EC, Rahman MH, Thorpe TA (1996) Comparative development of zygotic and microspore-derived embryos in *Brassica napus* L cv Topas .1. Histodifferentiation. Int J Plant Sci 157: 27-39

You XL, Yi JS, Choi YE (2006) Cellular change and callose accumulation in zygotic embryos of *Eleutherococcus senticosus* caused by plasmolyzing pretreatment result in high frequency of single-cell-derived somatic embryogenesis. Protoplasma 227: 105-112

Zagorska NA, Shtereva LA, Kruleva MM, Sotirova VG, Baralieva DL, Dimitrov BD (2004) Induced androgenesis in tomato (*Lycopersicon esculentum* Mill.). III. Characterization of the regenerants. Plant Cell Rep 22: 449-456

Zaki MA, Dickinson HG (1990) Structural changes during the first divisions of embryos resulting from anther and free microspore culture in *Brassica napus*. Protoplasma 156: 149-162

Zaki MAM, Dickinson HG (1991) Microspore-derived embryos in *Brassica*: the significance of division symmetry in pollen mitosis I to embryogenic development. Sex Plant Reprod 4: 48-55

Zarsky V, Garrido D, Rihová L, Tupy J, Heberle-Bors E (1992) Derepression of the cell cycle by starvation is involved in the induction of tobacco pollen embryogenesis. Sex Plant Reprod 5: 189-194

Zhang FL, Aoki S, Takahata Y (2003) RAPD markers linked to microspore embryogenic ability in *Brassica crops*. Euphytica 131: 207-213

Zhang FL, Takahata Y (2001) Inheritance of microspore embryogenic ability in Brassica crops. Theor Appl Genet 103: 254-258

Zhang GQ, He Y, Xu L, Tang GX, Zhou WJ (2006) Genetic analyses of agronomic and seed quality traits of doubled haploid population in *Brassica napus* through microspore culture. Euphytica 149: 169-177

Zhao JP, Newcomb W, Simmonds D (2003) Heat-shock proteins 70 kDa and 19 kDa are not required for induction of embryogenesis of *Brassica napus* L. cv. topas microspores. Plant Cell Physiol 44: 1417-1421

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5: 1411-1423

Żur I, Dubas E, Krzewska M, Janowiak F (2015) Current insights into hormonal regulation of microspore embryogenesis. Front Plant Sci 6