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Aspectos básicos y aplicados de la inducción de embriogénesis en microsporas de pimiento y colza

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“When I was 5 years old, my mother always told me that happiness was the key to life. When I went to school, they asked me what I wanted to be when I grew up. I wrote down ‘happy’. They told me I didn’t understand the assignment, and I told them they didn’t understand life.”

John Lennon

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Resumen

La embriogénesis derivada de microsporas es la ruta androgénica más empleada para la obtención de individuos dobles haploides (DHs), 100% homocigotos, que pueden ser utilizados como líneas puras. Este proceso es una alternativa biotecnológica a los programas clásicos de mejora que permite reducir el tiempo y los recursos necesarios para la obtención de híbridos comerciales. Gracias a los avances realizados en los últimos 30 años, hay determinadas especies que pueden ser consideradas modelo debido a la elevada eficiencia que presentan en la obtención de individuos DHs. Sin embargo, aun existen muchas especies, interesantes desde un punto de vista agrícola y comercial, que permanecen recalcitrantes a la embriogénesis de microsporas. Debido al elevado potencial biotecnológico de esta técnica es fundamental mejorar el proceso en este tipo de especies en las que la androgénesis no está puesta a punto. Para ello es esencial realizar estudios en los que se combine un enfoque más aplicado, tratando de conseguir las condiciones experimentales más adecuadas, y en paralelo, un enfoque más básico, orientado a investigar las bases de la reprogramación de las microsporas y de este modo, tener una mayor posibilidad de influir en el proceso. En esta Tesis se han empleado ambos enfoques, empleando la colza como especie modelo y el pimiento como especie recalcitrante a la androgénesis. En pimiento, el estudio de factores clave para la inducción de la androgénesis nos ha permitido optimizar un protocolo de cultivo de anteras aplicable a diferentes genotipos. Proponemos el uso combinado de la relación sépalo/yema (80-90% de la yema cubierta por los sépalos) junto con la pigmentación de las anteras (extremo apical morado) como marcadores morfológicos fiables y fáciles de medir para determinar qué yemas y anteras contienen las microsporas en su estadio idóneo para la inducción. También se ha demostrado que la presencia de callos de origen somático es más

dependiente de las condiciones de cultivo que del genotipo, lo cual nos ha permitido obtener un protocolo que reduzca la presencia de estos callos e incremente el número de embriones obtenidos.

En los estudios de investigación básica realizados con colza, el uso de la fijación por alta presión combinada con la criosustitución ha permitido observar cambios ultraestructurales en las microsporas inducidas nunca antes descritos. Se ha estudiado la arquitectura y composición de las paredes celulares que se forman *de novo* en las microsporas embriogénicas y se han observado paredes celulares incompletas y deformes que podrían estar favoreciendo fenómenos de fusión nuclear, que a su vez da lugar a la duplicación cromosómica típica de los DHs. Se ha comprobado que estas paredes presentan elevados niveles de calosa y ausencia de celulosa. También se ha estudiado la arquitectura de diferentes orgánulos presentes en las microsporas recién inducidas y se ha demostrado que los plastidios presentes en las microsporas embriogénicas actúan como plastolisomas. Estos estudios ultraestructurales han servido para extraer dos nuevos marcadores de embriogénesis: la presencia de calosa en las nuevas paredes y en las placas celulares en formación y plastolisomas que posiblemente actúen como parte de un mecanismo más general de limpieza del citoplasma, en paralelo a la reprogramación.

Abstract

Microspore-derived embryogenesis is the most common androgenic route to obtain doubled haploid (DH) individuals, 100% homozygous, which can be used as pure lines. This process is a biotechnological alternative to the classic breeding programs that allows for a reduction in the time and the resources needed to produce commercial hybrids. Due to the advances made in the last 30 years, some species can be considered as model organisms because of their high efficiency to produce DHs. However, there are still many species, interesting from a commercial and agronomical point of view, which remain recalcitrant to androgenesis induction. The biotechnological potential of this technique makes it essential to improve this process in those species where androgenesis is not yet optimized. For this, it is necessary to combine both applied and basic approaches, trying on the one hand to find the best experimental conditions, and on the other hand, exploring the basis of the reprogramming of microspores, in order to increase the possibilities of influencing the process. In this Thesis we have used both approaches, using rapeseed as model species and pepper as a recalcitrant species. In pepper, the study of key factors for androgenesis induction allowed us to optimize an anther culture protocol that can be applied to different genotypes. We propose the combination of calyx/bud ratio (80-90% of the bud covered by sepals) together with anther pigmentation (anthers with purple apical end) as reliable and easy-to-measure morphological markers to determine which buds and anthers contain the microspores at their optimal stage to induce androgenesis. We also demonstrated that the presence of somatic calli is more dependent on the culture conditions than on the genotype. This, in turn, allowed us to obtain a protocol that reduces the presence of somatic calli and increases the number of embryos obtained.

In the basic research studies made with rapeseed, the use of high pressure freezing followed by cryosubstitution allowed us to observe the ultrastructural changes undergone by the induced microspores. We studied the architecture and composition of the newly-formed cell wall in embryogenic microspores. We observed that these cell walls were deformed and incomplete, which could contribute to the well known phenomenon of nuclear fusion, which in turn leads to genome doubling, typical of DHs. We proved that these cell walls presented high levels of callose and absence of cellulose. We also studied the architecture of different organelles present in just induced microspores. We showed that plastids of embryogenic microspores behave as autophagic plastids (plastolysomes). These ultrastructural changes allowed us to define two new androgenic markers: the presence of callose in the first newly-formed cell walls and developing cell plates, and the presence of plastolysomes probably acting as part of a cytoplasmic cleaning mechanism, in parallel to microspore reprogramming.

Resum

L'embriogènesi derivada de microspores és la ruta androgènica més utilitzada per a l'obtenció d'individus dobles haploides (DHs), 100% homozigots, que poden ser utilitzats com a línies pures. Aquest procés és una alternativa biotecnològica als programes clàssics de millora, que permet reduir el temps i els recursos necessaris per a l'obtenció d'híbrids comercials. Gràcies als avanços realitzats en els últims 30 anys, hi ha determinades espècies que poden ser considerades models, per l'elevada eficiència que presenten en l'obtenció d'individus DHs. No obstant això, encara hi ha moltes espècies, interessants des d'un punt de vista agrícola i comercial, que son recalcitrants a l'embriogènesi de microsporas. A causa de l'elevat potencial biotecnològic d'aquesta tècnica, és fonamental millorar el procés en aquest tipus d'espècies en les quals la androgènesi no està posada a punt. Per a això és essencial realitzar estudis en els quals es combine una aproximació més aplicada, tractant d'aconseguir les condicions experimentals més adequades, i en paral·lel, una aproximació més bàsica, orientada a investigar les bases de la reprogramació de les microspores i d'aquesta manera, tindre una major possibilitat d'influir en el procés. En aquesta Tesi s'han utilitzat aquestes dos aproximacions, emprant la colza com a espècie model i el pimentó com a espècie recalcitrant a la androgènesi. En pimentó, l'estudi de factors clau per a la inducció de la androgènesi ens ha permès optimitzar un protocol de cultiu d'anteres aplicable a diferents genotips. Proposem l'ús combinat de la relació sèpal/rovell (80-90% del rovell cobert pels sèpals) juntament amb la pigmentació de les anteres (extrem apical morat) com a marcador morfològic fiable i fàcil de mesurar per determinar quins rovells i anteres contenen les microspores al seu estadi idoni per a la inducció. També s'ha demostrat que la presència de calls d'origen somàtic és més dependent de les condicions de cultiu que del genotip, la qual cosa ens ha

permès obtindre un protocol que reduïsca la presència d'aquests calls i incremente el nombre d'embrions obtinguts.

En els estudis d'investigació bàsica realitzats amb colza, l'ús de la fixació per alta pressió combinada amb la criosustitució ha permès observar canvis ultraestructurals, mai abans descrits, en les microspores induïdes. S'ha estudiat l'arquitectura i composició de les parets cel·lulars que es formen *de novo* en les microspores embriogéniques i s'han observat parets cel·lulars incompletes i deformes que podrien estar afavorint fenòmens de fusió nuclear, que a la seua volta originen la duplicació cromosòmica típica dels DHs. S'ha comprovat que aquestes parets presenten elevats nivells de cal·losa i absència de cel·lulosa. També s'ha estudiat l'arquitectura de diferents orgànuls presents a les microspores recentment induïdes i s'ha demostrat que els plastidis presents en les microspores embriogèniques actuen com plastolisomes. Aquests estudis ultraestructurals han servit per extraure dos nous marcadors de embriogènesi: la presència de cal·losa a les noves parets i les plaques cel·lulars en formació, i la presencia de plastolisomes que possiblement actuen com a part d'un mecanisme més general de neteja del citoplasma, en paral·lel a la reprogramació.

Índice

Introducción

1. Los híbridos en la Mejora Vegetal

La producción de cultivares híbridos revolucionó en su día la industria de semillas y sigue siendo uno de los métodos más utilizados en Mejora Genética Vegetal (Martín, 2002). Un cultivar híbrido es el producido por el cruzamiento entre dos o más parentales totalmente homocigóticos, denominados *líneas puras*. Una adecuada selección de los parentales es fundamental para garantizar el máximo rendimiento y la máxima homogeneidad fenotípica. Los híbridos comerciales pueden obtenerse en todos los grupos vegetales, incluyendo las hortícolas, que debido a su alto valor comercial hacen que sea rentable obtener estos híbridos de manera convencional. En el mercado existen excelentes cultivares híbridos de maíz (los más antiguos), sorgo, cebolla, tomate, pimiento, berenjena y coles, entre otras muchas especies (Cubero, 2003).

1.1. Importancia de los híbridos

El uso de semilla híbrida ofrece dos ventajas importantes para el agricultor: una elevada homogeneidad y un alto rendimiento en parcela. Estas características se van manteniendo a lo largo de las distintas generaciones debido al cruce de los mismos parentales cada generación. Debido a que el híbrido (F_1) es heterocigoto (al menos para los caracteres de interés comercial), su descendencia (F_2) segregará y generará una variabilidad de caracteres indeseable. Esto supone una clara ventaja para las empresas productoras de semilla ya que el agricultor se ve en la necesidad de comprar la semilla cada año (Cubero, 2003). No obstante, el elevado coste de la semilla híbrida es

compensado por un claro incremento en la producción, la homogeneidad en la parcela y la garantía de que el agricultor estará adquiriendo un producto con las mismas características a lo largo de los años.

1.2. Obtención convencional de los híbridos

La obtención de cultivares híbridos pasa por producir previamente unos parentales adecuados que formarán parte del híbrido. Los pasos a seguir para el desarrollo de un nuevo híbrido son: obtención y evaluación de los parentales, mantenimiento de estos parentales garantizando su identidad a lo largo del tiempo y la producción de la semilla híbrida comercial.

Los híbridos se obtienen mediante el cruce de dos, tres o cuatro líneas puras. La obtención de líneas puras con un alto grado de homogeneidad en todos los caracteres, requiere de 7 a 9 generaciones de autofecundación y selección de los caracteres deseados (Cubero, 2003). La identificación de combinaciones de parentales que produzcan híbridos con un rendimiento superior es el paso más importante en el desarrollo de los mismos y se realiza mediante lo que se conoce como *pruebas de aptitud combinatoria*. En estas pruebas, es necesario cruzar las líneas de mejora de las que se dispone y seleccionar las dos líneas puras concretas que produzcan el híbrido con mayor rendimiento. Una vez seleccionadas las mejores líneas puras parentales, estas se cruzan para obtener la semilla híbrida que las empresas de semillas venden a los agricultores.

Este método de obtención de semilla híbrida lleva utilizándose durante décadas por las casas comerciales y presenta la gran ventaja de que no es dependiente del genotipo, ya que funciona en todos los grupos vegetales. Sin

embargo, la obtención convencional de semilla híbrida presenta una importante limitación que reside en el elevado gasto en tiempo y recursos que supone lanzar un nuevo híbrido al mercado. Es fácil intuir que las 7-9 generaciones de autofecundación y selección de las líneas suponen un proceso largo, que requiere una gran inversión en espacio y tiempo. Además, las líneas puras que se obtienen mediante la mejora convencional no llegan a ser 100% homocigotas, ya que los mejoradores realizan rondas de autofecundación hasta alcanzar un grado de homocigosis en torno al 99%. Afortunadamente, existen técnicas biotecnológicas, como la obtención de dobles haploides, que proporcionan una alternativa al método convencional de obtención se semilla híbrida.

2. Haploides y dobles haploides: Importancia y utilidad en Mejora

Los haploides son individuos con la mitad de cromosomas que el número básico de la especie, es decir, presentan el mismo número de cromosomas que el gameto. Debido a que los cromosomas no tienen sus homólogos, no se llega a producir un apareamiento absoluto y no pueden realizar la meiosis. Este hecho les convierte en individuos estériles, cuyo genoma ha de ser duplicado para hacerlos fértiles. Cuando un haploide duplica su carga cromosómica, espontánea o artificialmente, se genera un organismo doble haploide (DH), que es viable, fértil y totalmente homocigoto para todos sus *loci* (Cubero, 2003).

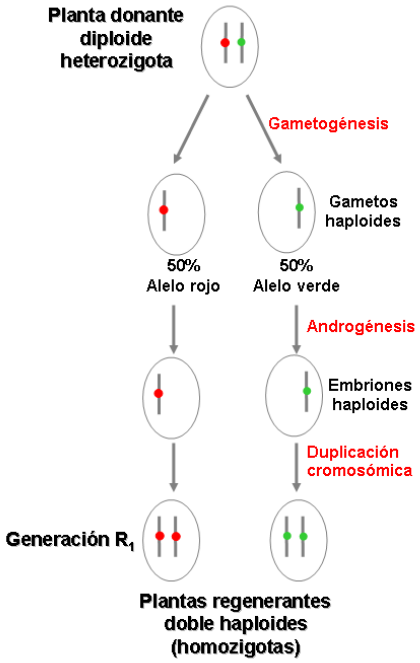

Figura 1. Esquema simplificado de la obtención de individuos DHs mediante androgénesis (Seguí-Simarro, 2010b)

La principal utilidad de los dobles haploides (DHs) es que pueden ser usados como líneas puras para la obtención de híbridos, lo cual supone una gran ventaja frente a los métodos de mejora clásica (largos y costosos), pues logran reducir el proceso de obtención de líneas puras a tan solo una generación y con un grado de homocigosis del 100% (Seguí-Simarro, 2010a). Además, la selección de plantas individuales en generaciones tempranas es más eficaz, ya que se facilita la fijación de alelos recesivos beneficiosos y la selección de caracteres cuantitativos, principalmente debido a la eliminación de los efectos de la dominancia y la segregación dentro de las familias (Snape, 1989).

2.1. Otras utilidades de los DHs

Además del uso como líneas puras para la producción de híbridos, los individuos DHs pueden ser utilizados en otros ámbitos de investigación:

- Elaboración de mapas genéticos: Debido al carácter homocigoto de los DHs, caracteres heredables cuantitativos de gran interés económico como son el rendimiento y la calidad, pueden ser mapeados genéticamente con mayor velocidad y precisión (Seguí-Simarro, 2010a). Los DHs han tenido una gran importancia en el establecimiento de mapas genéticos en un amplio rango de especies, como son cebada (*Hordeum vulgare*), arroz (*Oryza sativa*), colza (*Brassica napus*) y trigo (*Triticum aestivum*) (Forster et al., 2007).

- Asociación de marcadores moleculares y caracteres fenotípicos: Los DHs juegan un papel importante en genómica, pudiendo agilizar y simplificar las asociaciones entre carácter observable y marcador. Esto permite a los mejoradores evaluar líneas DHs con mayor velocidad que otras líneas con menor grado de homocigosis (Wedzony et al., 2009).

- Transgénesis: El uso de transgénesis combinado con la obtención de DHs ofrece la posibilidad de transformar millones de microsporas que provienen de la misma planta y obtener cientos de eventos de transformación distintos. Aunque probablemente, la principal ventaja es que, como las microsporas son haploides, cuando se obtenga el individuo DH por duplicación del genoma, el transgén también estará duplicado (Goedeke et al., 2007).

- Selección y detección de mutaciones recesivas: El procedimiento consiste en aplicar primero los tratamientos mutagénicos para, posteriormente, obtener los individuos DHs. De este modo se crea una población de líneas mutantes homocigotas en las que los caracteres

determinados por genes recesivos pueden ser fácilmente identificados (Dunwell, 2010; Seguí-Simarro, 2010a).

- Mejora inversa o "Reverse Breeding": El "Reverse Breeding" es una técnica novedosa en el ámbito de la mejora genética vegetal mediante la cual, partiendo de un individuo heterocigoto, se pueden obtener las líneas parentales de las que procede. Esta técnica se basa en bloquear la recombinación en el heterocigoto seleccionado, bloqueando genes esenciales para el entrecruzamiento de los cromosomas homólogos durante la meiosis. Los gametos obtenidos de estas plantas, que contienen combinaciones de los cromosomas parentales no recombinados, son cultivados *in vitro* para obtener plantas DHs. De esos DHs, se pueden seleccionar los parentales complementarios y usarlos para reconstruir el heterocigoto (Dirks et al., 2009). Debido al papel crucial de los DHs, esta técnica solo puede llevarse a cabo en aquellas especies en las que la tecnología de los DHs está puesta a punto.

2.2. Métodos de obtención de los DHs

Los DHs pueden ser obtenidos a partir del gametofito masculino o del femenino (Dunwell, 2010). En algunas especies monocotiledóneas y en patata, ambas rutas pueden ser empleadas. Sin embargo, en la mayoría de las especies dicotiledóneas la elección se limita a una de las dos vías (Wedzony et al., 2009). Los diferentes procedimientos para conseguir la obtención de individuos DHs son los siguientes:

- Hibridación interespecífica: Técnica en la que se provoca la polinización entre especies sexualmente incompatibles. Existen dos variantes en esta técnica: el método *bulbosum* y la hibridación "lejana" (*wide*

hybridization). Con el método *bulbosum* se generan barreras de incompatibilidad postzigótica tras la fecundación que eliminan progresivamente los cromosomas del parental masculino, con el resultado de un embrión con el fondo genético del parental femenino. En la actualidad, esta técnica es empleada en cebada, trigo, triticale, centeno y avena (Forster et al., 2007; Dunwell, 2010). En la *wide hybridization* el polen germina pero no fecunda a la célula huevo. En su lugar, las dos espermátidas fecundan a los núcleos polares (Tai 2005). Se forma un endospermo que además de proliferar con normalidad, induce y promueve el desarrollo de un embrión haploide cuyo fondo genético proviene exclusivamente del parental femenino (Seguí-Simarro, 2010b). En el caso de la patata, por ejemplo, gracias a la *wide hybridization* se pueden superar las barreras de incompatibilidad provocadas, entre otros factores, por el endospermo (Rokka 2009). Este método ha sido utilizado con éxito además de en patata, en especies como tabaco, pera o sorgo (Dunwell, 2010).

- Ginogénesis: Técnica empleada en la regeneración de un individuo haploide a partir de algunas de las células del gametofito femenino, incluyendo la célula huevo no fecundada. En este último caso, tan solo sería necesario activar la ruta embriogénica. En el caso de otras células haploides del saco embrionario, sería además necesario reprogramarlas previamente. La técnica consiste en cultivar in vitro óvulos, ovarios o incluso flores completas inmaduras y no polinizadas, hasta completarse la maduración in vitro del saco embrionario, donde se desarrolla un embrión haploide. Esta ruta es empleada mayoritariamente en especies en las que no se obtienen resultados suficientemente buenos mediante otras técnicas de obtención de DHs. La especie modelo para el estudio de esta técnica es la cebolla, aunque también se

utiliza en otras especies de elevada importancia económica como la remolacha azucarera, el pepino, el girasol y en algunos frutales (Germanà, 2006; Froelicher et al., 2007; Seguí-Simarro, 2010b).

- Eliminación del genoma uniparental inducida por cambios en la histona CENH3: Esta técnica ha sido descubierta recientemente por Ravi y Chan (2010) en *Arabidopsis thaliana*. Está basada en la creación de un individuo transformado con una versión alterada de la histona centromérica CENH3, sobre un fondo genético mutante knock out para dicha histona. Al cruzar un genotipo silvestre con el genotipo transformado tiene lugar una mitosis defectuosa. El centro de unión del centrómero de los cromosomas del mutante no es reconocido por los microtúbulos del huso. Estos cromosomas no migran hacia los polos de la célula y son eliminados, originando que parte de la descendencia que se genere sea haploide. Este método tiene un gran potencial debido a que la CENH3 es una histona universal en eucariotas, y en principio podría ser aplicado a la producción de haploides en cualquier especie. Sin embargo, dicha aplicación no parece fácil en muchos cultivos de interés agronómico, genéticamente más complejos que Arabidopsis.

- Androgénesis: Está técnica ha sido la más profundamente estudiada y la que ha demostrado ser más efectiva en la obtención de DHs (Wedzony et al., 2009). Debido a su gran importancia y a que es la técnica en la que se basa la presente Tesis Doctoral, se explica con mayor detalle en el apartado siguiente.

3. Androgénesis

La androgénesis es el conjunto de vías que dan lugar a un individuo cuyo fondo genético proviene exclusivamente de un núcleo de origen

masculino. Esta técnica está basada en el desvío o reprogramación de la ruta normal de desarrollo del gametofito masculino (microesporogénesis y microgametogénesis) hacia una nueva vía de desarrollo esporofítico (Seguí-Simarro, 2010a). Para entender los fundamentos de esta técnica, es necesario conocer previamente el proceso de formación de dicho gametofito en condiciones de desarrollo normal de la planta.

3.1. Microsporogénesis y microgametogénesis

El desarrollo del gametofito masculino consta de dos fases: microsporogénesis y microgametogénesis (Figura 2, ruta 0). La microsporogénesis sucede en primer lugar y consiste en la formación y el posterior desarrollo de la microspora (Seguí-Simarro, 2010b). El proceso comienza cuando la célula madre de las microsporas sufre el proceso de meiosis, dando como resultado una tétrada de microsporas haploides. Inicialmente las cuatro microsporas haploides están unidas por una pared de calosa, (1-3) β-glucano. Esta pared es degradada por acción de la enzima (1-3) β-glucanasa que es producida por el tapete (Reynolds, 1997; Seguí-Simarro, 2010b). Al disolverse la pared de calosa, las microsporas jóvenes son liberadas manteniendo una morfología tetraédrica. Presentan una fina y poco definida pared celular y el núcleo centrado. Al continuar el desarrollo, las microsporas mantienen la posición del núcleo y una pared de exina más gruesa y con forma poligonal característica de la fase de microspora media. Las microsporas en su estadio de desarrollo más tardío se caracterizan por tener una pared de exina bien desarrollada y trilobulada. El núcleo está desplazado hacia un lado debido

a la presencia de una gran vacuola, característica de esta etapa (microspora vacuolada).

En el momento que tiene lugar la primera mitosis comienza la segunda fase, la microgametogénesis. Esta etapa consiste en la formación y desarrollo del grano de polen. La primera mitosis también es denominada división asimétrica, debido a la formación de las células vegetativa y generativa. Tras la primera división mitótica, el grano de polen en su estadio joven incrementa su tamaño en relación al de la microspora, mantiene la gran vacuola que ocupa gran parte del grano y adquiere de nuevo una morfología redondeada. En el estadio de polen medio, la célula vegetativa, de mayor tamaño, migra al centro del grano de polen y la generativa, con forma alargada y de menor tamaño, está incluida dentro del citoplasma de la anterior y permanece junto a la pared del grano de polen. A su vez, la vacuola va desapareciendo gradualmente y al mismo tiempo se van acumulando granos de almidón en el citoplasma (Seguí-Simarro y Nuez, 2005; Seguí-Simarro, 2010b). La célula vegetativa es la encargada de dirigir el desarrollo restante del grano de polen y de la síntesis de la maquinaria necesaria para la formación del tubo polínico. Al contrario que la célula vegetativa, que es biosintéticamente muy activa, la célula generativa permanece inactiva hasta el estadio de polen maduro. Es en este momento cuando entra en ciclo celular y tiene lugar la segunda mitosis del polen. De esta división mitótica se originan las dos células espermátidas o gametos masculinos. En la mayoría de las especies, entre las que se incluye el pimiento o el tabaco, las espermátidas se originan durante la emisión del tubo polínico. En otras especies, como maíz, gramíneas o colza, la segunda mitosis del polen ocurre antes de la dehiscencia de la antera, dando lugar a un grano de polen maduro tricelular. El estadio de polen maduro se caracteriza

fundamentalmente por el aumento de tamaño y la forma ovalada, además de una gran acumulación en el citoplasma de granos de almidón que le dan un aspecto denso (Seguí-Simarro, 2010b).

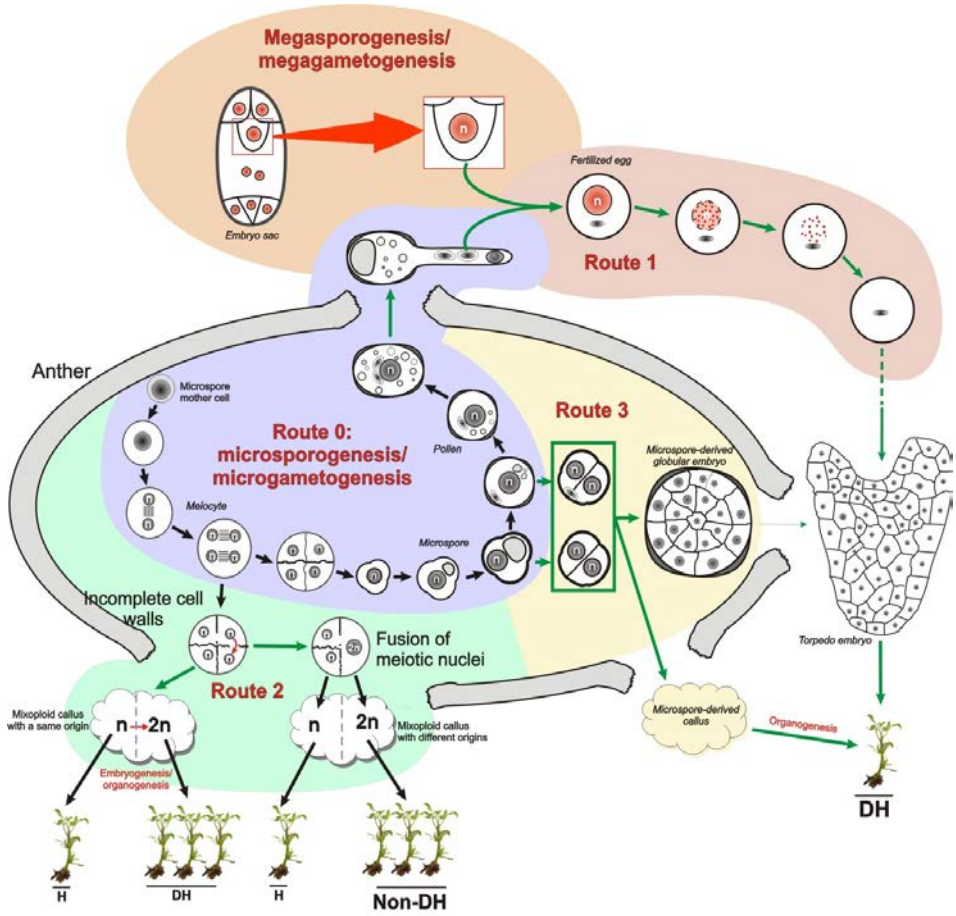

Figura 2. Desarrollo del gametofito masculino (Ruta 0: microsporogénesis y microgametogénesis) y las diferentes rutas de desarrollo androgénico (Rutas 1, 2 y 3). Imagen de Seguí-Simarro (2010b).

3.2. Rutas androgénicas

La base del proceso de androgénesis deriva de la totipotencialidad de las células. Este rasgo permite que una célula diferenciada de cualquier órgano o tejido se desdiferencie y sea capaz de diferenciarse de nuevo potencialmente en cualquier otro tipo celular del organismo vegetal. A partir de los procesos vistos en el apartado anterior (3.1. Microsporogénesis y microgametogénesis) que llevan a cabo la formación del grano de polen y los gametos masculinos, existen tres rutas androgénicas que pueden originar individuos haploides o DHs (Seguí-Simarro, 2010a):

3.2.1. Desarrollo de un embrión haploide dentro del saco embrionario

Tras la fecundación, el embrión se origina a partir de una célula huevo o zigoto donde el núcleo femenino se ha, de algún modo, inactivado o eliminado (Figura2, ruta 1). De esta forma, el fondo genético proviene tan sólo del gametofito masculino. Se trata de un fenómeno natural descrito por primera vez en 1929 (Clausen y Lammerts, 1929; Kostoff, 1929). La aplicación práctica de este fenómeno natural es nula, debido a la frecuencia extremadamente baja con la que tiene lugar este proceso (Seguí-Simarro, 2010a). En maíz, que es una de las especies donde más se ha observado y estudiado este fenómeno, la máxima incidencia observada ha sido de un individuo haploide por cada 80.000 individuos utilizados (Chase, 1969), siendo de un haploide por cada 2.500 en el caso del tabaco (Burk, 1962). Por estos motivos, esta posibilidad se descarta desde un punto de vista biotecnológico.

3.2.2. Callogénesis derivada del meiocito

Esta ruta está basada en la obtención de callos haploides a partir de meiocitos (Figura 2, ruta 2). A partir de los callos obtenidos se pueden originar plantas haploides o DHs mediante organogénesis o embriogénesis indirecta (Seguí-Simarro, 2010a). Esta ruta, al igual que la anterior, es muy poco frecuente y apenas se ha estudiado, debido entre otras cosas, a que se origina a partir de células difíciles de utilizar en cultivo *in vitro*, los meiocitos. Este método fue empleado en especies como *Arabidopsis thaliana* (Gresshoff y Doy, 1972), *Vitis vinifera* (Gresshoff y Doy, 1974) y *Digitalis purpurea* (Corduan y Spix, 1975), aunque estos estudios nunca fueron continuados. La especie en la que más se ha estudiado esta ruta ha sido el tomate (*Solanum lycopersicum)*. A pesar de conseguir la regeneración de plantas completas, la eficiencia fue muy baja y los regenerantes presentaron un elevado porcentaje de mixoploidía (Shtereva y Atanassova, 2001; Zagorska et al., 2004; Seguí-Simarro y Nuez, 2007; Corral-Martínez et al., 2011). Pese a la importancia de esta especie, los resultados obtenidos hasta ahora son insuficientes para ser trasladados a las empresas productoras de semilla híbrida de tomate.

3.2.3. Embriogénesis derivada de microsporas

Esta ruta fue empleada por primera vez en *Datura inoxia*, consiguiendo la regeneración de plantas haploides mediante el cultivo de anteras (Guha y Maheshwari, 1964). Después de este estudio, han sido numerosas las especies en las que se ha usado esta técnica llegando a ser la más explotada por producir DHs en un corto periodo de tiempo y con un rendimiento aceptable (Seguí-Simarro, 2010a). Esta técnica consiste en la reprogramación y desvío de

los precursores del grano de polen, las microsporas, desde su ruta original de desarrollo gametofítico hacia una nueva ruta de desarrollo embriogénico (Figura 2, ruta 3) (Seguí-Simarro y Nuez, 2008; Seguí-Simarro, 2010a). Algunos autores proponen que este cambio en el programa de desarrollo podría ser una vía de escape ante la imposibilidad de proseguir con su ruta original debido al tratamiento de estrés aplicado (Shariatpanahi et al., 2006). Tras la exposición al tratamiento de inducción, las microsporas se desvían de su ruta gametofítica original (Figura 3, ruta azul) hacia las siguientes direcciones (Seguí-Simarro y Nuez, 2008):

- Muchas microsporas a pesar de ser inducidas, detienen su crecimiento y/o se mueren. Algunas de ellas, siguen un desarrollo similar a la maduración *in vivo* del polen, comúnmente denominado *pollen-like*, para finalmente detener su crecimiento y morir (Figura 3, ruta rosa).
- Algunas de las microsporas que han sido inducidas, en función del genotipo, generan estructuras multicelulares, tipo callos haploides que bajo condiciones adecuadas pueden originar plantas haploides o DHs a través de un proceso de organogénesis (Figura 3, ruta verde).
- Otras microsporas, en este caso, correctamente inducidas, sufren una serie de cambios que acaban dando lugar a la formación de un embrión que, tras germinar, dará lugar al individuo correspondiente haploide o DH (Figura 3, ruta amarilla).

Los cambios más importantes que sufren las microsporas cuando son correctamente inducidas (Figura 3, ruta amarilla) son los siguientes: Aumento de tamaño de las células, recolocación del núcleo en el centro de la microspora y disgregación de la vacuola en pequeños fragmentos. Todo ello genera en las

microsporas inducidas una morfología típica, denominada tipo estrella. Se le ha dado ese nombre porque el núcleo permanece en el centro de la célula rodeado de canales citoplasmáticos que se asemejan a los brazos de una estrella. Hasta la fecha, también se había asumido como claro marcador de inducción en las microsporas, la formación de un nuevo plano de división simétrico (Kim et al., 2004; Shariatpanahi et al., 2006; Seguí-Simarro y Nuez, 2008). Sin embargo, recientes estudios han demostrado que la división simétrica no es un marcador exclusivo de la embriogénesis de microsporas, puesto que modelos de división irregular o asimétricos, así como la ausencia de suspensor en los embriones, pueden promover también el desarrollo y diferenciación de embriones derivados de microsporas (Soriano et al., 2014).

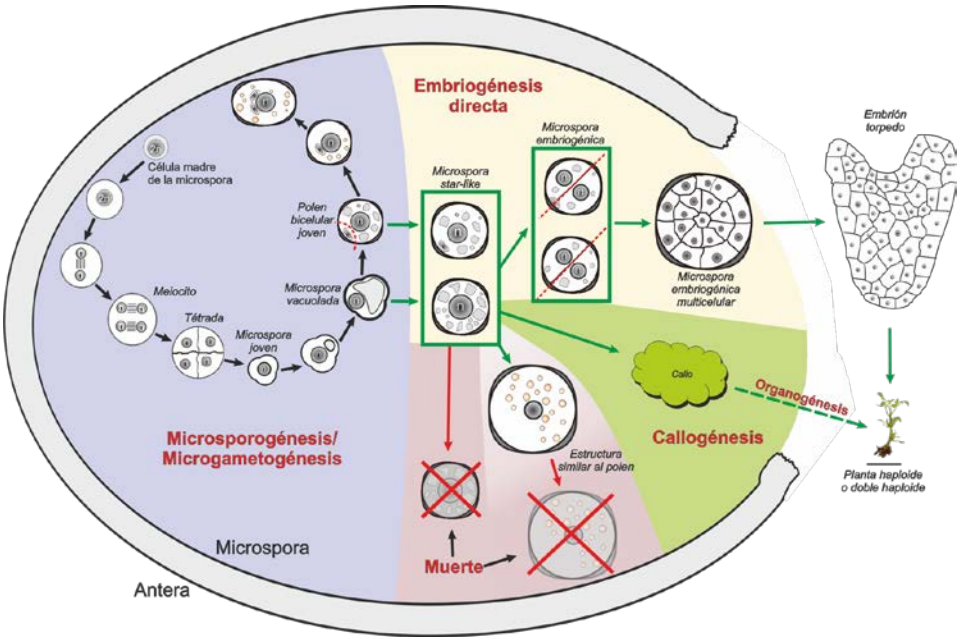

Figura 3. Esquema de la inducción de androgénesis a partir de microsporas/polen. Adaptado de Segui-Simarro y Nuez 2008.

Los DHs derivados de microsporas abren una nueva dimensión para la producción de líneas homocigotas debido a la gran cantidad de microsporas que se producen en una sola antera (Maraschin et al., 2005). Se han desarrollado protocolos para la producción de DHs mediante embriogénesis de microsporas en más de 250 especies, entre las que se incluyen herbáceas como trigo, cebada, arroz, colza, tabaco o maíz (Maluszynski et al., 2003), y leñosas como mandarino, naranjo amargo o alcornoque, entre otras (Germanà, 2006). Las leñosas suponen menos del 10% de las especies en las que se ha desarrollado esta técnica (Srivastava y Chaturvedi, 2008). La eficiencia de obtención de DHs es muy variable en las distintas especies en las que se han establecido estos protocolos, llegando algunas de ellas a considerarse especies modelo debido a su excelente respuesta androgénica. Este es el caso de colza (*Brassica napus*), tabaco (*Nicotiana tabacum*), trigo (*Triticum aestivum*) y cebada (*Hordeum vulgare*) (Seguí-Simarro, 2010a).

3.3. Ventajas de la androgénesis frente a otras técnicas de producción de DHs

De los cuatro métodos de obtención de DHs, la androgénesis, y más concretamente, la embriogénesis derivada de microsporas, es el método más eficaz y el que más se ha utilizado hasta la fecha, debido a las múltiples ventajas que presenta frente a las otras técnicas de producción de DHs:

- Puesta a punto en un elevado número de especies. En comparación con el resto de técnicas de producción de DHs, la androgénesis presenta protocolos optimizados en un mayor número de especies.

- Elevada eficiencia en la obtención de DHs. La gran cantidad de microsporas/polen generadas por una única yema permite obtener un elevado número de individuos por cada cultivo en comparación con los otros métodos de obtención de DHs.

- Elevado porcentaje de individuos que duplican espontáneamente su material cromosómico. Debido al alto número de individuos DHs espontáneos, en ocasiones puede no ser necesario duplicar el genoma haploide. Otras técnicas, como la hibridación interespecífica, siempre requieren la aplicación de colchicina para duplicar el genoma.

Todas estas ventajas demuestran la gran aportación que la androgénesis puede realizar en el campo de la mejora genética vegetal, por lo que resulta de vital importancia la optimización de protocolos para poder usar esta técnica en el mayor número de especies posible. Solo en el caso de que la especie con la que se trabaje no responda a la androgénesis, es decir, sea recalcitrante a este método, se hará necesario el estudio de vías alternativas a la androgénica para obtener DHs.

3.4. Factores que afectan a la inducción de la embriogénesis de microsporas

Hay muchos factores que influyen en la respuesta de las microsporas a la embriogénesis. Estos factores pueden ser endógenos y exógenos (Seguí-Simarro y Nuez, 2008). Los más relevantes que influyen en la inducción de la androgénesis se pueden agrupar en las siguientes 3 categorías:

3.4.1. Factores relativos a la planta donante

Los resultados obtenidos mediante embriogénesis de microsporas son altamente dependientes de los genotipos utilizados, así como de las condiciones de crecimiento y calidad de las plantas donantes (Wang et al., 2000). Entre estos factores, el genotipo es, con diferencia, el más influyente. El papel crucial del genotipo en la respuesta a la androgénesis quedó claramente demostrado cuando en *Brassica oleracea* var. *capitata* se comprobó que el potencial androgénico se transmitía a la descendencia (Rudolf et al., 1999). Algunos cultivares de especies como la colza (*Brassica napus*), el tabaco (*Nicotiana tabacum*), la cebada (*Hordeum vulgare*) o el trigo (*Triticum aestivum*) presentan una respuesta tan elevada que pueden ser consideradas como sistemas modelo. Sin embargo, otras especies económica y científicamente interesantes como Arabidopsis o tomate, aun permanecen extremadamente recalcitrantes a la inducción de embriones haploides (Seguí-Simarro, 2010a). Aparte de las diferencias entre especies, también se dan diferentes respuestas en cultivares dentro de una especie, así como individuos dentro de un mismo cultivar (Seguí-Simarro y Nuez, 2008). En el caso concreto de colza, hay cultivares de alta respuesta (cultivar 'Topas') y de baja respuesta (cultivar 'Westar') (Seguí-Simarro, 2010a). Por todo ello, a pesar de los enormes esfuerzos realizados hasta la fecha, no existe un protocolo universal efectivo para la obtención de DHs, ya que el éxito de estos protocolos es altamente dependiente del genotipo (Wedzony et al., 2009).

Con respecto al resto de condiciones de la planta donante, las condiciones naturales óptimas de cada especie suelen ser las mejores (intensidad de luz, duración de los días, régimen de temperaturas, humedad,

etc.). Cualquier infección o estrés causado a las plantas, conllevará una disminución en la eficiencia de la androgénesis. La edad del material vegetal puede influir en la eficiencia de regeneración. Por ejemplo, en cebada, las 5 primeras espigas muestran entre un 15 y un 20% más de eficiencia en la regeneración que las últimas espigas (Wang et al., 2000).

3.4.2. Factores relativos a la microspora

El estadio de desarrollo de las microsporas es otro de los factores cruciales en el proceso de cultivo, debido a que pequeñas diferencias en su desarrollo, provocan grandes diferencias en el rendimiento del proceso (Dunwell, 2010). Como se ha citado anteriormente, para la mayoría de las especies, la etapa en la que la microspora tiene mayor sensibilidad a los tratamientos de inducción es en la transición de microspora vacuolada a polen bicelular joven, es decir, en torno a la primera mitosis del polen (González-Melendi et al., 1996; Maraschin et al., 2005; Seguí-Simarro y Nuez, 2008). Esto puede ser debido al hecho de que ambos estadios se encuentran en un estado de proliferación y no diferenciación completa, al contrario que el polen maduro donde ya está activado un programa específico para la maduración del polen (Dunwell, 2010).

3.4.3. Factores relativos al cultivo in vitro

En el momento que las microsporas están en el estadio adecuado, deben ser sometidas a un conjunto de factores físico-químicos (agentes estresantes) que desencadenarán su reprogramación hacia embriogénesis (Shariatpanahi et al., 2006). De hecho, la necesidad de aplicar un estrés a los cultivos es lo único que tienen en común todos los protocolos de inducción de

las distintas especies estudiadas. Los métodos más comúnmente empleados como tratamiento inductor son:

- Calor: Los tratamientos de choque térmico con calor se han utilizado para inducir la embriogénesis en microsporas de colza, trigo, tabaco y berenjena entre otras especies (Shariatpanahi et al., 2006). Se ha visto que el tratamiento con calor influye en procesos del ciclo celular (Seguí-Simarro y Nuez, 2008), generando cambios estructurales en las microsporas, concretamente en los microtubulos y el citoesqueleto (Shariatpanahi et al., 2006), sintetizando proteínas de choque térmico (HSP) (Seguí-Simarro et al., 2003) y produciendo cambios en la regulación de la actividad quinasa (Seguí-Simarro et al., 2005).
- Reducción de la fuente de carbono y/o nitrógeno: El ayuno inducido ha sido utilizado con éxito en especies como tabaco, trigo, arroz, cebada y manzano. Se han observado cambios a nivel citoplásmico y nuclear, como la desdiferenciación de plastidios, cambios en la cromatina y estructura nuclear o disminución en el tamaño del nucleolo entre otros (Shariatpanahi et al., 2006).
- Frío: Se han empleado tratamientos de inducción con bajas temperaturas (alrededor de 4 °C) sobre plantas completas, espigas cortadas o yemas florales para inducir la embriogénesis de microsporas. Los periodos de tiempo son variables, dependiendo de la especie. Las especies en las que se ha empleado con éxito un tratamiento inductor en frío son, entre otras, cebada, arroz, trigo, avena, triticale, clementina y lino (Shariatpanahi et al., 2006). Se ha visto que los tratamientos con frío mejoran la viabilidad de las

microsporas y se asegura una mayor supervivencia del polen. El ayuno inducido también se ve favorecido, debido a que las microsporas desconectan del tapete. También se ha visto que incrementa el contenido total de aminoácidos libres y se favorece la síntesis de HSP. Además, en trigo duro se ha visto que los tratamientos con frío pueden incrementar el número de plantas verdes obtenidas, probablemente debido al retraso en la senescencia de los plastidios (Shariatpanahi et al., 2006).

- Aplicación de colchicina: La colchicina, además de ser utilizada en los tratamientos de duplicación del genoma, es utilizada a bajas concentraciones como tratamiento estresante. Se ha utilizado para la inducción de androgénesis en colza, maíz y café (Shariatpanahi et al., 2006).

Además de estos tratamientos, también se han utilizado otros como aplicación de etanol, radiaciones, centrifugación, ácido abscísico, agentes feminizantes… etc. (Shariatpanahi et al., 2006). Estos tratamientos pueden ser aplicados individualmente durante la inducción, o mediante una combinación de varios (Kim et al., 2008; Seguí-Simarro, 2010a).

La composición del medio en el que se cultivan las microsporas es otro factor que influye en la respuesta celular a la androgénesis. Como ejemplo de factores potenciadores del proceso podríamos citar el uso de proteínas de arabinogalactanos, o de reguladores de crecimiento para conseguir un correcto desarrollo embrionario, similar al del embrión zigótico (Letarte et al., 2006). Aunque cabe destacar que la presencia de fitohormonas en el medio de cultivo no parece ser un requerimiento esencial, pues todos los sistemas considerados

modelos para el estudio de la androgénesis, se caracterizan por no incluir fitohormonas en la composición del medio (Custers, 2003; Hayes et al., 2003; Kasha et al., 2003; Touraev y Heberle-Bors, 2003).

3.5. Métodos de inducción de la embriogénesis de microsporas

La obtención de individuos DHs mediante la inducción de la embriogénesis de microsporas puede llevarse a cabo mediante el uso de dos métodos: el cultivo de anteras y el cultivo de microsporas aisladas.

3.5.1. Cultivo de anteras

Es el método más utilizado para la producción de DHs en plantas cultivadas y está puesto a punto en un mayor número de especies que el cultivo de microsporas aisladas. Fue puesto en práctica por primera vez en la década de los sesenta (Guha y Maheshwari, 1964). Este método se basa en extraer las anteras de las yemas florales y cultivarlas *in vitro* directamente sobre un medio sólido o semisólido, permaneciendo las microsporas dentro del saco polínico. Este método es técnicamente más sencillo que el cultivo de microsporas, siendo el medio de cultivo menos complejo debido a la presencia del tejido de la antera, que aporta elementos necesarios para el metabolismo de las microsporas y permite una mejor inducción embriogénica (Seguí-Simarro y Nuez, 2008). Las plantas haploides regeneradas pueden provenir además de embriones, de callos androgénicos, aunque es conveniente conseguir una embriogénesis directa, para acelerar y abordar el proceso, y para eliminar la fase de crecimiento desdiferenciado (callo) que puede promover la variación gametoclonal en los regenerantes (Forster et al., 2007). Entre sus desventajas

cabe destacar la falta de control sobre lo que ocurre exactamente en la antera, pues resulta difícil discernir entre la aportación del medio de cultivo y la contribución de la antera sobre la nutrición de la microspora. Además, esta técnica tiene una menor eficiencia en la obtención de DHs en comparación con el cultivo de microsporas y la mayoría de individuos obtenidos son haploides, por lo que es necesario aplicar tratamientos de duplicación artificial del genoma (Forster et al., 2007). Otro inconveniente del cultivo de anteras es la posibilidad de que aparezcan callos somáticos que proceden del tejido conectivo de la pared de la antera, por lo que resulta necesario analizar el origen del callo.

3.5.2. Cultivo de microsporas

Esta técnica está basada en cultivar las microsporas aisladas en medio líquido. Este método es más complejo que el del cultivo de anteras y más exigente, técnicamente hablando, pues requiere de técnicas adicionales como el filtrado o el centrifugado de las microsporas. Este método se aplica a un rango limitado de especies. Sin embargo, en aquellas especies donde ha sido puesto a punto, es el método por excelencia debido a las ventajas que presenta en comparación con el cultivo de anteras. Con el cultivo de microsporas se evita la formación de callos y embriones a partir del tejido somático de la antera y por lo tanto regenerantes somáticos diploides. Al no estar presente el tejido de la antera, se evita el efecto excretor incontrolado del tapete, teniendo un mayor control de las condiciones del medio (Kim et al., 2008). Permite que el efecto del medio de cultivo sobre las microsporas sea más directo y rápido debido a que los componentes no deben atravesar el tejido de la antera. La eficiencia de obtención de DHs suele ser mayor, ya que se ha visto que en

cultivos de alta densidad de microsporas pueden obtenerse más de 1.000 embriones por mililitro de medio. Esta ventaja hace que el cultivo de microsporas sea un buen sistema experimental para estudiar la embriogénesis debido a la elevada cantidad de embriones que se producen en diferentes etapas de su desarrollo (Forster et al., 2007).

A un nivel más básico, el cultivo de microsporas ha proporcionado una gran cantidad de información sobre los mecanismos que rigen la inducción de embriogénesis de microsporas, los tipos de estreses útiles para activar la vía embriogénica, los cambios que tienen lugar en la expresión génica durante la androgénesis, el aislamiento de diversos genes implicados en la reprogramación de las microsporas hacia la vía esporofítica, etc. (Revisado en Maraschin et al., 2005; Pauls et al., 2006; Forster et al., 2007; Seguí-Simarro y Nuez, 2008; Dunwell, 2010; Soriano et al., 2013).

Una variante del cultivo de microsporas aisladas es el método de liberación de microsporas, denominado "shed-microspore culture". Este método presenta características intermedias entre el cultivo de anteras y el cultivo de microsporas. Las anteras son colocadas en un medio bifásico, formado por medio de cultivo sólido al que se le añade carbón activado, cubierto por medio de cultivo líquido. Las anteras flotan en el medio bifásico y son estimuladas para liberar sus microsporas (Dolcet-Sanjuan et al., 1997, Morrison et al., 1986). El método se ha utilizado en varias especies, como la cebada (Ziauddin et al., 1990), el trigo (Touraev et al., 1996), el tabaco (Dunwell, 1985) y el pimiento (Supena et al., 2006b). Este método tiene algunas de las ventajas del cultivo de microsporas entre las que destacan el paso directo del medio a las microsporas y una mayor cantidad de embriones que

con el cultivo de anteras. Sin embargo, el hecho de que la antera permanezca en el cultivo no elimina la posibilidad de formación de callos y embriones a partir del tejido somático de la antera y por lo tanto regenerantes somáticos diploides. Por ello, el cultivo de microsporas aisladas sigue siendo más empleado por las múltiples ventajas que ofrece.

3.6. Limitaciones de la embriogénesis de microsporas

Pese a las múltiples ventajas que ofrece la embriogénesis de microsporas (ver apartado 3.3. Ventajas de la androgénesis frente a otras técnicas), en ocasiones puede no ser una opción viable debido a las limitaciones que presenta:

- Malformaciones en los embriones obtenidos. Las más comunes son las relacionadas con una formación deficiente del meristemo apical, como la aparición de cotiledones múltiples, soldados, ausentes, etc. Pese a esto, una correcta puesta a punto de las condiciones del medio de cultivo en la fase de regeneración de embriones puede ayudar a reducir la tasa de embriones con malformaciones (Kim et al., 2008).

- Aparición de regenerantes albinos. En algunas plantas obtenidas mediante androgénesis se detiene el desarrollo normal de los plastidios y no se forman cloroplastos funcionales. Este fenómeno se ha observado exclusivamente en cereales (Torp y Andersen, 2009). Aunque los mecanismos moleculares de esta anomalía no están claros, se sabe que la frecuencia de regenerantes albinos tiene un fuerte

componente genético y está determinada también por los componentes del medio de cultivo (Dunwell, 2010).

- Elevado número de especies recalcitrantes. La eficiencia de obtención de DHs es todavía muy baja en la mayoría de las especies que se ha empleado, salvo en las consideradas modelo para este sistema (Touraev et al., 2001; Palmer et al., 2005). Este es el caso de especies hortícolas de elevado interés agronómico como las solanáceas. De los cinco principales cultivos de solanáceas (pimiento, tabaco, patata, berenjena y tomate), únicamente tabaco y patata pueden considerarse no recalcitrantes. En tabaco existen desde hace años sistemas puestos a punto para obtener DHs a partir de cultivos de antera y microsporas aisladas con una eficiencia aceptable (Chupeau et al., 1998; Touraev et al., 2001; Maluszynski et al., 2003) y en patata se han empleado protocolos para inducir la embriogénesis mediante cultivos de microsporas aisladas (Rihova y Tupy, 1999). El resto de solanáceas siguen estando consideradas como recalcitrantes, porque a pesar de que en algunos casos se han obtenido DHs, como en berenjena (Miyoshi, 1996; Corral-Martínez y Seguí-Simarro, 2014) y pimiento (Kim et al., 2008, 2013; Lantos et al., 2009, 2012) , todavía se está muy lejos de la eficiencia obtenida en tabaco o colza. Es precisamente este escaso desarrollo de la tecnología de obtención de DHs en solanáceas recalcitrantes lo que motiva la presente Tesis Doctoral.

Objetivos

El objetivo principal de la presente Tesis Doctoral es el estudio de la androgénesis, abordando esta técnica desde varios frentes. Un primer enfoque aplicado a una especie de interés agronómico (pimiento), y un segundo enfoque más básico y fundamental, que ayude a comprender las bases de esta ruta alternativa mediante el estudio del proceso en una especie modelo (colza). Para ello se aborda la presente Tesis Doctoral mediante dos subobjetivos o bloques: El primero se basa en optimizar factores clave para la inducción hacia androgénesis, como son la determinación de marcadores morfológicos que identifiquen las microsporas/polen en su estadio de desarrollo óptimo, o la optimización de las condiciones del cultivo *in vitro*. El segundo bloque se centra en el estudio de un sistema modelo mediante el cual se pretende conocer en profundidad las bases de la androgénesis y así poder buscar nuevas vías para abordar la optimización de esta técnica en especies recalcitrantes.

Bloque I. Optimización del protocolo para la obtención de DHs en pimiento dulce.

Capítulo 1: Marcadores morfológicos que relacionan el desarrollo de las anteras y yemas con la microsporogénesis y microgametogénesis. Se determinaron los marcadores morfológicos más precisos y fáciles de medir, que relacionan cambios visibles en anteras y yemas durante todo su desarrollo, con los estadios correspondientes de las microsporas y polen que contienen. El objetivo principal de este capítulo es establecer marcadores morfológicos que permitan seleccionar las anteras que presenten las microsporas en su estadio idóneo para la inducción a androgénesis.

Capítulo 2: Influencia de los tratamientos de estrés y las condiciones de cultivo *in vitro* en el desarrollo de callos en cultivo de anteras de pimiento. En este capítulo se comparó la influencia que tiene en la producción de callos y embriones el uso de diferentes medios de cultivo y la exposición a elevadas temperaturas (35 °C) durante periodos prolongados de tiempo. El objetivo fue estudiar la influencia de estos factores en la producción de callos para tratar de minimizar su presencia en el cultivo de anteras.

Capítulo 3: Protocolo optimizado de cultivo de anteras. En este capítulo se ponen en conjunto los resultados de los dos capítulos anteriores y se describe con detalle un protocolo de cultivo de anteras para genotipos de pimiento dulce.

Bloque II. Estudio de un sistema androgénico modelo mediante el cultivo de microsporas aisladas de *Brassica napus*.

Capítulo 4: Asociación de la inducción hacia embriogénesis con la presencia de paredes celulares con niveles alterados de calosa y celulosa. El objetivo de este capítulo es analizar la dinámica de la deposición de la calosa y la celulosa en las paredes celulares formadas *de novo* durante las primeras etapas de inducción hacia embriogénesis.

Capítulo 5: Estudio de los cambios estructurales que tienen lugar en los plastidios presentes en las microsporas de *B. napus* durante las primeras etapas de inducción hacia embriogénesis. El objetivo de este capítulo es estudiar los cambios que tienen lugar en estos orgánulos, y si estos cambios pueden estar relacionados con procesos de autofagia y/o con la propia inducción.

Bloque I

Optimización del protocolo para la obtención de DHs en pimiento dulce

Todas las variedades de pimiento pertenecen al género *Capsicum.* Este género está incluido dentro de la familia de las Solanáceas y está formado por 25 especies, 5 de las cuales son especies cultivadas por el hombre: *C. pubescens*, *C. annuum* var. *annuum*, *C. baccatum* var. *pendulum*, *C. chinense* y *C. frutescens* (Nuez et al., 2003).

El pimiento ha sido una de las primeras plantas domesticadas por el hombre y su expansión se ha hecho universal, siendo difícil encontrar algún lugar del mundo donde no se utilicen productos derivados de este cultivo. Su producción y consumo ha ido incrementándose a nivel mundial durante el s. XX debido a los múltiples usos, como especia y como hortaliza (Crosby, 2008). Su importancia económica es cada vez mayor, ocupando el duodécimo lugar en el ranking mundial de hortalizas en 2012, con una producción de 31.171.567 t y una superficie cultivada de 1.914.685 ha (FAOSTAT, 2014). En cuanto a la respuesta a la inducción de la androgénesis, el pimiento, junto con el tomate y la berenjena, es uno de los tres cultivos de solanáceas de interés agronómico que pueden definirse como recalcitrantes.

Las primeras plantas haploides de pimiento generadas a partir del cultivo de anteras fueron obtenidas por George y Narayanaswamy (1973), Kuo et al. (1973), Wang et al. (1973) y Novak (1974). En estos estudios iniciales, la composición del medio no estaba bien definida y las plantas se obtenían por regeneración a partir de los callos que se formaban. A raíz de estos estudios, han sido numerosos los cultivares en los que se ha probado el cultivo de anteras en pimiento, la mayoría de estos cultivares pertenecientes a la especie *C. annuum* aunque también se ha probado en híbridos interespecíficos (Sibi et al., 1979; Morrison et al., 1986; Kristiansen y Andersen, 1993; Mityko et al.,

1995; Nowaczyk et al., 2006; Gémes Juhász et al., 2009). El cultivo de anteras de pimiento ha sido usado como herramienta para estudios básicos (González-Melendi et al., 1995; Barany et al., 2010), pero la aplicación más importante ha sido la producción de DHs para programas de mejora. Dumas de Vaulx et al. (1981) establecieron las bases de un protocolo para el cultivo de anteras en pimiento, adaptable a diferentes variedades aplicando ligeras modificaciones. Este método se basaba en aplicar a las anteras un estrés por calor (35 °C) y en emplear condiciones de cultivo diferentes en el momento de la inducción y de la regeneración. En 1983, Vagera y Havranek añadieron carbón activo en el medio de cultivo y observaron un incremento significativo en la producción de plantas haploides. Estudios previos habían probado que el carbón activo absorbe del medio sustancias perjudiciales para la embriogénesis de microsporas, como fenoles y sustancias tóxicas producidas por la pared de la antera (Kohlenbach y Wernicke, 1978). Dolcet-Sanjuan et al. (1997) publicaron un método con el que se inducía la embriogénesis en un único paso. Emplearon un sistema con dos fases, una sólida (con carbón activo y gelrita como agente gelificante) y una líquida. El medio base que utilizaron fue el descrito por Nitsch y Nitsch (1969), sustituyeron la sacarosa por maltosa y emplearon una atmósfera rica en CO_2. Este método permitió la producción de embriones en genotipos que mostraban una pobre o nula respuesta al método desarrollado por Dumas de Vaulx. Sin embargo, la dificultad técnica que conlleva crear una cámara de cultivo con aporte de CO_2 ha impedido a muchos laboratorios emplear este método de manera rutinaria. En la actualidad, la mayoría de los protocolos de cultivo de anteras en pimiento están basados en el método descrito por Dumas de Vaulx. De hecho, muchas empresas del sector de la

producción de semillas continúan empleándolo para generar DHs que utilizan como líneas puras para obtener híbridos comerciales de pimiento.

A pesar de su popularidad y sencillez, el cultivo de anteras de pimiento presenta los problemas descritos anteriormente en la Introducción, en el apartado 3.5.1 Cultivo de anteras, como son la secreción incontrolada del tapete, una baja eficiencia y posibilidad de regenerantes somáticos. Por ello, diversos grupos han trabajado en la optimización del cultivo de microsporas aisladas en las últimas décadas. Sin embargo, en la mayoría de los casos el desarrollo de los embriones no ha sido posible más allá del estadio globular (revisado en Regner, 1996). Fue en 2006 cuando Supena et al. (2006a, b) fueron capaces de regenerar plantas haploides a partir de microsporas aisladas. Este método es el denominado "shed-microspore culture" (descrito en la Introducción apartado 3.5.2 Cultivo de microsporas), que demostró ser eficaz en 10 variedades asiáticas de pimiento picante. En 2008, Kim et al. elaboró un protocolo eficaz para aislar directamente las microsporas en medio líquido. Estos experimentos se llevaron a cabo en variedades asiáticas de pimiento picante con una elevada eficacia en términos de producción de embriones pero no de la calidad de los mismos. En 2009, Gémes Juhász et al. probaron unos 2000 genotipos en los que se incluían variedades picantes y dulces de pimiento y Lantos et al. (2012) optimizaron el cultivo de microsporas para variedades húngaras y turcas. Debido a que el paso limitante es la calidad de los embriones obtenidos, se han seguido implementando protocolos para mejorar los defectos presentes en su morfología, como son la ausencia de cotiledones y la inexistencia del meristemo apical entre otros (Supena y Custers, 2011; Kim et al., 2013).

Actualmente hay disponibles 4 métodos para inducir la embriogénesis de microsporas en pimiento: el cultivo de anteras descrito por Dumas de Vaulx, el método bifásico descrito por Dolcet-Sanjuan, el método denominado "*shed-microspore culture*" y el cultivo de microsporas aisladas en medio líquido. Antes de comenzar un programa de mejora basado en la producción de DHs es recomendable valorar en primer lugar, qué método puede ser el mas efectivo para los cultivares que se vayan a emplear. Los 4 métodos han resultado útiles para obtener plantas DHs. Sin embargo, la presencia de embriones con morfologías irregulares y la escasa aplicación en genotipos de pimiento dulce, interesantes para los mercados europeos, hacen que el cultivo de microsporas aun no se pueda considerar la mejor opción para inducir la embriogénesis en pimiento. De los otros 3 métodos, el de Dumas de Vaulx (DDV) y el "shed-microspore culture" (SM), son los más simples a la hora de emplearlos como técnica rutinaria en un laboratorio de cultivo *in vitro* y además, han demostrado ser efectivos en un mayor número de cultivares (revisado en Irikova et al., 2011). Una vez seleccionados los protocolos a usar, deberíamos centrarnos en sus limitaciones para intentar corregirlas o al menos minimizarlas. Uno de los objetivos a conseguir sería la reducción en el número de callos de origen somático y/o el incremento en el número de embriones obtenidos. Además, para lograr un protocolo eficaz para la obtención de DHs es importante valorar la respuesta de cada cultivar con los diferentes protocolos a emplear, así como optimizar las variables inherentes al propio cultivo, y los relativos a la identificación de las yemas adecuadas en las plantas donantes.

Bloque I

Capítulo 1

Morphological markers to correlate bud and anther development with microsporogenesis and microgametogenesis in pepper (*Capsicum annuum* L.)

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Keywords: Anther length, bud length, flower development, microsporogenesis, microgametogenesis, morphological markers.

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Abstract

The identification of microspores or pollen grains at particular developmental stages during microsporogenesis or microgametogenesis is an important step for different basic and applied purposes. Among them, the most relevant example from a biotechnological perspective is the production of androgenic doubled haploids. For this and other techniques, precise, fast, easy and reliable criteria to identify flower buds carrying microspores or pollen at particular stages are essential. In anthocyanin-producing pepper types, the particularities of flower development allow for the identification of several morphological markers potentially useful as criteria for such an identification. In this work, our aim was to determine the easiest and more accurate criterion to correlate visible, measurable traits of bud and anther development with each of the individual stages of microsporogenesis and microgametogenesis. For this, we used three Spanish sweet pepper F1 hybrids (‘Herminio’, ’Gacela’ and ’Águila’). We analyzed and discussed the accuracy and practical usefulness of using anther length, bud length, anther purple pigmentation and the ratio between calyx length and bud length (calyx/bud ratio) as predictors of individual microspore/pollen developmental stages. According to our results, we propose a combination of calyx/bud ratio and anther pigmentation as an easy, fast and accurate criterion potentially applicable to anthocyanin-producing pepper cultivars to determine their particular markers.

Introduction

During microsporogenesis and microgametogenesis, development of the microspore and the pollen grain is paralleled with changes in the anther

and in the flower bud. These changes relate to bud and anther size, shape and color, among others. The study of these correlative changes are important for the identification of visible morphological markers that allow us to infer what are the developmental stages of the microspores/pollen grains contained within an anther, in a non invasive manner. In other words, with no need for excision and opening of the corresponding flower bud. These morphological markers may be useful for different practical applications where microspores/pollen at particular developmental stages must be identified and used. e.g., for developmental studies of microsporogenesis and microgametogenesis where samples at different stages must be isolated to extract proteins or RNA, or to be observed with a microscope. Other examples with a more applied perspective could include techniques that need the isolation of pollen for storage or for viability or vigour tests, which include the isolation and in vitro culture of mature pollen grains to promote the development of pollen tubes (Shivanna 2003). However, among the biotechnological applications of these morphological markers, the most important would be the identification of buds and anthers with microspores/pollen at the optimal stage to be induced towards androgenesis.

Induction of androgenesis is the most convenient way to obtain doubled haploids, which are valuable tools in breeding programs. For an efficient production, either through anther culture or through isolated microspore culture, it is essential to have a protocol previously optimized. In such a protocol, a central issue is the accurate selection of donor flower buds containing microspores or pollen grains at the right stage of development. For the vast majority of inducible species, it is widely agreed that induction can only be achieved in a narrow time frame revolving around the first pollen

mitosis (Seguí-Simarro 2010). This means that buds and anthers containing mostly late, vacuolate microspores (VM) and just divided, young bicellular pollen (YBP) must be precisely identified. This is not a trivial issue, since out of this time frame induction efficiency drops dramatically or is nearly impossible (Seguí-Simarro 2010). Thus, it is essential to identify suitable morphological markers, predictors of the precise developmental stage of the microspore or pollen grain within the anther. However, this is not always easy, since different species have different flower anatomies, with different sizes, shapes and colors for their different organs. This also applies to different cultivars within a species.

Pepper (*Capsicum annuum*) has been traditionally considered a species recalcitrant to androgenesis induction. Anther cultures have been used in a number of cultivars with relative efficiency (reviewed in Irikova et al. 2011; Seguí-Simarro et al. 2011), but successful application of microspore cultures has only been reported in few hot (asian) pepper types (Kim et al. 2008; Supena et al. 2006b). Nevertheless, the stages most sensitive to inductive treatments in pepper revolve around the VM and YBP (Irikova et al. 2011), exactly the same described for most of the known inducible species. Pepper is not an exception in this sense. In pepper, the search for suitable morphological markers has been approached from different perspectives. The length of specific flower whorls has been used by some authors to estimate bud size. The most frequently measured whorls have been corolla (Dunwell 2010) and calyx, where the ratio between calyx and corolla has been the most used parameter (Koleva-Gudeva et al. 2007; Ltifi and Wenzel 1994; Mityko et al. 1995; Nowaczyk and Kisiala 2006). Other authors opted for a direct estimation of the degree of purple antocyanin pigments that anthers of many pepper types accumulate during

their development (Regnet 1994; Kim et al. 2008; Kim et al. 2004) or by a combination of this marker with the calyx/corolla ratio (Barany et al. 2005; Barany et al. 2010; Buyukalaca et al. 2004; Dumas de Vaulx et al. 1981; Supena et al. 2006a, b; Ercan et al. 2006; Lantos et al. 2009). From these reports it can be concluded that there is no a wide consensus with respect to the most suitable marker to define which pepper flower buds should be used to maximize the percentage of VM and YBP. In this work we addressed this issue in sweet pepper. Our goal was to determine the easiest and more accurate morphological markers to correlate the visible changes of anthers and buds during flower development with the corresponding stages of the microspores and pollen grains contained in them. To approach this, we first characterized the changes undergone by buds and anthers during microspore and pollen development in three Spanish sweet pepper cultivars ('Herminio', 'Gacela' and 'Águila'). According to the described changes, we evaluated the correlation of different morphological criteria (anther length, bud length, pigmentation level of anthers and calyx/bud ratio) with microspore/pollen development, and the convenience of using them to identify anthers enriched in a particular developmental window: the transitional stage of the first pollen mitosis (from VM to YBP). We chose these stages due to the importance of their precise identification for anther and microspore culture experiments.

Material and methods

Plant material

The following three anthocyanin-producing, commercial F1 hybrids of pepper (*Capsicum annuum* L.) were used: 'Herminio' (Lamuyo type), 'Gacela' and 'Águila' (California type), all of them from Syngenta Seeds. Plants were

grown in a growth chamber at 25°C under controlled conditions of humidity (60%) and photoperiod (16/8) during 12 consecutive months from October to September.

Microspore stage determination

Flower buds ranging from 2 to 8 mm long were selected and excised manually. Bud, anther (two anthers per bud) and calyx lengths were measured with an electronic digital caliper. For buds, measurements were performed from the pedicel insertion point to the tip of the bud, as illustrated in Fig. 1a. For anthers, measurements were performed from the filament insertion (basal end) to the anther tip (apical end), as illustrated in Fig. 1b. Anther purple pigmentation was categorized into four pigmentation levels as illustrated in Fig. 1c: (1) no pigmentation, (2) pigments in the apical end, (3) pigments covering most of the distal surface and only traces at the borders of the proximal surface of the anthers, and (4) full pigmentation of the distal surface and almost full pigmentation of the proximal surface of the anthers. For calyx/bud ratio, calyx length, considered from pedicel insertion to the topmost part of the calyx (Fig. 1d) was divided by the corresponding bud length.

A minimum of ten buds were considered for each of the intervals used. For each bud, the parameters above described were measured. From them, two anthers were measured, their pigmentation level estimated, and then used for microspore/pollen staging. When different, the length of both anthers was averaged. The different microspore/pollen developmental stages present in a single anther were observed and counted with a Zeiss (Axiovert 40 CFL) inverted microscope. A minimum of 300 randomly chosen microspores/pollen grains per anther preparation were counted. For each anther, the total microspores/pollen population was categorized into seven stages, as illustrated

in Fig. 2, according to the staging published for other solanaceous species (Seguí-Simarro and Nuez 2005; Salas et al. 2012): (1) meiocytes and tetrads, (2) young microspores, (3) mid microspores, (4) vacuolate microspores, (5) young, (6) mid and (7) mature bicellular pollen grains. For each stage, percentages from the total population were calculated. For simplification, the young and mid microspore stages were joined into a single stage (YM+MM). Numerical data were processed and graphically represented using a Microsoft Excel spreadsheet.

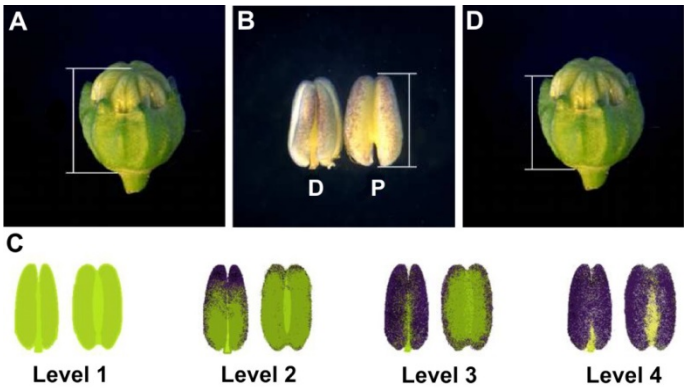

Fig. 1 Criteria used to measure the four morphological parameters used in this study. a: Bud length. b: Anther length. c: Anther pigmentation. d: Calyx length. Anthers in b and c are presented at their distal, petal-facing side (D) and proximal, pistil-facing side (P). See text for further details.

Results and discussion

First, we studied the parallel development of microspores/pollen grains (Fig. 2a-g), flower buds (Fig. 2a'-g') and anthers (Fig. 2a''-g''), covering from the meiocyte to the mature pollen stages. Dividing meiocytes or tetrads, characterized by four independent microspores still enclosed within the post-meiotic cell walls (Fig. 2a), were observed in all flower buds, which were

rounded and fully covered by sepals (Fig. 2a'). At this stage, corresponding anthers were small and green (Fig. 2a''). Young microspores, just released from the tetrad, showed a spherical shape and a still thin pollen coat (Fig. 2b). At this stage, buds remained rounded and closed (Fig. 2b'). Anthers slightly elongated with respect to the previous stage, but with a similar shape and color (Fig. 2b''). Mid microspores (Fig. 2c) showed marked differences with young microspores, mainly consisting on the lobulation of the thicker, polygonal and more sculptured coat, which began to be evident. These changes correlated with changes in the corresponding buds (Fig. 2c'), which increased in size. Besides, sepals began to open in the still rounded bud, allowing for the observation of the corolla at the top of the bud. Anthers (Fig. 2c'') increased in size as well, while keeping similar morphologies and colors. Vacuolate (mature) microspores presented a thicker exine wall where apertures were clearly visible (Fig.e 2d). At this stage, flower buds continued their increase in size and petals were partially visible, with sepals covering approximately three-fourths of the bud (Fig. 2d'). Anthers turned to dark yellow and presented a slightly purple pigmentation at the apical end, only on their distal, petal facing side (Fig.e 2d''). The transition of VMs to YBP (Fig. 2e) was characterized by changes in bud size, a widening of the bud receptacle, and the clear emergence of petals out of the calyx (Fig. 2e'). Purple pigmentation of the distal side of the yellow anthers was rather evident at this stage (Fig. 2e''). At the mid bicellular pollen stage (Fig. 2f), enlarged buds began to elongate due to petal growth, which reached a size approximately equal to that of sepals (Fig. 2f'). Anthers became purple through most of their distal surface (Fig. 2f''). Finally, at the mature pollen stage, these enlarged, densely filled grains (Fig. 2g) correlated with long buds, where the exposed petal length usually exceeded that of sepals (Fig. 2g'), and anthers were fully purple at the distal side and the borders of the proximal side (Fig.

2g’’). Further developmental stages consisted on anthesis, petal growth and anther dehiscence, with no changes in pollen grains other than desiccation (data not shown).

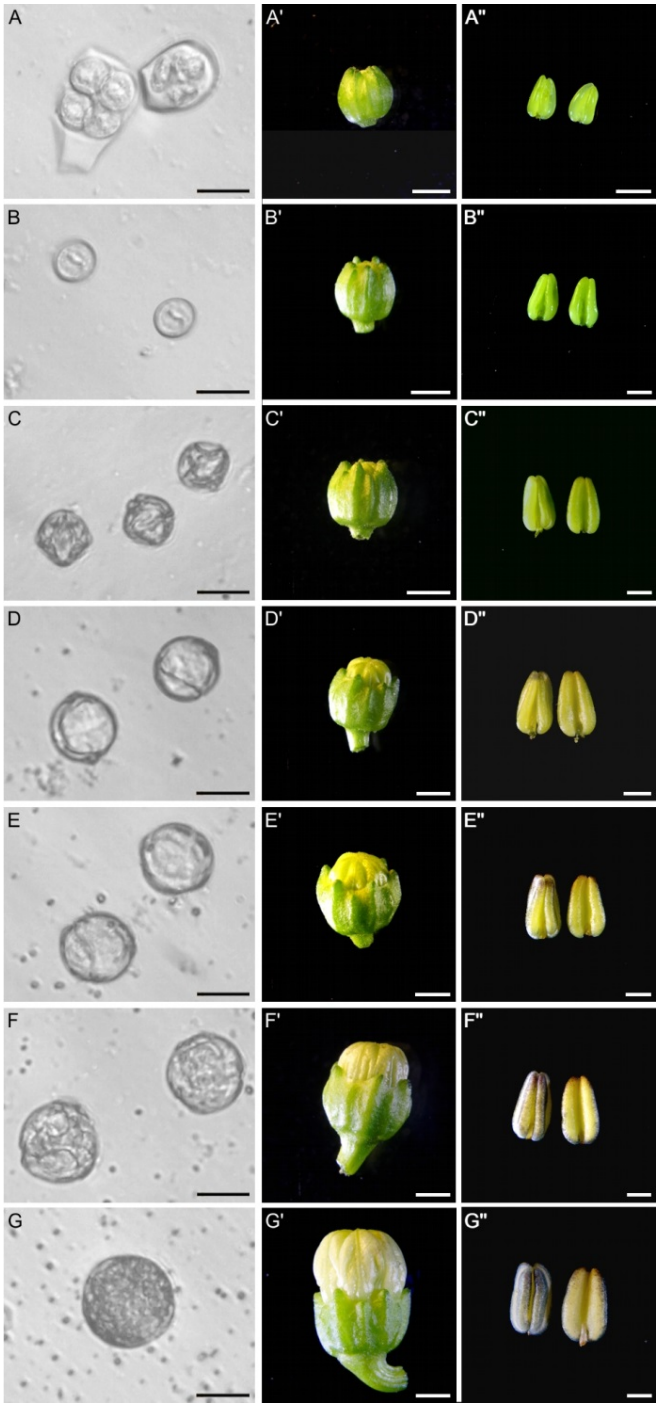

Fig. 2 Changes during microspore/pollen (a-g), bud (a'-g'), and anther development (a''-g''). a: meiocytes and tetrads. b: Young microspores. c: Mid microspores. d: Vacuolate microspores. e: Young bicellular pollen. f: Mid pollen. g: Mature pollen. Anthers are presented in a''-g'' at their distal (left) and proximal sided (right). Bars: a-g: 10 µm; a'-g'': 1 mm.

Next, we assessed the convenience of using anther length as a criterion. We correlated the evolution of microsporogenesis and microgametogenesis with anther increases in length in the 'Herminio' (Fig. 3a), 'Gacela' (Fig. 3b) and 'Águila' (Fig. 3c) cultivars. The percentage of microspores/pollen at each developmental stage is represented by the size of the corresponding bubble. These figures evidenced that anther grows in parallel to microspore development, and also to pollen development up to the mid pollen stage, where anthers did not grow further. Shorter (younger) anthers were characterized by the coexistence of microspores at few different stages, with one of them clearly predominating. In contrast, heterogeneity was more characteristic of longer (older) anthers, with up to five stages in 3.00-3.50 mm anthers of cvs. 'Herminio' and 'Gacela'. Cues of small bubbles, corresponding to low percentages of microspores, could be observed in older anthers of the three genotypes. They were most likely indicating the presence of dead and/or delayed microspores, unable to develop synchronously with the rest of microspores. With respect to the identification of the first pollen mitosis interval, we looked for the ranges containing mostly VM and YBP (green bubbles in Fig. 3). In case of doubt, we selected the range with a majority of VM with respect to YBP, since they were at a stage immediately prior to mitosis, and would enter it soon. 'Herminio' anthers contained these stages at a length range from 2.50 to 2.99 mm (Fig. 3a), whereas in 'Gacela' and 'Águila' it was from 2.0 to 2.49 mm (Fig. 3b, c). Such a slight discrepancy (0.5 mm) could be attributable to genotype-dependent differences in growth, and it is likely that by using less restrictive length intervals, differences would disappear. In light of these results, it was clear that anther length correlates with microspore/pollen development with very slight variations between cultivars,

confirming that anther length can be used to accurately identify microspore/pollen developmental stages, at least up to the mid pollen stage.

Then, we focused on the usefulness of bud length as a convenient criterion. From a practical perspective, this parameter would be faster and easier to measure. However, bud length might not correlate with anther growth and microspore/pollen development. For example, this occurs in related species such as tomato or eggplant, where sepals grow considerably at the onset of bud development, while anthers do not grow at the same pace (Seguí-Simarro and Nuez 2005; Salas et al. 2012). Thus, a previous mandatory study consisted on the verification of the relationship between anther and bud lengths. We studied this by analyzing the linear correlation between anther and bud length values for all three cultivars. In all cases, a positive linear correlation was clearly observed, with linear regression coefficients (R) higher than 0.9 (data not shown). Then, we compared bud lengths with the different stages of microsporogenesis and microgametogenesis (Fig. 3d-f). Due to the larger size of buds with respect to anthers, larger (1 mm) length intervals were established. In this case, a clear correlation was also observed at the different stages, confirming bud length as another reliable criterion. With respect to the VM and YBP stages, the three cultivars (‘Herminio’ in Fig. 3d, ‘Gacela’ in Fig. 3e and ‘Águila’ Fig. 3f) presented the highest percentages at the interval ranging from to 4.0 to 4.99 mm. Thus, a length between 4 and 5 mm could be an accurate marker to identify ‘Herminio’, ‘Gacela’ and ‘Águila’ buds with highest percentages of VM and YBP.

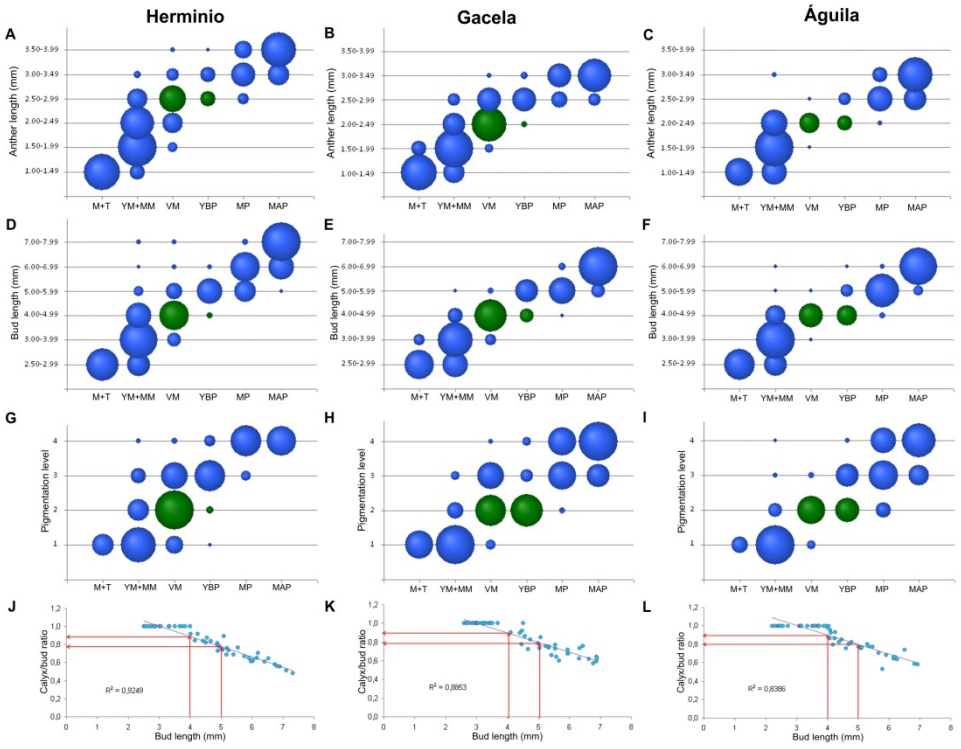

Fig. 3 Correlation of anther length (a-c), bud length (d-f), anther pigmentation (g-i) and calyx/bud ratio (j-l) with microspore/pollen development in cvs 'Herminio' (a, d, g, j), 'Gacela' (b, e, h, k) and 'Águila' (c, f, i, l). Percentages of microspores/pollen at each stage are proportionally represented by bubbles. Green bubbles correspond to the stages where most vacuolate microspores and young bicellular pollen are found. For j-l, blue dots are the data points used to calculate the regression line. *M+T* meiocytes and tetrads. *YM+MM* Young and mid microspores. *VM* Vacuolate microspores. *YBP* Young bicellular pollen. *MP* Mid pollen. *MAP* Mature pollen.

The correlation between anther purple pigmentation patterns and microspore/pollen development was subsequently studied in a way similar to that described above for anther and bud lengths. As observed in Fig. 3g for 'Herminio', Fig. 3h for 'Gacela' and Fig. 3i for 'Águila', pigmentation patterns also correlated well with microspore/pollen development. According to the pigmentation levels established in "Materials and methods", the higher

percentage of VM and YBP was observed exclusively in anthers with a level 2 of pigmentation (purple pigmentation at the apical end of the distal surface) for all the genotypes studied. Thus, the differential anther pigmentation pattern would also be a valid criterion to identify anthers enriched in VM and YBP.

As the last morphological criterion, we assessed the convenience of using the proportion of calyx length with respect to the whole bud. This criterion allows for a quick visual identification of suitable buds, provided that their exact values are studied for each stage. As for bud length, it was necessary to verify whether this parameter correlated with microspore/pollen development. For this, we analyzed the linear correlation between bud length and calyx/bud ratio values for all three cultivars. In all cases, a clearly positive linear correlation was observed, with linear regression coefficients (R) ranging between 0.84 and 0.92 (Fig. 3j-l). Therefore, the calyx/bud ratio could also be a reliable criterion to be used. Since the highest concentration of VM and YBP was found at the bud length interval of 4.0 to 4.99 mm, we extrapolated the corresponding calyx/bud ratios for the three cultivars and obtained a similar calyx/bud ratio between 0.8 and 0.9 (red arrows in Fig. 3j for 'Herminio', Fig. 3k for 'Gacela' and Fig. 3l for 'Águila'). Thus, buds with a calyx covering about 80-90% of the total bud length should be selected to maximize the presence of VM and YBP.

If we consider that externally visible criteria to identify microspore/pollen stages must be precise, all four criteria analyzed would be valid, being anther and bud length the most precise. However, criteria should also be easy to use and quickly measurable. According to this, bud and anther length would not be the most useful in practice due to the time needed to excise and measure every single bud and anther. These time-consuming

procedures would delay significantly the experiments aimed to do with the microspores/pollen, which may in turn compromise their viability. The small size of pepper anthers would be an added difficulty. Furthermore, these invasive processes imply that after measurement, many useless excised and open buds will be discarded, thus reducing the efficiency of these procedures. Anther pigmentation has been suggested as the most reliable criterion for anthocyanin-producing cultivars, since it is less genotype-dependent (Regner 1996). However, it is based on the visual estimation of pigmentation, which may reduce its accuracy, and is a time-consuming and destructive procedure as well. All this considered, these three markers would have little practical utility. For a large-scale practical implementation of a criterion to identify microspores/pollen, it is preferable to rely on easier and faster criteria, even if they imply less accuracy. Besides, it would be desirable to apply this criterion on the buds still *in planta*, avoiding excision. According to this, the calyx/bud ratio would be the best criterion. Compared to others, its only limitation would be a reduced accuracy, since in practice it may be difficult to visually distinguish between ratios of 70%, 80% or 90%. Although for an experienced observer this may not be a problem, we propose in case of doubt a combination of the two external criteria hereby described, calyx/bud ratio and anther pigmentation, as follows. First, optimal buds could be quickly identified *in planta* according to their calyx/bud ratio. Then, once in the laminar flow hood, anthers could be excised and quickly checked on the basis of their pigmentation pattern. Restricting selected anthers to those matching both criteria would compensate for the reduced accuracy of these criteria when used separately.

In conclusion, we propose that the combined use of calyx/bud ratio and anther pigmentation may provide easy, fast and accurate identification of

buds and anthers with microspores/pollen at particular developmental stages in anthocyanin-producing pepper cultivars. In the cultivars used in this study and for the identification of VM and YBP stages, this would mean the selection of anthers from buds having a calyx/bud ratio of about 80-90%, and then the exclusion of those not having purple pigments only at the apical end of the distal surface.

Author contribution

Verónica Parra-Vega and Beatriz González García performed all the experimental work required for this study. Jose M. Seguí-Simarro was responsible for the experimental design, interpretation of results and writing of the manuscript.

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Bloque I

Capítulo 2

Stress treatments and *in vitro* culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (*Capsicum annuum* L.)

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Keywords: Androgenesis; *Capsicum annuum*; heat stress; microspore embryogenesis; *in vitro* culture.

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Abstract

Production of doubled haploids (DHs) is a convenient tool to obtain pure lines for breeding purposes. Until now, the easiest and most useful approach to obtain pepper DHs is via anther culture. However, this method has an associated possibility of producing calli from anther wall tissues that would be coexisting in the anther locule with embryos derived from microspores. Using two established protocols for anther culture, Dumas de Vaulx et al. (1981) and Supena et al. (2006a, b) callus and embryo development was assessed in four sweet pepper cultivars. For all genotypes tested, the protocol of Dumas de Vaulx et al. (1981) promoted both embryo development and callus growth, whereas the protocol of Supena et al. (2006a, b) produced no callus but only embryos. However, differences in embryo production were observed among these genotypes. In parallel, anthers were exposed to a 35 °C inductive heat shock for 4, 8, 12 and 16 days, prior to culture at 25 °C. The duration of the heat shock had significant effects in embryo production, but also in callus generation. Callus generation increased with prolonged exposures to 35 °C. Embryo and callus origin was analyzed by flow cytometry, light microscopy and molecular markers. Tests conducted demonstrated a gametophytic origin for all of the embryos tested, and a sporophytic origin for all of the calli. Together, our results reveal that culture conditions have a significant influence on the presence of calli derived from anther walls, which could be minimized by reducing heat shock exposure and/or using a shed-microspore approach.

Introduction

Induction of androgenesis is one of the most convenient ways to obtain haploid and doubled haploid (DH) individuals. Pepper (*Capsicum annuum*) is, together with tomato and eggplant, one of the three solanaceous crops most recalcitrant to the induction of androgenic DHs (Seguí-Simarro et al. 2011). Apart from the spontaneous occurrence of some cases of *in vivo* androgenesis with no practical relevance (Campos and Morgan 1958), haploids in pepper were first obtained through parthenogenesis (reviewed in Regner 1996). Soon after their discovery by Guha and Maheshwari (1964), anther cultures were explored as a way to haploidy, and since then, they have been used as a tool to produce pepper DHs for breeding programs (Arnedo Andrés et al. 2004; Dumas de Vaulx and Pochard 1986; Jiang and Li 1984; Hwang and Paek 1998). Despite the recent progress made on isolated microspore culture (Kim et al. 2013; Lantos et al. 2012; Lantos et al. 2009; Ferrie and Caswell 2011), anther culture is still considered the method of choice for pepper DH production due to its simplicity (Germanà 2011). However, this technique carries a number of drawbacks. Among others, these include: a limited efficiency, producing only a few embryos per cultured anther; the uncontrollable secretory effect of the tapetum, which precludes a strict control of culture conditions; and the undesirable presence of calli produced from anther wall tissues (Seguí-Simarro et al. 2011).

The study of callus formation in anther cultures is not a trivial issue, at least in recalcitrant solanaceous species. Whereas in some species calli may have a gametophytic origin, in many others a sporophytic origin has been demonstrated for some of the calli produced. It was recently demonstrated that, under certain experimental conditions, microspore-derived embryos in

eggplant may transform into haploid or DH calli (Corral-Martínez and Seguí-Simarro 2012). However, callus generation from anther wall cells is also frequent in eggplant anther cultures, as demonstrated in 12 different eggplant accessions (Salas et al. 2011). In tomato, haploid and DH plants could be regenerated from calli produced by anthers containing meiocytes (Seguí-Simarro and Nuez 2007). Regrettably, 83% of these calli were produced by anther wall tissues (Corral-Martínez et al. 2011). Although there are no specific studies addressing this topic, callus production has been frequently reported in the literature about pepper anther culture, with many protocols producing both calli and embryos, and few producing only calli (reviewed in Irikova et al. 2011). Interestingly, the first attempts to culture pepper anthers produced calli, from which plants were regenerated through organogenesis (Wang et al. 1973; George and Narayanaswamy 1973; Kuo et al. 1973). As deduced from the literature, it would be important to know where calli come from, to what extent they may constitute a drawback, as well as to find ways to overcome or at least minimize such a drawback.

In this work, we addressed these issues. In order to shed light on the relationship between culture conditions and callus production, we compared the influence on callus and embryo production of two successfully used media. We also checked the effect of different durations of a 35 °C heat shock. Embryo and callus origin was also analyzed by flow cytometry, light microscopy and microsatellite molecular markers. Our results demonstrate that culture conditions have a significant influence not only on the production of microspore-derived embryos, but also of calli derived from anther wall tissues. We propose ways to minimize such callus presence.

Material and methods

Plant material

The following four commercial F1 hybrids of pepper (*Capsicum annuum* L.) were used: 'Herminio' (Lamuyo type, from Syngenta Seeds), 'Coyote', 'Quito' (California types, both from Syngenta Seeds), and 'Vélez' (California type, from Enza Zaden). Plants were grown in 30 cm pots at COMAV greenhouses (Universitat Politècnica de València), at a minimum of 18 °C under natural light during 9 months from March to November.

Anther culture

We cultured anthers of the four genotypes above mentioned. The number of anthers cultured for each genotype was as follows: for 'Herminio', 491 anthers (corresponding to 90 buds); for 'Coyote', 381 anthers (64 buds); for 'Quito', 378 anthers (64 buds); and for Vélez, 389 anthers (68 buds). Selection of buds was based on a sepal length being around 80% of petal length, according to Parra-Vega et al. (2013). Anthers with purple distal tips, containing mostly vacuolate microspores and young bicellular pollen grains (Parra-Vega et al. 2013), were extracted from selected buds. After surface sterilization with 10% commercial bleach (40 g l^{-1}) for 5 min, anthers were plated and cultured according to two previously published, different protocols: (1) the protocol of Dumas de Vaulx et al. (1981) for anther culture in agar-based solid medium (hereinafter referred to as the DDV protocol), and (2) the protocol published by Supena et al. (2006a, b) for anther culture in a biphasic (solid-liquid) medium, also known as the shed-microspore method (hereinafter the SM method). Five repetitions with five dishes per repetition (6 anthers per dish) were performed at different months from March to November for each culture method. Mean

and standard deviation were calculated. Additionally, anthers of the four genotypes were cultured according to the DDV method with modification of the duration of the 35 °C exposure to 4, 8, 12 and 16 days. A minimum of three repetitions with five dishes per repetition (6 anthers per dish) were performed for each combination of genotype and exposure time, and the mean and standard deviation were calculated. For all comparisons, data of the corresponding experiments were subjected to standard analysis of variance using the Sigmastat software (Systat Software, Inc. Germany) and means were separated using a Holm-Sidak test with $p \leq 0.05$.

Flow cytometry

Small pieces of anther-derived calli and embryos were processed for flow cytometry as described in Abdollahi et al. (2012). Additionally, young leaf samples from donor plants were analyzed and used as standards for 2C DNA content. Briefly, samples were chopped with a razor blade and processed using the CyStain UV Precise P kit for nuclear extraction and staining (Partec GmbH, Münster, Germany) according to manufacturer's specifications. Extracted nuclei were filtered through 30 µm CellTricks filters (Partec GmbH, Münster, Germany) and immediately analyzed in a Partec PA-I Ploidy Analyzer.

Genetic analysis with microsatellite molecular markers

Prior to their use to determine the origin of the plants obtained from anther cultures, donor plants of the commercial F1 hybrids 'Herminio' and 'Vélez' were screened using microsatellite markers (SSR). Young leaf tissue was sampled from 5 different, randomly chosen donor plants, and genomic DNA was isolated from 50 mg of tissue using the modified CTAB (hexadecyl trimethylammonium bromide) method described in Ferriol et al. (2003). Each

donor plant was analyzed using the following 16 SSR markers known to be polymorphic in other pepper materials (Portis et al. 2007; Minamiyama et al. 2006): EPMS670, EPMS755, EPMS643, EPMS716, EPMS689, EPMS747, EPMS924, EPMS704, EPMS725, EPMS757, EPMS745, EPMS650, EPMS749, CAMS117, CAMS340 and CAMS806. The forward primers were labeled with different fluorescent dyes and six loci were simultaneously detected using an ABI PRISM 310 Genetic Analyzer. Heterozygous loci were consistently found for six SSR markers (CAMS117, CAMS340, EPMS650, CAMS806, EPMS670 and EPMS643) in all 'Herminio' and 'Vélez' donor plants analyzed. These markers were used as described above to check the origin of embryo-derived plants.

Light microscopy

Cultured anthers at different culture days were picked up and processed for light microscopy as described in Seguí-Simarro and Nuez (2005). Briefly, anthers were fixed in Karnovsky fixative (4% formaldehyde+5% glutaraldehyde in 0.025 M cacodylate buffer, pH 7), dehydrated in ethanol series and embedded in Technovit 7100 according to manufacturer's specifications. Thin (1.5 μm) sections were obtained with a Leica UC6 ultramicrotome and observed under phase contrast with a Nikon Eclipse E1000 light microscope.

Results

Callus and embryo production with different anther culture methods

In order to elucidate the influence of culture conditions on embryo and callus production, we cultured anthers of the 'Herminio', 'Coyote', 'Quito' and

'Vélez' genotypes using two markedly different culture conditions: the DDV and the SM protocols. In the DDV method, flower buds at the appropriate stage of development (Figure 1A) were excised and their anthers extracted and cultured in semisolid, agar-based culture medium. Three to four weeks after culture initiation, the first embryos were observed emerging out of the walls of the necrosing anther (Figure 1B). In parallel, a whitish, callus-like mass of cells emerged from the anther locule (Figure 1C). Upon separation from the anther, individualized embryos germinated (Figure 1D), giving rise to *in vitro* plantlets (Figure 1E). After acclimatization, these developed into normal pepper plants (Figure 1F). In quantitative terms (Figure 1G), all four genotypes responded to *in vitro* induction by producing both calli and embryos. However, the embryo-to-callus ratio was remarkably different among genotypes, being embryo formation more frequent than callus production in 'Herminio', similarly frequent in 'Coyote' and less frequent in 'Quito' and 'Vélez'. Production of embryos was heterogeneous as well, ranging from 3.3 embryos/100 anthers for 'Vélez' to 22 for 'Herminio'. In contrast, production of calli was more homogeneous, ranging from 3.9 calli/100 anthers in 'Herminio' to 8.5 in 'Quito'. Thus, it appeared that embryo production was more genotype-dependent than callus production.

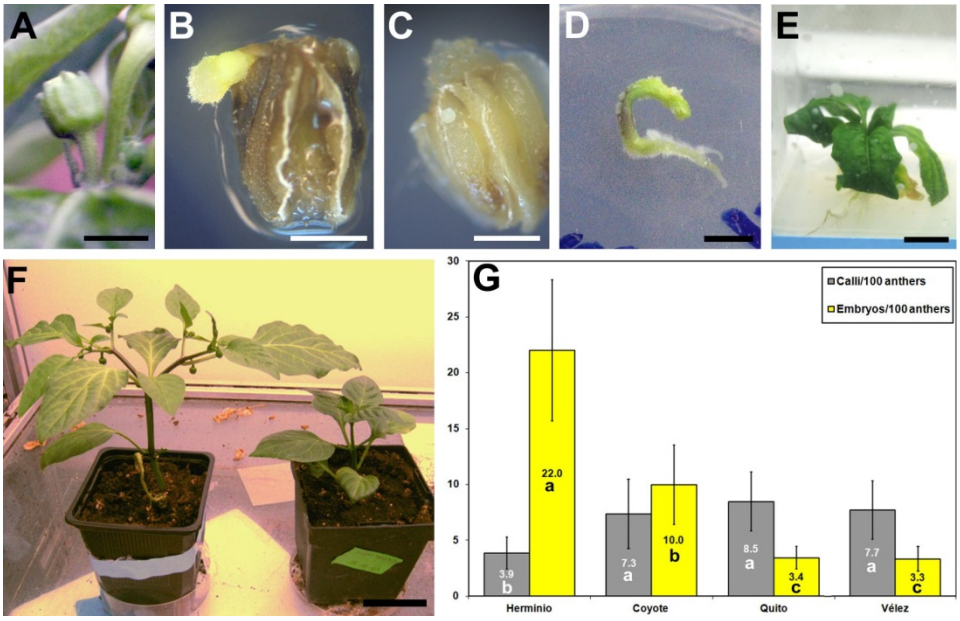

Figure 1: Anther culture in cv 'Herminio' using the DDV method. A: flower bud at the right stage for anther isolation. B: Cultured anther producing a microspore-derived embryo. C: Cultured anther producing calli. D: Germinating microspore-derived embryo. E: *In vitro* microspore-derived plantlet. F: *Ex vitro*, fully acclimated microspore-derived plantlets. G: Comparison between callus (dark bars) and embryo production (light bars) in the four genotypes studied. Results are expressed as number of callus or embryos produced per 100 cultured anthers. Different letters indicate statistically significant ($p \leq 0.05$) differences among genotypes. Bars: A, D: 5 mm. B, C: 1 mm. E: 1 cm. F: 5 cm.

In the SM method, anthers were extracted from flower buds at the appropriate stage of development and cultured in liquid medium (Figure 2A). Upon dehiscence, open anthers released microspores to the liquid medium (Figure 2B), sinking to the solid-liquid interphase and then transforming into microspore-derived embryos (Figure 2C). Some embryos germinated and upon acclimatization transformed into entire plants (Figure 2D). Quantitative analysis of these cultures (Figure 2E) revealed a pattern of embryo and callus production markedly different from the DDV method. Although embryo

production was similar using both methods for the 'Herminio' (22.0 vs 27.4 embryos/100 anthers) and 'Vélez' genotypes (3.3 vs 4.0 embryos/100 anthers), 'Coyote' and principally 'Quito' showed a dramatically increased response, nearly doubled for 'Coyote' (10.0 vs 19.2 embryos/100 anthers) and 17 times higher for 'Quito' (3.4 vs 60.7 embryos/100 anthers). Production of calli was not observed in any case. Thus, as observed with the DDV method, with the SM method callus production was less influenced by the genotype than embryo production.

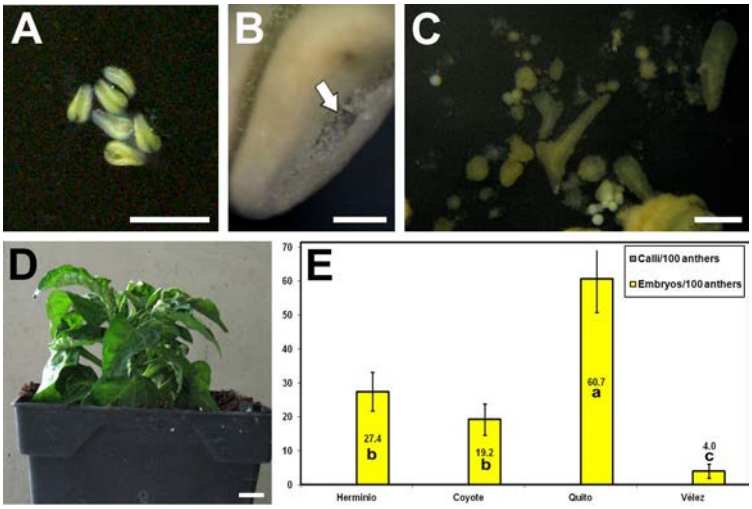

Figure 2: Anther culture in cv 'Herminio' using the SM method. A: anthers floating on the liquid upper phase. B: Anther opening at the dehiscence line (arrow), releasing microspores to the liquid medium. C: Microspore-derived embryos originated from induced microspores. D: Ex vitro, fully acclimated microspore-derived plantlet. E: Comparison between callus and embryo production in the four genotypes studied. Results are expressed as number of callus or embryos produced per 100 cultured anthers. Different letters indicate statistically significant ($p \leq 0.05$) differences among genotypes. Bars: A: 5 mm; B: 500 µm; C: 200 µm; D: 1 cm.

Callus and embryo response under different pretreatment durations

Anthers of the 'Herminio', 'Coyote', 'Quito' and 'Vélez' genotypes were cultured according to the DDV method but modifying the duration of the 35 °C

exposure to 4, 8, 12 and 16 days. The embryogenic response was highly dependent of the genotype for the four durations assayed. 'Herminio' (Figure 3A) showed the highest levels of embryo production, followed by 'Coyote' (Figure 3B), 'Quito' (Figure 3C) and 'Vélez' (Figure 3D). Quantitative differences in terms of embryo production, probably due to seasonal effects, were observed. Nevertheless, the pattern of differences observed among genotypes when cultured for 8 days at 35 °C (as in the original DDV protocol) was consistent with that shown in Figure 1G. Equally consistent was the observation that callus formation was less dependent on the genotype, with differences smaller than for embryo production.

The effect of prolonged 35 °C treatments on embryo production was found to be detrimental in all cases, with a general trend pointing to a reduced embryo production with increased durations. Conversely, the shortest duration (4 days) was found to be the most effective in all four genotypes studied. However, it must be noted that in the case of 'Herminio' the difference between 4 and 8 days was not significant. In terms of callus production it was difficult to extract any defined trend for 4, 8 and 12-day treatments, since some genotypes responded with a rather discrete or even null callus production rate (Figures 3A-D). Interestingly, all four genotypes responded to 16-day treatments with a similar rate of callus production, ranging between 8 and 14 calli/100 anthers, and similar to the rate of embryos/100 anthers. It appeared that prolonged exposures to 35 °C increased the rate of callus production in a genotype-independent manner.

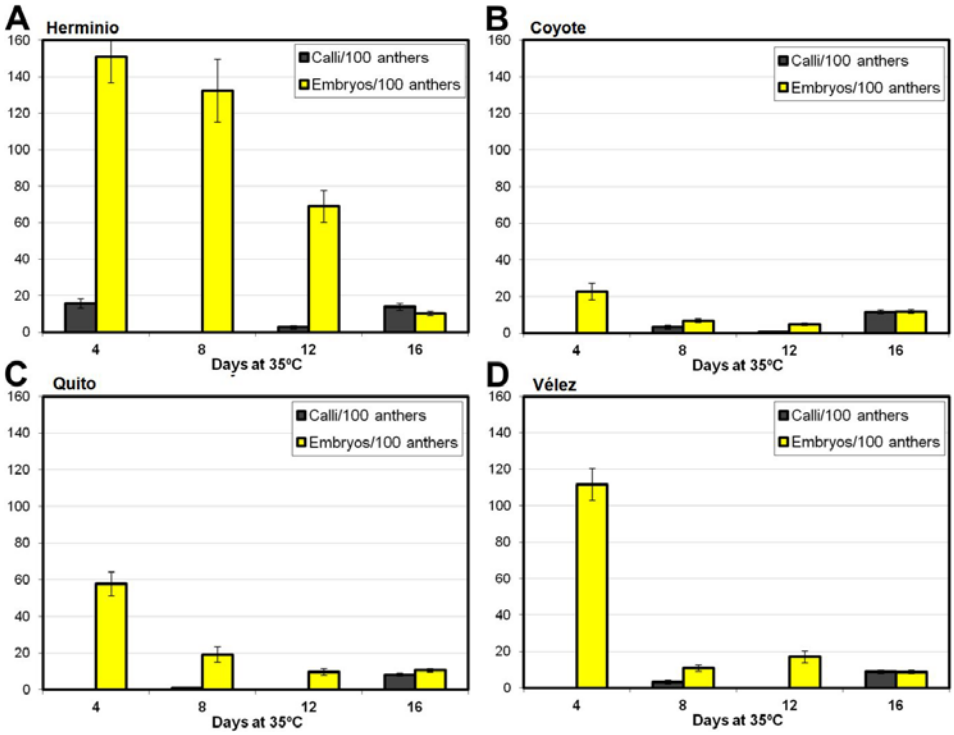

Figure 3: Comparison between callus and embryo production in anthers of 'Herminio' (A), 'Coyote' (B), 'Quito' (C) and 'Velez' (D), cultured according to the DDV method but exposed to 35 °C during 4, 8, 12 and 16 days. Results are expressed as number of callus or embryos produced per 100 cultured anthers.

Analysis of callus and embryo origin

Calli from 'Herminio' and 'Vélez' obtained using the DDV protocol were analyzed in order to clarify their cellular origin. For this, we analyzed by flow cytometry 20 randomly selected calli. All of them presented a diploid DNA content, identical to the diploid parental plants used as controls. Not a single haploid signal was observed in any histogram (data not shown). During processing for flow cytometry, all of the calli had to be mechanically detached from the anther, indicating that they were physically connected to the anther tissue. Light microscopy sections of fixed and embedded anthers (Figure 4)

revealed that these calli originated by proliferation of the connective tissues of the anther. Proliferating calli first invaded and collapsed the anther locule (Figure 4A) and then ruptured the anther walls, emerging out of the anther (Figure 4B). These observations, together with the absence of haploid cells demonstrated by flow cytometry, indicated that calli were originated from tissue layers of the anther walls.

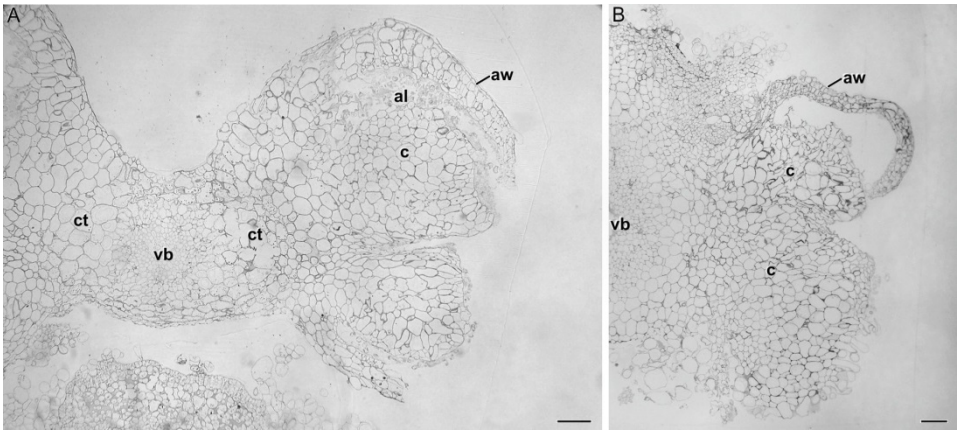

Figure 4: Light microscopy analysis of callus growth in cultured 'Herminio' anthers. A: anther with a callus (c) derived from connective tissues (ct) invading the anther locule (al). B: anther with developing calli that tear off the anther wall (aw) and emerge out of the anther. vb: vascular bundle. Bars: 100 µm.

We also analyzed by flow cytometry 20 plants produced from anther-derived MDEs of 'Herminio' hybrids'. From them, 12 (60%) showed a 1C DNA content, equivalent to a haploid genome, 7 (35%) showed a 2C DNA content, equivalent to diploid controls (donor plants) and 1 (5%) showed a 3C, triploid DNA content. According to this, a gametophytic origin could be unambiguously assigned to the 12 haploid plants. The other 8 were analyzed using microsatellite (SSR) molecular markers. All 8 plantlets were homozygous for the six SRR markers found heterozygous for their corresponding donor plants.

These data, together with those coming from flow cytometry indicated that all the embryos produced had a microspore origin, i.e. they were true androgenic embryos.

Discussion

It is widely accepted that anther culture promotes the formation of microspore-derived embryos, and that the genotype has a remarkable influence in the percentage of microspores deviated towards embryogenesis and effectively transformed into embryos (Dunwell 2010; Irikova et al. 2011; Seguí-Simarro 2010; Seguí-Simarro and Nuez 2008). It is also known that in addition to embryos, calli may also be formed in anther cultures. The results presented in this work, using four different sweet pepper cultivars subjected to two different anther culture methods, are consistent with these notions. However, we also showed that under the same conditions and for the same four cultivars, callus induction from sporophytic anther tissues appeared less genotype-dependent. In the case of the DDV method, all four cultivars produced calli, in similar amounts. In the case of the SM method, no calli were produced in any of the four sweet pepper cultivars we tested. Furthermore, no callus (just embryo) presence was reported by Supena et al. neither in their original publications of the shed-microspore method (Supena et al. 2006a, b) nor in a further refinement of the method (Supena and Custers 2011). An additional confirmation to this notion was provided by the experiment where different durations of the 35 °C treatment led to different rates of callus production, ranging from zero to 15 calli/100 anthers. Thus, we could speculate that at least in pepper, callus induction seems more dependent upon culture conditions than upon the genotype. Similar results have been described in

other solanaceous species such as tomato and eggplant. In tomato, anthers of 6 out of 8 cultivars produced callus when cultured using a defined protocol but not with other protocols (Seguí-Simarro and Nuez 2007). The other two cultivars did not produce callus under any circumstances. Later on, it was demonstrated that 83% of the calli produced originated from anther wall tissues (Corral-Martínez et al. 2011), as we showed hereby for pepper. In eggplant, anthers of 11 out of 12 genotypes cultured using the Dumas de Vaulx and Chambonnet (1982) method produced anther-derived callus, whereas only 5 of them produced embryos under the same experimental conditions (Salas et al. 2011).

Our flow cytometry and light microscopy analysis demonstrated that in pepper, calli are produced by proliferation of cells from anther wall tissues. It might be argued that these calli could potentially be a problem in terms of producing callus-derived embryos (for example, through secondary embryogenesis). These calli might give rise to non-DH plants. This, in turn, would imply the consumption of time and resources to identify and dispose of the useless individuals. However, in our genotypes this possibility seemed unlikely since for all of the embryos analyzed, a haploid origin was clearly assigned. Notwithstanding this, the occurrence of calli from anther wall tissues should be minimized in order to maximize the availability of resources for microspore growth and conversion to embryos.

A straightforward way to achieve this would be to use a method that minimizes or excludes callus formation. For example, the shed-microspore method produced no callus. However, we showed that this method may not work efficiently in some genotypes. It is likely that in other cultivars, not tested by us, this method may not work at all. Thus, a previous validation of the

method should be required. A second suggestion would be to use a method with a wider range of application, such as the DDV, but modifying particular conditions in order to minimize callus production. For example, the duration of the heat treatment could be optimized. We showed that prolonged periods at 35 °C stimulate callus formation. It is likely that other conditions would promote similar effects. For example, in *Capsicum annuum* var. Grossum Sendt, it was shown that when cultured in solid MS medium, 25 °C promoted callus growth but 35 °C prevented it (Mythili and Thomas 1995). In the same study it was also shown that anthers cultured under continuous darkness produced more calli than those cultured under a 16/8 photoperiod. Thus, photoperiod could well be another candidate factor.

In conclusion, we showed that culture conditions have a notable influence, even higher than the genotype, on the formation of callus from sweet pepper anther walls. This influence could be minimized in general by reducing heat shock exposure to a minimum when cultured in solid medium, or by using the shed-microspore approach in particularly sensitive genotypes.

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Bloque I

Capítulo 3

Anther culture in pepper

(*Capsicum annuum* L.)

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Summary

Anther culture is the most popular of the techniques used to induce microspore embryogenesis. This technique is well set up in a wide range of crops, including pepper. In this chapter, a protocol for anther culture in pepper is described. The protocol presented hereby includes the steps from the selection of buds from donor plants, to the regeneration and acclimatization of doubled haploid plants derived from the embryos, as well as a description of how to analyze the ploidy level of the regenerated plants.

1. Introduction

Androgenesis can be defined as the generation of an individual derived from a nucleus of male origin, usually a haploid microspore or young pollen grain (Seguí-Simarro 2010). Haploid embryos or calli are produced through the deviation of the microspore from its original gametophytic pathway towards a new sporophytic pathway. Haploid embryos may become doubled haploid individuals by themselves or through the application of treatments for genome doubling (Seguí-Simarro and Nuez 2008b). Doubled haploid individuals can be used as pure lines to produce hybrid seeds, which reduces considerably the time and resources needed to obtain pure lines when compared with conventional breeding methods (Wedzony et al. 2009).

For most of the studied species the optimal stage of male gamethophyte development to induce embryogenesis is the transition between vacuolate microspores and young bicellular pollen (Seguí-Simarro 2010; Soriano et al. 2013). Technically, microspore embryogenesis can be induced through anther culture or isolated microspore culture. Isolated microspore culture is based on

the isolation of the microspores in liquid medium. Since the maternal tissue is removed, microspores are directly in contact with the medium components. Therefore, the possible formation of somatic embryos coming from the anther walls is avoided. Despite these advantages, isolated microspore culture is more complex than anther culture and therefore it is well set up just in a few species. Just tobacco (*Nicotiana tabacum*), rapeseed (*Brassica napus*), wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) can be considered as model systems for microspore culture (Forster et al. 2007). For most crops of agronomic interest, the most used technique is still anther culture. Anther culture consists on the cultivation of the anthers in a solid or semisolid medium. It can be applied to a wide range of crops and it is the preferred technique used to produce doubled haploids due to its simplicity (Germanà 2011), and to the possibility of culturing large amounts of anthers per isolation. In some species, the presence of the anther walls in the culture medium seems to provide a proper environment for microspore development, allowing for the induction of the microspores towards embryogenesis (Seguí-Simarro and Nuez 2008a). Anther culture in pepper (*Capsicum annuum* L.) has been used to produce doubled haploid plants for breeding programs since the mid 80's (reviewed in Seguí-Simarro 2014).

In this chapter, a protocol for anther culture of sweet pepper is explained according to Dumas de Vaulx et al. (1981) with some modifications. The protocol was adapted for commercial F1 hybrids of sweet pepper (Parra-Vega et al. 2013b) and the selection of donor flower buds was made according to Parra-Vega et al. (2013a). In this protocol, the combination of two morphological markers (calix-bud length ratio and anther pigmentation) is used to select the optimal flower buds.

2. Materials

2.1. Plant material

Commercial F1 hybrids of pepper (*C. annuum* L.): 'Herminio' (Lamuyo type, from Syngenta Seeds), 'Coyote', 'Quito' (California type, both from Syngenta Seeds), and 'Vélez' (California type, from Enza Zaden).

2.2. Equipment

- Plastic tubes of 50 mL
- Box with melting ice
- Laminar flow hood
- Sterile Whatmann paper
- Sterile forceps and scalpel
- Sterile Petri dishes 90 x 25 mm (Ø x height)
- Parafilm
- Inverted or light microscope
- Microscope slides and cover slips
- Aluminum paper
- Incubator at 35 °C and 25 °C
- Sterile baby food jars with plastic caps (200 ml)
- Plastic plant pots 90 x 100 mm (width x height)
- Composite soil
- Transparent plastic cup
- Growth chamber at 25 °C
- Pasteur pipettes 3 ml
- Razor blades
- Filters of 30 µm pore (CellTricks, Partec)

- Plastic tubes 3.5 ml, 55 x 12 mm (Ø x height)
- Flow cytometer Partec Ploidy Analyzer I

2.3. Solutions and culture media

- Ethanol 70% (v/v)
- Sodium hypochlorite 4 g/L with Tween 0.05% (v/v)
- Sterile distilled water (3 glass jars) autoclaved at 121 °C for 20 minutes
- Induction medium: C medium (Table 1) supplemented with 0.01 mg/L kinetin and 0.01 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D)
- Regeneration medium: R medium (Table 1), supplemented with 0.1 mg/L kinetine. Adjust the pH of media C and R to 5.9. Autoclave media at 121 °C for 20 minutes, and then pour it in 90 x 25 mm sterile Petri dishes
- Rooting medium: V3 medium (table 1). Adjust the pH to 5.9. Autoclave medium at 121 °C for 20 minutes and pour it in 90 x 25 mm sterile Petri dishes and sterile baby food jars (200 ml).
- Lysis buffer (LB01) (Dolezel et al. 1989): 5 mM Tris(hydroxymethyl)aminomethane, 2 mM Na_2EDTA, 0.5 mM espermine, 80 mM KCl, 20 mM NaCl, 15 mM β-mercaptoethanol and 0.1% (v/v) Triton X-100. The pH is adjusted at 7.5
- Staining buffer: 4,6-diamidino-2-phenylindole (DAPI) (Partec CyStain UV precise P, PARTEC GmbH)

Table 1. Macroelements, microelements and vitamins used in the three basal media in pepper anther culture (mg/L). C and R media from Dumas de Vaulx et al. (Dumas de Vaulx et al. 1981). V3 medium from Chambonnet (Chambonnet 1988).

	Medium C	Medium R	Medium V3
Macroelements			
KNO_3	2,150	2,150	1,900
NH_4NO_3	1,238	1,238	1,650
$MgSO_4 \cdot 7H_2O$	412	412	370
$CaCl_2 \cdot 2H_2O$	313	313	440
KH_2PO_4	142	142	170
$Ca(NO_3)_2 \cdot 4H_2O$	50	50	-
$NaH_2PO_4 \cdot H_2O$	38	38	-
$(NH_4)_2SO_4$	34	34	-
KCl	7	7	-
Microelements			
$MnSO_4 \cdot H_2O$	22.130	20.130	0.076
$ZnSO_4 \cdot 7H_2O$	3.625	3.225	1.000
H_3BO_3	3.150	1.550	1.000
KI	0.695	0.330	0.010
$Na_2MoO_4 \cdot 2H_2O$	0.188	0.138	-
$CuSO_4 \cdot 5H_2O$	0.016	0.011	0.030
$CoCl_2 \cdot 6H_2O$	0.016	0.011	-
$AlCl_3 \cdot 6H_2O$	-	-	0.050
$NiCl_2 \cdot 6H_2O$	-	-	0.030
Vitamins and aminoacids			
Myo-Inositol	100.00	100.00	100.00
Pyridoxin HCl	5.500	5.500	5.500
Nicotinic acid	0.700	0.700	0.700
Thyamine HCl	0.600	0.600	0.600
Calcium panthotenate	0.500	0.500	0.500
Vitamin B12	0.030	-	-
Biotin	0.005	0.005	0.005
Glycin	0.100	0.100	0.200
Chelated iron			
Na_2 EDTA	18.65	18.65	37.30
$FeSO_4 \cdot 7H_20$	13.90	13.90	27.28
Sucrose	30,000	30,000	30,000
Bacto-agar	8,000	8,000	8,000

3. Methods

3.1. Donor plant growth conditions

Donor plants are grown in a growth chamber at 25 °C, light intensity of 200 $\mu mol.s^{-1}.m^{-2}$ with a 16/8 photoperiod and 60-65% relative humidity.

3.2. In vitro culture of anthers

1. Select by eye the optimal buds for anther culture. In our genotypes, they are covered approximately the 80% of them by the sepals (Fig. 1A), according to Parra-Vega et al. (2013a) (see Note 1). Excise the buds from the plant. Bring them to the lab in plastic tubes immersed on melting ice (*see* Note 2).
2. Take the buds to the laminar flow hood.
3. Surface sterilize the buds with 70% ethanol for 30 sec, and then with sodium hypochlorite 4 g/L for 5 min, and finally 3 washes of 4 min each with sterile distilled water (see Note 3).
4. Place the buds over sterile Whatmann paper and excise them to extract the anthers (*see* Note 4). At this step, make a second selection of the buds. Culture only buds containing anthers with purple distal tips (Fig. 1E), according to Parra-Vega et al. (2013a). In case the optimal stage of anther development has not been well set up in advance for the genotype used, it is highly recommended, at this point, to check the microspores/pollen stage of every bud before culturing them (*see* Note 5).
5. Place the selected anthers in Petri dishes with C medium. Place them with their concave part in contact with the medium. Seal the dishes with Parafilm and introduce them in the incubator at 35 °C in darkness for 4 days (*see* Note 6).

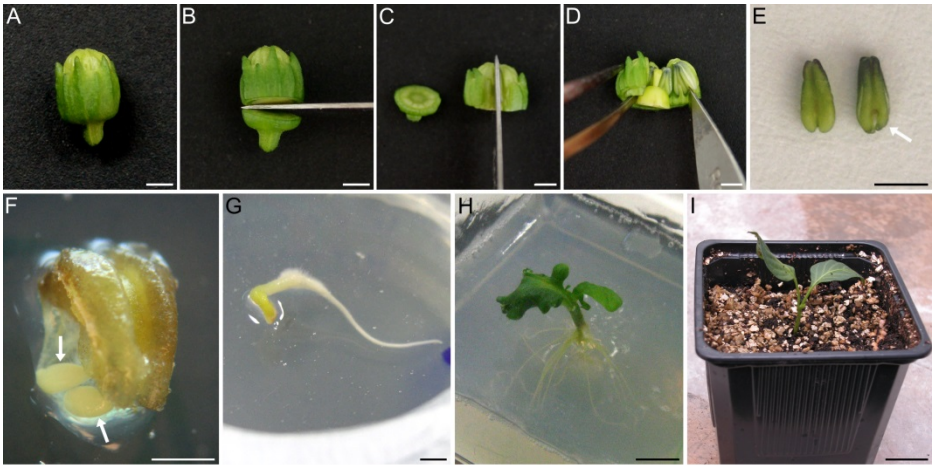

Figure 1. Process of anther culture in pepper. (A) Flower bud at the right stage for anther isolation. (B-D) Anther extraction out of the bud: (B) Transversal cut of the flower bud, (C) longitudinal cut of the flower bud surface and (D) opening of the flower bud with scalpel and forceps to extract the anthers. (E) Anthers at the right stage for isolation. White arrow points the right position to culture the anthers in medium (concave part). (F) Anther cultured in vitro producing two microspore-derived embryos (white arrows) in C medium. (G) Microspore-derived embryo germinated in V3 medium. (H) Microspore-derived seedling cultured *in vitro* in V3 medium. (I) Acclimated seedling cultured *ex vitro* in a plastic plant pot. Bars: A-E 2 mm; F, G 5 mm; H 1 cm; I 2 cm.

6. At day 4, place the dishes in the incubator at 25 °C, light intensity of 32 $\mu mol.s^{-1}.m^{-2}$ and 12/12 photoperiod for 4 days more.

7. At day 8, transfer the anthers to R medium and incubate them at 25 °C, light intensity of 32 $\mu mol.s^{-1}.m^{-2}$ and 12/12 photoperiod. Every 2 months, change the anthers to fresh R medium.

8. As soon as the embryos pop out of the anthers, pick them with forceps and transfer them to V3 medium in 90 x 25 mm Petri dishes, incubate them at 25 °C, light intensity of 32 $\mu mol.s^{-1}.m^{-2}$ and 12/12 photoperiod. Transfer the embryos that germinate correctly to sterile baby food jars with V3 medium (*see* Note 7).

9. When seedlings develop a proper root system (one or two primary roots and some secondary roots), transfer them to plastic plant pots with wet soil.

10. Acclimate the seedlings in the growth chamber at 25 °C and 16/8 photoperiod (*see* Note 8).

3.3. Analysis of the ploidy level

1. Analyze the nuclear DNA content with a flow cytometer (Partec Ploidy Analyzer I) according to its commercial specifications. Use DAPI as the fluorescent stain.

2. Use donor plants as control for 2C DNA content. Plants derived from embryos will be analyzed in order to know the ploidy level (*see* Note 9).

3. Excise young leafs from the plant and place them in a box with ice (*see* Note 10).

4. Chop with a razor blade a piece of 1 cm^2 of a young leaf in a plastic Petri dish containing 0.5 ml of lysis buffer (*see* Note 11).

5. Filter the extracted nuclei with a 30 µm pore filter into a 3.5 ml plastic tube.

6. Add 1.5 ml of DAPI staining buffer with a 3 ml Pasteur pipette.

7. Keep the tubes on ice for 2 minutes prior to analyze the samples using the flow cytometer. Count a minimum of 10,000 cells per sample.

4. Notes

1. The selection of anthers is one of the critical steps of anther culture. The anthers must contain vacuolate microspores and young bicellular pollen grains to efficiently induce embryogenesis. As this parameter determination is highly genotype-dependent, it is recommended to study previously, in each genotype,

the right size and appearance of anthers containing the appropriate stage of microspore/pollen development to be induced towards embryogenesis.

2. Once the buds are excised from the plant, keep them on ice in order to slow down the development of the microspores/pollen. Also, keep the sterilized solutions at 4 °C before using them to reduce the degradation process of anthers.

3. Pour the sterilized solutions into the plastic tube, close the lid and shake the solutions during the corresponding time for each solution. After that, open the lid and remove the liquid keeping the buds. Pour the next solution into the tube and repeat the process. An alternative to the plastic tubes is to use tea filter sieves.

4. Excise the anthers with a scalpel avoiding to break them. First, make a transversal cut at the basal part of the bud (near to the pedicel), removing the basal part of the floral bud (Fig. 1B). Second, make a longitudinal cut, only at the surface of the bud (Fig. 1C), to open the sepals. Later, take away the sepals and petals with forceps, and extract the anthers (Fig. 1D). It is important to remove the anther filament as much as possible, just to avoid callus formation from this tissue, which is especially prone to proliferate.

5. After extracting the anthers from the bud, take one anther to observe it under the microscope and keep the remaining anthers waiting in the laminar flow hood. Place the anther onto a microscope slide with a drop of water, chop the anther with a razor blade in order to extract the microspores/pollen and cover it with a standard cover slip. Observe the preparation under a light or inverted microscope checking the stages of microspores contained. If the anther contains mostly vacuolate microspores and young bicellular pollen, the rest of anthers from the same bud will be used for anther culture.

6. Cover the Petri dishes with aluminum paper to create a darkness environment inside the incubator.

7. Transfer the germinated embryos to baby jars in order to increase the space to develop the roots and aerial parts of the new plant.

8. In order to avoid drastic change in humidity conditions, use a transparent plastic cup to protect the seedlings. Pinch holes in the cup every 2 days, to gradually reduce the humidity inside the cup down to the levels of the growth chamber. Then, remove the glass.

9. The flow cytometer is used to analyze the ploidy level, but when a 2C individual appears, molecular analysis marker (preferentially SSRs) has to be performed in order to clarify whether this individual has a somatic or an embryogenic origin. For donor plants polymorphic for the SSR used, if the regenerated samples analyzed are homozygous for the used molecular markers, the origin of these plants will be gametophytic. However, if the samples are heterozygous for the SSRs used, their origin will likely be somatic (most likely coming from anther wall tissues).

10. Young tissues are used to analyze the ploidy level because these tissues present more cells in G2 phase; therefore the second peak of the histogram appears clearer.

11. The nucleic extraction buffer from Partec (CyStain UV precise P, PARTEC GmbH) may be used at this step. However, with pepper is recommended to use the lysis buffer in order to slow down the oxidizing process of pepper samples.

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Bloque II

Estudio de un sistema androgénico modelo: *Brassica napus*

La colza (*Brassica napus* L.) es un cultivo perteneciente a la familia *Brassicaceae*. Esta especie tiene una gran importancia económica a nivel mundial debido a los múltiples usos que presenta: Es utilizada como forrajera para alimento de ganado, se está empleando para la producción de biodiesel y sus semillas son empleadas para la producción de aceite vegetal para el consumo humano. La producción a nivel mundial en 2012 ascendió a 64.563.586 t., siendo Canadá el país que presentó una mayor producción con 15.409.500 t. Dentro de los cultivos oleaginosos ocupa el cuarto puesto en producción mundial y el tercer puesto en términos de área cosechada (34.101.512 ha) (FAOSTAT, 2014). Además de su importancia económica como cultivo oleaginoso, la colza también resulta muy interesante desde un punto de vista científico, ya que puede ser empleada para el estudio de diferentes procesos biológicos relacionados con la embriogénesis (Chupeau et al., 1998; Friedt y Zarhloul, 2005; Seguí-Simarro y Nuez, 2008; Soriano et al., 2013). Hace mas de 30 años que se publicó el primer protocolo para obtener DHs en esta especie (Lichter, 1982). Desde entonces, se han ido implementando los protocolos que permiten obtener, de forma rutinaria, un elevado número de embriones derivados de microsporas que regeneran plantas completas (Huang et al., 1991; Telmer et al., 1992; Mollers et al., 1994; Custers, 2003). Esta elevada eficiencia embriogéncia, junto con la facilidad de obtener buenos controles no embriogénicos, explica que los cultivos de microsporas aisladas de colza se utilicen en múltiples estudios de investigación básica (Custers et al., 2001). Los cambios que sufren las microsporas, tanto a nivel molecular como ultraestructural, debido a la inducción de la androgénesis, han sido ampliamente estudiados en las últimas décadas (Boutilier et al., 2005; Maraschin et al., 2005; Seguí-Simarro, 2010a). Mediante estudios

ultraestructurales realizados en colza, se ha demostrado que las microsporas recién inducidas sufren una serie de cambios, celulares y moleculares, típicos de esta nueva ruta de desarrollo. Algunos de estos estudios confirmaron que las microsporas inducidas sufren una reestructuración del patrón de división, y relacionaron la división simétrica de las microsporas con el proceso de inducción (Zaki y Dickinson, 1991; Telmer et al., 1995). Se vio que se formaba una banda pre-profásica de microtúbulos en el centro de la microspora. Esta banda estaba marcando la posición de la nueva pared celular que separaría las dos células hijas. Se comprobó que durante la primera mitosis del polen esta banda no se desarrollaba, dejando claro que esta primera división simétrica era un rasgo típico de la inducción hacia embriogénesis (Simmonds y Keller, 1999; Gervais et al., 2000). Estudios recientes han demostrado que la división simétrica no es una característica esencial para que tenga lugar la embriogénesis derivada de microsporas, pudiendo obtenerse embriones en aquellas estructuras que sufren divisiones asimétricas (Tang et al., 2013; Soriano et al., 2014). Otros autores han empleando la colza para estudiar los cambios generados en la expresión génica durante la inducción a embriogénesis. Se han identificado genes, como el *Baby Boom* (BBM), que se expresa en embriones cigóticos y en semillas, y también durante el desarrollo temprano de los embriones derivados de microsporas. Se ha visto que tiene un papel esencial en su desarrollo, promoviendo la división celular y la morfogénesis (Boutilier et al., 2002). Se ha comprobado también, que durante la reprogramación se están expresando genes implicados en la respuesta celular al estrés. Un ejemplo de ello, es la familia de proteínas de choque térmico Hsp70, que se demostró que incrementaba sus niveles de translocación debido a la inducción a embriogénesis (Cordewener et al., 1995; Testillano et

al., 2000; Seguí-Simarro et al., 2003). También se vio que en el momento de la inducción y hasta 5 días más tarde, en microsporas inducidas se expresaban de manera simultánea, genes específicos de polen y de embriones, demostrando que durante los primeros días de la reprogramación, están conviviendo los dos programas de desarrollo, el gametofítico y el embriogénico (Malik et al., 2007). *B. napus* ha sido empleada también para elaborar estudios en los que se ha llevado a cabo una comparativa, a nivel transcriptómico y metabolómico, entre embriones derivados de microsporas con suspensor, que siguen un modelo de desarrollo similar al cigótico, y embriones sin suspensor, que presentan un patrón de desarrollo inicial mas desorganizado (Joosen et al., 2007). Se cree que el papel del suspensor en los embriones derivados de microsporas juega un papel esencial en el correcto desarrollo del embrión y en el establecimiento de su polaridad (Supena et al., 2008). Estudios recientes han demostrado que, en los embriones derivados de microsporas que presentan suspensor, el transporte de auxinas es impulsado desde el suspensor y juega un papel esencial en el desarrollo embriogénico. Al mismo tiempo, se ha descubierto que en aquellos embriones que no presentan suspensor y sufren una diferenciación celular irregular, este crecimiento es independiente del transporte de auxinas y la polaridad del embrión parece estar determinada por la ruptura y posición de la exina (Soriano et al., 2014). Li et al. (2014) publicaron recientemente un importante avance en el conocimiento de los mecanismos que provocan la inducción de la androgénesis, demostrando que la totipotencia del gametofito masculino está controlada por la expresión o represión de ciertos genes, a su vez mediada por un mecanismo epigenético de acetilación de histonas. Las histonas deacetilasas (HDAC) son los enzimas encargados de la desacetilación de las histonas y por tanto de inactivar dichos genes. Mediante el uso de

Tricostatina A (TSA, inhibidor de las HDAC) bloquearon la actividad de las HDAC, logrando incrementar el porcentaje de células que se inducían, además de ampliar el rango del estadio óptimo para la inducción. Estos estudios concluyen que los tratamientos de estrés empleados para inducir la reprogramación hacia embriogénesis, pueden estar incidiendo (aunque quizás en distinta medida) en los mismos procesos que se activan al inhibir la función de la HDAC.

En la presente Tesis Doctoral se ha profundizado en el estudio de la colza como modelo para conocer mejor el proceso de inducción. En los capítulos de este bloque, se ha empleado la colza para analizar en profundidad algunos de los cambios celulares que sufren las microsporas cuando son reprogramadas hacia una nueva ruta embriogénica. Nos hemos centrado en los cambios que tienen lugar a nivel de la pared celular y a nivel del citoplasma, centrándonos en los plastidios y las mitocondrias.

Bloque II

Capítulo 4

The induction of embryogenesis in *Brassica napus* microspores is associated to the occurrence of abnormal cell walls with altered levels of callose and cellulose

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Keywords: *A*ndrogenesis, cytokinesis, confocal microscopy, electron microscopy, microspore embryogenesis.

Abstract

In this work we analyzed the composition of the cell walls present in all the structures formed in *B. napus* microspore cultures. We focused on the deposition of two polysaccharides essential for cell wall biosynthesis, callose and cellulose, at different stages of microspore embryogenesis, including before, during and after the inductive treatment. Samples fixed by high pressure freezing and freeze substitution (HPF/FS) were observed by transmission electron microscopy (TEM) and revealed that embryogenic microspore presented discontinuous cell walls and incomplete cell plates. Anti-callose immunogold labeling and confocal microscopy of samples stained with callose and cellulose-specific stains, revealed abnormal development of the new cell walls of the induced microspores. Massive deposition of callose and absence of cellulose was observed in embryogenic microspores. Callose was localized in a thick layer between the intine and the plasma membrane (the subintinal layer), in the developing cell plates and in the new cell walls. Pollen-like structures and microspore-derived embryos showed cell walls with cellulose only, as expected for somatic-type cell walls. Our work reveals that, at the initial stages of embryogenesis, callose is not replaced by cellulose during cell wall formation, which leads to the formation of callose-rich, abnormal cell walls. These cell walls may be used as early markers of microspore embryogenesis, and could explain some of the features observed in induced microspores, including nuclear fusion and genome duplication.

Introduction

Microspore embryogenesis is a fascinating experimental process whereby a haploid microspore is reprogrammed to become a haploid or doubled haploid (DH) embryo. This inducible androgenic pathway has a great biotechnological potential, since it allows for faster and cheaper ways to obtain pure lines for hybrid seed production. In addition, it offers the possibility to be used as an *in vitro* model to study different basic and induced processes. Indeed, the androgenic switch is induced by the application of different types of abiotic stresses, including heat shock, cold, and starvation, among others (Shariatpanahi et al. 2006). Once induced, the cellular responses to abiotic stresses coexist with a developmental switch towards embryogenesis, and with the cessation of the old gametophytic program. Conceivably, all these changes must imply a profound remodeling at the genetic and molecular levels, and also in cell architecture. Among all the changes undergone by the embryogenic microspore, one of the aspects that attracted the attention of the first cell biologists that studied this process was how induced cells are divided.

In conventional, somatic-type Arabidopsis plant cells, the first structural marker of cell division is the microtubular pre-prophase band (PPB), which defines the future division plane (Pickett-Heaps and Northcote 1966). Later on, cytokinesis starts in parallel to the end of mitosis. By late anaphase, chromosome separation is paralleled by the formation of the phragmoplast initials from groups of residual spindle microtubules, and of dumbbell-shaped cell plate-forming vesicles (Austin et al. 2005; Seguí-Simarro et al. 2004). At early telophase, a tubulo-vesicular cell plate network is assembled by a solid phragmoplast that forms a cylinder at the cell equatorial plane. At mid

telophase, a ring-shaped transitional phragmoplast marks the transformation of the central region of the cell plate into a wide tubular network and then into a maturing, planar fenestrated sheet, while the actively growing peripheral growth zone expands centrifugally and eventually fuses with the mother cell wall. Finally, at late telophase the peripheral zone matures too, and the cell plate is transformed into a new cell wall (reviewed in Seguí-Simarro et al. 2008). These orchestrated changes in cell plate structure are accompanied by the deposition of different polysaccharides in a timely manner. Whereas some of them, such as pectins, hemicelluloses and arabinogalactan proteins (AGPs), are produced by enzymes in the Golgi apparatus and delivered via vesicles to the cell plate, others like callose and cellulose are produced *in situ* by callose and cellulose synthase complexes, respectively, located in the cell plate membrane (reviewed in Worden et al. 2012). Therefore, the first polysaccharides present in the nascent cell plate would be pectins and hemicelluloses. Then, the tubulo-vesicular network (TVN) cell plate is transformed into a maturing tubular network by the deposition of copious amounts of callose in the cell plate lumen (Seguí-Simarro et al. 2008), which is also responsible for the widening of the cell plate tubules into fenestrated sheets (Samuels et al. 1995). It was recently proposed that in addition to callose, small amounts of cellulose are also produced and deposited at the TVN stage, which could contribute to flatten and strengthen this already unstable structure (Miart et al. 2013). The final transformation of the planar fenestrated sheet-type cell plate into a new primary cell wall involves the progressive replacement of callose deposits by cellulose fibrils (Kakimoto and Shibaoka 1992; Otegui and Staehelin 2000; Samuels et al. 1995). Proper cellulose deposition appears essential for cell plate stabilization, as revealed by the aborted cell plates present in cellulose-deficient mutants (Beeckman et al. 2002; Zuo et al. 2000). Finally, cellulose

combines with the already secreted hemicellulose molecules into a cellulose-hemicellulose network, while pectic polysaccharides reorganize to form the pectin-rich middle lamella (Carpita and McCann 2000). In the final, somatic-type primary cell wall, callose is restricted to the region around plasmodesmata, where it is supposed to play a regulatory role in cell-to-cell movement of molecules (Levy et al. 2007).

Based on this canonical pattern, some specialized cell types have developed alternative division mechanisms adapted to their function. This is the case, for example, of microspores. The first pollen mitosis (PMI) that transforms a microspore into a young pollen grain is characterized by the absence of a previous PPB (van Lammeren et al. 1985), and by the building of an asymmetric phragmoplast (Brown and Lemmon 1991), giving rise to the large, vegetative cell and the small, generative cell of the pollen grain. The cell wall formed around the generative cell is also special, since it is hemispherical and transiently rich in callose (Park and Twell 2001). However, soon it was found that microspores committed to *in vitro* embryogenesis significantly diverge from these cell division patterns. First, embryogenic microspores usually divide symmetrically, as opposed to *in vivo* microspores, in a mechanism more similar to somatic-type cytokinesis that to PMI cytokinesis (Zaki and Dickinson 1991). Accordingly, it was found that embryogenic microspores show the typical PPB of somatic-type dividing cells (Simmonds and Keller 1999). The cell walls of induced microspores and microspore-derived embryos (MDEs) present other unique features. For example, it was recently shown that embryogenic microspores exhibit abundant deposits of excreted cytoplasmic material (Corral-Martínez et al. 2013). These walls are also characterized by altered levels of certain cell wall components, such as

xyloglucans, pectins (Barany et al. 2010) and arabinogalactan proteins (El-Tantawy et al. 2013). The major cell wall polymer, cellulose, has also been investigated, but unfortunately, the results are somehow confusing. For example, Schulze and Pauls (2002) assumed that the first cell walls produced in *B. napus* embryogenic microspores are somatic-type and therefore, cellulose-rich. In olive, Solis et al. (2008) described the presence of cellulose in some embryogenic microspores, at both the inner cell walls and the *"thick peripheral wall localized below the exine"*. However, Dubas et al. (2013) recently demonstrated by calcofluor white staining that cellulose was absent from the first cell walls formed in few-celled *B. napus* embryogenic microspores, whereas older MDEs presented abundant cellulose signal in their walls.

The other major cell plate-forming polymer, callose, is a β-1,3-glucan essential not only for somatic-type cell plate formation. Callose synthesis is typically triggered as a cellular response to biotic and abiotic stresses including pathogen attack, wounding, or high temperatures (reviewed in Chen and Kim 2009; Stone and Clarke 1992). During microspore and pollen development, callose is the main component of the transient post-meiotic cell walls at the tetrad, prior to the release of the microspores (Heslop-Harrison and Mackenzie 1967; Otegui and Staehelin 2004). During microspore development, the deposition of a thin callose layer below the exine was proposed to have a role in exine patterning (Dong et al. 2005). In pollen grains, callose forms a transient wall around the generative cell (Park and Twell 2001; Toller et al. 2008), and during pollen tube growth it is actively synthesized at the growing apex (Meikle et al. 1991) as well as in the plugs formed along the tube after pollen germination (Steer and Steer 1989). In the particular case of the first embryogenic divisions of the microspore, Telmer et al. (1995) found aniline

blue staining at the connections of the new cell wall with the mother cell wall, attributing it to alterations in the plasma membrane or the cell wall physiology. Other than that, little is known about the role of callose in this process.

In this work we present a detailed study of the cell walls present in *B. napus* microspores during the initial stages of their induction towards embryogenesis. We analyzed a collection of EM images obtained from samples processed by high pressure freezing and freeze substitution (HPF-FS), which is known to be the best procedure to preserve the even the most labile and/or transient ultrastructural elements of *in vivo* physiological processes (Gilkey and Staehelin 1986), as well as during the androgenic switch (Corral-Martínez et al. 2013). Our samples covered all the stages of *Brassica napus* microspore embryogenesis, the different cell types formed, and the subcellular changes undergone as a consequence of embryogenesis induction. Together, our results shed light on the role and interactions of callose and cellulose during the initials of the androgenic switch.

Material and methods

Plant materials

B. napus L. donor plants of the highly embryogenic cv. Topas were grown as previously described (Custers 2003). Plants were grown in the greenhouses of the COMAV Institute (Universitat Politècnica de València, Valencia, Spain), the University of Colorado (Boulder, CO, USA), and the Plant Research International (Wageningen, The Netherlands), at 20ºC under natural light.

B. napus microspore culture

Flower buds containing mostly vacuolate microspores were selected as previously described (Custers 2003), surface sterilized with 2% sodium hypochlorite for 10 min, and washed three times in sterile distilled water. To release the microspores, buds were gently crushed in filter sterilized NLN-13 medium with the back of the plunger of a disposable 50 ml syringe. NLN-13 medium (Lichter 1982) consists of NLN medium + 13% sucrose. Then, the slurry was filtered through 30 µm nylon cloths. The filtrate was transferred to 15 ml conical tubes and centrifuged at 800 rpm for 3 min. After discarding the supernatant, the pellet of microspores was resuspended in 10 ml of fresh NLN-13 medium. This procedure was additionally repeated twice for a total of three centrifugations and resuspensions. Before the last centrifugation step, microspore concentration was calculated using a hemacytometer. The required volume of NLN-13 medium was added to adjust suspension to a concentration of 4×10^4 microspores per ml. Adjusted microspore suspension was distributed in sterile culture dishes. Dishes were incubated in darkness for 24h at 32ºC to induce embryogenesis, and then continuously at 25ºC for embryogenesis progression. Culture dishes at different stages, as described in Results, were collected and processed by HPF/FS.

Callose and cellulose detection for confocal laser scanning microscopy

Samples of *B. napus* microspore cultures were collected at day 1, 2, 3, 4 and 6 after isolation, and fixed with 4% formaldehyde in PBS pH 7.4. Samples were placed in glass slides with 0.9% agarose. For callose detection, samples were stained with 0.1% aniline blue (Evans et al. 1984) dissolved in 0.1 M PBS for 20 min. After three washes with PBS, some of the samples were stained

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

For cellulose detection, we used two different fluorescent stains, Calcofluor White ST and Pontamine Fast Scarlet (S4B). Calcofluor White was used diluted to 0.05% in water (Dubas et al. 2013). PI was also used as described above to provide a better identification of the developmental stage of the microspore. Both stains were added directly to the fixed microspores and slides were kept in darkness for at least 15 min before microscopic analysis. For S4B staining, samples were stained with 0.01% S4B in 0.1 M PBS for 30 min (Anderson et al. 2010), then washed thrice with PBS, and finally incubated with DAPI according to Custers et al. (1994) to stain the nuclei. Double staining with aniline blue and S4B was also performed as described above but combining both stains at 0.05% and 0.01%, respectively. Finally, slides were mounted with Vectashield and observed with a Leica CTR 5500 Confocal Laser Scanning Microscope equipped with 40x (n.a. 1.15) and 63x (n.a. 1.30) oil-immersion lenses. For visualization of aniline blue staining, samples were excited at 405 nm and the emission window was set at 455-489 nm. For visualization of PI staining, samples were excited at 532 nm and the emission window was set at 583-703 nm. For visualization of DAPI and calcofluor white staining, samples were excited at 365 nm and the emission window was set at 397 and 420 nm respectively. For visualization of S4B staining, samples were excited at 566 nm and the emission window was set at 566-719 nm. Digital images were

processed with Leica Application Suite Advanced Fluorescence (LAS AF) software.

Processing of B. napus anthers and microspore cultures for transmission electron microscopy

Anthers carrying microspores and pollen grains at different stages of microsporogenesis and microgametogenesis were excised, transferred to aluminum sample holders, cryoprotected with 150 mM sucrose, frozen in a Baltec HPM 010 high-pressure freezer (Technotrade, Manchester, NH), and then transferred to liquid nitrogen. Cultured microspores and small MDEs were recovered from culture dishes by gently spinning culture media. Larger MDEs were manually picked up from cultures. These samples were transferred to aluminum sample holders, cryoprotected with their same glucose-rich culture medium and frozen in a high-pressure freezer as described above. The samples were then freeze substituted in a Leica AFS2 system (Leica Microsystems, Vienna) in 2% OsO_4 in anhydrous acetone at -80ºC for 7 days, followed by slow warming to room temperature over a period of 2 days. After rinsing in several acetone washes, they were removed from the holders, incubated in propylene oxide for 30 min, rinsed again in acetone, and infiltrated with increasing concentrations of Epon resin (Ted Pella, Redding, CA) in acetone according to the following schedule: 4 h in 5% resin, 4 h in 10% resin, 12 h in 25% resin, and 24 h in 50, 75, and 100% resin, respectively. Polymerization was performed at 60ºC for 2 days in a vacuum oven. 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-coated copper, 200 mesh grids, stained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron

microscope at the Electron Microscopy Service of Universitat Politècnica de València.

Processing of tomato meiocytes for transmission electron microscopy

Tomato anthers carrying meiocytes were selected according to the criteria used in Seguí-Simarro and Nuez (2005) and processed according to Seguí-Simarro and Nuez (2007). Briefly, samples were fixed in Karnovsky fixative + 2% OsO4 for 4 h at room temperature, dehydrated in acetone series and embedded in Epon resin. Ultrathin (80 nm) sections were obtained, mounted onto copper grids, counterstained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron microscope.

Immunogold labeling

For the immunodetection of callose we used an anti-callose monoclonal antibody (mouse IgG) crossreacting with linear β-1,3-oligosaccharide segments in β-1,3-glucans (Meikle et al. 1991). Immunogold labeling was performed in OsO_4-treated, epoxy-embedded samples. This type of processing is not usually employed for immunogold labeling because it may preclude the immunolocalization of protein epitopes. Since all the antibodies we used in this work were raised against carbohydrate epitopes, we used these samples for immunolocalization in order to combine specific immunolabeling with the excellent ultrastructural images provided by the use of OsO_4 and epoxy resins when combined with HPF-FS. However, it must be noted that the hydrophobic nature of epoxy resins makes gold particle labeling density relatively lower when compared with samples embedded in hydrophilic resins. Epon sections (80-100 nm) were deposited on Formvar and carbon-coated, 200-mesh nickel grids (Electron Microscopy Sciences). Sections were hydrated with distilled

water for 1 min, 1x PBS for 1 min, and blocked with 5%BSA in PBS for 5 min. Then, sections were incubated for 1 h at 25ºC with anti-callose antibody, diluted 1:5,000 in 1%BSA. Next, sections were subjected to three 4-min washes with PBS and incubated for 45 min at 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 subjected to three 4-min washes with PBS and one with distilled water. Finally, sections were counterstained with 0.5% uranyl acetate in 70% methanol and lead citrate, 10 min each.

Results

Embryogenic microspores occasionally present discontinuous, incomplete cell plates and nuclear fusion profiles

Embryogenic microspores were characterized by a larger size, compared with non-induced microspores, and by the presence of 2 or more cells separated by visible cell walls. Some of these embryogenic microspores presented continuous cell walls morphologically similar to conventional walls with the only exception of the irregularities and deposits of excreted material (arrowheads in Figure 1A) previously described (Corral-Martínez et al. 2013). However, we also observed often dividing cells with abnormal cell plates and cell walls. Abnormal cell plates were characterized by an irregular architecture, with numerous tubular profiles and abundant openings (fenestrae) that permitted the contact between the cytoplasm of daughter cells (arrows in Figure 1B). In conventional cell plates, planar fenestrated profiles usually arise at mid stages of cytokinesis. These stages are defined by the presence of a transitional, ring-shaped phragmoplast where microtubules depolymerize at the central cylinder but remain at the periphery where the cell plate connects

with the maternal cell wall (Austin et al. 2005). At these stages, Golgi-derived vesicles are principally targeted to the growing edges of the cell plate, and not to the maturing central part (Seguí-Simarro et al. 2004). However, in the unusual cell plates of embryogenic microspores such a ring phragmoplast was absent, as revealed by the close proximity of cell organelles to both central and peripheral domains of the cell plate (Figures 1A, B). In addition, the few Golgi-derived vesicles identified were observed randomly dispersed throughout the tubular cell plate (Figure 1B). These data would be indicating that in these cells, the final stages of cytokinesis (the formation of a planar fenestrated sheet) would be disturbed. In parallel, we identified embryogenic, yet divided microspores with binucleated cells (Figure 1C). We also found cells containing nuclei larger than usual, and occasionally with a peanut-like morphology (Figure 1A), indicative of the recent occurrence of nuclear fusion events (Corral-Martínez et al. 2011; Seguí-Simarro and Nuez 2007, 2008).

Thus, we analyzed embryogenic microspores at previous stages of cell division in order to identify the initials of this unusual cytokinetic behavior. A detailed observation of mitotic cells (Figure 2A) did not reveal any structural abnormality with respect to what is considered the normal architecture of the mitotic machinery. As seen in Figure 2B, metaphasic cells presented the typical condensed chromosomes and an organelle-free region where the abundant parallel microtubules of the mitotic spindle could be identified. Images of cells showing the microtubular scaffold of the mitotic spindle and the solid phragmoplast appeared normal, as well as the cell plate initials that appeared correctly assembled (data not shown). Later on, at early telophase, the tubulo-vesicular network typical from cell plates at the solid phragmoplast stage, was

clearly identified together with abundant vesicles and microtubules near the cell plate (Figure 2C).

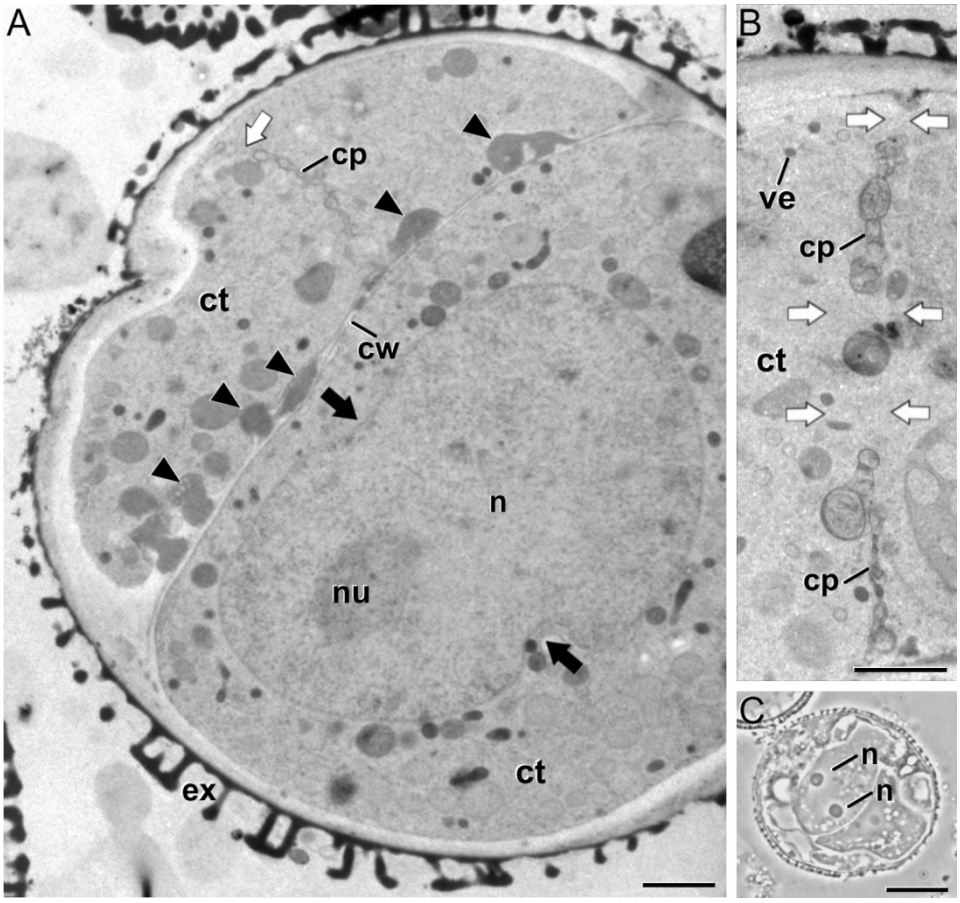

Figure 1: Embryogenic *B. napus* microspores with abnormal cell plates and signs of nuclear fusion. A: Induced microspore with a complete cell wall (cw) with numerous deposits of excreted material (arrowheads), a gapped cell plate (cp), and a peanut-shaped nucleus (n). The black arrows point to a nuclear constriction indicative of a recent nuclear fusion. B: Abnormal, incomplete cell plate with abundant gaps (white arrows) that connect the cytoplasms of the daughter cells. C: Embryogenic microspore with two nuclei coexisting in the same cytoplasm. ct: cytoplasm; ex: exine; nu: nucleolus; ve: Golgi-derived vesicle. Bars: A: 10 µm; B: 1 µm; C: 200 nm.

Together, these observations pointed to the existence of problems during late stages of cytokinesis, but not before. It seemed that at the ring

phragmoplast stage, where cell plate flattening and maturation starts, cytokinesis was somehow blocked and incomplete cell walls were finally formed.

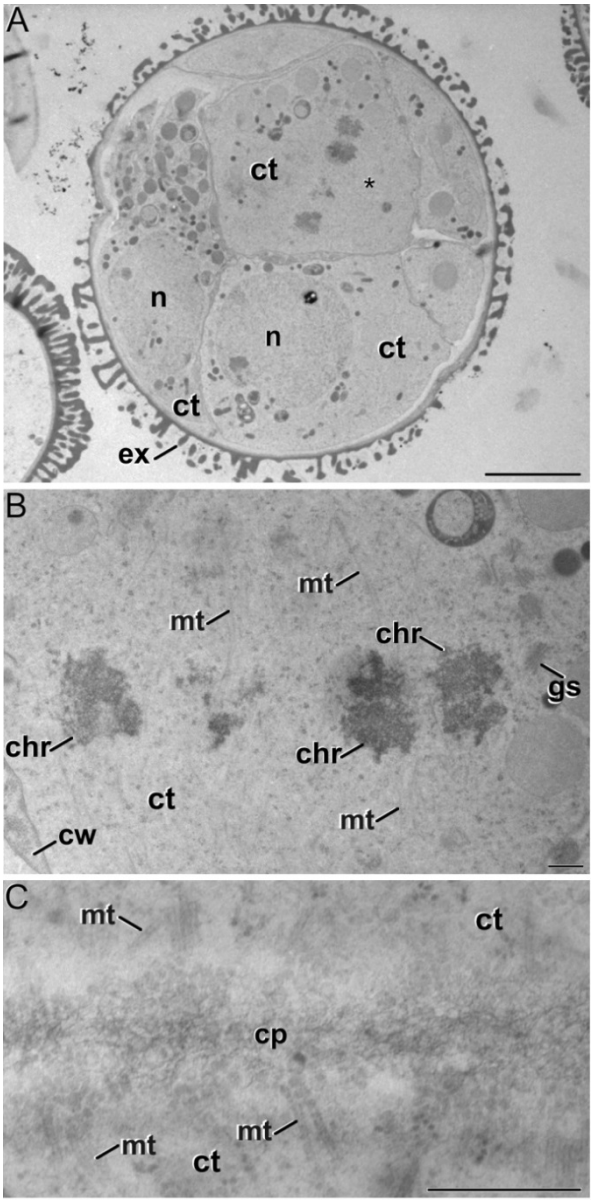

Figure 2: Dividing cells of *Brassica napus* embryogenic microspores. A: Overview of a multicellular microspore with a dividing cell (asterisk) at metaphase. B: Magnification of the metaphasic chromosomes (chr) at the cell equator. C: Tubulo-vesicular network cell plate (cp) of a dividing cell at early telophase. ct: cytoplasm; cw: cell wall; ex: exine; gs: Golgi stack; mt: microtubules; n: nucleus. Bars: A: 5 µm; B-C: 500 nm.

The presence of a thick, distinct layer beneath the intine is a differential feature of embryogenic microspores

Four days after induction, we observed that cultured microspores presented an extra layer deposited just between the intine and the plasma membrane (Figure 3A). From now on, we will refer to this layer as the subintinal layer. The subintinal layer is defined as an additional layer, continuous but of irregular thickness, alternating thick and thin domains, and with an electron light appearance in osmium-treated, Epoxy-embedded samples. Such a layer, with all of the mentioned features, was exclusively observed in all of the studied embryogenic microspores (Figure 3B). Some embryogenic microspore cells also developed an additional layer of electron dense material just beneath the subintinal layer (arrows in Figure 3C). This layer was discontinuous or even absent in certain cells. Its appearance, absolutely different from the subintinal layer, indicated the subsequent deposition of a material different from that composing the subintinal layer. Pollen-like structures also presented a thickened inner layer (Figure 3D). However, this layer was homogeneous in thickness and very similar to the intine, at least in terms of electron density. Indeed, at low magnification the coat of pollen-like structures appeared to be composed by the exine and a thickened intine, in contrast to the three layers of embryogenic microspores, i.e. exine, intine and subintinal layer (compare Figures 3D and 3B). This suggested an independent nature for the subintinal layer. As expected, this layer was absent from young microspores and pollen grains not subjected to isolation and induction treatments (Figures 5A and B, respectively). Therefore, it seemed that the subintinal layer is a structure assembled exclusively in embryogenic microspores. Since both pollen-like structures and embryogenic microspores

were exposed to the inductive heat-shock and only the latter developed an additional subintinal layer, we deduced that this layer would not be a direct consequence of the exposure to the heat shock. Instead, it should be related to the change in developmental fate.

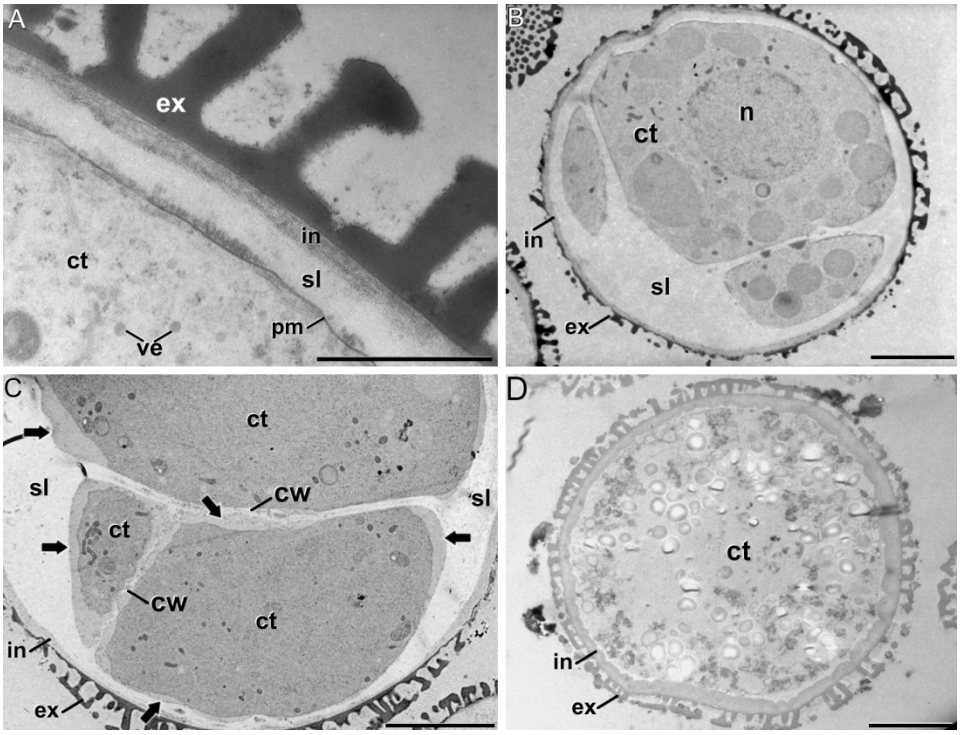

Figure 3: The subintinal layer of *B. napus* embryogenic microspores four days after induction. A: Electron light layer (sl) deposited beneath the intine (in) of a embryogenic microspore. B: Overview of an embryogenic microspore showing a continuous subintinal layer. C: Embryogenic cells with dense cell wall material (arrows) deposited after the subintinal layer. D: Pollen-like structure showing a thickened intine, uniform and more electron dense than the subintinal layer. ct: cytoplasm; cw: cell wall; ex: exine; n: nucleus; ve: Golgi vesicules; pm: plasma membrane. Bars: A: 200 nm, B-D: 10 µm.

Embryogenic microspores present callose-rich subintinal layers and cell walls

The subintinal layer of embryogenic microspores had in general a texture resembling the callose-rich wall of meiocytes (Supplementary Figures S1A, B), as it can be deduced by comparing Figure 3B and Supplementary Figure S1C. For this reason, we investigated the callosic nature of this layer by two parallel approaches: staining with aniline blue, a callose-specific fluorescent dye, and immunogold labeling with anti-callose antibodies. Just after the induction treatment, abundant aniline blue fluorescence was found at specific regions throughout the embryogenic microspore. Figure 4 shows a series of confocal slices from a representative embryogenic microspore three days after induction, covering different planes along it. In this series, it appears clear that callose accumulated at the subintinal layer below the exine. However, this accumulation was not uniform. As also observed in TEM images of whole embryogenic microspores (Figure 3B), wide regions alternated with thin regions. Wide regions showed bright fluorescence signal and thin regions presented a faint or even null fluorescence (Figure 4). After studying different aniline blue-stained embryogenic microspores, we were unable to identify any particular pattern of accumulation at specific regions of the subintinal layer. Interestingly, we also found aniline blue fluorescence at the newly formed cell walls separating daughter cells in embryogenic microspores (Figure 4). As for the subintinal layer, fluorescence at the new cell walls was not uniform, indicating a differential callose accumulation at different cell wall domains. On the contrary, no evident aniline blue fluorescent signal was observed at the thickened coat of pollen-like structures (Supplementary Figure S2A). In cell walls of small globular MDEs no spots were observed in general (Supplementary Figure S2B). Therefore, we confirmed the unusual and

transient accumulation of callose in embryogenic microspores, both in the subintinal layer as well as in the first newly formed cell walls.

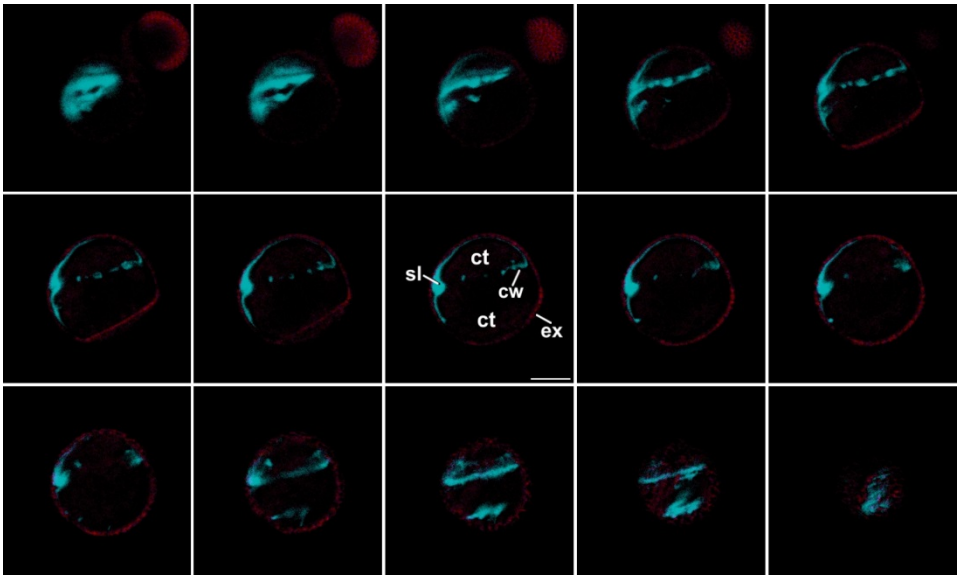

Figure 4: Callose staining with aniline blue. Confocal Z-series of a *B. napus* embryogenic microspore at day 3 after isolation. The blue signal corresponds to aniline blue staining and the red signal corresponds to exine (ex) autofluorescence. ct: cytoplasm; cw: cell wall; sl: subintinal layer. Bar: 10 µm.

Then, we performed a more comprehensive study at different stages before induction (during *in vivo* microspore development), 3-4 days after induction, and 10 days after induction (in MDEs) by means of immunogold labeling with anti-callose antibodies. As a control of the specificity of the anti-callose antibodies we used tomato meiocytes (Supplementary Figure S1C), where immunogold particles intensely decorated the callosic walls, both inner and peripheral, with a nearly null signal (background) in any other cell compartment (Supplementary Figure S1D). As expected, controls excluding the primary antibody provided no labeling at all (data not shown). Vacuolate

microspores, during their in vivo development within the anther, showed almost no callose at their coat (Figure 5A). The exine was completely devoid of labeling and only clusters of 2-3 gold particles could be rarely observed at the intine, usually close to the plasma membrane, and probably corresponding to the thin callose layer necessary for exine formation and pollen viability (Dong et al. 2005). Pollen grains maturing within the anther showed no labeling at any layer of the pollen coat (Figure 5B). Only in the case of mature pollen, anti-callose labeling was observed at the intine region just below the pollen apertures (data not shown). After induction, the few cells enclosed within the embryogenic microspores presented a specific anti-callose labeling throughout the subintinal layer (Figure 5C). In contrast, the thickened inner layer of pollen-like structures (Figure 5D) showed no labeling (except for the original aperture region, as observed in pollen grains within the anther). This different labeling pattern between embryogenic microspores and pollen-like structures clearly correlated with their previously mentioned differences at the ultrastructural level. In addition to their different thickness and electron density, these layers also differed in callose contents.

Anti-callose antibodies also decorated the new cell walls created after the first embryogenic divisions. Young, developing cell plates (Figure 5E) but also older, mature cell walls (Figure 5F) showed immunogold labeling dispersed throughout the wall. Callose is a common component of developing somatic-type cell plates, but is progressively replaced by cellulose during the maturation stages (Samuels et al. 1995). With the particular exception of the plasmodesmata, callose is not present in mature cell walls. Therefore, its persistence in mature cell walls should be considered an abnormal feature of embryogenic microspores. In walls with deposits of excreted material (Corral-

Martínez et al. 2013), callose was always absent from these deposits (Figure 5G). This suggested that callose deposition and secretion of cytoplasmic material were independent processes. In older stages of microspore embryogenesis, the cell walls of suspensor-bearing MDEs did not show decoration with anti-callose antibodies (Figure 5H), as expected for mature cell walls derived from somatic-type cytokinesis. In other words, the unusual presence of callose in mature cell walls of few-celled embryogenic structures appeared to be a transient phenomenon, associated to the first stages of this developmental switch, and absent from MDEs with a clearly established embryogenic pattern.

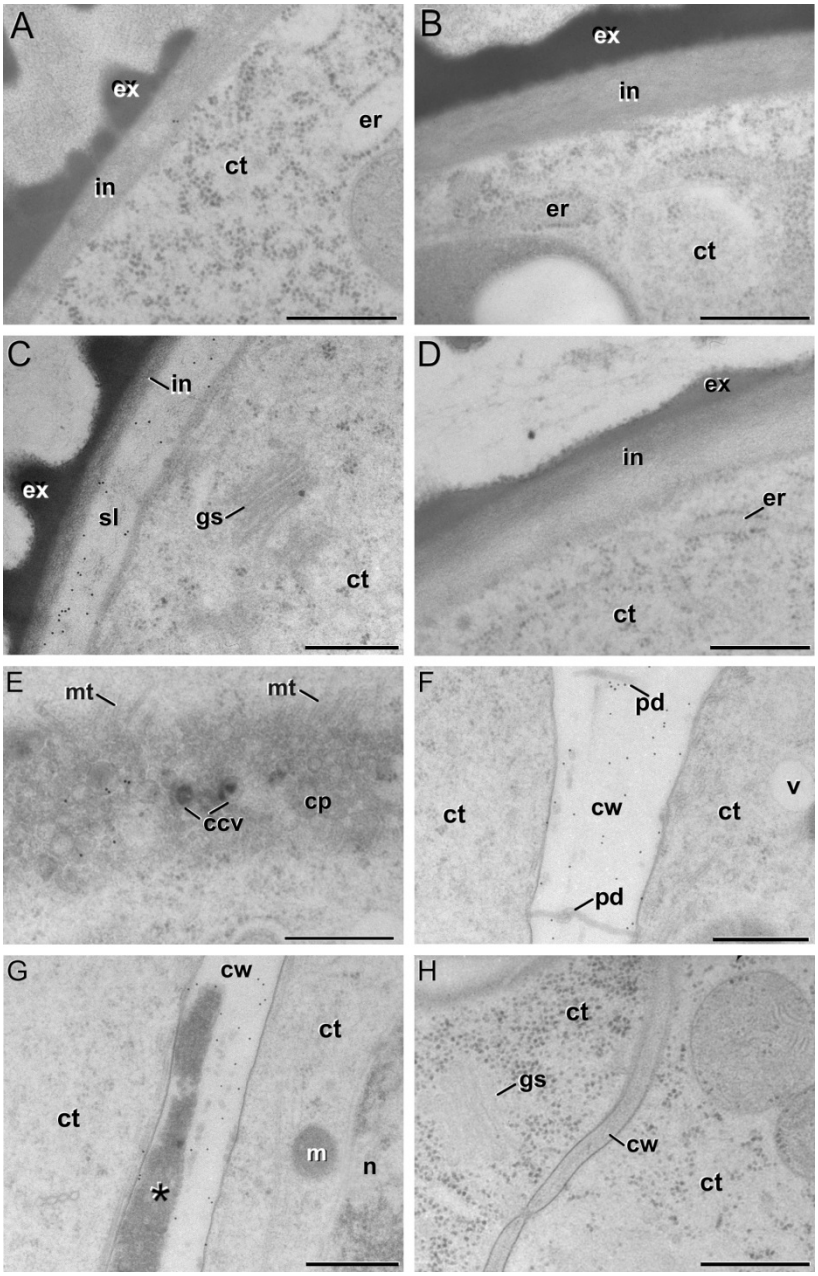

Figure 5: Anti-callose immunogold labeling. A, B: *In vivo* vacuolate microspore (A) and pollen grain (B). Note the absence of a subintinal layer in these cell types. C: Embryogenic microspore at day 4 after isolation with abundant gold particles decorating the subintinal layer (sl). D: Pollen-like structure with a thickened intine

without anti-callose gold particles. E-G: Developing cell plate (E) and mature cell wall (F, G) of embryogenic microspore cells, decorated with anti-callose gold particles. Note in G that callose labeling is absent from the deposits of excreted material (asterisk). H: MDE cell with no anti-callose gold particles in the cell wall. ccv: clathrin-coated vesicles, ct: cytoplasm; cp: cell plate; cw: cell wall; er: endoplasmic reticulum; ex: exine; gs: golgi stack; in: intine; m: mitochondria; n: nucleus; mt: microtubulues; pd: plasmodesma; v: vacuole; Bars: 500 nm.

Cellulose is replaced by callose in the first divisions of embryogenic microspores

Due to the abnormal pattern of callose deposition observed in embryogenic cells, we next studied whether this might have an influence on its subsequent replacement by cellulose. For this, we stained microspore cultures with two cellulose-specific stains: calcofluor white, conventionally used to stain cellulose, and Pontamine Fast Scarlet 4B (S4B), described as more specific for cellulose than calcofluor white (Thomas et al. 2013). Calcofluor white staining revealed diverse patterns of cellulose presence in the cell walls of the different structures present in cultures. Pollen-like structures showed a noticeable and continuous cellulose signal at the intine layer, being more intense at the regions just below the pollen apertures (Supplementary Figure S4A). In contrast, the induced microspores observed exhibited variable patterns of cellulose deposition. To further investigate this unusual observation and the possible interaction between cellulose and callose, we performed a double staining of just induced microspore culture samples (4 days after induction) with aniline blue and S4B. We analyzed 289 cultured microspores. From them, 217 (75%) showed no staining, only exine autofluorescence, when excited at both 405 nm and 566 nm wavelengths (data not shown). Most likely, these unstained microspores would be either dead or arrested in development. The presence of a majority of non-responding microspores is a common feature of the species inducible to androgenesis, which accounts for the relatively low

percentages of MDEs obtained compared to the microspores cultured. The remaining 72 microspores (25%) presented any type of fluorescent signal (aniline blue positive, S4B positive, or both). We carefully analyzed these 72 microspores and categorized them into four groups according to their morphology, internal architecture and double staining pattern (Figure 6).

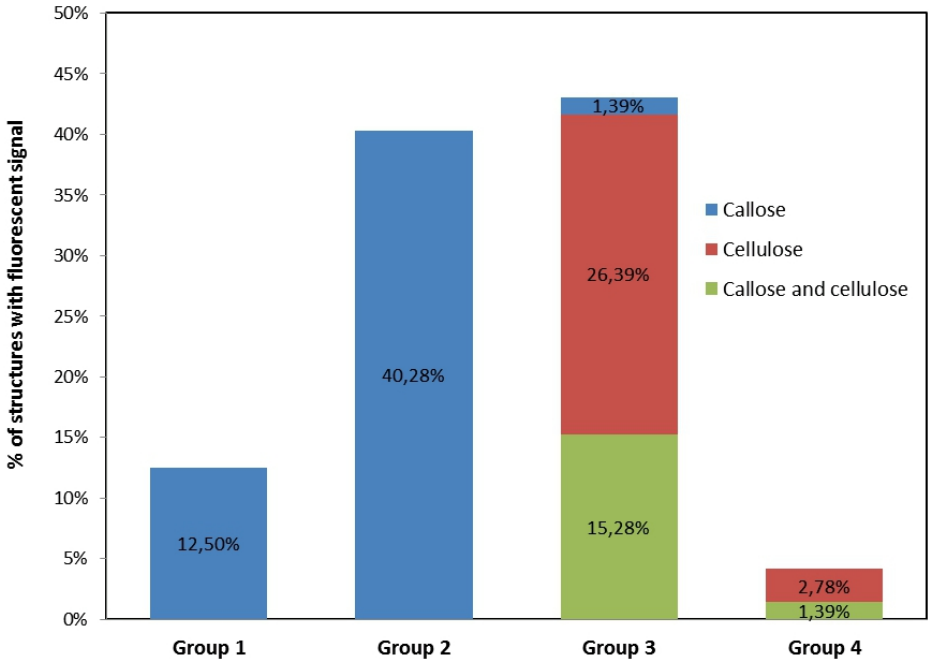

Figure 6: Distribution of callose and cellulose in *B. napus* cultured microspores. Callose and cellulose double staining with aniline blue and S4B, respectively, was observed by confocal microscopy in four different microspore types: microspores before the first embryogenic division (Group 1), 2-4-celled embryogenic microspores (Group 2), pollen-like structures (Group 3) and microspore-derived structures with unusual patterns of development (no embryogenic, no pollen-like; Group 4).

- **Group 1:** 12.5% of the microspores presented a slightly lobulated shape and a size similar to vacuolate microspores (Figures 7A-D). No embryogenic divisions were identified in their cytoplasm. No S4B staining was observed in any part of any of these cells. However, we detected in them aniline blue fluorescence not

previously present in vacuolate microspores. Such a fluorescence was consistently found at the region below the apertures (Figure 7A), in some cases extended beyond these regions (Figure 7B), and even along the entire thickened subintinal layer (Figure 7C). Some of them also presented small fluorescent stubs penetrating into the cytoplasm (Figure 7D), although no a clear cell plate was observed in phase contrast images. Together, these microspores with increasing levels of aniline blue staining at their periphery were suggestive of different stages in the formation of the subintinal layer, prior to the first embryogenic division. Such formation would start at the region of the apertures, and would extend centrifugally to eventually cover the whole microspore.

- **Group 2:** 40.28% of the microspores presented the typical features of embryogenic microspores. In addition to the morphological features mentioned above for the first group, we clearly observed in all of them a cell wall separating two distinct cells (Figure 7E), both of them surrounded by a continuous subintinal layer. The subintinal layer was variable in thickness and aniline blue fluorescence intensity, consistently with the results obtained from TEM structural analysis and anti-callose immunolocalization. With respect to S4B staining, none of them showed fluorescence. This same pattern of callose abundance and cellulose absence was also observed in few-celled (3-4) embryogenic microspores (Figure 7F).

- **Group 3:** 43.06% of the microspores clearly showed pollen-like features, such as enlarged size, oval shape, dense cytoplasm, absence of symmetric divisions, and abundance of starch granules. When observing the S4B staining, these cells revealed another common feature: all of them but one presented a clear and continuous staining at the thickened intine layer (Figure 7G). This trait was

remarkably different from the embryogenic microspores, devoid of cellulose at any cell wall region. Some of the cellulose-containing, pollen-like structures (15.28% of the total) were also positive for aniline blue staining, principally at regions devoid of exine (data not shown), and only one (1.39% of the total) was positive only for aniline blue. In some double stained pollen-like structures, starch granules were clearly delineated by aniline blue staining (Figure 7G). Although unexpected, this is not a surprising finding (Supplementary Figure S3).

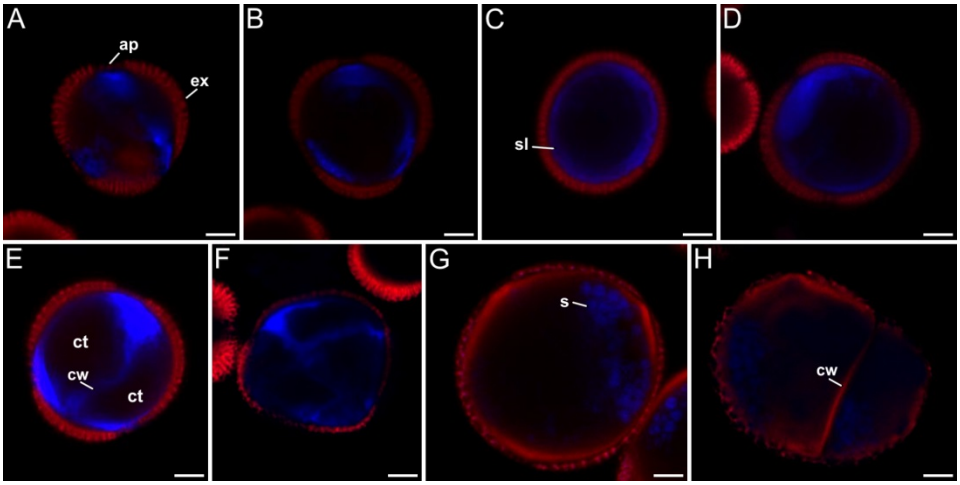

Figure 7: Callose (blue) and cellulose (red) double staining with aniline blue and S4B. A-D: Unicellular (not yet divided) microspores developing a callose-rich subintinal layer (Group 1). E-F: Two-celled (E) and four-celled (F) microspores (Group 2) with callose-rich subintinal layers (sl) and inner cell walls (cw). Note the absence of red cellulose signal. G: Pollen-like structure (Group 3) showing cellulose signal (red) below the exine, but not callose signal (blue). H: Microspore with unusual patterns of development (Group 4) showing cellulose signal (red) below the exine and in the inner cell wall. The dark red signal corresponds to exine (ex) autofluorescence. The blue spots in G-H correspond to unspecific staining of starch granules (s) by aniline blue. ap: aperture; ct: cytoplasm. Bars: 5 µm.

- **Group 4:** 4.17% of the microspores presented abnormal patterns of development, mixing features of pollen-like structures such as starch grains and

large sizes with features of embryogenic microspores, such as cell divisions and new cell walls (Figure 7H). In addition, these cells presented high levels of cytoplasmic vacuolation, exine rupture at different regions of their surface, and asymmetric division patterns. These structures have been previously described as non-embryogenic, which after one or few rounds of division, arrest and die (Corral-Martínez et al. 2013). Two microspores of this group (2.78% of the total) showed only cellulose staining, which delineated both the inner cell walls and the thickened intine (Figure 7H), and only one (1.39% of the total) presented both, cellulose and callose signal, mostly at the regions below the apertures.

As for MDEs, in young (quadrant and octant) suspensor-bearing MDEs, cellulose deposition was observed only occasionally in the outer walls of the embryo proper domain, and absent in the inner walls. As an example, Supplementary Movie S1 shows a 3D reconstruction of a suspensor-bearing quadrant MDE made from serial confocal slices, where S4B staining can be clearly observed in the cell walls separating suspensor cells, but is barely noticeable in the cell of the embryo proper, except for the region that contacts the outern walls. A similar pattern was observed in octant MDEs, with cellulose almost absent from the embryo proper, but delineating the outern and inner cell walls of the suspensor (Supplementary Figure S4B). This pattern, however, changed in further developmental stages. From globular MDEs onwards (Supplementary Figure S4C), calcofluor white staining consistently decorated all the cell walls, both inner and outern, in a pattern identical to a conventional, mature cell wall of vegetative cells.

Together, these results indicated that the first embryogenic divisions of the induced microspore are defined by abnormal patterns of callose and cellulose deposition, establishing a clear correlation between the morphological

features that define a microspore as embryogenic, the presence of callose at both the subintinal layer and the cell walls, and the total absence of cellulose. This pattern, however, changes in MDEs, where cellulose presence is progressively more present in cell walls of developing MDEs, being abundant at the stages of globular and beyond, as expected for normal somatic-type cell walls.

Discussion

It is clear from this work, as well as from others (Barany et al. 2010; Dubas et al. 2013; Solis et al. 2008), that the cell wall is a highly dynamic structure whose architecture, arrangement and composition changes dramatically as the microspore undergoes embryogenesis induction and then transforms into a MDE. We have demonstrated that direct consequences of the exposure of microspores to the inductive treatment are the production of unusual walls with a unique composition. The microspore intine, described to have a pectocellulosic nature (Sitte 1953), retained a cellulose-rich composition in pollen-like structures, as previously described in rye pollen (Heslop-Harrison 1979). In contrast, the subintinal layer presented a composition dramatically different, indicating that it is not an extension of the intine, but a different structure originated *de novo* upon induction. Interestingly, the two cell walls created *de novo* in embryogenic microspores (inner cell walls and the subintinal layer) had the same appearance and composition, which was different from previously existing walls (the intine), but also from the walls of MDEs. It seems that during the first stages of the switch, the mechanisms of synthesis and/or deposition of cell wall components are altered, giving rise to the transient formation of disorganized walls where cellulose is absent. In the following

sections we will discuss about the possible origins, implications and consequences of these walls.

The subintinal layer is an early marker of the androgenic switch

The presence of additional layers beneath the coat of embryogenic microspores has been previously mentioned in the literature. Three classical examples can be found in *B. napus*, where it was defined as a thick (0.75-1.5 µm) fibrillar carbohydrate wall beneath the intine (Zaki and Dickinson 1990), in pepper, where it was described as a *"fibrillar layer between the plasmalemma and the exine"* (González-Melendi et al. 1995), and in tobacco, where Rashid et al. (1982) described *"the formation of a fibrillar wall around the pollen cytoplasm and within the intine"* as the first noticeable feature of embryogenesis induction. The most recent example is the work of Dubas et al. (2013), who showed by scanning electron microscopy the presence of an extracellular matrix-like layer beneath the exine of *B. napus* embryogenic microspores, which disappears in late globular MDEs. Perhaps, the reason why this layer has not been studied more in detail relays on the fact that TEM images of chemically-fixed androgenic microspores frequently showed cell retraction and waviness of plasma membrane, leaving empty spaces between the plasma membrane and the microspore coat (González-Melendi et al. 1995; Maraschin et al. 2005; Seguí-Simarro et al. 2006; Telmer et al. 1993). According to our own experience with chemically fixed samples, in this type of images it is difficult to figure out whether these spaces are real structures or just artifacts due to retraction. Most likely, all the classical TEM images of embryogenic microspores have inadvertently shown such a layer, although only in some cases it has been noticed as a real entity. This would not be surprising, since we

recently showed that the use of chemical fixatives has been traditionally precluding the identification of other major processes associated to embryogenesis induction, such as the massive autophagy and excretion undergone by *B. napus* embryogenic microspores (Corral-Martínez et al. 2013).

In plant development, the synthesis of a callose-rich layer is generally related to the developmental fate of the cells that synthesize it. During meiosis, callosic walls isolate developing microspore and megaspore mother cells from the surrounding diploid tissues, acting as a 'molecular filter' and allowing for the expression of their specific developmental programs (formation of meiocytes and then of microspores and megaspores respectively) without the interference of the surrounding environment (Abramova et al. 2003; Heslop-Harrison and Mackenzie 1967). Aside of natural processes, callose has been observed in internodal cells of *Humulus lupulus* committed to organogenesis (Fortes et al. 2002), and in cells of *Trifolium* (Maheswaran and Williams 1985) and *Cichorium* (Dubois et al. 1991) reprogrammed to somatic embryogenesis. The deposition of a callose layer covering *in vitro*-induced young globular embryos was reported during somatic embryogenesis in *Camellia japonica* leaves (Pedroso and Pais 1992). Most importantly, it was found that when zygotic embryos of *Eleutherococcus senticosus* are exposed to osmotic stressors such as mannitol or sucrose, single epidermal cells develop a surrounding callose layer between the plasma membrane and the cell walls, similar to the subintinal layer we hereby describe, and then enter somatic embryogenesis (You et al. 2006). The authors stated that plasmolysis-induced callose would interrupt cell-to-cell communication, which in turn would stimulate the embryogenic reprogramming. In line with these findings, we observed that all the dividing, embryogenic microspores developed a callosic subintinal layer,

whereas those non-embryogenic did not develop it. We even found some microspores that developed the callosic layer even before the first clear evidence of inner cell walls was found in them. Thus, we propose that the callosic subintinal layer would be formed in microspores committed to embryogenesis prior to the first embryogenic division, and this would isolate them from the outern environment (the liquid culture media or the cultured anther tissue), creating a suitable environment to start the embryogenic program. It can also be concluded that the deposition of a callose-rich layer, as a step previous to *in vitro* embryo development, would be a common feature associated to reprogrammed cells during different types of *in vitro* morphogenesis, including somatic and microspore embryogenesis. In our system, it would be an early marker of microspore embryogenesis.

The unusual callose-rich and cellulose-free inner cell walls are due to altered cytokinesis

Aside of its presence in the subintinal layer, we demonstrated that callose is abundant in the first mature cell walls of embryogenic microspores, where cellulose is absent. In conventional, somatic-type cytokinesis, callose is an essential component of somatic-type developing cell plates, where it is needed for the progression of cytokinesis. However, callose is in general absent from conventional mature cell walls, where it is replaced by cellulose (Samuels et al. 1995). Therefore, it is pertinent to ask why callose persists and cellulose is not deposited in the newly formed somatic-type cell walls of embryogenic microspores.

For us, the most reasonable explanation is that cytokinesis proceeds normally up to the fenestrated sheet-transitional phragmoplast stage of

cytokinesis, and then it is disrupted. Our results are consistent with this hypothesis, since we never observed in our samples any structural alteration at the initial stages (phragmoplast initials and tubule-vesicular network stages, according to Samuels et al. (1995), Seguí-Simarro et al. (2004) and Seguí-Simarro et al. (2008). Once the cell plate is established at the cell equator in the form of a tubulo-vesicular network (Seguí-Simarro et al. 2004), callose synthesis is the driving force that transforms the architecture of the developing cell plate. Callose is an amorphous polymer proposed to provide the fluidity needed to rapidly develop an initial cell plate membranous scaffold (Samuels et al. 1995) and to respond to the guidance mechanisms that insert it at specific sites on the parental wall (Xie et al. 2012). Newly synthesized callose widens the cell plate membranous tubules, as it spreads over the membrane inner surface (Samuels et al. 1995). At the end of this spreading phase, conventional somatic-type cell plates resemble irregular sheets with numerous openings (Samuels et al. 1995; Seguí-Simarro et al. 2004; Seguí-Simarro et al. 2008). This is exactly how many final, mature cell walls look like in our micrographs of abnormally walled *B. napus* embryogenic microspores (Figures 1A, 1B). Once this spreading phase is accomplished, the transition from a fluid and wrinkled immature cell plate to a stiff and straight cell wall is associated to deposition of cellulose and pectins and the parallel removal of callose (Kakimoto and Shibaoka 1992; Samuels et al. 1995). However, we showed that callose is not replaced by cellulose in the gapped and incomplete walls of embryogenic microspores.

Callose does not form crystalline associations with adjacent strands, as cellulose does (Samuels et al. 1995). Thus, a callose-rich cell wall would in principle be more fluid that a conventional, cellulose-rich cell wall with no callose. In addition, β-1,3-glucans such as callose tend to gelate. It was

suggested that the formation of hydrogels by callose would allow insertion or co-gelation with other polymers in the cell plate (Brown and Lemmon 2009). It is also known that callose gelation can be increased upon heating (Stone and Clarke 1992). Conceivably, the combination of a mild heat stress, callose accumulation and reduced cellulose contents would confer the newly formed cell walls of embryogenic microspores new properties, different from those of somatic-type walls. These walls would be more difficult to flatten, straighten and close through the conventional mechanisms of cell plate maturation, giving rise to irregular final walls, wrinkled and incomplete, as those shown in Figures 1A and 1B.

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

Increased Ca2+ levels might be responsible for cellulose inhibition and prolonged CalS1 and CalS5 activity

In contrast to other cell wall forming polymers, callose and cellulose are produced *in situ* by plasma membrane-bound callose and cellulose synthases, respectively (Northcote et al. 1989; Verma 2001). A heat stress treatment is necessary to induce microspore embryogenesis in *B. napus*, as well as in many other inducible species. Heat stress is also known to alter the properties of plasma membranes in plant cells in general (reviewed in Horvath et al. 2012) and in *B. napus* embryogenic microspores in particular (Pauls et al. 2006). Thus, it could be speculated that the heat stress is acting at the plasma membrane level to alter the regulation of these enzymes. One signaling candidate to mediate in callose persistence and inhibition of cellulose synthases is Ca^{2+}. Ca^{2+} signaling is known to play an important role in inducing embryogenesis in *B. napus* cultured microspores (Pauls et al. 2006). In turn, high levels of Ca^{2+} are required for callose synthesis in sporophytic cells (Kauss 1987). Indeed, an increased concentration of Ca^{2+} is known to cause the shift towards callose production in somatic-type cell walls. On the other hand, the Ca^{2+} levels must be reduced for cellulose synthesis to initiate during cell plate maturation. As stated by Verma (2001), a prolonged presence of high levels of Ca^{2+} at the nascent cell plate may promote the continuation of callose synthesis and the delay of cell plate maturation by inhibiting the shift from callose synthesis to cellulose synthesis. A similar scenario might be occurring during the formation of the first cell walls in *B. napus* embryogenic microspores. Heat shock conditions tend to cause plasma membranes to become leaky to ions, favoring uncontrolled influxes of Ca^{2+}. Indeed, heat shock induce rapid, transient and proportional elevations of cytosolic Ca^{2+} in both moss cells (Saidi et al. 2009)

and higher plants (Gong et al. 1998). Since Ca^{2+} signaling is a highly conserved mechanism for temperature sensing among land plants (Horvath et al. 2012), it is very likely that *B. napus* embryogenic microspores also undergo such Ca^{2+} increases. According to Pauls et al. (2006), the mild heat shock used to induce embryogenesis triggers a series of intermediate steps that would finally allow for the release of additional Ca^{2+} to the cytoplasm. Such an increased level of Ca^{2+} would allow for the function of callose synthases and at the same time, would inhibit cellulose synthases. As a consequence, cell walls enriched in callose but with abnormally low levels of cellulose would be created.

The presence of callose-rich cell walls may promote chromosome doubling

As pointed out by De Storme et al. (2013), reproductive structures are particularly sensitive to cytokinetic aberrations, which typically induce the occasional formation of diploid (unreduced) gametes. Incomplete cell walls with large holes allow for the contact between haploid nuclei of adjacent cells. Even those with smaller gaps may end up producing larger holes, since they are prone to break and collapse into pieces. In other words, the instability and fluidity of callose-rich cell walls, and the severe cell wall deformations produced by the accumulation of excreted cytoplasmic material (Corral-Martínez et al. 2013) seem to be behind the occurrence of incomplete, broken or fenestrated cell walls. These abnormal walls are known to facilitate the coexistence of nuclei of different cells in the same cytoplasm (Sunderland and Dunwell 1974) and their eventual fusion, as demonstrated to occur in many other *in vitro* culture systems and mutants with defective cytokinesis (Corral-Martínez et al. 2011; De Storme et al. 2013; González-Melendi et al. 2005; Kasha et al. 2001; Mayer et al. 1999; Muller et al. 2002; Seguí-Simarro and Nuez 2007; Seguí-

Simarro and Nuez 2008; Strompen et al. 2002; Testillano et al. 2004;). Hence in some embryogenic microspores, callose-mediated impairment of cytokinesis would facilitate nuclear fusion and therefore genome duplication (Figure 8), a necessary step for producing DH cells, embryos and plants.

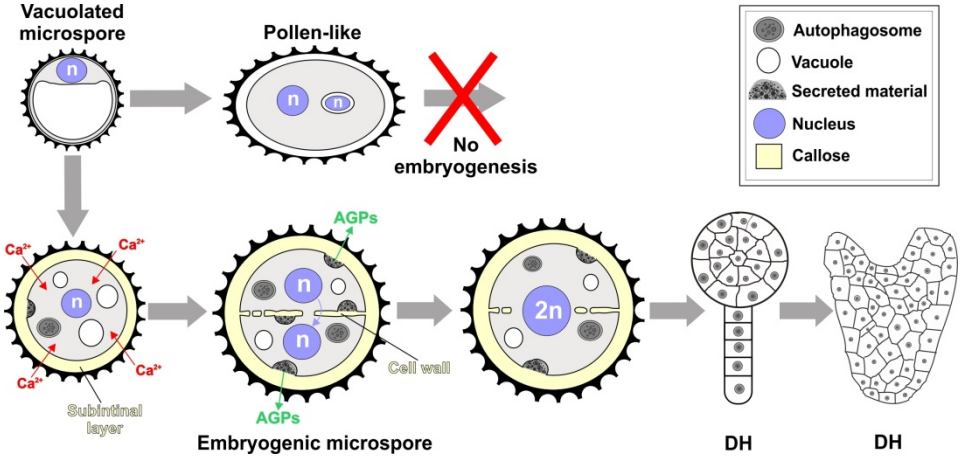

Figure 8: Proposed relationship between callose-rich cell walls and chromosome doubling in *B. napus* embryogenic microspores. Vacuolate microspores after the inductive treatment may enter two alternative pathways: (1) the pollen-like pathway, similar to the *in vivo* gametophytic pathway, and (2) the embryogenic pathway. In this pathway, the stress treatment would increase the intracellular Ca^{2+} levels, activating callose-synthases and producing callose-rich, fluid and incomplete cell walls. These abnormal cell walls would facilitate the coalescence and eventual fusion of nuclei of different cells, producing double haploid (DH) cells.

In parallel, those embryogenic microspore cells developing normal walls would not undergo nuclear fusion during these initial stages, remaining haploid and giving rise to haploid embryos. On the other hand, it is reasonable to assume that there will also be embryogenic microspores whose initial phase of defective cytokinesis would last longer, being their cells more prone to undergo more than one round of nuclear fusions. It can also be possible that in a single embryogenic microspore, normally developing cells coexist with others

undergoing defective cytokinesis, giving rise to MDEs with cells with different ploidy. Therefore, the combined occurrence of normal and defective cell walls would explain the common occurrence of DHs, but also the occasional presence of polyploid and mixoploid *Brassica* embryos and plantlets (Abdollahi et al. 2012; Sato et al. 2005).

All the mechanisms proposed hereby are associated to the induction of microspore embryogenesis by a transient heat shock. This implies that upon cessation of the heat shock effects, the cell wall-associated phenomena (abundance of callose, absence of cellulose, incomplete cell walls, presence of excreted material, nuclear fusion, etc.) should cease as well, and cell wall formation should progressively adopt the patterns typical of embryos of this species, as we hereby showed. A similar reversible behavior has previously been described for other phenomena associated to embryogenesis induction, once the heat shock has finished (Barany et al. 2010; Corral-Martínez et al. 2013; Dubas et al. 2013;). It would be interesting to know whether these or similar phenomena are triggered in other species where microspore embryogenesis is induced by means of stresses other than heat.

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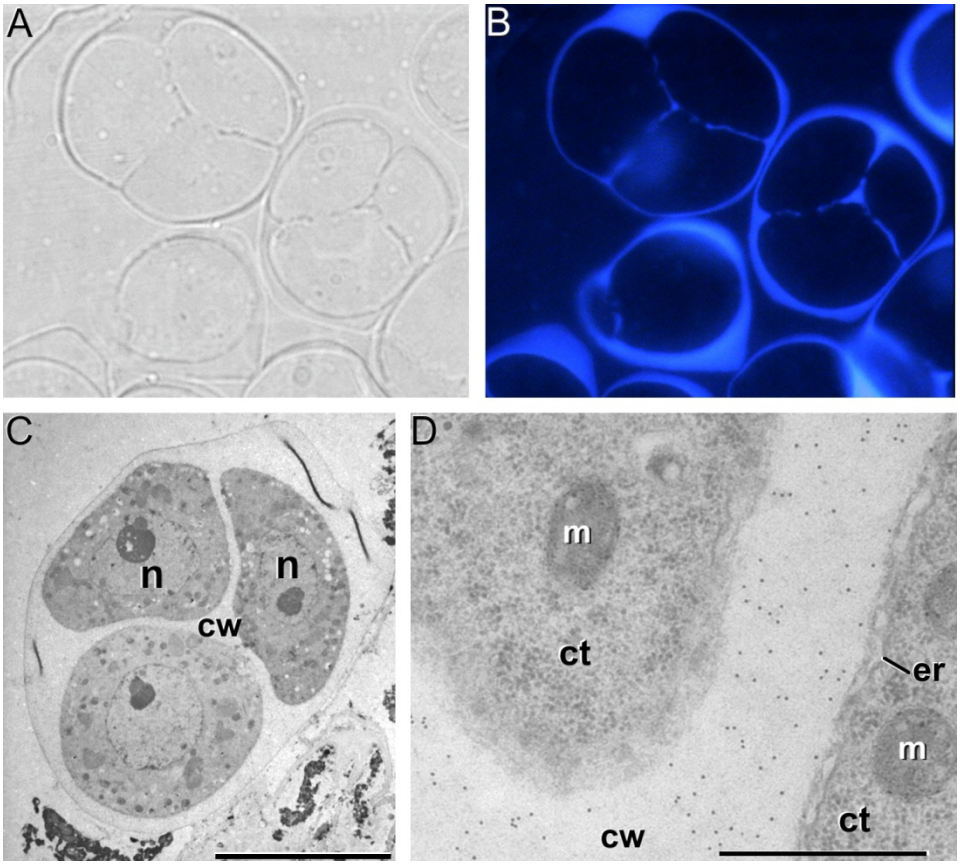
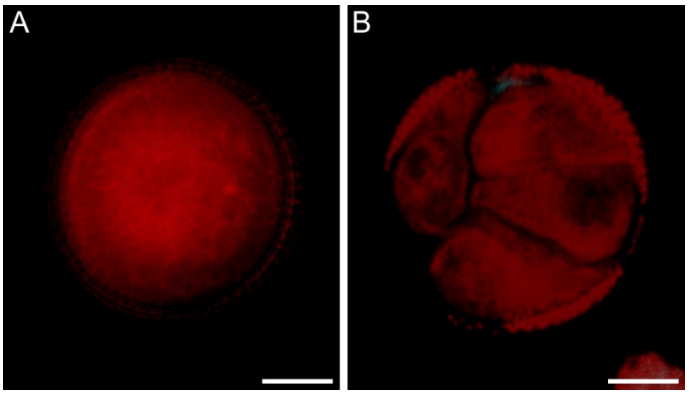
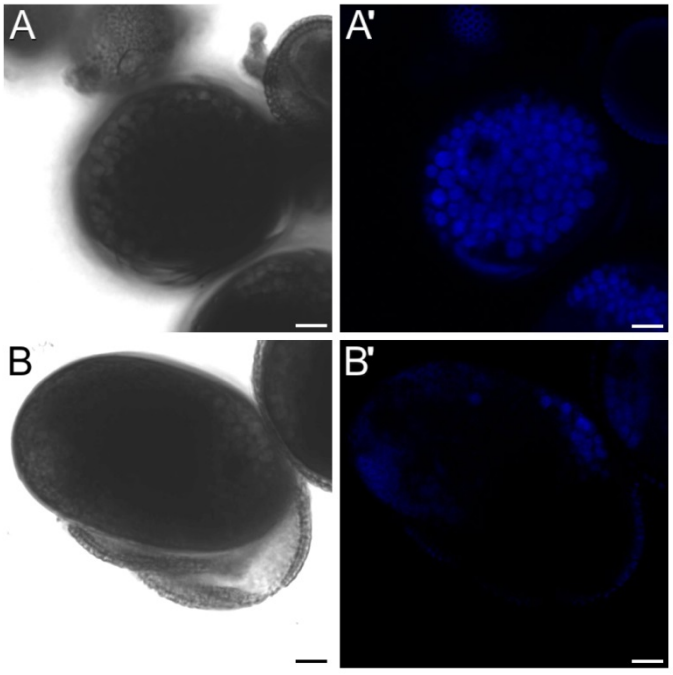
Supplementary figures



Supplementary Figure S1: Callose staining in tomato meiocytes. A, B: DIC (A) and fluorescence (B) images of meiocytes stained with aniline blue. C, D: Immunogold labeling of meiocytes with anti-callose antibody. Note the abundant presence of gold particles in the callose-rich cell wall (cw). ct: cytoplasm; er: endoplasmic reticulum; m: mitochondria; n: nucleus. Bars: C: 10 µm, D: 1 µm.

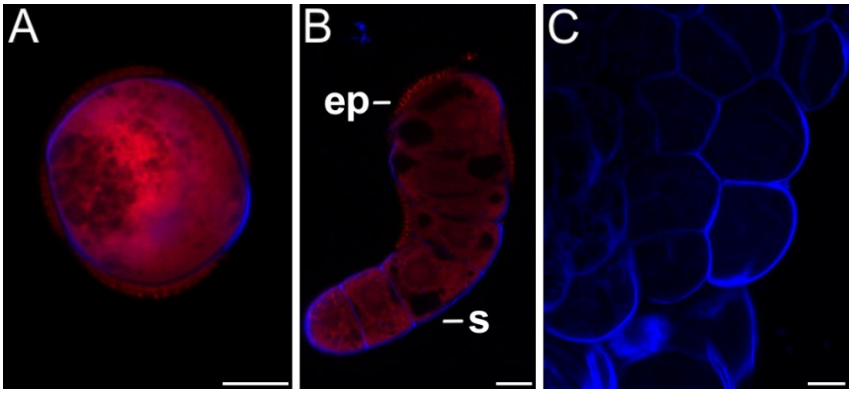


Supplementary Figure S2: Staining of *B. napus* cultured microspores with aniline blue and PI. A: Pollen-like structure with no aniline blue staining. B: Multicellular embryogenic microspore where only a small blue spot can be observed beneath the intine. The dark red signal corresponds to exine autofluorescence. Bars: 10 µm.



Supplementary Figure S3: *B. napus* pollen grain stained with aniline blue. Note that the blue cytoplasmic spots correspond to starch granules. Aniline blue is not a starch-specific stain, but it has a demonstrated affinity for multiple kinds of biological molecules, including not only callose but also laminarin and substituted β-1,3-glucans

(Albersheim et al. 2011). This is probably due to the fact that aniline blue is not a pure chemical, and together with its two main components, it may contain traces of other molecules capable of staining different carbohydrates with branching residues or links similar to those of callose. In parallel, starch granules are widely known to bind different kinds of molecules, from actin to antibodies, in a non-specific manner. Bars: 5000 nm.



Supplementary Figure S4: Staining of *B. napus* cultured microspores with calcofluor white (blue) and PI (red). A: Pollen-like structure with cellulose staining (blue) at the intine. B: Octant suspensor-bearing MDE with cellulose staining at the outer and inner cell walls of the suspensor (s). Note that the cell walls of the embryo proper (ep) show a barely detectable blue staining, and only at the outer walls. C: Transitional MDE (globular to heart-shaped) showing abundant cellulose staining in all the cell walls. The dark red signal in A and B corresponds to exine autofluorescence. Bars: 10 µm

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Bloque II

Capítulo 5

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

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Abstract

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

Introduction

Microspore embryogenesis is the most efficient biotechnological approach to obtain haploid individuals and doubled haploid (DH) lines. DHs are used as pure lines in breeding programs to produce hybrid seeds. They are also extremely valuable tools for plant genetic research (Forster et al. 2007; Seguí-Simarro 2010). Microspore embryogenesis consists of the reprogramming of the microspores (the pollen precursors) towards embryogenesis. This developmental switch (also known as androgenesis) is generally induced through the application of stress. The microspores of some species can be induced by starvation, others by the application of cold temperatures to the inflorescences prior to in vitro culture, and others by the application of a heat shock to the in vitro cultured microspores, as is the case for Brassica napus microspores (reviewed in Shariatpanahi et al. 2006). Once microspores are reprogrammed, they undergo multiple changes to readapt themselves to the new developmental scenario. These changes include, among others, a profound remodeling of gene expression, the triggering of a (stress) response as a consequence of the inductive (stressing) treatment, the suppression of the ongoing gametophytic program, and the initiation of embryogenesis (Maraschin et al. 2005; Seguí-Simarro and Nuez 2008; Dunwell 2010). At the subcellular level, there is also an extensive remodeling of cell ultrastructure, including a displacement of the nucleus to the center of the cell, a rearrangement of the cytokinetic machinery, a switch from an asymmetric to a symmetric division pattern, and a reduction in the number of plastids (Hause et al. 1993; Testillano et al. 2000; Telmer et al. 1995; Zaki and Dickinson 1991; Shariatpanahi et al. 2006; Makowska and Oleszczuk 2013).

The study of the ultrastructural changes associated to the androgenic switch started approximately 40 years ago. During these decades, these studies have been traditionally done by using transmission electron microscopy (TEM) in samples preserved with aldehyde-based chemical fixatives. The main disadvantage of these fixatives is the parallel generation of structural disorders in membranous elements of different subcellular compartments and organelles (McDonald and Auer 2006). Among these artifacts, chemical fixatives may generate membrane retraction, fusion and/or swelling, as well as vesiculation of large membranous elements (Gilkey and Staehelin 1986). Such a change of the original cell ultrastructure frequently precludes the accurate identification and analysis of complex membranous structures (McDonald and Auer 2006; Gilkey and Staehelin 1986). Fortunately, there is an alternative to avoid the artifacts of chemical fixatives, which consists on the combined use of two cryotechniques for sample preservation: High Pressure Freezing and Freeze Substitution (HPF/FS). HPF consists on freezing the sample within milliseconds while subjected to high pressure (2100 bar). HPF/FS prevents the formation of ice crystals derived from freezing and provides an excellent ultrastructural preservation, much better than chemical fixation (Gilkey and Staehelin 1986). These features make HPF/FS the method of choice for fine ultrastructural analysis. Using this method, Corral-Martínez et al. (2013) found evidence for the extensive formation of autophagosomes engulfing from small to large regions of cytoplasm, and the occurrence of massive autophagy prior to excretion of the partially digested cytoplasmic material to the apoplast. These were exclusive features of just induced microspores, not present in cells neither before nor long after the inductive stage. It seemed that in induced cells, the autophagosomes and the vacuolar system worked together as a cytoplasmic cleaning mechanism to adapt the cell to a new embryogenic scenario.

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

Materials and methods

Plant material

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

Isolated microspore culture of Brassica napus

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

Processing of samples for TEM

Three different types of *B. napus* samples were processed for TEM, including anthers containing microspores and pollen at different stages of microsporogenesis and microgametogenesis, 4-day-old cultured microspores, and MDEs at different developmental stages: globular, heart-shaped and torpedo embryos. All samples were randomly selected and three different sample batches were processed as previously described (Corral-Martínez et al. 2013; Seguí-Simarro 2014a). Briefly, samples were fixed with a Baltec HPM 010 (Technotrade, Manchester, NH, USA) and a Leica EM HPM 100 (Leica Microsystems, Vienna, Austria) high pressure freezers. After HPF, samples were freeze substituted in a Leica AFS2 system. Substitution was performed with 2% OsO_4 in anhydrous acetone. Infiltration was carried out with increasing concentrations of Epon resin (Ted Pella, Redding, CA, USA) diluted in acetone, and polymerization was performed at 60 °C for 2 days. A minimum of five resin blocks were randomly selected and sectioned for further analysis. A Leica UC6

ultramicrotome was used to obtain thin sections (~1 µm) and ultrathin (~80 nm) sections for observation at light microscopy and TEM, respectively. Ultrathin sections were mounted on formvar and carbon-coated, 200-mesh copper grids, stained with uranyl acetate and lead citrate, observed and photographed in a Philips CM10 TEM. A minimum of 100 electron micrographs were taken and analyzed at each of the stages studies. For the stage presenting atypical plastids, 242 electron micrographs were taken and studied. For the quantitative analysis of atypical plastids, 107 of these images, containing at least one plastid per image, were analyzed.

FESEM-FIB three-dimensional reconstruction of subcellular volumes

The 3-D study of the plastids and mitochondria contained within the induced microspores was carried out with a FESEM-FIB (Auriga Compact, Zeiss) as described in Seguí-Simarro (2014b). This technique combines a Field Emission Scanning Electron Microscope (FESEM) and a Focused gallium Ion Beam (FIB) to sequentially mill the sample surface. Briefly, the sample-containing part of HPF/FS-processed resin blocks was smoothened with a diamond knife, separated, and placed into the specimen stage of the FESEM-FIB. The areas of interest (embryogenic microspores) were visualized using the secondary electron detector. Then, the sample was tilted to 52º. The ion beam was used to mill a window exposing the areas of interest. Then, the samples were milled with the FIB (operating at 1 nA) removing 20 nm thick layers from the sample surface. Images were acquired every 40 nm. Two different stacks from two different areas of interest were acquired. The images were aligned with MIDAS and the reconstruction was made with 3dmod, both included in the IMOD software package (Kremer et al. 1996).

In situ detection of acid phosphatase activity

The assay of acid phosphatase activity was performed over ultrathin sections according to Gärtner and Nagl (1980). The nature of this cytochemical reaction imposes the use of hydrated samples and temperatures above 0 °C. For these reasons, this assay was performed on chemically fixed specimens, instead of using HPF for fixation. 3 day-old *B. napus* microspore cultures were collected and fixed with 6.25% glutaraldehyde and 3% DMSO in 0.05 M cacodylate buffer (pH 7.4) at 4 °C for 45 min. After three washes with 0.05 M cacodylate buffer, samples were incubated for 1 hour at 37 °C in a solution containing 10 ml 0.2M Tris-maleate buffer (pH 5.2), 6 ml 0.02M lead nitrate, 4 ml 0.1M β-glycerophosphate disodium salt and 30 ml distilled water. Control incubation was made replacing the substrate (β-glycerophosphate) by distilled water. After incubation, the samples were washed three times with 0.04M Tris-maleate buffer (pH 5.2) at 4 °C, 30 min each. Then, samples were postfixed with 1% OsO_4 in 0.05M cacodylate buffer for 4 hours, and washed three times with same buffer, 30 min each. Samples were dehydrated through increasing series of ethanol at 4 °C, and then infiltrated with increasing concentrations of Spurr resin (Ted Pella, Redding, CA) in ethanol. Polimerization was carried out at 70 °C for 24 hours. Thin (~1 µm) and ultrathin (~80 nm) sections were obtained for light microscopy and TEM, respectively, using a Leica UC6 microtome. Ultrathin sections were mounted on formvar and carbon-coated, 200-mesh copper grids, stained with uranyl acetate and lead citrate, and finally observed and imaged in a Philips CM10 TEM.

Results

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

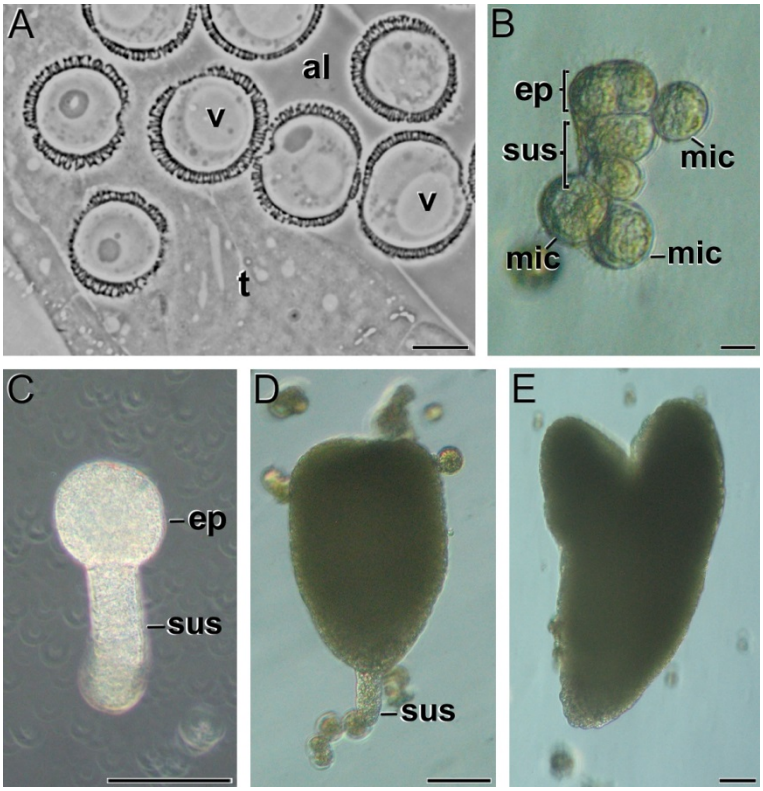

Figure 1: Stages of B. napus microspore embryogenesis. A: Vacuolate microspores during in vivo development within the anther. B: Just induced, embryogenic microspore showing two clearly differentiated domains, the embryo proper domain (ep) and the suspensor (sus). Other microspores (mic) are not sensitive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

Non induced cells presented normal development of plastids

Vacuolate microspores, still within the anther or just after isolation (not yet induced; Figure 2A), presented only proplastids, still undifferentiated. Proplastids were typically round. Their stroma appeared less electron dense than in the rest of stages studied, and presented very few thylakoids. Pollen-like structures presented plastids clearly transformed into amyloplasts, as revealed by the large starch deposits present in most of the plastids (Figure 2B).

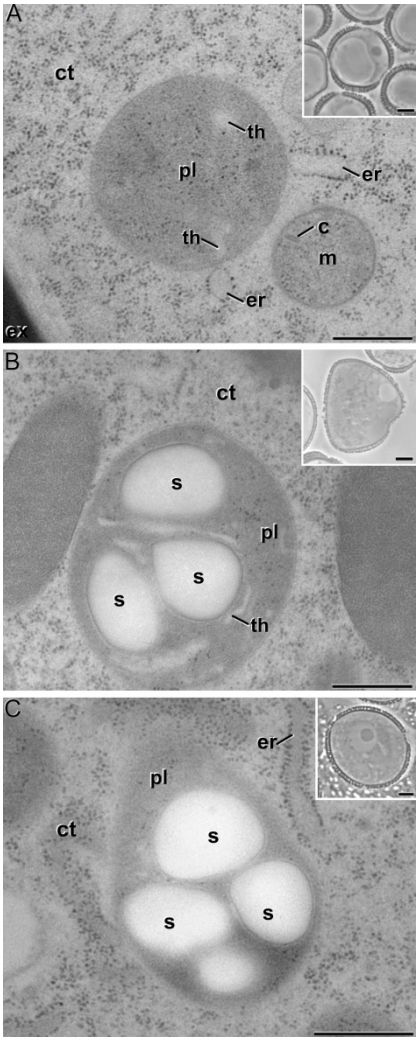

Figure 2: Plastids (pl) of non-embryogenic B. napus cells. A: Proplastid of a vacuolate microspore during in vivo development within the anther. B: Amyloplast of an in vitro cultured, pollen-like structure. C: Amyloplast of a pollen grain within the anther. Insets show light microscopy sections of the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

Amyloplasts of pollen-like cells presented different sizes and shapes, rounded and/or elongated, and accumulated one or more starch granules. These amyloplasts were remarkably similar to those found in in vivo pollen grains within the anther (Figure 2C). In summary, non-induced cells presented conventional proplastids and amyloplasts, similar to those previously described

for equivalent stages in this and other species (Sangwan and Sangwan-Norreel 1987; Zaki and Dickinson 1990; Satpute et al. 2005).

Embryogenic microspores presented atypical plastids

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

Table 1: Quantitative analysis of plastids of embryogenic microspores

	Number	Percentage (from total)	Percentage (from atypical)
Conventional	142	60,7%	
Atypical	92	39,3%	
Engulfing (open profiles)	14	6,0%	15,2%
Engulfed (closed profiles)	63	26,9%	68,5%
Concentric membranes/ disorganized contents/ multilamellar	15	6,4%	16,3%
Total	234	100%	100%

However, many other plastids (39.3%) exhibited morphologies remarkably different from conventional (Figures 3B-I). The differences pertained principally to plastid shape and contents. As for plastid shape, the most striking difference was the presence of open plastid profiles surrounding cytoplasmic portions Based on this observation, we identified two types of open plastid profiles. In the first type, we included elongated and curled dumbbell-shaped plastids (Figures 3B, C). Their extreme bending trapped portions of cytoplasm, as revealed by the presence of ribosomes and vesicles embedded in a matrix identical to that of the outer cytoplasm (Figure 3B). In some of these plastids, the connection between both cytoplasms was reduced to a thin channel or a pore of few nanometers (arrows in Figures 3C- E). In other plastids the channel was absent, suggesting that the membranes of opposite ends of the plastids were fused (Figures 3F), leaving a cytoplasmic portion isolated at the center of the closed plastid profile. The envelope of the internal cytoplasm-containing compartment was formed by a double membrane system identical to that found at the outer plastid envelope (insets in Figures 3C, F and I). Together, these observations suggested a dynamic process of plastid curling to engulf small regions of cytoplasm. The second type of profiles consisted of a relatively round plastid body engages in the process of engulfing larger cytoplasmic areas (Figures 3G-H), or having them entirely engulfed (Figure 3I). In these cases, the large cytoplasmic area appeared in a lateral position within the deformed plastid, suggesting that the engulfment of large cytoplasmic areas imposes dramatic changes to the typical structure of the plastid. Overall, open plastid profiles suggesting engulfment of cytoplasm accounted for 15.2% of the atypical plastid profiles we identified.

Among all the atypical plastid profiles, the most frequent (68.5%) were those containing isolated cytoplasm portions. Most of these closed plastid profiles showed no structural abnormalities other than the engulfed cytoplasm. However, some of them presented more electron dense contents, indicating the onset of a change in the engulfed cytoplasm. Occasionally, plastids with an electron dense content (asterisk in Figure 3G) were engaged in a second round of cytoplasmic engulfment, suggesting that this might be a recurrent process. In addition, others showed one or more concentric membranous structures surrounding the cytoplasmic compartment (Figure 3J). Most of the plastids with several concentric membranes presented dark and disorganized contents as well (Figure 3K). Interestingly, multilamellar bodies of a size similar to the plastids with concentric membranes were also found in the cytoplasm, close to the plasma membrane, and in the apoplast (Figure 3L), together with cellular debris excreted as a consequence of embryogenesis induction (Corral-Martínez et al. 2013). These multilamellar bodies presented an internal compartment with fibrillar material, similar to that present in lytic compartments. Closed plastid profiles with concentric membranes, dark, fibrillar and disorganized contents, together with cytoplasmic and apoplastic multilamellar bodies, accounted for 16.3% of the atypical profiles observed. Altogether, these plastid profiles suggested the occurrence of plastid degradation and excretion out of the cell.

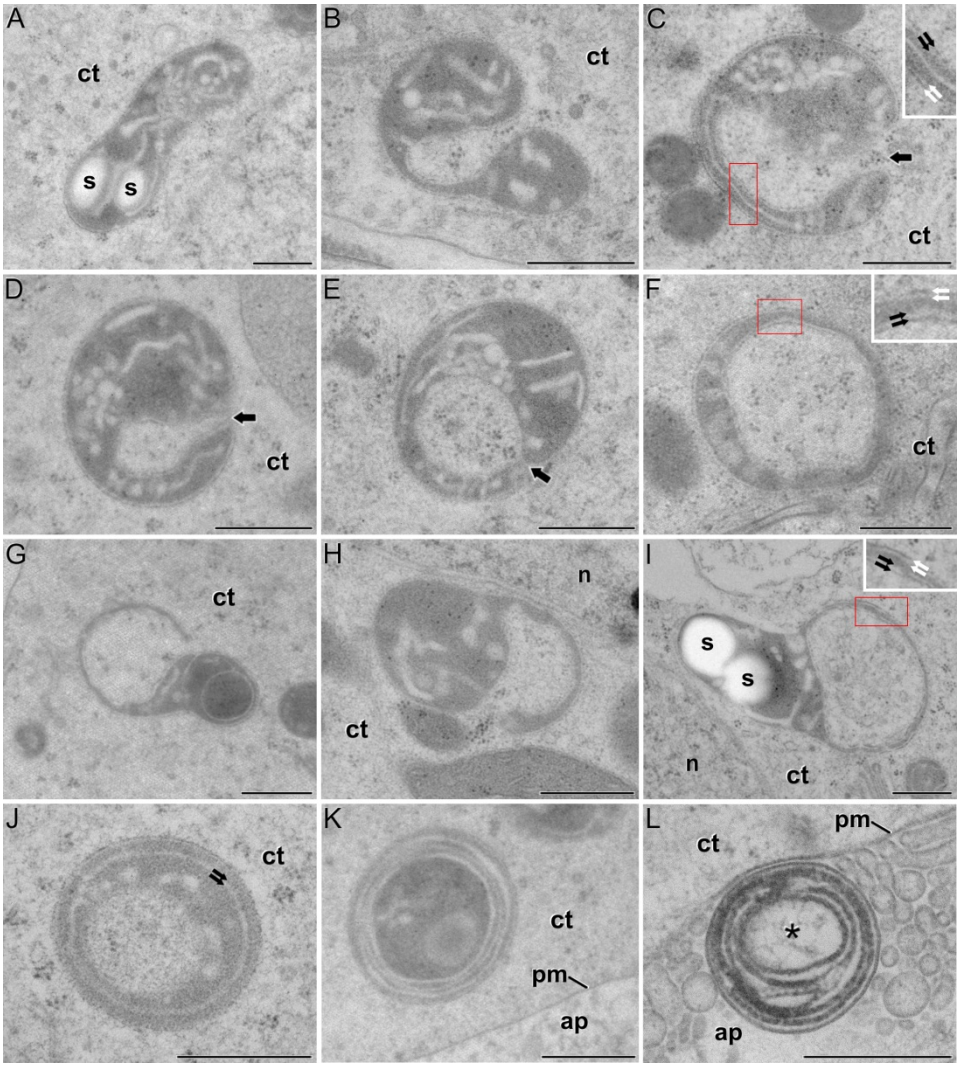

Figure 3: Plastids of *B. napus* embryogenic microspores. A shows a conventional, elongated plastid, with two small starch deposits (s). B: Dumbbell-shaped plastid curled to engulf a small cytoplasm region. C, D: Plastids wrapped around a cytoplasmic region. Arrows indicate the thin cytoplasmic channel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

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

3-D reconstruction of subcellular volumes of embryogenic microspores

Theoretically, it might be possible that the atypical plastid profiles observed in TEM micrographs of embryogenic microspores correspond to polar sections of cup-shaped plastids. Alternatively, these plastid profiles might correspond to equatorial sections of ring-shaped plastids. In other words, the atypical plastid profiles we observed might be artifactual, and might not engulf cytoplasm actually. In order to rule out this possibility, and to figure out the actual 3-D structure of these plastids, we performed FESEM-FIB-based 3-D reconstructions and models of large cytoplasmic areas of embryogenic microspores (Fig. 4A). These models confirmed the presence of three morphologically different plastid types (Fig. 4B), as previously observed in TEM micrographs. We modeled each plastid type in different colors. Plastids with conventional morphologies (oval, round or elongated, not engulfing cytoplasm) were modeled in light green (Figs. 4B-D). Some of them were round or slightly oval (Fig. 4C), and others exhibited a disc-like morphology with a slight central depression (arrow in Fig. 4D), suggesting the onset of a process of membrane invagination. Plastids engulfing cytoplasm were modeled in dark green (Figs. 4B, E-F'). These plastids presented different sizes and shapes. Some of them, similar in shape to the disc-shaped conventional plastid of Fig. 4D, showed a

profound invagination of their envelope, generating a cytoplasm-containing open pocket (arrow in Fig 4E). Others were oval or elongated, and engulfed large portions of cytoplasm. In these plastids, the cytoplasm outside and inside the plastid was connected by a narrow channel of a few nanometers, leaving a pore at the surface of the plastid (arrows in Figs 4F, F'). The third plastid type, modeled in yellow (Figs. 4B and G) showed an oval or elongated shape, and included one or more cytoplasm portions (modeled in white) fully isolated from the outer cytoplasm, as evidenced by the absence of connecting channels (Fig. 4G). Together, the different plastids observed in 3-D models (Figs. 4C-G) confirmed our observations from 2-D TEM micrographs, and indicated the occurrence of a mechanism whereby some plastids surround and engulf discrete cytoplasm regions, isolating them from the outer cytoplasm.

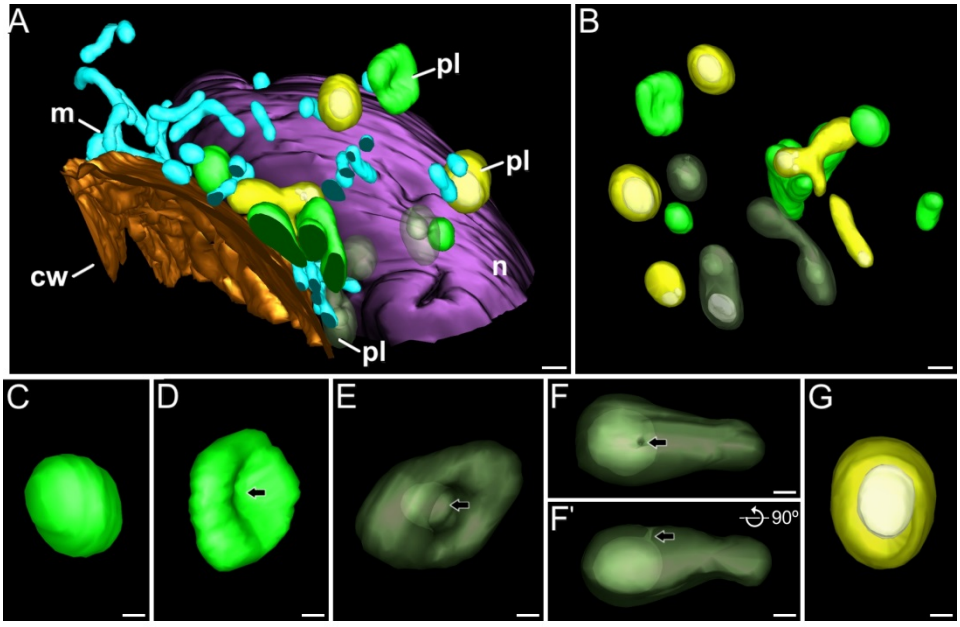

Figure 4: 3-D model of a reconstructed cytoplasmic region of an embryogenic microspore. A: Global model including the nucleus (n), different plastid types (pl), mitochondria (m) and the cell wall (cw). B: Model showing only the different plastid

types: conventional (light green), open profiles engulfing cytoplasm (dark green) and closed profiles (yellow) containing cytoplasmic compartments (white). C-G: Morphological sequence of the changes undergone by the plastids during cytoplasm engulfment. C: Conventional plastid. D: Conventional disc-like plastid with a central slight invagination (arrow). E: Plastid with a deep invagination of its envelope (arrow), creating a cytoplasmic pocket. F: Plastid engulfing a cytoplasm portion but not yet closed. The cytoplasmic compartment is still connected with the outer cytoplasm by a thin channel that creates a pore at the plastid surface (Arrow). F' illustrates a 90º tilt of the same plastid for a better visualization of the thin cytoplasmic channel (arrow). G: Round plastid with a closed cytoplasmic compartment. Bars: A, B: 500 nm; C-G: 200 nm.

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

In addition to conventional and atypical plastids, we also modeled the multilamellar bodies excreted to the apoplast (Fig. 5). Fig. 5A shows a model of an apoplast region and Fig. 5B shows one of the several FESEM-FIB micrographs used to reconstruct it. This region corresponds to a swollen apoplast area with numerous membranous bodies embedded in a matrix of dense material (Corral-Martínez et al. 2013). Close to this area but separate from it, a

multilamellar body (inset in Fig. 5A, modeled in red) similar to those observed in TEM images (Figs. 3L, 5B) is observed. The multilamellar body is similar in size and shape to the atypical plastids and multilamellar bodies observed in the cytoplasm. The 3-D model showed that nearly half of the body is facing the apoplast, while the other half is still tightly wrapped by the plasma membrane, which clearly delineates the shape of the body (inset in Fig. 5A). These observations are suggestive of a process of excretion of the multilamellar body, likely mediated by the fusion of its outer membrane with the plasma membrane, and independent of the excretion of the membranous and dense material.

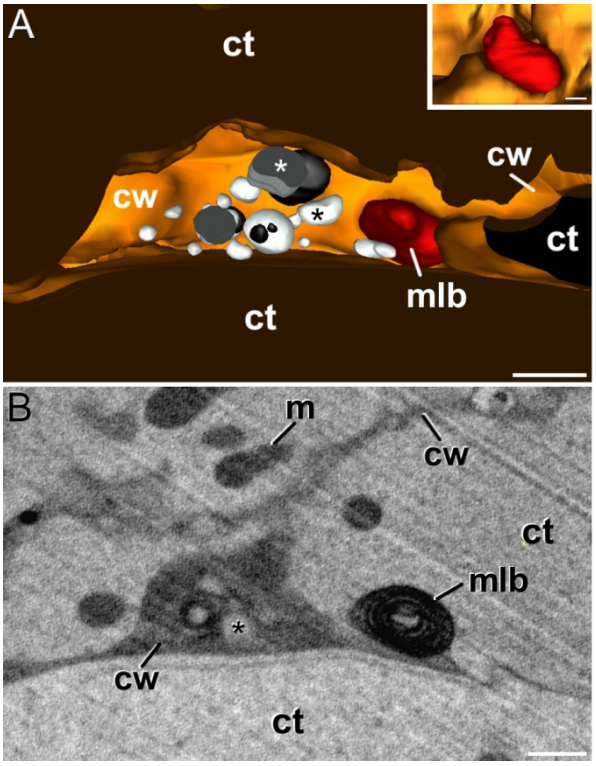

Figure 5: 3-D reconstruction of a cell wall region of an embryogenic microspore. 3-D model (A) and FESEM-FIM slice (B) of a swollen cell wall region (cw) including numerous excreted vesicular bodies (asterisks) and a contiguous multilamellar body (mlb). Inset in

A shows the multilamellar body oriented for a better visualization of its shape. See text for further details. ct: cytoplasm, m: mitochondrion. Bars: 500 nm.

Cytoplasm-containing plastids showed acid phosphatase activity

As seen in Figs. 3J-L, some of the cytoplasm-containing plastids showed signs of degradation of their cytoplasmic contents and of the entire plastid. In order to elucidate a putative lytic activity in these organelles, similar to that found in autophagosomes of the cytoplasm of embryogenic microspores (Corral-Martínez et al. 2013), we performed an *in situ* acid phosphatase cytochemical assay (Fig. 6). In embryogenic microspores we observed cytoplasm-containing plastids with different amounts of electron dense precipitates, indicative of different levels of acid phosphatase activity. Fig. 6A shows a plastid containing cytoplasm similar to that found out the plastid, together with few small precipitates distributed throughout the plastid, indicating a mild lytic activity. As a reference, this figure also includes a lytic cytoplasmic vacuole with an electron light lumen and numerous precipitates, indicating a more intense lytic activity. Fig. 6B shows a plastid where most of the precipitates concentrate in the engulfed cytoplasm, suggesting that lytic activity is first initiated in the cytoplasmic cargo. Fig. 6C shows a plastid where the cytoplasmic content seems already digested, as revealed by the electron translucent internal compartment, similar to that of lytic vacuoles (Fig 6A). This plastid exhibits electron dense precipitates dispersed throughout the stroma. Together, Figs. 6A-C are suggestive of a process whereby cytoplasm-containing plastids first digest their cytoplasm, and then enter an auto-lytic process conducing to the entire degradation of the plastid. In contrast, pollen-like structures present in the same sections and therefore exposed to the same cytochemical assay did not show any precipitate, neither in their amyloplasts

nor in their cytoplasmic vacuoles (Fig. 6D). Controls excluding β-glycerophosphate did not show any comparable precipitate in any of the studied cell types (Fig. 6E).

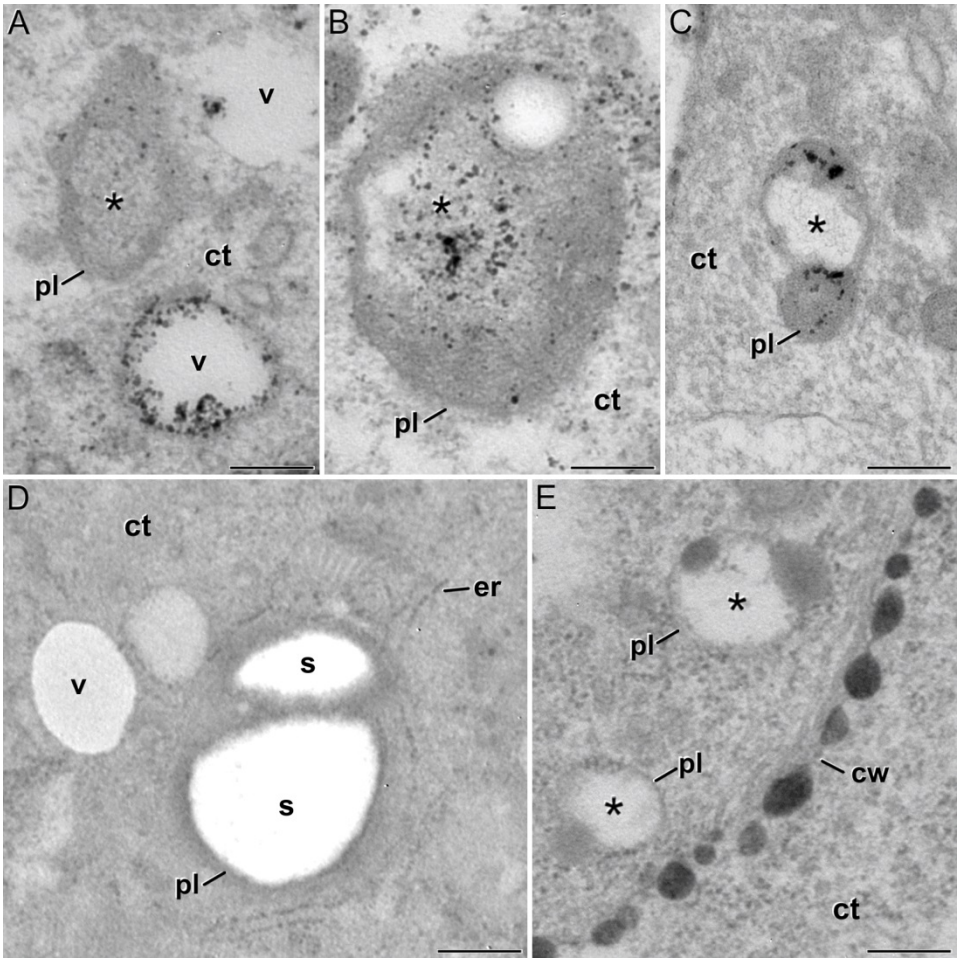

Figure 6: Detection of acid phosphatase activity in cytoplasm-containing plastids of *B. napus* embryogenic microspores. A: Cytoplasm-containing plastid (pl) showing a discrete acid phosphatase activity as revealed by the small and scarce dense precipitates. The cytoplasm compartment (asterisk) is devoid of precipitates. As a reference, two adjacent vacuoles (v) show different amounts of precipitates. B: Cytoplasm-containing plastid where most of the dense precipitates concentrate within the cytoplasm compartment (asterisk). C: Plastid where all the cytoplasmic contents seems digested, and the dense precipitates (lytic activity) D: Vacuoles and amyloplasts

of a non-embryogenic, pollen-like structure. Note the total absence of dense precipitates. E: Cytoplasm-containing plastids of a control sample without β-glycerophosphate. Note the total absence of dense precipitates. ct: cytoplasm; cw: cell wall; er: endoplasmic reticulum; s: starch. Bars: 250 nm.

MDEs presented conventional plastids

Embryogenic structures progressed as MDEs through the different stages of embryo development, including globular (Figure 1C), transitional (Figure 1D), heart-shaped and torpedo (Figure 1E) MDEs. Plastids from cells of these MDE stages were also analyzed in order to check whether the unusual plastid profiles found in induced microspores persisted during further MDE development, or they were transient structures, exclusive of the first stages of MDE induction. As seen in Figure 7, the plastids found in the embryo proper domain (Figure 7A) and in the suspensor (Figure 7B) of globular MDEs were similar to those found in pollen grains (Figure 2C). All of them exhibited a round or oval shape, dense stroma, tubular and/or lamellar thylakoids, and starch granules. No engulfed cytoplasmic regions were observed in any case. Cells of heart-shaped, transitional and torpedo MDEs (data not shown) presented only conventional starch and thylakoid-containing plastids, structurally equivalent to those described for globular MDEs. Thus, it seemed that the unusual features observed in plastids of embryogenic microspores did not persist during MDE development. Instead, plastids adopted a conventional architecture, characterized by the presence of starch and thylakoids.

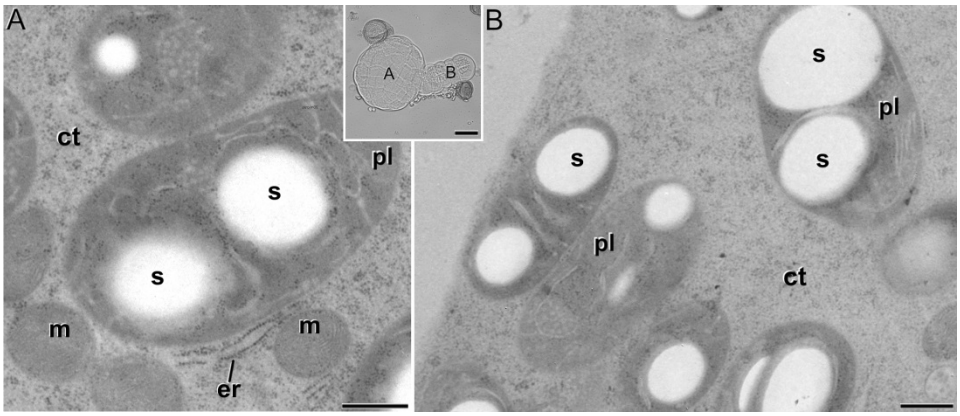

Figure 7: Starch-containing plastids (pl) from *B. napus* globular MDEs. The inset shows a light microscopy section of a globular MDE where the embryo proper (A) and the suspensor (B) domains can be clearly differentiated. Figures A and B show electron micrographs of plastids of embryo proper (A) and suspensor (B) cells. ct: cytoplasm; er: endoplasmic reticulum; m: mitochondria; s: starch. Bars: A, B: 500 nm. Inset: 20 µm.

Mitochondria present a conventional architecture during all the stages of microspore embryogenesis

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

presented exactly the same morphology and architecture than cells of any other culture stage, as well as of non-cultured *B. napus* cells.

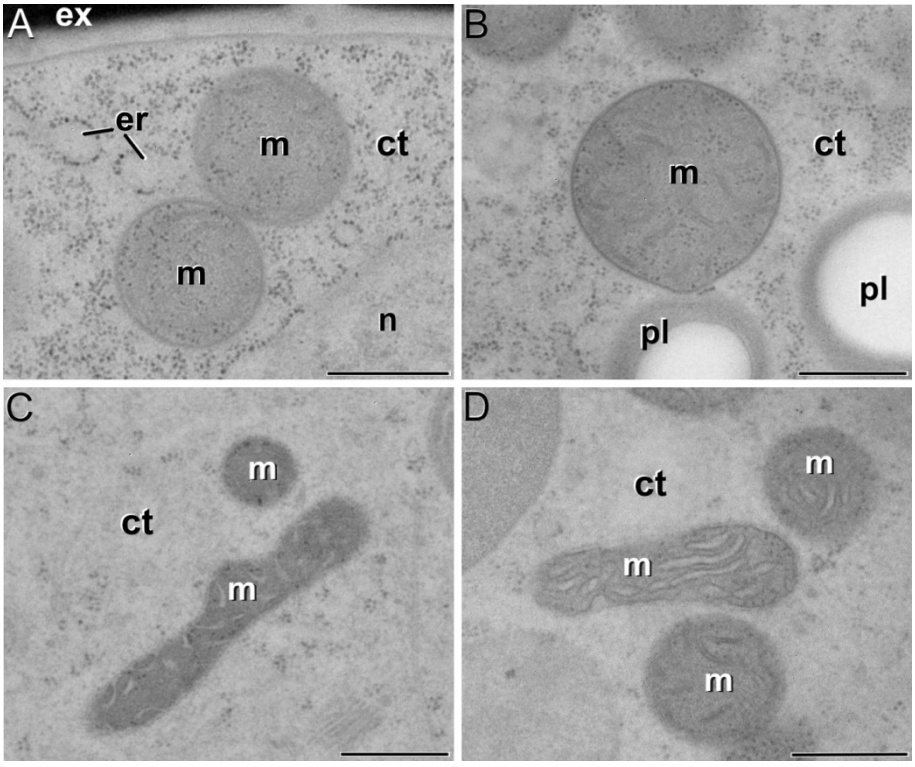

Figure 8: Mitochondria (m) of *B. napus* vacuolate microspores during *in vivo* development within the anther (A), pollen grains within the anther (B), embryogenic microspores (C), and globular MDEs (D). ct: cytoplasm; er: endoplasmic reticulum; ex: exine; pl: plastid; n: nucleus. Bars: 500 nm.

Discussion

The androgenic switch produces atypical plastids

We showed in this work that induction of embryogenesis produces dramatic changes in proplastids of embryogenic microspores, diverting them from their original fate (pollen amyloplasts), and transforming them into

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

Cytoplasm-containing plastids of embryogenic microspores are engaged in autophagy

A plastidial architecture similar to that described hereby for *B. napus* embryogenic microspores has been rarely reported in the literature. Similar observations have only been reported in plastids of suspensor cells of *Phaseolus coccineus* (Nagl 1977) and *Phaseolus vulgaris* (Gärtner and Nagl 1980), where plastids transformed into autophagic vacuoles during the senescence of the suspensor, and in petal cells of *Dendrobium*, where it was

shown that plastids adopt autophagic functions, engulfing and digesting portions of the cytoplasm (van Doorn et al. 2011). As seen, all these reports established a clear link between these plastid transformations and their engagement in autophagy. Our TEM images and 3-D reconstructions demonstrated that a significant percentage of the plastids of embryogenic microspores engulf and isolate entire cytoplasmic portions, creating an independent intraplastidial compartment. Structural changes such as the reduction in the stromal volume and the number of thylakoids, suggests the onset of a new role for the cytoplasm-containing plastids. As seen in Figure 3, the compartmentalized cytoplasm is surrounded by a double membrane system structurally identical to the plastid envelope. This suggests that the intraplastidial compartment is originated from the plastidial double membrane. The 3-D models confirmed the physical continuity between the plastid envelope and the cytoplasmic pockets or internal cytoplasmic compartments. Such double membrane-bound compartments are remarkably similar to the widely described plant autophagosomes (Lundgren Rose et al. 2006; Aubert et al. 1996; Reyes et al. 2011; Otegui et al. 2005). The abundance of C-shaped and dumbbell-shaped plastid profiles found in embryogenic cells, together with the 3-D plastid models, suggests that the intraplastidial compartment is formed by curling and protrusion of a disc-shaped plastid, which wrap around a cytoplasm portion and eventually fuse their opposite ends to engulf it. Mechanistically, this process is also resembling the process of autophagosome formation in plant cells (Li and Vierstra 2012), and specifically in *B. napus* embryogenic microspores, as a consequence of embryogenesis induction (Corral-Martínez et al. 2013). Furthermore, the acid phosphatase activity demonstrated not only in the internalized cytoplasm but also in the stroma, confirmed the lytic activity of these organelles. Based on all these evidences, it is reasonable to assume that

we are observing plastids acting as autophagosomes and developing internal autophagic compartments that eventually lead to the digestion of the entire plastid. According to the term coined by Nagl (1977), they would be *plastolysomes*.

Plastolysomes of embryogenic microspores are excreted to the apoplast

In *Dendrobium* and *Phaseolus*, the plastidial lytic compartments were filled with material of different levels of electron density, from dark to very light (van Doorn et al. 2011; Nagl 1977; Gärtner and Nagl 1980). This, together with the demonstration of acid phosphatase activity, led the authors to propose that the engulfed cytoplasm was digested and recycled. In embryogenic microspores, despite that many of the atypical plastids presented few or no structural evidences of digestion, evidences of acid phosphatase activity was detected in many of them (Figures 6A, B). Furthermore, full plastid degradation, similar to those of *Dendrobium* and *Phaseolus* cells, was found in ~16% of the atypical plastids, and multilamellar bodies, similar in size to plastids, were found in the cytoplasm and also out of it, in the apoplast. Figure 5 illustrates one of these bodies being excreted to the apoplast in a way that indicates that the body has been excreted independently, and not as part of a massive excretion of cytoplasmic material. All these observations made us think that the fate of cytoplasm-containing plastids would not only be their degradation, but also their excretion out of the cell. We propose that cytoplasm engulfment affects not only the structure of the plastid, but also its fate and subsequently, its function. The cytoplasm engulfment, the lytic activity, and the loss of stroma and grana may be related to a functional shift of the plastid, transforming itself into a vehicle for autophagy-like cytoplasm degradation and subsequent

excretion of the digested, or partially digested, material. Recently, similar processes of autophagy associated to excretion of the digested material were demonstrated to occur in embryogenic microspores of *B. napus* (Corral-Martínez et al. 2013). It was proposed that these combined processes would be acting as a cleaning mechanism for massive removal of useless cytoplasmic material. Thus, it seems reasonable to speculate that both mechanisms could be related as parallel parts of a *cleaning program* aimed to adapt the embryogenic microspore to its new developmental scenario. In this context, the cellular mechanisms, still unknown, that mediate the excretion of entire autophagosomes, might also govern the excretion of cytoplasm-containing plastids, even before their contents have been entirely digested and recycled. This would explain the relative low amount of plastids found with empty (fully digested) compartments.. In turn, these mechanisms would account for the reduction in the number of plastids previously described as a consequence of embryogenesis induction (reviewed in Makowska and Oleszczuk, 2013 and Shariatpanahi et al. 2006). Further research should focus on elucidating such putative mechanisms.

Is there a possible link between plastolysome formation and androgenic recalcitrance?

It is known that, in general, angiosperm microspores have only proplastids, which transform into amyloplasts in pollen grains (Clément and Pacini 2001). This general rule, however, may have some exceptions. In 1987, Sangwan and Sangwan-Norreel studied the relationship between the androgenic response of several species and the plastid types present in their microspores and pollen grains. They observed that responsive species present proplastids up to the bicelullar pollen stage (the latter inducible stage). In

contrast, in recalcitrant species proplastids differentiate to amyloplasts at the microspore stage or even before. Indeed, the differentiation of proplastids into amyloplasts has been related to the lack of embryogenic response of late pollen stages (Maraschin et al. 2005) and of recalcitrant, non-responsive species (Sangwan and Sangwan-Norreel 1987). Our results would entirely fit into this theory, since we showed that *B. napus* vacuolate microspores presented proplastids exclusively, whereas amyloplasts were typical for *in vivo* pollen grains and *in vitro*, non-induced pollen-like structures. Furthermore, in this work we provided evidences for a process, exclusive for embryogenic microspores, by which plastids transform into plastolysomes that engulf discrete portions of cytoplasm, digest themselves, and are then excreted out of the cytoplasm. This process might add a new piece to the theory of Sangwan and Sangwan-Norreel (1987), explaining why amyloplasts-containing microspores cannot undergo embryogenesis: an amyloplast would not be able to transform into a plastolysome by the time when the stress treatment is applied. Therefore, amyloplast-containing cells would not be able to execute the cytoplasmic cleaning process as efficiently as those having proplastids, not yet differentiated. This way, the link found hereby between plastid transformation and embryogenic competence could possibly be extended to other species beyond *B. napus*. We are currently examining HPF/FS-processed embryogenic microspores from other species, including recalcitrant crops, in order to validate this hypothesis and to verify to what extent the formation of plastolysomes is a common feature of microspore embryogenesis.

Concluding remarks

This is not the first time that cytoplasm-containing plastids have been described in plant cells. Aside of the present study and the works of Nagl (1977), Gärtner and Nagl (1980) and van Doorn et al. (2011), electron micrographs showing this plastid type have been occasionally published. A good example is the work of González-Melendi et al. (2008), who showed pictures of fertile and Ogu-INRA male sterile tetrads of *Brassica napus* whose plastids contained round compartments filled with cytoplasm. However, the authors did not make any mention in their article to this atypical plastid architecture. Although very rarely, similar plastid morphologies have also been observed in Arabidopsis shoot apical meristem cells (unpublished results of the authors). We speculate that perhaps, plastolysomes have not been more widely identified and described due to the uncertainty that chemical fixation imposes to the interpretation of electron micrographs. To illustrate this, compare the sharp structures and well-defined membranous elements of the plastids shown in Fig. 3 (processed by HPF/FS) with those of Fig. 6 (chemically fixed), where internal structures other than the cytoplasmic compartment are barely seen. It is likely that in many studies using chemical fixatives, the authors have assumed that the unusual plastid morphology might be caused by the fixatives, known to promote structural disorders in membranous elements (see introduction). Therefore, it seems likely that researchers might have assumed during years that the intraplastidial compartments could somehow be an artifact created by the chemical fixatives. This study adds to the increasing number of evidences pointing to this plastid type as a real entity present in some plant cells under particular developmental circumstances, possibly with a role similar to that assigned by this and other previous works.

Conflict of Interest Statement

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

Authors and Contributors

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

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Discusión general

Optimización del protocolo para la obtención de DHs en *Capsicum annuum* L.

El primer paso para poder llevar a cabo la obtención de DHs es identificar el estadio óptimo de desarrollo de las microsporas, a partir del cual se puede inducir la androgénesis. Para la mayoría de las especies estudiadas, incluyendo el pimiento (*Capsicum annuum* L.), este estadio óptimo se encuentra en torno a la transición de microspora vacuolada a polen bicelular joven (la primera mitosis del polen) (Touraev et al., 2001; Maraschin et al., 2005; Seguí-Simarro, 2010a; Irikova et al., 2011). En el capítulo 1 de la presente Tesis Doctoral, se buscaron marcadores morfológicos fácilmente reconocibles que identificaran este estadio idóneo para inducir las microsporas hacia una nueva ruta de desarrollo embriogénico. Los marcadores evaluados fueron la longitud de yema, longitud de antera, proporción de la longitud de los sépalos con respecto a la longitud total de la yema y pigmentación de las anteras. La longitud de yema y antera son los marcadores que aportan mayor precisión, pero es fundamental que los marcadores a utilizar sean también fáciles de usar y medir para agilizar el proceso de extracción de las anteras sin comprometer la viabilidad de las microsporas. En especies que presentan yemas florales grandes, como el caso de la berenjena que presenta tamaños de anteras óptimas para la inducción entre 5 y 6 mm (Salas et al., 2012), el parámetro longitud de antera se aplica de manera rutinaria, ya que su gran tamaño facilita la medición y no ralentiza la puesta en cultivo de las mismas. En otras especies que presentan yemas florales de pequeño tamaño, como el caso de la colza que presenta longitudes óptimas de yema comprendidas entre 3,2 y 3,5 mm (Custers, 2003), se miden directamente las yemas florales sin necesidad de extraer las anteras. En el caso del pimiento, que presenta tamaños de yema

florares comprendidos entre estas dos especies, muchos autores han optado por emplear marcadores que por ser visuales podrían parecer menos precisos que la longitud de yema y antera, pero que agilizan la identificación de las yemas y anteras óptimas sin comprometer la viabilidad de las microsporas. La proporción sépalo/yema es uno de los marcadores mas empleados en pimiento, en variedades tanto dulces como picantes (Qin y Rotino, 1993; Ltifi y Wenzel, 1994; Irikova y Rodeva, 2005; Ercan et al., 2006; Supena et al., 2006a; Koleva-Gudeva et al., 2007). Debido a que la pigmentación de las anteras es un parámetro poco dependiente del genotipo (Regner, 1996), hace que el uso de este marcador pueda ser aplicado a un rango mas amplio de genotipos. Por ello, algunos autores se han decantado por el uso combinado de la proporción sépalo/yema junto con la pigmentación de las anteras para genotipos productores de antocianos (Dumas de Vaulx et al., 1981; Dolcet-Sanjuan et al., 1997; Buyukalaca et al., 2004; Barany et al., 2005). En el caso de los genotipos empleados en el capítulo 1, también se propone el uso combinado de ambos marcadores para identificar los estadios idóneos para la inducción hacia embriogénesis para aumentar la precisión en la identificación de la etapa adecuada. En los tres genotipos estudiados, las yemas idóneas son aquellas que presentan el 80-90% de las mismas cubiertas por el cáliz, denominado por algunos autores como "*pétalos un poco más largos que los sépalos*". De estas yemas se hace una segunda selección, escogiendo todas aquellas que contengan anteras con pigmentación morada en el extremo apical. Estos mismos marcadores se han empleado en muchas de las investigaciones previas en embriogénesis de microsporas de pimiento, utilizándose tanto para variedades dulces como picantes (Dolcet-Sanjuan et al., 1997; Buyukalaca et al., 2004; Supena et al., 2006b; Koleva-Gudeva et al., 2007). Aunque en principio,

estos marcadores indirectos no serían aplicables a otras especies debido a las diferentes morfologías florales de cada especie (Irikova et al., 2011), el hecho de que los mismos marcadores se hayan empleado en diferentes genotipos, indica que existe una correlación de la proporción sépalo/yema con el estadio de las microsporas que se conserva en los diferentes cultivares de pimiento. Por lo tanto, podría decirse que estos marcadores, con pequeños ajustes, se podrían aplicar a diferentes variedades, tanto de pimiento dulce como picante.

Una vez establecidos estos marcadores, se estudió la relación que existe entre las condiciones de cultivo y la producción de callo en el cultivo de anteras. Se sabe que una de las principales desventajas del cultivo de anteras es la producción de callos. La formación de plantas a través de organogénesis es un proceso mas lento que la embriogénesis directa, consume mas recursos y además incrementa la posibilidad de promover la variación gametoclonal (Forster et al., 2007). Pero el principal problema proviene del posible origen somático de los callos obtenidos. Por ejemplo, en el cultivo de anteras de tomate, se vio que un 83% de los callos generados provenía directamente del tejido de la antera (Corral-Martínez et al., 2011). Los regenerantes obtenidos a partir de estos callos no podrán ser utilizados para obtener DHs por su origen no haploide. Por ello, es tan importante minimizar la aparición de callos en este sistema. Para lograr este objetivo, en el capítulo 2 se compara la influencia que tiene el uso de dos protocolos diferentes de cultivo de anteras en la producción de callos y embriones, así como la influencia de someter a las anteras a diferentes tiempos de choque térmico (35 °C). Se hicieron cultivos de anteras en 4 variedades de pimiento dulce (Herminio, Coyote, Quito y Velez) y se emplearon dos de los métodos más usados para inducir la embriogénesis de microsporas en pimiento: el método descrito por Dumas de Vaulx et al. (1981)

(DDV) y el método descrito por Supena et al. (2006a, b), más conocido como "*shed-microspore culture*" (SM). En los resultados obtenidos, la formación de callo a partir del tejido de la antera parece independiente del genotipo, puesto que manteniendo las mismas condiciones para las 4 variedades, con el método DDV todas ellas generaron un número similar de callos, mientras que con el método SM no se indujo la formación de callos en ninguna de las 4 variedades. Esto demuestra que la formación de callo está más ligada a las condiciones de cultivo que al genotipo. Estudios previos de cultivo de anteras realizados en 12 cultivares de berenjena, demostraron también la independencia de la formación de callo con el genotipo utilizado (Salas et al., 2011). En el caso concreto de nuestros resultados, la presencia o ausencia de callo a partir del tejido de las anteras podría estar condicionada por el balance de reguladores de crecimiento en el medio de cultivo. Con el método DDV la concentración de auxinas y citoquininas es casi idéntica (0,046 µM de kinetina y 0,045 µM 2,4-Dichlorophenoxyacetic acid), lo cual es sabido que favorece la formación de callo (van Staden et al., 2008). En el caso del método SM, el balance auxinas/ citoquininas empleado se decanta en favor de las auxinas (5 µM de ácido indolacético y 2,5 µM de zeatina), lo cual promueve los primeros estadios de la embriogénesis (van Staden et al., 2008). Al someter las anteras a diferentes tiempos de exposición a elevadas temperaturas, se confirma que el origen de los callos es más dependiente de las condiciones de cultivo que del genotipo, puesto que a medida que se incrementa el tiempo de exposición al calor, el número de callos se incrementa de manera similar en los 4 genotipos, mientras que el número de embriones se reduce de manera significativa. Estos resultados confirman que la obtención de callos en el cultivo de anteras es más dependiente de las condiciones del cultivo que del genotipo. Del mismo modo,

demuestran una relación entre las condiciones de cultivo de los dos procesos morfogénicos posibles en este caso: inducción de embriogénesis y proliferación celular indiferenciada.

En el momento de elaborar un protocolo eficaz de cultivo de anteras que evite la formación de callos de origen somático, se podría emplear el método SM, sin embargo, este método puede no funcionar de manera eficaz en todos los genotipos y sería imprescindible validarlo previamente, ya que hasta la fecha solo se ha empleado con éxito en variedades asiáticas de pimiento picante (Supena et al., 2006b; Supena y Custers, 2011). El método DDV ha sido empleado por muchos autores en un amplio rango de cultivares, tanto dulces como picantes (Irikova et al., 2011). Por ello, en el capítulo 3 se han adaptado los resultados extraídos de los capítulos 1 y 2 al método DDV para obtener un protocolo detallado de cultivo de anteras en pimiento. Una de las modificaciones es la reducción en el tiempo de exposición al choque térmico, lo cual ha servido para incrementar la producción de embriones y reducir la aparición de callos de origen somático. De esta manera, se reduciría el tiempo y recursos necesarios para la obtención de plantas DHs al no ser necesaria la regeneración a partir del tejido indiferenciado para obtener un mismo número de individuos DHs. O dicho de otro modo, aumentaría la eficiencia del proceso. Además, se ha visto que la frecuencia de DHs que surgen de manera espontánea a partir de embriones derivados de microsporas en pimiento, es aproximadamente un 30-40% del total de los regenerantes obtenidos, dependiendo del genotipo empleado (revisado en Irikova et al., 2011). Por ello, cuanto mayor sea el número de embriones obtenidos, mayor serán las posibilidades de obtener un elevado número de plantas regeneradas y en consecuencia, se producirá un mayor número de DHs de forma espontánea.

Esto podría evitar la aplicación de tratamientos para la duplicación del genoma, como puede ser la colchicina, que es tóxica para las plantas y los operarios que la aplican e incrementa el gasto y el tiempo de obtención de los DHs. Además, el pimiento ha mostrado una eficiencia de duplicación del genoma en planta adulta del 50%, aunque si se aplica en regenerantes haploides de 1 mes, el éxito de duplicación alcanza el 95% (revisado en Irikova et al., 2011). No debemos olvidar que cuanto mayor sea el número de plantas DHs obtenidas, mayor será la variabilidad genética que puede ser utilizada en los programas de mejora, ya que cada planta proviene de una microspora haploide diferente, debido a la recombinación en los productos meióticos.

La presencia de calosa y la aparición de plastolisomas son dos nuevos marcadores de la reprogramación hacia embriogénesis

Los estudios de investigación básica acerca de los procesos que actúan como causas de la reprogramación hacia embriogénesis, o que se disparan a consecuencia de ella, son una herramienta fundamental a la hora de descubrir nuevas vías para mejorar la eficiencia del proceso de obtención de DHs. Estos estudios permiten obtener una gran cantidad de información acerca de los acontecimientos que suceden en el interior de las microsporas cuando son inducidas hacia embriogénesis, y ayudan a entender los procesos que están teniendo lugar durante el cambio en el programa de desarrollo de las microsporas. La información obtenida mediante este tipo de estudios, es empleada para mejorar la eficiencia en especies consideradas recalcitrantes a la inducción. Estos estudios de investigación básica son realizados con especies modelo, debido principalmente a dos razones. En primer lugar, presentan una eficiencia de inducción y de regeneración de embriones muy elevada, y en

segundo lugar, en los sistemas modelo generalmente no es necesario emplear reguladores del crecimiento para inducir la reprogramación, lo cual favorece la embriogénesis directa sin pasar por la fase de callo (Joosen et al., 2007). *Brassica napus* es una de las especies consideradas modelo. Se ha venido empleando para la obtención de DHs desde hace mas de 30 años (Lichter, 1982), siendo numerosos los estudios de investigación básica acerca del desarrollo embriogénico en los que se ha empleado, tanto a nivel molecular (Boutilier et al., 2005; Maraschin et al., 2005; Joosen et al., 2007), como ultraestructural (Zaki y Dickinson, 1990; Telmer et al., 1993, 1995; Testillano et al., 2000; Seguí-Simarro et al., 2003) (ver Introducción del Bloque II). En la presente Tesis Doctoral nos hemos centrado en los cambios ultraestructurales que sufren las microsporas cuando tiene lugar la inducción de la embriogénesis. Debido a que en esta ruta androgénica se desarrollan muy diversas estructuras, tanto inducidas como no inducidas, los estudios ultraestructurales juegan un papel muy importante porque permiten diferenciar *in situ* todos estos tipos estructurales e identificar qué está pasando en cada uno de ellos. La mayoría de los estudios de este tipo realizados hasta la fecha están basados en el análisis de muestras preservadas con fijadores químicos. El principal inconveniente de estos fijadores es la creación de artefactos en el citoplasma que pueden generar una visión distorsionada de los eventos celulares (Gilkey y Staehelin, 1986; McDonald y Auer, 2006). Nosotros hemos empleado una técnica alternativa a la fijación química, la fijación por alta presión combinada con la criosustitución (HPF/FS). La combinación de bajas temperaturas y presión muy elevada (2100 bares), evita la formación de cristales de hielo y proporciona una excelente preservación a nivel ultraestructural (Gilkey y Staehelin, 1986). Además, esta técnica ya ha sido empleada previamente con

éxito en cultivos de microsporas de *B. napus,* obteniéndose resultados novedosos nunca antes descritos (Corral-Martínez et al., 2013). En el capítulo 4, se presenta un estudio detallado de las paredes celulares presentes en las microsporas de *B. napus* durante los estadios iniciales de la inducción hacia embriogénesis. En las microsporas correctamente inducidas, gracias al uso de HPF/FS se pudieron ver anomalías en la formación de la placa celular, como la presencia de paredes celulares discontinuas, incompletas y núcleos fusionándose. Mediante inmunodetección de calosa y tinciones específicas de calosa y celulosa, se observaron patrones inusuales de acumulación de calosa y celulosa en las paredes celulares y placas celulares en formación. En la formación normal de una pared celular vegetal, la red túbulo-vesicular de la placa celular se transforma en una red tubular madura debido a la deposición de grandes cantidades de calosa en el lumen de la placa celular. Al mismo tiempo que la placa celular alcanza la pared de la célula originaria, la calosa comienza a ser eliminada y sustituida por la deposición de fibras de celulosa, formándose la nueva pared celular que separa las dos células hijas (Seguí-Simarro et al., 2008). En el caso de las microsporas inducidas de *B. napus*, la formación de las paredes no siguió este patrón de acumulación de calosa y posterior sustitución por celulosa. Lo que se vio realmente, fue una acumulación discontinua de calosa en la capa subintinal, en las paredes nuevas y en las ya maduras de las microsporas inducidas. Se vio que la celulosa no sustituía a la calosa en el momento de formar las nuevas paredes, sino que la calosa permanecía en ellas. Solo en embriones más desarrollados se pudieron ver paredes con una composición convencional en cuanto a celulosa y ausencia de calosa. En los estudios realizados hasta la fecha, no se ha llegado a un consenso con respecto a la aparición de la celulosa en microsporas

embriogénicas. Algunos autores, que han usado calcofluor para detectar este polímero, afirman que la celulosa está presente en las primeras etapas de desarrollo de las microsporas inducidas y en las estructuras tipo polen (Schulze y Pauls, 2002; Solis et al., 2008). Sin embargo, en estudios recientes de las fases iniciales de la reprogramación a embriogénesis en *B. napus* no se detectó celulosa en las paredes hasta la fase de embrión (Dubas et al., 2013). Estas diferencias en los resultados podrían ser debidas a la especificidad de la tinción, ya que se ha visto que el calcofluor es menos específico que otras tinciones, como la pontamina (S4B) o el congo red, pudiendo unirse, además de a la celulosa, a otros carbohidratos presentes en la pared celular (Anderson et al., 2010; Thomas et al., 2013). En este sentido, nuestro estudio contribuye a esclarecer este aspecto de la formación y composición de las paredes celulares en las microsporas inducidas. Se han empleado diferentes técnicas para la detección de la calosa (inmunomarcado y tinción fluorescente), y la detección de la celulosa se ha realizado con S4B, tinción que ha demostrado ser mas específica que el calcofluor o el congo red (Anderson et al., 2010). Sería interesante para poder confirmar totalmente este patrón de comportamiento de la calosa y la celulosa, realizar también una inmunodetección de la celulosa a nivel ultraestructural para confirmar definitivamente los resultados obtenidos. Por desgracia, no existen actualmente anticuerpos específicos para la celulosa, y la única posibilidad de detectarla es mediante la enzima celobiohidrolasa I conjugada con oro coloidal (Samuels et al., 1995). La celobiohidrolasa es una enzima que corta la cadena β-1,4-D-glucano a partir del extremo no reductor de la molécula de celulosa, dando lugar a unidades de celobiosa (2 moléculas de glucosa). Esta técnica es mucho más compleja y menos específica que las tinciones utilizadas. Además, nuestro trabajo aporta una nueva dimensión en

este estudio, pues relaciona estas paredes de composición alterada con la ocurrencia de fenómenos de fusión nuclear, y por tanto, de aparición espontánea de DHs y/o mixoploides.

Las alteraciones a nivel de la pared celular en las microsporas inducidas podrían estar causadas por el choque térmico al que se someten las microsporas durante el periodo de inducción. Se sabe que temperaturas elevadas provocan alteraciones de los niveles de Ca^{2+} citosólico (Gong et al., 1998), y que la presencia prolongada de elevados niveles de Ca^{2+} en la placa celular inmadura, puede activar de forma permanente las calosa-sintasas (dependientes del Ca^{2+}), y por tanto promover la síntesis continuada de calosa. Además, el Ca^{2+} y la propia presencia de calosa pueden inhibir la síntesis de celulosa, provocando un retraso en la maduración de la placa celular (Verma y Hong, 2001). En el capítulo 2 hemos visto que al someter a las anteras a periodos prolongados a 35 ºC se promueve una disminución en el número de embriones. Esto podría ser debido a la formación de paredes exclusivamente de calosa, poco consistentes y fluidas, que impidan el desarrollo y maduración de las paredes celulares propias de los embriones. Para corroborar esta hipótesis, sería interesante probar factores que alterasen los canales del Ca^{2+} y comprobar si incrementar o disminuir los niveles de Ca^{2+} citosólico afecta a la formación de las paredes celulares en las microsporas inducidas y en consecuencia, al número de embriones obtenidos. La cafeína es un ejemplo de compuesto químico que podría ser utilizado para realizar estos estudios, puesto que se ha demostrado que inhibe la formación de la placa celular. La cafeína disminuye los niveles de Ca^{2+} intracelular lo cual hace que se inhiba la síntesis de calosa (Samuels y Staehelin, 1996). En los cultivos de microsporas, el estrés por calor estaría incrementando los niveles de Ca^{2+} citosólico. Por tanto, la

adición de cafeína podría disminuir los niveles de Ca^{2+} hasta valores cercanos a los normales, de modo que no se alterara la dinámica normal de deposición de la calosa y se formaran paredes celulares normales, con celulosa. También podría suceder que los niveles de Ca^{2+} disminuyeran hasta tal punto que no fuera posible ni tan siquiera la síntesis de calosa, con lo que tampoco podrían formarse paredes celulares normales, pero esta vez no por ausencia de celulosa, sino de calosa. En cualquiera de estos dos casos, la cafeína nos estaría confirmando el papel que tiene la regulación del Ca^{2+} intracelular en la síntesis de calosa y en la aparición de paredes celulares anormales e incompletas como las que hemos descrito.

Otros compuestos químicos como la digitonina y el chitosán podrían usarse como inductores de la formación de calosa, ya que parece que actúan favoreciendo la entrada del Ca^{2+} a través de la membrana plasmática e incrementan temporalmente los niveles del Ca^{2+} intracelular (Albersheim et al., 2011). La presencia permanente de estos compuestos en el cultivo de microsporas favorecería una síntesis de calosa continua, y en consecuencia la inhibición de manera permanente de la síntesis de celulosa. Esto daría lugar a la formación de paredes celulares inmaduras, fluidas e incompletas compuestas exclusivamente por calosa. Se estarían favoreciendo fenómenos de fusión nuclear y se impediría la formación de las paredes de los embriones, debido a que la celulosa es un polímero esencial en la composición de las paredes celulares (Seguí-Simarro et al., 2008). Debido a que estos compuestos parecen tener un efecto similar al de someter las microsporas a elevadas temperaturas (en ambos casos se incrementan los niveles de Ca^{2+} intracelular), sería interesante comprobar también, si el uso de estos compuestos podría sustituir al estrés por calor en el cultivo de microsporas lo cual confirmaría el papel clave

del Ca^{2+} en la inducción a embriogénesis. Sería importante mantener la digitonina o el chitosán el tiempo suficiente para que tuvieran lugar las primeras fases de la inducción, para después eliminarlos y que se pudieran recuperar los niveles normales de Ca^{2+} que permitan la activación de las celulosa-sintasas, y se formen las paredes celulares normales de los embriones. Todos estos estudios servirían para confirmar si realmente la presencia de calosa y la ausencia de celulosa tienen un papel clave en las primeras etapas de la inducción de las microsporas hacia embriogénesis, en la formación de las paredes celulares y finalmente en el número de embriones obtenidos.

Si asumimos que la presencia de calosa es un marcador de los primeros estadios de la inducción hacia embriogénesis, podemos emplear esta información para obtener poblaciones enriquecidas de microsporas inducidas. Mediante el uso de un "*Fluorescence-activated cell sorter*" (FACS), citómetro que separa poblaciones de células en función de la fluorescencia y tamaño, podríamos separar las células inducidas de las no inducidas mediante una tinción con azul de anilina. Schulze y Pauls (1998) emplearon esta técnica en cultivos de microsporas aisladas de *B. napus*, separando las microsporas viables y embriogénicas, empleando fluorescein diacetate (FDA) como tinción fluorescente. Las poblaciones homogéneas que se extraen del citómetro, pueden utilizarse para estudios moleculares, celulares o ultraestructurales entre otros, en los que solamente estarán presentes las microsporas que realmente interesan para el estudio, pudiendo realizarse comparativas entre las poblaciones inducidas y no inducidas, como por ejemplo, variación en los niveles endógenos de hormonas o de diferentes genes que se expresan en cada población.

También podrían emplearse (1,3)β-glucanasas, enzimas encargadas de la degradación de la calosa, para disgregar las células que se han formado en las microsporas inducidas. Como en estas células ya estará activado el nuevo programa de desarrollo embriogénico, continuarían posiblemente dividiéndose al igual que lo harían en las microsporas inducidas. Si esta técnica funcionara, se multiplicaría el número de embriones que provienen de la misma microspora haploide. Estos embriones serían genéticamente idénticos y el resultado final no se traduciría en un incremento de la variabilidad genética, pero sí se lograría un mayor porcentaje de plantas regeneradas, ya que de los embriones obtenidos no todos llegan a regenerar planta completa. En el caso del método SM, del total de los embriones obtenidos, solamente entre un 15 y 33% regeneraron planta completa (Supena et al., 2006b).

En el capítulo 5, también se han empleado muestras procesadas con HPF/FS para estudiar, en este caso, los posibles cambios que sufren plastidios y mitocondrias durante la reprogramación de las microsporas, y que pudieran estar relacionados con procesos de autofagia. Los resultados demuestran que las mitocondrias mantienen su estructura convencional durante todo el proceso de embriogénesis. Sin embargo, en las primeras fases de la inducción de las microsporas, se ha visto que casi un 40% de los plastidios presentan morfologías atípicas, diferentes de los perfiles elongados, redondeados u ovalados, típicos de los plastidios convencionales. La reconstrucción tridimensional, realizada a partir de imágenes seriadas ha permitido demostrar que estos plastidios presentan un comportamiento diferente de lo habitual y que captan y engloban porciones del citoplasma en su interior. Estos resultados confirman las imágenes vistas al TEM y la clasificación realizada de los tres tipos de plastidios presentes en las microsporas embriogénicas: con perfiles abiertos

que rodean parcialmente porciones de citoplasma, con perfiles cerrados en los que el citoplasma forma parte del interior del orgánulo y plastidios que presentan estructuras membranosas multilamelares. La detección de la fosfatasa ácida ha permitido confirmar que en estos orgánulos están teniendo lugar procesos de digestión, similares a los observados en *Dendrobium* y *Phaseolus*, donde se vio que los plastidios engullían y digerían porciones del citoplasma (Nagl, 1977; Gärtner y Nagl, 1980; van Doorn et al., 2011). Sin embargo, a diferencia de estas especies, nuestros resultados en microsporas embriogénicas de *B. napus* indican que estos plastolisomas acaban siendo excretados, junto con su contenido, total o parcialmente digerido, al apoplasto, de forma similar a lo que sucede con los autofagosomas descritos también en microsporas inducidas de *B. napus* (Corral-Martínez et al., 2013).

En las pocas especies en las que se ha documentado la presencia de estos plastidios (células del suspensor y de pétalos en senescencia (Nagl, 1977; Gärtner y Nagl, 1980; van Doorn et al., 2011), en todas ellas estaban teniendo lugar procesos de muerte celular programada. Se sabe que la muerte celular programada puede originarse como una respuesta a la diferenciación celular o a un estrés abiótico (van Doorn y Woltering, 2005). En el caso de las microsporas inducidas, el desvío del programa gametofítico y el estrés por calor, podrían ser la causa de la transformación de los plastidios en plastolisomas. Las situaciones de estrés también pueden provocar que los proplastidios se diferencien a amiloplastos (Clément y Pacini, 2001). El hecho de que en las estructuras tipo polen no se hayan detectado plastolisomas podría ser debido a que no han sufrido la reprogramación hacia embriogénesis y no necesitan adaptarse a un nuevo programa de desarrollo esporofítico.

Se sabe que la presencia de amiloplastos en las microsporas/polen es mayor en especies recalcitrantes a la inducción de la embriogénesis (Sangwan y Sangwan-Norreel, 1987). Este hecho podría deberse a la incapacidad de los amiloplastos para comportarse como plastolisomas. De ser así, podría decirse que la presencia de amiloplastos estaría bloqueando procesos esenciales para la reprogramación de las microsporas hacia embriogénesis. El tomate es una de las especies más recalcitrantes y se ha visto que presenta proplastidios y amiloplastos desde la fase de tétrada a la de polen bicelular joven. En cambio el pimiento, solo presenta proplastidios en estos estadios (Sangwan y Sangwan-Norreel, 1987). En base a esto podríamos especular que la manipulación experimental de las rutas de biosíntesis del almidón, podrían ser una vía potencial de aumentar la sensibilidad a la inducción de las especies recalcitrantes.

De los estudios a nivel ultraestructural realizados en la presente Tesis Doctoral se puede extraer una secuencia hipotética de eventos celulares necesarios para que tenga lugar la inducción a embriogénesis. Cuando las microsporas son sometidas a un estrés por calor, algunas de las que responden a ese estrés sufrirían alteraciones en los niveles de Ca^{2+}. Se activarían las calosa sintasas que sintetizan la calosa que se va depositando en forma de capa subintinal. A partir de este momento, estas microsporas ya habrían entrado en el nuevo programa de desarrollo embriogénico y es cuando tiene lugar la primera división. En el estadio de anafase tardía comienza la formación de la placa celular bajo niveles elevados de Ca^{2+} intracelular. Esto a su vez daría lugar a la formación de una pared celular anormal, incompleta y compuesta principalmente por calosa y no por celulosa. Es en estos momentos donde podrían darse las fusiones nucleares y los fenómenos de duplicación

cromosómica. Las microsporas necesitarían deshacerse del antiguo programa gametofítico y empezaría el proceso de limpieza del citoplasma incluyendo los plastolisomas. En el momento en que el nuevo programa de desarrollo está en marcha, las microsporas inducidas continúan con su desarrollo hacia embriogénesis. Entonces, las nuevas células formadas en las siguientes rondas de mitosis recuperarían los niveles normales de Ca^{2+}, la dinámica normal de deposición y eliminación de la calosa, y la síntesis normal de celulosa, formándose las paredes celulares maduras, tal como ocurre en la citocinesis de células somáticas. Los plastidios dejarían de actuar como compartimentos líticos, desempeñando su función normal en el embrión.

De los resultados obtenidos, se han logrado extraer dos posibles marcadores de la inducción a embriogénesis: presencia de paredes con calosa y los plastidios actuando como plastolisomas. Sería interesante extrapolar estos marcadores para su uso en especies que han resultado ser recalcitrantes a la androgénesis, como es el caso del pimiento, o incluso el tomate. Se debería comprobar si en estas especies también está presente la calosa en los primeros estadios de la inducción, si en los estadios óptimos para la inducción hay presentes amiloplastos o si los plastidios presentan porciones de citoplasma en su interior. Con esos primeros resultados, se podrían tomar medidas para tratar de solucionar problemas como la baja eficiencia de inducción. Algunas posibles soluciones podrían ser el uso de compuestos químicos que incrementen los niveles de Ca^{2+} citosólico, o que disminuyan o impidan la acumulación de almidón en los plastidios en aquellas especies que presentes amiloplastos en los estadios óptimos para la inducción.

Conclusiones

En la presente Tesis Doctoral se han estudiado diferentes factores que influyen en la reprogramación hacia embriogénesis de las microsporas/polen y se ha logrado optimizar un protocolo de cultivo de anteras para la obtención de DHs en pimiento.

- Para determinar las yemas que contienen las microsporas en su estadio idóneo para la inducción de la androgénesis en pimiento, se propone el uso combinado de la relación sépalo/yema junto con la pigmentación de las anteras. La combinación de ambos criterios proporciona rapidez a la hora de seleccionar las yemas y anteras óptimas, sin perder precisión, ni perjudicar la viabilidad de las microsporas/polen.
- La aparición de callos de origen somático en el cultivo de anteras de pimiento, es más dependiente de las condiciones del medio de cultivo que del genotipo.
- Reducir el tiempo que las anteras son sometidas al estrés por calor a 4 días genera un mayor número de embriones y un menor número de callos de origen somático.
- El protocolo utilizado comúnmente para el cultivo de anteras en pimiento (Dumas de Vaulx et al., 1981), junto con las modificaciones descritas en esta Tesis, se traduce en un protocolo más eficaz para la obtención de DHs en pimiento.
- Mediante los estudios de investigación básica realizados en *B. napus* se confirma que la combinación de alta presión y criosustitución (HPF/FS) es el mejor método descrito hasta la fecha para la preservación ultraestructural de las células.

- Se han establecido dos nuevos marcadores de embriogénesis en las microsporas recién inducidas que abren nuevas posibilidades de manipulación del proceso para su optimización. Estos marcadores embriogénicos son:

    1. Formación y excreción de plastolisomas (plastidios con morfología atípica y actividad lítica, que capturan y digieren porciones del citoplasma).
    2. Formación de paredes celulares deformes, incompletas y con una composición anormal, que presentan altos niveles de calosa y ausencia de celulosa.

- La presencia de estas paredes favorece los fenómenos de fusión nuclear y por lo tanto, la aparición de DHs espontáneos sin necesidad de aplicar tratamientos para la duplicación del genoma.

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