Universidad Politécnica de Valencia

Tesis para la obtención del título de Doctor en Biotecnología

OBTENCIÓN DE DOBLE HAPLOIDES EN ESPECIES DE INTERÉS AGRONÓMICO: ANÁLISIS DE AGENTES Y MECANISMOS CELULARES IMPLICADOS EN LA INDUCCIÓN ANDROGÉNICA EN BERENJENA, COLZA Y TOMATE

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CERTIFICA:

Que Dª **Patricia Corral Martínez**, Licenciada en Biología por la Universitat de València, ha realizado bajo mi dirección la Tesis Doctoral titulada "O**btención de doble haploides en especies de interés agronómico: análisis de agentes y mecanismos celulares implicados en la inducción androgénica en berenjena, colza y tomate"** y constituye su Memoria de Tesis para optar al grado de Doctora en Biotecnología.

Y para que así conste, firmo la presente
en Valencia, a 31 de Mayo 2013

Dr. Jose María Seguí Simarro.

A mi hermano

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Abstract

Androgenesis is an inducible process that allows to obtain doubled haploid, pure lines through embriogenesis or callogenesis, starting from male gametophytes (young bicellular pollen) or their precursors, the microspores. From a biotechnological perspective, this possibility has become very relevant because pure lines are the base of hybrid seed production, and doubled haploid technology reduces the 8 or 9 generations necessary obtain a pure line with the conventional selfing and selection approaches, to only one generation.

The present Dissertation focuses on the study of androgenesis applying in parallel two approaches: (1) a basic one, related to the study of the factors that influence this process and the changes undergone by the microspores when induced; and (2) an applied approach directed to improve induction efficiency in recalcitrant species. For this work, we aimed three plant species: rapeseed *(Brassica napus)*, used as a model plant for the study of androgenesis, and two species described as recalcitrant, tomato *(Solanum lycopersicum)*, and eggplant *(Solanum melongena).*

We used High Pressure Freezing (HPF) and Freeze substitution (FS) to process rapeseed isolated microspore cultures to study the ultrastructural changes undergone in the induced microspores. We also optimized a system for genetic transformation of microspores, and further induction of secondary embryogenesis over the microspore-derived primary embryos, with the purpose of using this system as a tool for basic studies.

In tomato, we have characterized the process of androgénesis induction through anther culture. We demonstrated that the meiocyte is the inducible stage, instead of the microspore, which is the most sensitive stage in many other androgenic systems. Upon induction, meiocytes produce calli, coming most of them from somatic cells or from the fusion of two haploid nuclei of the meiocyte. These observations could explain the difficulty to obtain doubled haploids from tomato anther cultures.

In eggplant we developed an efficient system for the production of double haploid plants through isolated microspore culture. In this case, doubled haploid plants were obtained through callogenesis and further organogenesis. We also investigated the role of several factors, previously used in other plant species, in the process of induction of the eggplant microspore. Most of the tested factors showed a positive effect in the androgenic response, in growth and in the quality of the material obtained.

In summary, the studies presented in this work help to increase our knowledge about the androgenic process through its study in both model and recalcitrant species. Besides, they allowed us to present a new and efficient method to obtain doubled haploids in eggplant through microspore culture.

Resumen

La androgénesis es un proceso que permite la obtención de líneas puras doble haploides partiendo, en la mayoría de los casos, del gametófito masculino o de su precursor, la microspora. Desde una perspectiva biotecnológica, esta posibilidad adquiere gran relevancia, pues las líneas puras son la base del proceso de obtención de semilla híbrida, y este proceso permite reducir notablemente el tiempo y los recursos necesarios para obtener líneas puras de forma convencional.

En la presente Tesis Doctoral se ha abordado el estudio de la inducción de androgénesis aplicando en paralelo dos enfoques: uno básico, mediante el estudio de los factores que influyen y los cambios que tienen lugar en la microspora al ser inducida, y uno aplicado, orientado a mejorar la eficiencia de la inducción en especies recalcitrantes. El estudio se ha llevado a cabo en tres especies: colza *(Brassica napus L.)*, utilizada como sistema modelo para estudios básicos, y dos recalcitrantes, tomate *(Solanum Lycopersicum Mill.)*, y berenjena *(Solanum melongena L.)*.

En esta Tesis Doctoral se ha utilizado la fijación por alta presión y la criosustitución para procesar cultivos de microsporas de colza y estudiar los cambios ultraestructurales que tienen lugar como consecuencia de la inducción del cambio en el programa de desarrollo. Hemos optimizado también un sistema para la transformación genética de microsporas de colza, como paso previo a la inducción androgénica y obtención de embriones secundarios, con el objetivo de ampliar su uso como herramienta para estudios básicos más allá de lo que se utilizaba actualmente.

En tomate se ha caracterizado el proceso de la inducción a partir del cultivo de anteras, determinando que el estadio óptimo de inducción no es el de microsporas, sino de una etapa muy anterior, el de meiocito. También se analizaron los callos obtenidos, obteniendo una mayoría de origen somático o procedentes de fenómenos de fusión nuclear dentro del meiocito. Estos procesos podrían explicar la dificultad para obtener doble haploides a partir del cultivo de anteras de tomate.

En berenjena se ha puesto a punto un sistema eficiente para la obtención de plantas doble haploides mediante cultivo de microsporas aisladas. En este caso, las plantas doble haploides se generan mediante callogénesis y posterior organogénesis en el callo obtenido. Por último, se analizó el potencial efecto inductor de una serie de factores, previamente utilizados en otras especies, en el proceso de inducción de las microsporas de berenjena. La ma-

yoría de los factores ensayados mostraron un efecto positivo en la respuesta androgénica, en el crecimiento o en la calidad del material obtenido.

En resumen, mediante los estudios realizados en esta Tesis Doctoral, se han identificado nuevos procesos asociados y factores que influyen en el cambio de programa de desarrollo que sufren las microsporas o sus precursores (en el caso del tomate), con un impacto potencial muy notable en el resultado final de este proceso experimental. Asimismo, se ha mejorado la eficiencia de la obtención de doble haploides de berenjena, y se ha avanzado en la posibilidad de combinar inducción de embriogénesis y transformación genética en un sistema modelo como colza.

Resum

L'androgénesi és un procés que permet l'obtenció de línies pures doble haploides partint, en la majoria dels casos, del gametòfit masculí o del seu precursor, la microspora. Des d'una perspectiva biotecnològica, esta possibilitat adquirix gran rellevància, perquè les línies pures són la base del procés d'obtenció de llavor híbrida, i este procés permet reduir notablement el temps i els recursos necessaris per a obtindre línies pures de forma convencional.

En la present Tesi Doctoral s'ha abordat l'estudi de la inducció d'androgénesi aplicant en paral·lel dos aproximacions: una bàsica, per mitjà de l'estudi dels factors que influïxen i els canvis que tenen lloc en la microspora al ser induïda, i una aplicada, orientada a millorar l'eficiència de la inducció en espècies recalcitrants. L'estudi s'ha dut a terme en tres espècies: colza (*Brassica napus*), utilitzada com a sistema model per a estudis bàsics, i dos recalcitrants, tomaca (*Solanum lycopersicum*) , i albergina (*Solanum melongena*) .

En esta Tesi Doctoral s'ha utilitzat la fixació per alta pressió i la criosubstitució per a processar cultius de microspores de colza i estudiar els canvis ultraestructurals que tenen lloc com a conseqüència de la inducció del canvi en el programa de desenrotllament. Hem optimitzat també un sistema per a la transformació genètica de microspores de colza, com a pas previ a la inducció androgénica i obtenció d'embrions secundaris, amb l'objectiu d'ampliar el seu ús com a ferramenta per a estudis bàsics més enllà del que s'utilitzava actualment.

En tomaca s'ha caracteritzat el procés de la inducció a partir del cultiu d'anteres, determinant que l'estadi óptim d'inducció no és el de microspores, sinó d'una etapa molt anterior, el de meiòcit. També es van analitzar els cal.lus obtinguts, obtenint una majoria d'origen somàtic o procedents de fenòmens de fusió nuclear dins del meiòcit. També es van analitzar els cal.lus obtinguts, obtenint una majoria d'origen somàtic o procedents de fenòmens de fusió nuclear dins del meiòcit. Estos processos podrien explicar la dificultat per a obtindre doble haploides a partir del cultiu d'anteres de tomaca.

En albergina s'ha posat a punt un sistema eficient per a l'obtenció de plantes doble haploides per mitjà de cultiu de microspores aïllades. En este cas, les plantes doble haploides es generen per mitjà de callogénesi i posterior organogènesi en el cal.lus obtingut. Finalment, es va analitzar l´efecte inductor d'una sèrie de factors, prèviament utilitzats en altres espècies, en el

procés d'inducció de les microspores d'albergina. La majoria dels factors assajats van mostrar un efecte positiu en la resposta androgénica, en el creixement o en la qualitat del material obtingut.

En resum, per mitjà dels estudis realitzats en esta Tesi Doctoral, s'han identificat nous processos associats i factors que influïxen en el canvi de programa de desenrotllament que patixen les microspores o els seus precursors (en el cas de la tomaca), amb un impacte potencial molt notable en el resultat final d'este procés experimental. Així mateix, s'ha millorat l'eficiència de l'obtenció de doble haploides d'albergina, i s'ha avançat en la possibilitat de combinar inducció d'embriogènesi i transformació genètica en un sistema model com la colza.

Índice

Introducción

1- Los híbridos en la Mejora Vegetal

El aumento constante de la población ha llevado a la necesidad de obtener una mayor producción para resolver los problemas de la carencia de alimentos. Esta necesidad, junto con los conocimientos científicos que se han ido adquiriendo, ha permitido poder seguir avanzando en el campo de la Mejora Vegetal. Uno de estos avances ha consistido en la obtención de híbridos. Un híbrido es el individuo obtenido a partir del cruzamiento entre dos o más parentales, elegidos de tal forma que se garanticen la máxima producción y homogeneidad fenotípica en la explotación comercial. La producción de híbridos revolucionó en su día la industria de las semillas, y sigue siendo en la actualidad uno de los métodos más utilizados en Mejora Genética Vegetal.

1.1- Importancia de los híbridos en Mejora Genética Vegetal

La semilla híbrida tiene un elevado valor comercial (Martín 2002). Esto es debido a que los híbridos ofrecen dos grandes ventajas para el agricultor:

- Homogeneidad: Los híbridos presentan una extraordinaria homogeneidad en todas las fases del cultivo, como consecuencia de que las plantas del híbrido son genéticamente idénticas y heterocigóticas para todos los *loci*.
- Vigor híbrido: El alto valor comercial se debe al aumento que se observa en el rendimiento del cultivo, que reside en el vigor híbrido o heterosis. La heterosis es el aumento en la expresión de ciertos caracteres que surge tras el cruzamiento entre especies, variedades o líneas puras (Cubero 2003) y que ocurre como resultado de la heterocigosidad.

Otra de las grandes ventajas del uso de híbridos, esta vez para el productor, es la posibilidad de mantener las características de la semilla durante generaciones, a lo largo del tiempo de comercialización, ya que la obtención de semilla híbrida es repetible mediante el cruce de los mismos parentales. Esto dota al híbrido de una patente interna que pone a la casa de semillas en ventaja frente a la competencia y hace que el agricultor tenga que comprar la semilla cada año si quiere obtener el mismo rendimiento. No obstante, ese

gasto se compensa por el incremento en la producción que consigue el agricultor.

1.2- Obtención convencional de los híbridos

Los pasos a seguir para el desarrollo de un nuevo híbrido son: la obtención de los parentales que formarán parte del híbrido, la evolución de dichos parentales, su mantenimiento y finalmente la producción de la semilla híbrida comercial.

Los híbridos se obtienen mediante el cruce de dos o más líneas puras, seleccionadas de forma que garanticen la máxima producción y la máxima homogeneidad fenotípica en la explotación comercial. Por tanto, las líneas puras son la base del proceso de obtención de híbridos. Las líneas puras son conjuntos de individuos genotípicamente idénticos y homocigotos para todos los caracteres, obtenidos por vía sexual (Cubero 2003). Estas líneas se vienen obteniendo tradicionalmente por Mejora Genética Clásica, mediante ciclos de autofecundación y selección durante varias generaciones (7-9), para alcanzar un alto grado de homogeneidad en todos sus caracteres.

La identificación de combinaciones de parentales que produzcan híbridos con un rendimiento superior es el paso más importante en el desarrollo de híbridos. Sin embargo, es uno de los pasos más costosos tanto en tiempo como en dinero, dentro de cualquier programa de mejora. Se sabe que los distintos genotipos difieren en su capacidad para combinarse entre sí y con otros genotipos con el fin de obtener buenos híbridos, lo que hace que la identificación de las combinaciones más prometedoras de parentales sea esencial a la hora de explotar la heterosis en los cultivos agrícolas (Geleta et al. 2004). Esto se hace 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 evaluar los híbridos obtenidos en ensayos de rendimiento. Sin embargo, las limitaciones de espacio muchas veces hacen que sólo un escaso número de líneas de mejora puedan ser evaluadas como parentales. En primer lugar, los parentales se evalúan por su Aptitud Combinatoria General (ACG), y aquellas seleccionadas se cruzarán combinándolas de todas las formas posibles para elegir generalmente a las de mayor Aptitud Combinatoria Específica (ACE), que son las que formarán parte del híbrido.

Los parentales son mantenidos por autofecundación, y pueden continuar siendo mejorados durante el proceso. Mediante el cruce de los parenta-

les seleccionados obtendremos la semilla híbrida, que en el caso de especies hortícolas requiere de una hibridación manual.

2- Los haploides y doble haploides en la obtención de híbridos

Los haploides son individuos cuyo número de cromosomas es la mitad del número básico de la especie. Es decir, tienen igual número de cromosomas que el gameto. A partir de estos individuos haploides, es necesario que tenga lugar una duplicación cromosómica para poder obtener individuos doble haploides (Cubero 2003). Por tanto, los doble haploides son individuos totalmente homocigotos para todos sus *loci* debido a que provienen de la duplicación, inducida o no, del número de cromosomas gaméticos de un individuo haploide, originado a partir de un único núcleo gametofítico (Germanà et al. 2005).

Los diferentes pasos mencionados en el punto 1.2 suponen un gasto considerable en tiempo y recursos, lo que hace que lanzar un nuevo híbrido al mercado sea un proceso muy largo y costoso. Si, como se ha mencionado, son necesarias entre 7 y 9 generaciones, en plantas anuales este proceso requeriría entre 7 y 9 años. Es fácil imaginar que un proceso semejante en, por ejemplo, frutales, es prácticamente inabordable. Afortunadamente, las técnicas biotecnológicas de cultivo *in vitro* permiten acelerar mucho este proceso, al permitir la obtención de líneas puras mediante la tecnología de los doble haploides en una sola generación. Además, la selección utilizando doble haploides es más eficiente, 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). En la mejora genética clásica, las líneas puras que se obtienen no son 100% homocigotas. Los mejoradores realizan rondas de autofecundación hasta que aproximadamente el 99% de los caracteres están en homocigosis. En el caso de individuos haploides que posteriormente han duplicado su genoma, la homocigosis siempre será absoluta.

La líneas puras obtenidas mediante la tecnología de los doble haploides pueden tener distintas aplicaciones en Mejora Genética Vegetal. Dependiendo del tipo de cultivar que se pretenda conseguir, esas líneas puras serán

utilizadas directamente como un nuevo cultivar, o como parentales para la obtención de un híbrido o una variedad sintética.

2.1- Otras utilidades de los doble haploides

Además de su aplicación en la obtención de líneas puras, los doble haploides son también herramientas muy útiles en otros campos, dentro y fuera de la mejora:

2.1.1- Elaboración de mapas genéticos:

Los mapas genéticos son una herramienta para el cartografiado de los caracteres, lo cual es de especial interés para los mejoradores (Forster et al. 2007). Disponer de caracteres en homocigosis permite mapear a mayor velocidad y con más precisión caracteres heredables como el rendimiento y la calidad, que son caracteres cuantitativos de gran interés económico. En este contexto, los doble haploides han tenido una gran importancia en el establecimiento de mapas genéticos en una gran cantidad de especies, como son cebada, arroz, colza y trigo por su elevado grado (100%) de homocigosis. Los doble haploides, debido a que pueden autoperpetuarse por autofecundación y a que mantienen las combinaciones de marcadores al ser homocigotos, son también útiles para estudios básicos de ligamiento y estimación de fracciones de recombinación (Wedzony et al. 2009).

2.1.2- Asociación de marcadores moleculares y caracteres fenotípicos:

Los doble haploides son también muy útiles agilizando y simplificando el establecimiento de asociaciones entre marcador y carácter observable. Esto permite por un lado que los mejoradores puedan evaluar líneas doble haploides con mayor velocidad y precisión que otras líneas con menor grado de homocigosis (Wedzony et al. 2009).

Por otro lado, desde un punto de vista más básico, estas asociaciones entre marcador y carácter también pueden ser útiles para la identificación de genes que controlan un determinado carácter. Por ejemplo, un método que se utiliza de forma común es la búsqueda de ESTs ("Expressed Sequence Tags", fragmentos del genoma que están siendo expresados en un determinado momento) y el cartografiado de su posición relativa respecto al carácter en cuestión. Los genes candidatos que son localizados en la misma posición pueden ser cartografiados físicamente utilizando genotecas BAC (genotecas de cromo-

somas bacterianos artificiales) y así verificar su asociación. Los doble haploides juegan un papel muy importante en la integración de mapas genéticos y físicos, aportando precisión en la identificación de genes candidatos (Forster et al. 2007).

2.1.3- Selección y detección de mutantes recesivos:

Los doble haploides permiten la identificación de mutaciones recesivas, tanto espontáneas como inducidas, que pueden estar enmascaradas en los individuos diploides heterocigotos (Forster et al. 2007), tanto en mutaciones espontáneas como inducidas. La mutación inducida proporciona otro sistema para relacionar los genes con los fenotipos. Es importante que las poblaciones mutantes deriven de una línea pura (como un doble haploide) para evitar la detección de falsos positivos debido a la variación del material de partida. El procedimiento consistiría en primer lugar en aplicar primero los tratamientos mutagénicos y posteriormente la inducción para la obtención de doble haploides, creando así una población de líneas mutantes homocigotas, y descartando los efectos del quimerismo y la heterocigosidad que podrían enmascarar las mutaciones. Esto a su vez permite la expresión y la identificación de caracteres tanto dominantes como recesivos (Dunwell 2010).

2.1.4- Transgénesis:

La utilidad de los doble haploides para la transformación genética (o transgénesis) radica en que permite la fijación rápida del transgén en homocigosis, evitando así la presencia de hemicigotos. Además, los homocigotos transgénicos pueden identificarse en la primera generación transformante (Figura 1). De esta forma, se ahorra tiempo y recursos en la obtención de plantas transformadas con el transgén en ambos alelos (Goedeke et al. 2007).

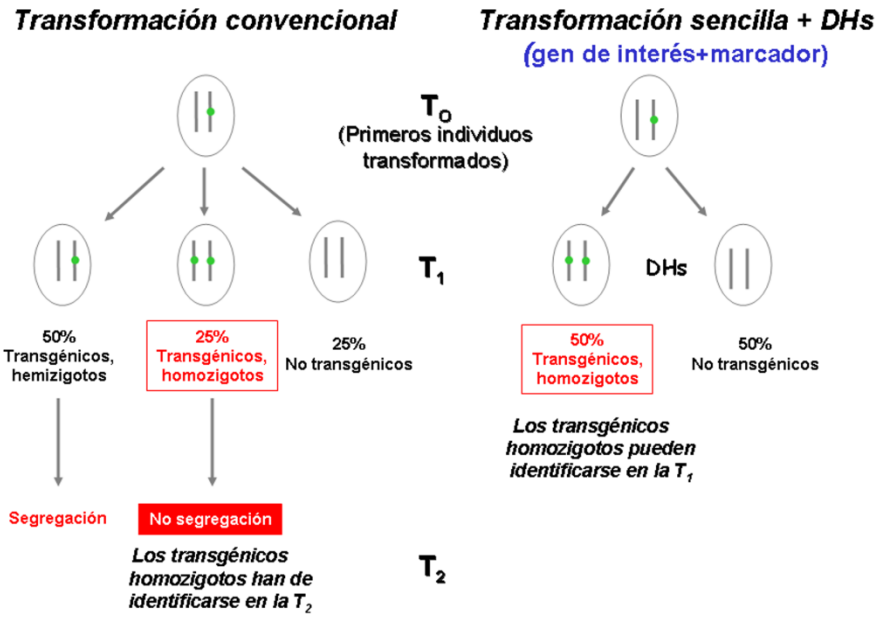

Figura 1. Esquema de las ventajas de combinar la transformación genética con la obtención de doble haploides. Imagen de (Seguí-Simarro 2010b). Reproducida con autorización.

2.2- Métodos de obtención de doble haploides

Todos los métodos conocidos de obtención de doble haploides se basan en la obtención previa de individuos que en algún momento de su desarrollo fueron haploides. En 1921 se descubrió la producción espontánea de individuos haploides en *Datura stramonium* (Dunwell 2010). Desde entonces, se han identificado más de cien especies que son capaces de producirlos *in vivo.* Sin embargo la frecuencia en la que tiene lugar en la naturaleza es muy baja. Por lo tanto, la producción artificial de individuos haploides y doble haploides puede ser una solución efectiva para obtenerlos en cantidades suficientes para abordar las aplicaciones mencionadas en los puntos anteriores. Pueden obtenerse individuos haploides y doble haploides de forma artificial de diferentes formas, pudiendo estar implicado el gametófito masculino o el femenino.

2.2.1- Hibridación interespecífica

Mediante esta técnica pueden llegar a formarse embriones haploides tras la polinización con polen de otras especies sexualmente incompatibles. Esto suele observarse sobre todo en cereales (Dunwell 2010; Sidhu et al. 2006). En ocasiones tiene lugar una doble fecundación normal que da lugar al cigoto y al endospermo. Después, debido a barreras de incompatibilidad post-cigótica, tiene lugar un proceso de eliminación cromosómica selectiva de la parte masculina, de forma que finalmente queda un embrión haploide con el fondo genético del parental femenino (Dunwell 2010). Las células del endospermo se dividen con rapidez y también sufren eliminación cromosómica. Esto provoca con frecuencia aborto en fases tempranas del desarrollo de la semilla, y hace necesario recurrir al rescate de embriones por medio de cultivo *in vitro*. El método más utilizado, dentro de los programas de mejora de cereales, es el "método bulbosum". Permite obtener embriones haploides de *Hordeum vulgare* o de *Triticum aestivum* después de polinizar plantas de estos cereales con polen de la especie *Hordeum bulbosum*, emparentada con *H. vulgare*. En algunos casos (trigo, triticale, centeno y avena), también se utiliza el polen de maíz para inducir la formación de embriones haploides (Forster et al. 2007). En patata (*Solanum tuberosum*) el sistema que se utiliza es similar. Los haploides en este caso pueden obtenerse al cruzar con algunos clones de la especie relacionada *Solanum phureja* (Carputo and Barone 2005; Peloquin et al. 1996). Aquí no tiene lugar una doble fecundación, sino que el núcleo secundario (4x) es fecundado por un núcleo diploide de restitución que se forma en el tubo polínico por fusión de las dos espermátidas. Esto provoca la formación de un endospermo funcional hexaploide que consigue inducir el desarrollo del embrión a partir de la célula huevo. La frecuencia de semillas que contienen embriones haploides es variable (Dunwell 2010).

2.2.2- Ginogénesis

Esta técnica consiste básicamente en el cultivo *in vitro* de óvulos, ovarios o flores inmaduras. En la ginogénesis, el desarrollo del embrión haploide tiene lugar en el saco embrionario, después de que las células haploides del gametofito femenino sean estimuladas para desarrollar un embrión en un proceso similar a la partenogénesis (Forster et al. 2007). Generalmente, el embrión deriva de la célula huevo, aunque en algunas especies se origina a partir de las células antípodas o sinérgidas. En la mayoría de los casos, lo que se obtiene son embriones haploides. Por lo tanto, habría que recurrir posteriormente a tratamientos para inducir la duplicación cromosómica y obtener

doble haploides. La especie en la que mejor se conoce esta técnica es la cebolla, aunque también se utiliza en remolacha y en varias cucurbitáceas, como el calabacín, el melón y el pepino (Wei-ping et al. 2009). En algunas especies, para que se desencadene el proceso, hay que añadir polen sobre los tejidos del gineceo. El polen dispara respuestas típicas de la germinación que pueden llevar a la inducción de ginogénesis, pero sin que la fecundación tenga lugar. Para que la inducción se desencadene, se utiliza polen de otra especie, o incluso de la misma, pero que previamente ha sido irradiado, para que no sea capaz de fecundar. Esto ha sido utilizado principalmente en frutales (Froelicher et al. 2007; Germanà 2006). En algunas variedades de cítricos, se utiliza polen de individuos triploides, que tiene el mismo efecto estimulador (Germanà 2006; Germanà and Chiancone 2001). La ginogénesis es la técnica menos utilizada para producir dobles haploides debido a su baja eficiencia, a la escasa tasa de duplicación cromosómica espontánea, y a la baja regeneración de los embriones obtenidos. No obstante, en especies que no responden a métodos más eficientes, esta es la única opción (Germanà 2011).

2.2.3- Androgénesis

De todas las posibles, la inducción de androgénesis es la técnica más eficiente y más ampliamente utilizada. De hecho, es la técnica en la que se basa la presente Tesis Doctoral. Por este motivo, se verá con más detalle en el siguiente apartado.

3- Androgénesis

La androgénesis se define como el conjunto de vías biológicas que dan lugar a un nuevo individuo haploide o doble haploide cuyo fondo genético proviene exclusivamente de un núcleo de origen masculino (Seguí-Simarro 2010a).

La androgénesis se basa, en la mayor parte de los casos, en el desvío o reprogramación de la ruta normal del desarrollo del gametofito masculino, permitiendo la obtención de individuos cuyo fondo genético proviene exclusivamente del gametofito masculino o de su precursor (Seguí-Simarro 2010a). Por ello, para una mejor comprensión del proceso de la reprogramación androgénica es necesario conocer brevemente los distintos pasos que dan lugar a la formación del gametofito masculino a partir de la célula madre de la mi-

crospora, a través de los procesos denominados microsporogénesis y microgametogénesis.

La microsporogénesis consiste en la formación y el posterior desarrollo de la microspora (Figura 2) (Seguí-Simarro 2010b). El proceso comienza cuando la célula madre de las microsporas sufre una meiosis, dando lugar a una tétrada de cuatro microsporas. Después de la citocinesis de la tétrada, las microsporas jóvenes son liberadas. Al principio mantienen la forma que tenían en la tétrada. Posteriormente van sufriendo una serie de cambios, aumentando su volumen y redondeándose, dando lugar a una microspora media. Estas microsporas siguen su desarrollo adoptando la morfología típica de la especie, y sufren cambios en el núcleo y el citoplasma. El más señalado es el comienzo de la formación de pequeñas vacuolas que acaban fundiéndose en una enorme vacuola que ocupa la mayor parte del volumen de la microspora, empujando hacia fuera el núcleo. Esta última etapa del proceso es la que se conoce como *microspora vacuolada.*

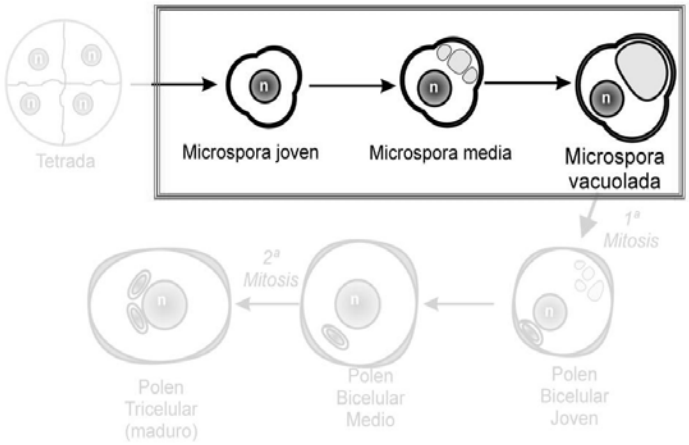

Figura 2. Etapas de la microsporogénesis. Imagen de Seguí-Simarro (2010b).Reproducida con autorización.

La microgametogénesis (Figura 3) comienza con la formación del grano de polen bicelular, una vez tiene lugar la división asimétrica o *primera mitosis del polen* (Seguí-Simarro 2010b). Después de esta primera división mitótica, el grano de polen joven aumenta de tamaño y adquiere una morfo-

logía redondeada u ovalada. Ya en este polen bicelular joven se observa la célula generativa alargada en la periferia de la célula vegetativa, que ocupa la mayor parte del volumen del grano de polen. En el estadio medio del grano de polen, la célula generativa migra hacia el interior del grano adquiriendo una morfología fusiforme. Tiene lugar también la desaparición de la vacuola y el inicio de la acumulación de almidón en forma de amiloplastos. El grano de polen continúa aumentando de tamaño y se va haciendo más ovalado. Posteriormente, continúa su desarrollo hasta su maduración, donde en la mayoría de las especies sigue siendo bicelular, con forma ovalada y presentando grandes acumulaciones de almidón.

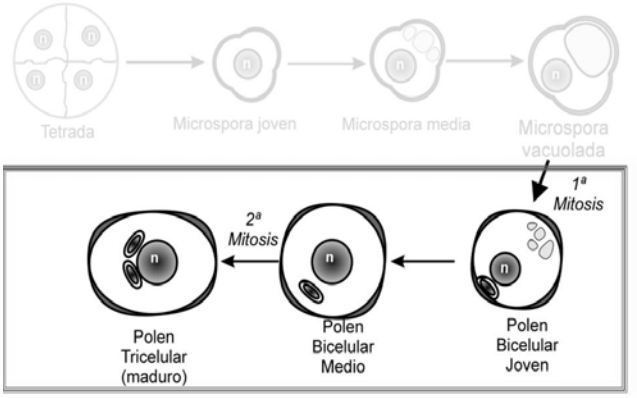

Figura 3. Etapas de la microgametogénesis.Imagen de Seguí-Simarro (2010b). Reproducida con autorización.

3.1- Rutas androgénicas

A partir de estos procesos que llevan a la formación del grano de polen y los gametos masculinos (Figura 4, ruta 0) hay tres rutas androgénicas que pueden originar individuos haploides o doble haploides (Seguí-Simarro 2010a).

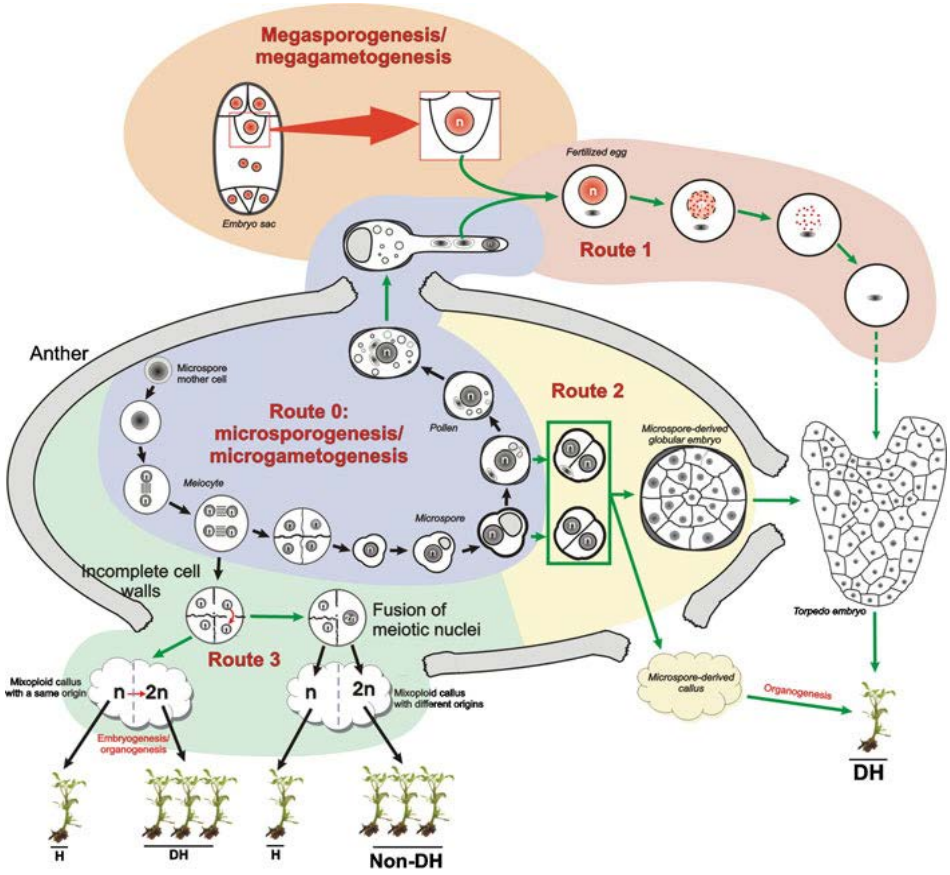

Figura 4. Las diferentes rutas androgénicas. Imagen de Seguí-Simarro (2010b). Reproducida con autorización.

3.1.1- Desarrollo de un embrión haploide dentro del saco embrionario (Figura 4, ruta 1):

Tras la fecundación, el embrión haploide se origina a partir de una célula huevo o cigoto donde el núcleo femenino ha sido inactivado o eliminado. De este modo queda un cigoto unicelular haploide, con cromosomas exclusivamente masculinos, que prosigue su desarrollo en el óvulo como si de un embrión cigótico normal se tratara. Se trata de un fenómeno androgénico natural descrito en 1929 por Kostoff, y Clausen y Lamberts. Una de las especies donde más se ha observado y estudiado este fenómeno es en maíz (Chase 1969). Su aplicación práctica es nula, debido a su frecuencia extremadamente

baja (Seguí-Simarro 2010a). Por ejemplo, en maíz la máxima incidencia observada es de un individuo por cada 80.000.

3.1.2- Callogénesis derivada del meiocito (Figura 4, ruta 3):

Se basa en la obtención de callos haploides o doble haploides a partir de meiocitos. A partir de los callos obtenidos, pueden regenerarse plantas completas haploides y doble haploides mediante organogénesis o embriogénesis indirecta (Seguí-Simarro and Nuez 2007). Esta ruta, es también muy poco frecuente debido, entre otras cosas, a su baja frecuencia y a que se induce a partir de células difíciles de utilizar en cultivo *in vitro*, los meiocitos. Sólamente ha sido documentada en *Arabidopsis thaliana, Vitis vinífera, Digitalis purpurea* y sobre todo *Solanum lycopersicum* (Corduan and Spix 1975; Gresshoff and Doy 1972; 1974; Seguí-Simarro and Nuez 2005; 2007; Shtereva and Atanassova 2001; Zagorska et al. 2004). De hecho, esta ruta androgénica se ha estudiado principalmente en tomate, llegando a conseguir en varios casos la regeneración de plantas completas (Seguí-Simarro and Nuez 2005; 2007; Shtereva and Atanassova 2001; Zagorska et al. 2004), aunque con una baja eficiencia y un porcentaje elevado de mixoploidía en los regenerantes.

3.1.3- Embriogénesis de microsporas:

Esta técnica consiste en la reprogramación de las microsporas hacia la embriogénesis, desviándolas de su ruta gametofítica (Seguí-Simarro 2010a) (Figura 4, ruta 2). Esta técnica fue descubierta por Guha (1964) en Datura innoxia, consiguiendo la regeneración de plantas haploides a partir del cultivo de anteras. En los años posteriores y hasta la actualidad, muchos grupos de investigación han estudiado esta ruta experimental en distintas especies debido a su gran utilidad para la obtención de doble haploides. De los métodos de obtención de doble haploides descritos anteriormente, este es el más eficiente. Por ello es de largo la técnica más estudiada y utilizada para producir doble haploides.

Esta ruta alternativa se puede inducir en diversas especies de angiospermas, tanto mono como dicotiledóneas aunque desgraciadamente no en todas. Tras la inducción (Figura 5), algunas de las microsporas quedan detenidas, mueren o pueden continuar durante un tiempo con un desarrollo similar al gametofítico aunque finalmente acabarán muriendo. Sin embargo, otras entrarán en la ruta embriogénica permitiendo la regeneración de plantas completas a partir de embriogénesis haploide directa, o indirectamente por

organogénesis a través de una fase intermedia de callo (Figura 5). Aunque útil y viable, esta última alternativa es menos eficiente que la embriogénesis directa, y solo se usa cuando la directa no es posible.

Los doble haploides derivados de microsporas abren una nueva dimensión para la producción de líneas homocigotas debido a la gran cantidad (miles) de microsporas que se producen en una sola antera (Maraschin et al. 2005). Existen desde hace años sistemas de producción de doble haploides en más de 250 especies de interés agronómico, desde herbáceas como trigo, cebada, arroz, colza, tabaco o maíz (revisado en Maluszynski et al. 2003; Dunwell 2010) hasta leñosas como mandarino, naranjo amargo o alcornoque, entre otras (revisado en Germanà 2006; Srivastava and Chaturvedi 2008), siendo la eficiencia variable en los distintos cultivos.

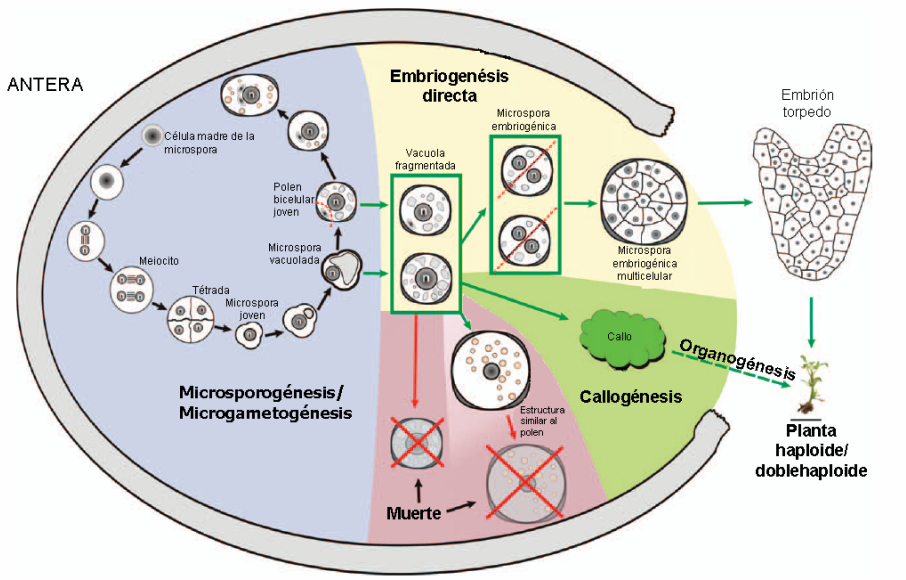

Figura 5. Esquema de la inducción de androgénesis a partir de polen/microsporas. Adaptado de Segui-Simarro y Nuez 2008).

3.2- Ventajas de la inducción de embriogénesis frente a otras técnicas de producción de doble haploides

La inducción de embriogénesis es la técnica de obtención de doble haploides más útil y ventajosa de entre las disponibles para obtener doble haploides porque:

- Está puesta a punto en un mayor número de especies en comparación con la hibridación interespecífica, que se utiliza en muchas menos especies (básicamente en cereales como cebada, trigo, centeno y avena), la ginogénesis que sólo se consigue en cebolla, remolacha, y ciertas cucurbitáceas, o la callogénesis de meiocitos (arabidopsis, uva, dedalera, tomate).
- Su eficiencia a la hora de obtener doble haploides es mucho mayor que con los otros métodos.
- Mediante inducción a embriogénesis puede obtenerse un mayor porcentaje de individuos que duplican de forma espontánea su material cromosómico, de modo que puede no ser necesario duplicar el genoma haploide. Con la hibridación interespecífica o la ginogénesis, siempre es necesario aplicar colchicina para duplicar el genoma.
- No es necesario que la floración de los parentales esté sincronizada como en la hibridación interespecífica.

3.3- Factores que afectan a la inducción de embriogénesis de microsporas

Hay una gran cantidad de factores que pueden afectar a la inducción de embriogénesis, y en general, también de androgénesis, muchos de los cuales son todavía desconocidos (Datta 2005). Los principales factores que se conocen pueden concentrarse en tres grandes grupos: los que dependen de la planta donante, los relativos al estado de la microspora y por último aquellos relativos al medio de cultivo utilizado (Seguí-Simarro and Nuez 2008b).

3.3.1- Factores relativos a la planta donante

Dentro de los factores relativos a la planta donante, el genotipo de la planta utilizada es el de mayor importancia. Diferentes especies, variedades e

incluso distintos individuos de la misma variedad pueden presentar diferencias importantes en la respuesta a inducción de embriogénesis de microsporas (Seguí-Simarro and Nuez 2008b). La capacidad de respuesta de un determinado genotipo viene referida tanto a la inducción de la célula haploide como a la posterior regeneración de la planta completa (Gémes-Juhasz et al. 2006). Dicha capacidad de respuesta puede ser heredada por la descendencia. Se han llegado a realizar análisis genéticos en diferentes especies que muestran que está controlada por más de un gen, normalmente de tipo recesivo (Rudolf et al. 1999; Smykal 2000).

La edad del material vegetal es otro de los factores que afectan a la respuesta. Normalmente, las plantas jóvenes ofrecen una mayor respuesta a la inducción pero en algunos casos excepcionales, como en la especie arbórea *Aesculus carnea*, los individuos adultos (mayores de 60 años) presentan una mayor respuesta que los jóvenes (Wang et al. 2000).

Las condiciones de cultivo de las plantas tienen también una gran importancia en la respuesta embriogénica (Smykal 2000). La época del año en la que se extraen las anteras para su cultivo es una de ellas. Diversos autores señalan que la eficiencia en la obtención de doble haploides disminuye durante los meses de verano (Datta 2005; Rotino 1996). Las condiciones naturales de floración (intensidad de luz, duración del día, régimen de temperaturas, humedad, etc.) parecen resultar las más adecuadas para el cultivo de plantas donantes de microsporas (Wang et al. 2000). Asimismo, la presencia de patógenos o la aplicación de tratamientos fitosanitarios, pueden hacer disminuir la respuesta embriogénica (Chambonnet 1988).

3.3.2- Factores relativos a la microspora

El estadio de desarrollo es el factor más importante de los relativos a la microspora. El periodo de sensibilidad a los tratamientos de inducción transcurre próximo a la primera mitosis polínica, es decir, entre el estadio de microspora vacuolada y el de polen bicelular joven. Esto se debe a que ambos estadios no presentan un estado transcripcional totalmente diferenciado, al contrario que en el caso del polen maduro, donde el programa específico para la maduración polínica y formación de gametos está activado. De cualquier modo, cuanto más difícil resulta conseguir la inducción de androgénesis en un genotipo, más pequeño es el periodo de sensibilidad, llegando en algunos casos a limitarse a un solo tipo de estadio de desarrollo (Seguí-Simarro and Nuez 2008b).

3.3.3- Factores relativos al medio de cultivo

De todos los factores relativos al medio de cultivo, el más importante es la aplicación de un estrés que permita la reprogramación de las células y desvíe su ruta de desarrollo gametofítico hacia el desarrollo esporofítico (Shariatpanahi et al. 2006). De hecho, la necesidad de un estrés inductor es lo único que tienen en común todos los protocolos de inducción de las distintas especies estudiadas. Los tipos de estrés más comúnmente utilizados son:

- **Frío**: Con el objetivo de mejorar la respuesta embriogénica, se han aplicado tratamientos a bajas temperaturas (alrededor de 4ºC) sobre plantas completas, espigas cortadas o yemas florales, antes de su puesta en cultivo *in vitro*. Los periodos de tiempo son variables, dependiendo de la especie. Han sido aplicados con éxito en alcornoque, cebada, arroz, trigo, avena y cítricos (Bueno et al. 2003; Shariatpanahi et al. 2006)

- **Calor**: Los tratamientos de choque de calor se han utilizado para inducir la embriogénesis en microsporas de colza, trigo, tabaco y berenjena entre otras especies. El estrés por calor provoca cambios estructurales en las microsporas, síntesis de proteínas de choque térmico (HSP) (Seguí-Simarro et al. 2003) y cambios en la regulación de la actividad quinasa (Seguí-Simarro et al. 2005), influyendo en procesos del ciclo celular (Seguí-Simarro and Nuez 2008a).

- **Reducción de la fuente de carbono y/o nitrógeno**: El ayuno inducido ha sido utilizado con éxito en especies como el tabaco, la cebada y el manzano. Parece ser que está relacionado también con la síntesis de proteínas de choque térmico (HSP) y con cambios en la actividad quinasa de las proteínas, que conducirían hacia una desdiferenciación celular (Touraev and Heberle-Bors 2003; Touraev et al. 1996).

- **Aplicación de agentes osmóticos**: También se ha estudiado la utilización de agentes osmóticos, principalmente manitol y polietilenglicol (PEG), solos o en combinación con sacarosa. El uso de estas sustancias para elevar el potencial osmótico del medio se debe a que la utilización de un modo de estrés adicional aplicado a las microsporas, puede ayudar a favorecer la inducción a embriogénesis (Ferrie and Keller 2007).

Además de estos, se han utilizado otros tipos de estreses, como la colchicina, radiaciones, etanol, utilización de medios hipertónicos, centrifugación, reducción de la presión atmosférica, agentes feminizantes, ácido abscísico, etc., pero no han dado tan buenos resultados. Otros estreses que pueden dar buenos resultados, pero en unas pocas especies concretas, y que necesitan ser probados para cada especie son: pH alto (8-8,5) durante cortos periodos, adición de carragenatos al medio de cultivo y adición de metales pesados, como el litio (Shariatpanahi et al. 2006).

Aunque en menor medida que el estrés, el tipo de medio de cultivo (líquido o sólido), concentración, tipo de fuente de carbono y los reguladores del crecimiento aplicados, afectan a la eficiencia de regeneración de embriones androgénicos (Wang et al. 2000). Como fuente de carbono, se ha observado que una alta concentración de sacarosa produce una adecuada respuesta en una gran cantidad de especies (Datta 2005). En la berenjena, una alta concentración de dicho azúcar es fundamental para conseguir una adecuada inducción (Rotino 1996). De cualquier forma, el tipo y la concentración de la fuente de carbono deben ser ajustados para cada especie. La fuente de nitrógeno también tiene importancia, ya que se ha demostrado que el incremento de glutamina y la disminución de nitrato de amonio mejoran el desarrollo de los embriones en la mayoría de los cereales (Datta 2005).

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 por presentar una alta eficiencia, se caracterizan por no incluir fitohormonas en la composición del medio. No obstante, los medios de cultivo de microsporas en la gran mayoría de las especies vegetales estudiadas necesitan auxinas, citoquininas, o una combinación de ambas en el medio de cultivo (Smykal 2000). Como auxinas, las más frecuentes son el ácido 2,4-diclorofenoxiacético (2,4-D), el ácido indolacético (IAA) y el ácido indolbutírico (IBA). Las citoquininas más utilizadas son la kinetina y la zeatina. Su concentración y momento de aplicación deben ser determinados experimentalmente para cada genotipo.

3.4- Metodologías de inducción de embriogénesis de microsporas

Desde un punto de vista técnico existen dos métodos para obtener individuos doble haploides de origen androgénico a partir de microsporas: el cultivo de anteras y el cultivo de microsporas.

3.4.1- Cultivo de anteras:

En este método se cultivan *in vitro* las anteras enteras, tal como son extraídas de las yemas florales, permaneciendo las microsporas dentro del saco polínico. Es el primer método que se llevó a la práctica y es el más sencillo y universal para la producción de doble haploides en plantas cultivadas, ya que está puesto a punto en un mayor número de especies que el otro método, el cultivo de microsporas aisladas. La inducción de androgénesis es más fácil cultivando la antera, ya que se requiere una menor manipulación del material vegetal y la presencia del tejido de la antera en el medio de cultivo proporciona un ambiente más adecuado para el desarrollo de las microsporas (Seguí-Simarro and Nuez 2008b).

3.4.2- Cultivo de microsporas:

Consiste en aislar las microsporas de la antera y ponerlas directamente en cultivo *in vitro* en medio líquido. La obtención de individuos doble haploides a partir de esta metodología está puesta a punto en menos especies que el cultivo de anteras. Además, técnicamente es más complejo ya que requiere una mayor manipulación y la utilización de técnicas adicionales como el filtrado, centrifugado, etc. Los medios de cultivo utilizados en este caso son también más complejos, debido a la ausencia de tejido somático de la antera. A pesar de ello tiene varias ventajas sobre el cultivo de anteras. Por un lado, al no estar presente el tejido de la antera, podemos evitar el efecto secretor incontrolado del tapete y tener así un control más estricto de las condiciones del medio. Además, los compuestos añadidos al medio estarán en contacto directo con las microsporas al no haber tejido que haga de barrera. Por otro lado, la eficiencia de obtención de doble haploides suele ser mayor, ya que mediante cultivos de alta densidad de microsporas pueden obtenerse más de 1.000 embriones por mililitro (Forster et al. 2007). Por último, al haber únicamente microsporas en el medio y no tejido de la antera, evitamos la aparición de individuos de origen somático.

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, o los tipos de estreses útiles para activar la vía embriogénica, el aislamiento de diversos genes implicados en la reprogramación de las microsporas hacia la vía esporofítica, etc. (Revisado en Dunwell 2010; Forster et al. 2007; Maraschin et al. 2005; Pauls et al. 2006; Seguí-Simarro and Nuez 2008b).

3.5- Origen y ploidía de las plantas obtenidas

Como en cualquier otra técnica de cultivo *in vitro* de células y tejidos vegetales, las plantas obtenidas a partir de embriogénesis de microsporas pueden presentar ploidías diferentes, siendo necesario el recuento del número de juegos cromosómicos para confirmar el éxito de la técnica. Algunos autores señalan que es posible diferenciar a las plantas haploides de las diploides por su morfología. En general, los haploides suelen ser más pequeños. Es lógico pensar que un núcleo con una sola copia del genoma haploide será más pequeño que otro con dos copias. En consecuencia, las células, órganos e individuos serán también más pequeños. Por ejemplo, en berenjena las plantas haploides son en principio más pequeñas, con hábito arbustivo y de hojas más largas y estrechas que las de los diploides de los cuales proceden (Rotino 1996). La distinción es más fácil cuando se comparan estructuras florales, ya que los individuos haploides producen flores más pequeñas y estambres con polen no funcional.

Existen diferentes métodos para conocer la ploidía de las plantas regeneradas. Tradicionalmente, se ha hecho mediante conteo de los cromosomas tras teñir células metafásicas de regiones meristemáticas. En estas células, los cromosomas están condensados en las placas metafásicas permitiendo así su conteo. Otro de los métodos utilizados es el conteo del número de cloroplastos presentes en las células guarda de los estomas. El número de estos cloroplastos está altamente correlacionado (r=0,92) con el nivel de ploidía (Rotino 1996). Pese a su sencillez metodológica, estas técnicas son muy tediosas, requieren mucho tiempo y esfuerzo.

Hoy en día, la técnica más utilizada para conocer la ploidía de las células es la citometría de flujo. Esta técnica consiste en hacer pasar un conjunto de células en suspensión por un conducto, en el que son obligadas a fluir de una en una y a pasar a través de un foco de luz fluorescente intensa. Los pulsos de luz reflejados y la fluorescencia son recogidos por sensores ópticos, convertidos en corriente eléctrica y analizados. Pueden medirse grandes conjuntos de células en un periodo de tiempo pequeño (Dolezel and Bartos 2005) permitiendo una gran precisión y rapidez a la hora de estimar la ploidía de las plantas regeneradas. Así, pueden distinguirse haploides de diploides, triploides, aneuploides o mixoploides, entre otros.

De las diferentes ploidías que pueden observarse en los individuos regenerados, es evidente que los individuos haploides han sido formados a partir

de células con un número cromosómico haploide, pero puede haber dudas sobre el tejido de origen de los individuos diploides o con ploidías superiores. Estos individuos pueden proceder de microsporas que hubieran sufrido una duplicación espontánea posterior, con lo que serían doble haploides (totalmente homocigóticos), pero también podrían proceder de la fusión de dos productos meióticos, por lo que no serían homocigotos, o de tejido somático de la antera, con lo que serían individuos diploides, genéticamente idénticos a la planta de la cual procede el tejido. Obviamente, estas dos últimas posibilidades suponen un problema cuando lo que interesa son los doble haploides. Este problema es mucho más importante en el caso de cultivo de anteras, ya que en el cultivo de microsporas, al haber sido separadas del tejido de la antera, la posibilidad de que tenga lugar un "escape" somático es altamente improbable. Para resolver esta duda, en el caso de individuos procedentes del cultivo de anteras es necesario comprobar el origen androgénico de las plantas regeneradas mediante algún tipo de marcador a nivel molecular. Un tipo de marcador útil para conocer el origen de las plantas regeneradas son los microsatélites o SSRs ("Simple Sequence Repeats"). Los SSRs son secuencias de ADN en las que un fragmento de dos a seis pares de bases se repite de manera consecutiva. La variación en el número de repeticiones crea diferentes alelos. El análisis molecular utilizando SSRs como marcadores se basa en la amplificación (producción de un alto número de copias) del marcador SSR con cebadores que tienen la misma secuencia que los extremos de las regiones flanqueantes a través de una reacción en cadena de la polimerasa (PCR). El diferente número de repeticiones es el origen del polimorfismo entre individuos. Estos marcadores son, por tanto, codominantes, puesto que dos alelos de diferente número de repeticiones producirán fragmentos de distinta longitud, lo que nos permitirá diferenciar a los individuos doble haploides de los diploides provenientes del tejido somático de la antera. En organismos eucariotas, los microsatélites son abundantes, pudiendo haber miles de ellos en el genoma de una especie. Además de la codominancia, los SSRs son muy reproducibles y fáciles de usar. Todo ello hace que sean muy utilizados en la identificación de doble haploides.

3.6- Duplicación cromosómica

Los individuos haploides obtenidos, además de presentar un menor tamaño y vigor, son estériles. Por todo ello, es necesario duplicar su genoma para obtener individuos doble haploides y fértiles (figura 6).

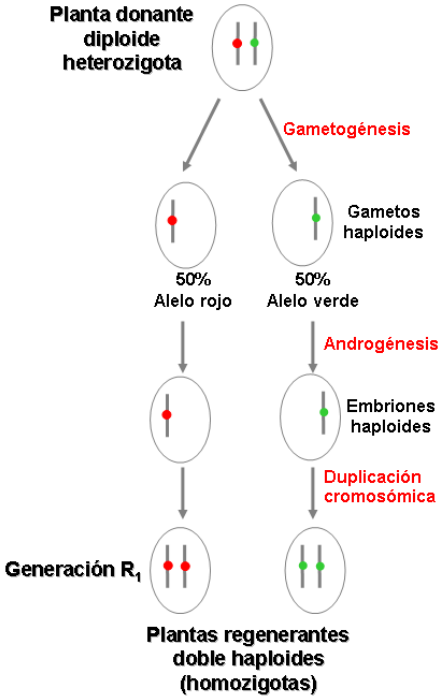

Figura 6 .Esquema simplificado de la obtención de doble haploides por la vía androgénica. Imagen de (Seguí-Simarro 2010b). Reproducida con autorización.

En la mayoría de las especies estudiadas, un porcentaje variable de los individuos haploides obtenidos duplican su genoma sin necesidad de inducir dicha duplicación *ex profeso*. Estas duplicaciones están muy influidas por las condiciones del cultivo *in vitro*, aunque también dependen de la especie e incluso del genotipo. Hasta hace unos años, la endorreduplicación se barajaba como uno de los mecanismos para la duplicación en sistemas androgénicos. En la endoreduplicación la célula sale del ciclo celular y sufre rondas adicionales de duplicación de las cromátidas (figura 7). Suele suceder en células especializadas en funciones específicas y es más frecuente con la edad. En cualquier caso, cuando una célula sale del ciclo para entrar en endorreduplicación no suele volver a entrar. En los últimos años, gracias a las técnicas microscópicas, se ha podido estudiar con más detalle el proceso androgénico y se ha visto que tienen lugar un gran número de eventos de fusión nuclear. Todas

estas evidencias, apuntaron claramente a que el mecanismo principal de duplicación cromosómica es la fusión nuclear (Seguí-Simarro and Nuez 2008b).

La fusión nuclear implica que durante la mitosis, la cariocinesis se produce normalmente, pero la posterior citocinesis no llega a completarse y los núcleos, una vez separados, vuelven a fusionarse (figura 7).

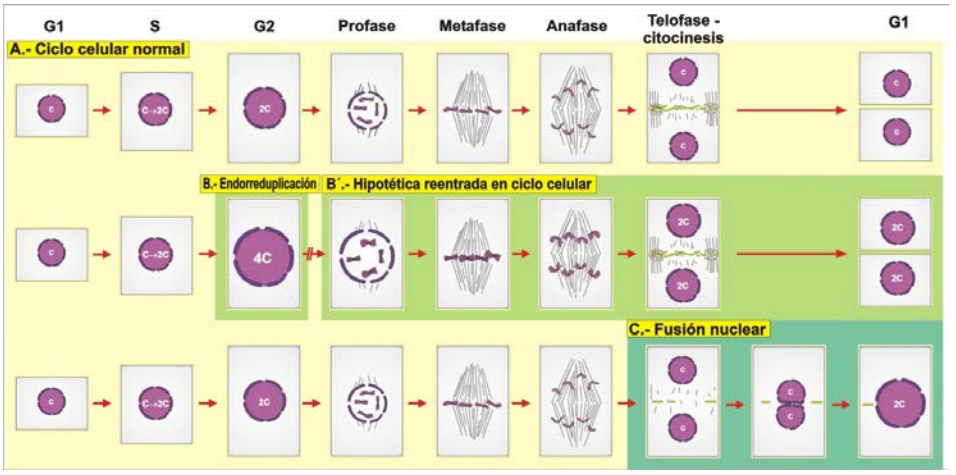

Figura 7. Comparación entre un ciclo celular normal (A), la endorreduplicación (B) y la fusión nuclear (C) como mecanismos celulares para la duplicación del genoma haploide. Imagen de Seguí-Simarro (2010b). Reproducida con autorización.

Si no es suficientemente alto el porcentaje de individuos que sufren la duplicación cromosómica de forma no inducida, se puede recurrir al uso de colchicina para incrementar la eficiencia en la obtención de doble haploides. La colchicina es un alcaloide extraído de varias especies del género Colchicum, que se une a los dímeros de tubulina, impidiendo su polimerización. Por lo tanto, el huso mitótico y el fragmoplasto no pueden formarse correctamente. El resultado es una duplicación genómica al producirse la replicación del ADN, pero no la separación de las cromátidas. Si el genoma de partida es haploide, se obtiene un individuo doble haploide totalmente homocigótico. La colchicina puede ser aplicada tanto a partes de la planta haploide regenerada, como al embrión en desarrollo o incluso al medio de cultivo en la fase de inducción de androgénesis. El tipo de aplicación, tiempo y concentración utilizados deben ser ajustados para cada genotipo. Además de la colchicina,

también se utilizan otros compuestos para conseguir la duplicación cromosómica, como los herbicidas trifluralin o amiprofosmetil (AMP), que se aplican en dosis más bajas que la colchicina, por lo que su efecto tóxico se reduce. No obstante, la aplicación de un citotóxico como la colchicina u otros agentes exógenos para promover la duplicación tiene varios inconvenientes, entre ellos los efectos negativos en el crecimiento, la regeneración y el subsiguiente desarrollo de los individuos (Wan et al. 1990), además de su efecto potencial en la variación somaclonal observada en los doble haploides (Baenziger et al. 1991).

3.7- Limitaciones de la androgénesis

Pese a las ventajas antes descritas, en ocasiones la androgénesis puede no ser una opción viable para obtener doble haploides debido a algunas de las siguientes limitaciones. Algunos problemas que podemos encontrar de forma frecuente son la aparición de variación gametoclonal (o "androclonal") por mutaciones en el proceso de cultivo *in vitro* y la aparición de individuos heterocigotos, debido a la regeneración de plantas a partir de gametos no reducidos (2n). Pero las principales limitaciones de este proceso son:

- **Distorsión en la segregación**: Comparando con la vía de desarrollo sexual, al obtener individuos doble haploides por duplicación del material genético proveniente de un gameto haploide, estamos eliminando la posibilidad de obtener heterocigotos y alterando por tanto la segregación mendeliana, obteniendo una proporción 1:1. Además, habrá genes recesivos deletéreos que en homocigosis resultarán letales, que también alterarán las frecuencias. Así, se han detectado distorsiones en la segregación en especies como trigo, cebada, raigrás, arroz y maíz (Yamagishi et al. 1996).
- **Malformaciones:** la aparición de embriones con malformaciones es muy común en los procesos de androgénesis. Las malformaciones más comunes son las relacionadas con una formación deficiente del meristemo apical; cotiledones múltiples, fusionados, 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; Seguí-Simarro 2010a).

- **Albinismo:** Entre los regenerantes obtenidos mediante este proceso suelen encontrarse un cierto número de individuos albinos (Dunwell 2010). El desarrollo de los plastidios para formar cloroplastos funcionales, queda detenido en estas plantas. Este fenómeno se ha observado generalmente en cereales (Torp and Andersen 2009), y se debe al proceso del cultivo *in vitro.* Aunque los mecanismos moleculares de esta anomalía no están claros, se sabe que la frecuencia de regenerantes albinos tiene un fuerte componente genético (Dunwell 2010) y está determinada también por los componentes del medio de cultivo.

- **Recalcitrancia:** Pese a que existen ya sistemas de producción de doble haploides en más de 250 especies de interés agronómico, salvo en especies modelo como colza, tabaco o cebada, la eficiencia en la obtención de doble haploides es todavía muy escasa (Palmer et al. 2005; Touraev et al. 2001). Este hecho es aún más crítico en especies hortícolas de elevado interés agronómico como las solanáceas. De los cinco principales cultivos de solanáceas (pimiento, tabaco, patata, berenjena y tomate), únicamente en tabaco se han hecho los progresos suficientes como para considerar esta especie como modelo, existiendo desde hace ya años sistemas puestos a punto para obtener doble haploides a partir de cultivos de anteras y de microsporas aisladas con una eficiencia aceptable (Chupeau et al. 1998; Maluszynski et al. 2003; Touraev et al. 2001). En patata ya existen protocolos para inducir cultivos de microsporas (Rihova and Tupy 1999). Sin embargo, el resto de solanáceas son consideradas recalcitrantes, pues aunque en algunos casos se han obtenido doble haploides, todavía se está muy lejos de la eficiencia obtenida en tabaco o colza. En berenjena y pimiento únicamente parece puesto a punto el cultivo de anteras, y en tomate todavía no existe ninguno de los dos métodos puesto a punto (Seguí-Simarro and Nuez 2007). Es precisamente este escaso desarrollo de la tecnología de obtención de doble haploides en solanáceas recalcitrantes lo que motiva la presente Tesis Doctoral.

Objetivos

El objetivo principal de la presente Tesis Doctoral es optimizar los protocolos de obtención de doble haploides androgénicos en Solanáceas recalcitrantes, tanto para la obtención de líneas puras como para su uso como herramientas en sus distintas aplicaciones biotecnológicas. Para ello, se han abordado en paralelo dos subobjetivos que definen los dos grandes bloques en los que se divide esta Tesis Doctoral:

Bloque I: El estudio concreto de dos de las Solanáceas más recalcitrantes, el tomate y la berenjena. En el caso del tomate en el Capítulo 1, abordamos un estudio del único método publicado de obtención de doble haploides, el cultivo de anteras. Dicho método se caracteriza, por un porcentaje de obtención de doble haploides muy bajo. En este capítulo se analizaron las causas de esta baja eficiencia y el origen de las plantas regeneradas, con el objetivo de plantear futuras vías de abordar la inducción androgénica de esta especie. En el caso de la berenjena, el objetivo consiste en poner a punto un protocolo eficiente de inducción de androgénesis y regeneración de plantas doble haploides mediante el cultivo *in vitro* de microsporas aisladas. Para ello, en el Capítulo 2 se probaron diferentes genotipos de interés para los diversos programas de Mejora Genética de la berenjena y se identificaron aquellos de mayor respuesta androgénica. El objetivo será por tanto obtener embriones o callos androgénicos y regenerar plantas doble haploides a partir de aquel o aquellos en los que la respuesta sea mayor. Se caracterizó el particular proceso de obtención de individuos doble haploides en esta especie, se analizó la ploidía del material obtenido y se pusieron a punto métodos de análisis genético mediante marcadores moleculares de tipo microsatélite (SSR) para tener certeza del origen androgénico de las plantas regeneradas.

En el Capítulo 6 se determinó el efecto en la inducción de androgénesis en berenjena, de determinados factores que tienen un papel demostrado en la eficiencia, en la inducción o el desarrollo del embrión en otras especies.

Bloque II: El estudio de un sistema modelo como *Brassica napus,* en el que se dispone de protocolos muy eficientes para la obtención de embriones y plantas doble haploides con los que abordar:

- Estudio de cambios citoplásmicos durante el desarrollo androgénico (Capítulo 4). El objetivo de este capítulo es la detección de cambios que tiene lugar en el citoplasma mediante la observación de imágenes de las células durante los primeros momentos de la

ruta androgénica. Esos cambios pueden ser debidos al cambio en el programa de desarrollo y también como consecuencia del tratamiento de inducción.

- Transformación genética (Capítulo 5). La transformación de microsporas que más tarde son sometidas a inducción a embriogénesis conseguiría evitar la presencia de hemicigotos al obtener plantas transformadas con el transgén en ambos alelos. El objetivo de este capítulo consiste en asentar las bases de un protocolo eficiente para la transformación genética de las microsporas y la expresión transitoria del transgén.

- Embriogénesis secundaria (Capítulo 6). En este capítulo se aborda la obtención de embriones secundarios a partir de embriones androgénicos transformados. Al provenir el embrión secundario de una única célula, si está transformada, todas las células del embrión tendrán el transgén. De esta forma no sólo podemos evitar la presencia de hemicigotos sino también la formación de quimeras.

Bloque I

La familia de las Solanáceas

La familia de las Solanáceas(angiospermas) comprende entre 3.000 y 4.000 especies y sobre 90 géneros (Knapp et al. 2004). Esta familia es una de las más importantes en términos de interés para la agricultura, e incluye cinco de las plantas más cultivadas, como es la patata (*Solanum tuberosum*), el pimiento (*Capsicum annum*), la berenjena (*Solanum melongena*), el tabaco (*Nicotiana tabacum*) y el tomate (*Solanum lycopersicum*). La familia de las Solanáceas es un ejemplo típico de familia etnobotánica, es decir, que ha sido explotada y utilizada por los humanos desde los inicios de la agricultura. Esta familia es una de las fuentes más importantes de alimento y especias, principalmente los cinco cultivos mencionados anteriormente. A pesar de la tremenda importancia de esta familia en el mundo de la agricultura, la tecnología de los doble haploides no se ha conseguido de forma eficiente en algunos de estos cultivos de interés. Curiosamente, la primera observación de embriogénesis de microsporas fue reportada en una solanácea por Guha y Maheshwarii en (1964), que describieron la formación *in vitro* de plantas a partir de polen contenido en anteras de *Datura innoxia.* Entre los cinco principales cultivos de solanáceas (pimiento, tabaco, patata, berenjena y tomate), solo en tabaco los avances han sido suficientes como para considerar esta especie un sistema modelo para el estudio de la embriogénesis de microsporas. Actualmente, se han perfeccionado protocolos para producir doble haploides a partir de cultivos de anteras y de microsporas aisladas de tabaco de forma rutinaria y eficiente (Belogradova et al. 2009). La patata no puede considerarse una especie modelo, pero sí hay disponibles protocolos tanto para el cultivo de anteras como de microsporas (Rokka 2009). El resto de estas solanáceas de interés (tomate, berenjena y pimiento) son consideradas recalcitrantes. Aunque en algunos casos se han obtenido doble haploides, todavía se está muy lejos de conseguir la eficiencia observada en tabaco. A pesar de la proximidad genética de estas solanáceas, parecen claramente distantes en términos de respuesta androgénica. Así pues, solamente el cultivo de anteras resulta útil en el caso de berenjena y pimiento. En tomate no existen protocolos eficientes disponibles.

Tomate

El tomate es el primer cultivo hortícola del mundo, tanto en términos de producción (151.699.405 Tm en 2010) como de área cultivada (4.412.757 Ha en 2010). Estos datos dan una idea de la gran importancia del tomate para la agricultura global. A pesar de ello, se conoce muy poco dentro del campo de doble haploides en tomate, donde se está lejos de disponer de métodos fiables y estandarizados. En los últimos 40 años, se han ensayado un gran número de condiciones de inducción y de cultivo (Bal and Abak 2007), aunque tan solo dos laboratorios han publicado resultados de regeneración completa de plantas de tomate a partir de anteras con un origen haploide o doble haploide demostrado (Seguí-Simarro and Nuez 2005; 2007; Shtereva et al. 1998; Zagorska et al. 2004). Estos regenerantes mostraron un grado de variabilidad morfológica que iba desde elevada (Zagorska et al. 1998) a baja (Seguí-Simarro and Nuez 2007). En todos los casos se observaron casos de mixoploidía y una baja eficiencia general. Por lo tanto, el tomate es extremadamente recalcitrante, y es esencial que se lleve a cabo un estudio en profundidad de los factores implicados en la androgénesis en esta especie para llegar a conseguir resultados en este campo.

Los factores más críticos son el genotipo y el estadio del desarrollo. Como ocurre en otros sistemas androgénicos, el genotipo juega un papel muy importante. En concreto, las líneas mutantes androestériles han demostrado ser especialmente sensibles a la inducción (Seguí-Simarro and Nuez 2007; Zagorska et al. 1998; Zamir et al. 1980). Hay que mencionar que los fenotipos androestériles se manifiestan generalmente en el estadio de meiocito tardío, el cual en la mayoría de casos se solapa con el periodo óptimo para el cultivo de anteras de tomate. Desde los primeros trabajos publicados, la identificación del estadio adecuado para la inducción del gametofito de tomate ha sido también una causa de debate. De hecho, hasta hace unos pocos años, el estadio de desarrollo más adecuado no estaba claro. En 1978, Dao y Shamina publicaron la generación de callos a partir del cultivo de anteras con microsporas en el estadio de tétradas, mientras que las anteras que contenían microsporas vacuoladas o polen bicelular joven, producían embriones directamente. Este trabajo sugirió que en tomate, diferentes estadios pueden responder bajo diferentes tratamientos. Hace pocos años, tres estudios publicados han reforzado esta hipótesis. En primer lugar, Seguí-Simarro y Nuez (2005), estrecharon el estadio óptimo para el cultivo de anteras al de meioci-

to tras la recombinación pero justo antes de que tenga lugar la tabicación de la tétrada.

En segundo lugar, se observó la formación de estructuras multicelulares a partir de microsporas aisladas, tras la exposición a ayuno, frío y colchicina (Bal and Abak 2005). En tercer lugar, en 2007, se demostró finalmente que en tomate, la androgénesis puede ser inducida en dos estadios diferentes (Seguí-Simarro and Nuez 2007), aunque con resultados e implicaciones muy diferentes, como se va a ver a continuación. Por un lado, este estudio confirmó que es posible inducir androgénesis a partir de meiocitos siempre que sean cultivados *in vitro* dentro de las anteras, como se vio previamente (Seguí-Simarro and Nuez 2005; Shtereva et al. 1998). De esta forma pueden obtenerse haploides, doble haploides y mixoploides a partir de meiocitos. Tras la inducción, los callos obtenidos de los meiocitos pueden provenir:

- De células haploides contenidas dentro de la tétrada, las cuales detienen su programa gametofítico y comienzan a proliferar.
- De células haploides, que provienen de la fusión de dos núcleos haploides separados por presentar paredes celulares defectuosas, incompletas o por ausencia total de las mismas.

En la primera alternativa, los callos se originan a partir de productos meioticos haploides, la microspora potencial, sufriendo después división y proliferación. De este modo, se podría sostener que las microsporas son las verdaderas precursoras del callo. Sin embargo, hay que remarcar que la inducción debe ser previa a la formación de la microspora como tal. Entonces, las células del callo haploide duplican su genoma por fusión nuclear, originando células doble haploides verdaderas que podrán regenerar una planta doble haploide (Seguí-Simarro and Nuez 2007). El segundo proceso implica la alteración de los mecanismos normales postmeióticos de citocinesis durante la compartimentalización de las tétradas, causadas por las condiciones de estrés aplicadas al cultivo de anteras. La compartimentalización defectuosa de núcleos haploides favorece su consiguiente fusión. Al contrario que en la primera alternativa, esta fusión podría no dar lugar a individuos doble haploides, ya que la fusión de dos células meioticas genera nuevas combinaciones de alelos no necesariamente homocigotas.

El hecho de que los meiocitos se cultiven dentro de sus anteras hace posible también la aparición ocasional de callos y regenerantes de origen somático, que provengan del tejido conectivo o del filamento de las paredes de

la antera. De hecho, los tejidos del filamento muestran una elevada proliferación cuando son cultivados *in vitro* (Seguí-Simarro and Nuez 2006), y se cree que los tejidos de la antera del tomate en los estadios meioticos son más sensibles al cultivo de tejidos que los de estadios más avanzados (Bal and Abak 2007).

Por otro lado, se ha visto también que cuando se aíslan las microsporas en el estadio de microsporas vacuoladas y se crecen en medio líquido, es posible inducir divisiones proliferativas en esas microsporas, generando estructuras tipo embrión (Seguí-Simarro and Nuez 2007). Esta alternativa elimina la influencia de los tejidos somáticos, evitando de ese modo los problemas mencionados anteriormente. Desafortunadamente, hasta la fecha se han evaluado muy pocos genotipos con este método, se han obtenido muy pocos resultados positivos y no ha sido posible promover el crecimiento más allá de esas primeras divisiones de la microspora embriogénica (Seguí-Simarro and Nuez 2007).

Nuestra opinión actual es que las condiciones de cultivo no son las más apropiadas para promover el desarrollo de los embriones en un ambiente *in vitro*, desprovisto del papel protector y nutritivo del endospermo cigótico. Esto no es un hecho sorprendente ya que los embriones rescatados a partir de cruces interespecíficos o intergenéricos abortan frecuentemente en diferentes estadios del desarrollo debido a alteraciones en el desarrollo del endospermo (Bhatia et al. 2004). Una posibilidad alternativa podría ser el desenmascaramiento de genes letales en las células haploides en división, lo cual podría influir en que el desarrollo no continúe. En resumen, por el momento es difícil concebir un método eficiente para la obtención de doble haploides de forma rutinaria, donde sólo ha sido probada la obtención de doble haploides en tomate a partir del cultivo de anteras y obteniendo una baja eficiencia.

Berenjena

La berenjena (*Solanum melongena*) está también entre los vegetales más importantes del mundo. En términos de producción y de área cosechada, la berenjena en 2010 se encontraba en el sexto y el octavo lugar en el ranking de cultivos vegetales con 43.891.773 Tm y 1.674.092 Ha, respectivamente (FAOSTAT 2010). La berenjena parece responder a la inducción de androgénesis mejor que el tomate. De hecho, mediante cultivo de anteras es posible inducir la desviación de la microspora de berenjena a embrión haploide o doble haploide y finalmente a planta. Los primeros resultados de la regeneración de plantas a partir de cultivos de anteras datan de 1973 (Raina and Iyer 1973). Con un tratamiento con colchicina se logró inducir la proliferación de callos y la regeneración de plantas a partir de los mismos. Sin embargo, de acuerdo con la propia interpretación del autor, es probable que los callos obtenidos fueran producidos desde el tejido conectivo, teniendo por tanto un origen somático. Al final de esa década, otros autores reportaron la producción de individuos haploides de berenjena (Isouard et al. 1979). Misra y colaboradores publicaron en 1983 la inducción de callos derivados de tejido microesporogénico, siendo capaces de regenerar brotes y raíces. Sin embargo, fue en 1982 cuando el trabajo de Robert Dumas de Vaulx y Daniel Chambonnet estableció la base de un protocolo general, fiable y reproducible para la producción de embriones haploides y la regeneración de plantas doble haploides a partir de cultivo de anteras de berenjena (Dumas de Vaulx and Chambonnet 1982). En su método original, la incubación de anteras a 35ºC durante 8 días en oscuridad cambiando después a 25ºC en un medio con 2,4-D y kinetina, se propuso como un protocolo capaz de promover el desarrollo de MDEs (embriones derivados de microsporas) dentro de las anteras en cultivo. Posteriormente, se vio que la androgénesis en berenjena es dependiente del genotipo de la planta donante y del estadio del desarrollo de la microspora, así como de las condiciones del cultivo, incluyendo temperatura, tipo, y concentración de los reguladores del crecimiento presentes en el medio (Rotino 1996; Rotino et al. 2005). Actualmente, este método es la base de muchos protocolos utilizados de forma rutinaria en programas de mejora para la producción de líneas puras doble haploides, adaptadas a cultivares particulares.

Como se ha visto, pueden inducirse con éxito embriones de berenjena a partir de microsporas contenidas en el interior de la antera. Sin embargo, este método no excluye la aparición ocasional de embriones somáticos provenientes del tejido de la antera, como se ha visto en tomate. También se debe

tener en cuenta el efecto secretor incontrolado del tapete que rodea el saco polínico, el cual imposibilita tener un control estricto de las condiciones de cultivo. Además el cultivo de anteras tiene una eficiencia limitada, produciendo sólo unos pocos embriones por antera cultivada. Estas limitaciones pueden ser solventadas por el aislamiento y el cultivo directo de microsporas. En aquellas especies donde el cultivo de microsporas aisladas está puesto a punto, es posible obtener cientos o incluso miles de embriones a partir de las microsporas contenidas en una única antera. Igual que en tomate, el desarrollo de un método para la inducción androgénica a partir de microsporas aisladas de berenjena podría ser muy ventajoso, y podría evitar los efectos mencionados anteriormente del efecto del tapete, la aparición de regenerantes somáticos y la baja eficiencia. Teniendo esto en cuenta, resulta sorprendente que haya tan pocos trabajos publicados referidos a la obtención con éxito de plantas doble haploides a partir de microsporas aisladas (Bal et al. 2009; Miyoshi 1996). En los estudios de Miyoshi, las plántulas fueron obtenidas a partir de callos derivados de microsporas, con una eficiencia de 20-65 callos/antera y 0,001-0,02 plantas/callo, mejorando los resultados de cultivos de anteras según el autor. Más recientemente, tras la combinación de un choque térmico con tratamientos de manitol se observaron divisiones en las microsporas de berenjena (Bal et al. 2009), aunque dicho estudio no reportó datos acerca de la regeneración a planta completa, ni a partir de callos ni de embriones.

Capítulo 1

Genetic, quantitative and microscopic evidence for fusion of haploid nuclei and growth of somatic calli in cultured *ms10*^{35} tomato anthers

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Abstract

In plant breeding, androgenic doubled haploids represent powerful tools to save time and resources for pure line generation. Unfortunately, the knowledge on induction of androgenesis in tomato (*Solanum lycopersicum*) is still very scarce, and little is known about the particularities of this highly recalcitrant species. The only known method to obtain haploid/doubled haploid tomato plants is the culture of meiocyte-containing anthers. However, this method has important limitations, including a low efficiency of induction and the presence of non doubled haploid regenerants. In order to shed light to the causes of these limitations, we have analyzed the process of callus formation in anthers of tomato lines carrying the *ms10*^{35} gene for male-sterility, using a multidisciplinary methodological approach, including light and electron microscopy, flow cytometry and genetic analysis with morphological and molecular markers. Our results demonstrate that haploid, doubled haploid and non-doubled haploid calli occur in tomato anthers, although at different frequencies. Non-doubled haploid callus, derived either from somatic cells or from the fusion of two different haploid meiotic cells accounts for more than 90% of the total of calli produced. Somatic calli are derived from the growth of the stubs of connective tissue present in the interlocular septa of the anther. This growth is markedly increased in *ms10*^{35} mutants, which explains the higher callogenic rates in these genotypes. Together, our results reveal serious drawbacks that explain the low efficiency of anther-derived, doubled haploid production in tomato, and stress the need for alternatives towards doubled haploidy.

Introduction

Androgenesis is defined as a set of biological processes leading to the development of individuals derived from the nuclei of male spores (Seguí-Simarro 2010). Androgenesis can be a powerful shortcut leading to the production of homozygous, doubled haploid (DH) pure lines in just one *in vitro* generation. Compared to traditional breeding methods, the considerable savings in terms of costs and time make this method the choice in those species where efficient androgenesis protocols are well established. In species such as tobacco, rapeseed, wheat or barley, it is relatively easy to produce androgen-

ic DHs in some cultivars (Seguí-Simarro and Nuez 2008). Unfortunately, this is not the case in tomato.

While tomato is the first vegetable crop worldwide in terms of production and cultivated area (FAOSTAT 2009), little is known about androgenesis in tomato. Since 1971 (Gresshoff and Doy 1972; Sharp and Dougall 1971), nearly all attempts to induce androgenesis in tomato have focused on the *in vitro* anther culture. A significant number of culture media types, conditions, and combinations of nutrients, vitamins, growth factors and supplements have been assessed reviewed in Bal and Abak (2007). Different laboratories have reported induction of calli (Dao and Shamina 1978; Jaramillo and Summers 1990; 1991; Sharp et al. 1972) or embryo-like multicellular structures (Dao and Shamina 1978; Varghese and Gulshan 1986), and regeneration of roots (Gresshoff and Doy 1972; Gulshan and Sharma 1981; Levenko et al. 1977; Sharp and Dougall 1971) or apical shoots (Ma et al. 1999). Only two laboratories have published on the regeneration of entire plants with a clear haploid origin (Seguí-Simarro and Nuez 2005; 2007; Shtereva et al. 1998; Zagorska et al. 1998; Zagorska et al. 2004), but also with a high percentage of mixoploid calli and at a low general efficiency of the process. At present, a detailed study about the causes of such a low efficiency and the impact of somatic tissues on the total of calli produced is still lacking.

In general, the most critical parameters in the induction of androgenesis are the genotype and the developmental stage of the microspore (Seguí-Simarro and Nuez 2008). Whereas in nearly all responsive species the inducible stage revolves around the first pollen mitosis (Touraev et al. 2001), in tomato, most studies based on anther culture point to the meiocyte as the inducible stage (Gresshoff and Doy 1972; Seguí-Simarro and Nuez 2005; 2007; Shtereva et al. 1998; Summers et al. 1992; Zamir et al. 1980). The second critical issue is the genotype. Some male-sterile mutant lines of different tomato cultivars have been reported as especially sensitive to the induction of androgenic calli (Seguí-Simarro and Nuez 2005; 2007; Shtereva et al. 1998; Zagorska et al. 1998; Zagorska et al. 2004; Zamir et al. 1980). Specifically, male-sterile tomato lines carrying the mutant gene *ms10*^{35} exhibited an increased production of callus masses compared to the fertile lines. It was suggested that somatic tissues of the *ms10*^{35} mutants' anthers also contribute to the total calli produced, since haploid origin could be demonstrated only for a small percentage of the calli. However, such aspects as to how and which

somatic cells are induced to proliferate in these mutants remain largely unknown.

Previous research on androgenic calli from the *ms10*^{35} mutants also indicated a major undesirable feature of this system, the presence of heterozygous calli. In addition to the proliferation of somatic (diploid) tissues of the anther walls, it is currently believed that heterozygous individuals may also originate from fusion of two different haploid meiotic cells, prior to their release as individual microspores (Seguí-Simarro and Nuez 2007). However, no genetic evidence for such fusions has ever been presented.

From all of these studies it is evident that tomato is extremely recalcitrant to androgenesis, and additional efforts have to be devoted to understand the specifics of the process in this species as a prerequisite step towards the design of an efficient haploid induction protocol. In this work, we provide evidence for the occurrence of nuclear fusions within meiocytes, and study to what extent such fusions of haploid nuclei and the presence of callus of somatic origin may be a drawback in this system. We used flow cytometry, molecular and morphological markers to evaluate the putative gametophytic/sporophytic origin of calli and regenerants. To reconstruct the process of haploid nuclear fusion, we used electron microscopy. Combining light and electron microscopy we reconstructed the process of microsporogenesis in the *ms10*^{35}/*ms10*^{35} male-sterile mutant of the 'Resaplus' cultivar as well as in the fertile genotype for comparison. The putative origin of callus and regenerated plants have been addressed through the morphological and molecular characterization of the callus and regenerants produced by the *in vitro* culture of anthers of the *ms10*^{35}/+ hybrid between 'Resaplus' (the fertile line) and *ms10*^{35}/*ms10*^{35} (the male-sterile mutant). The *ms10*^{35} gene is linked to the *aa* gene for the absence of anthocyanin in the vegetative parts, with a recombination frequency ranging from 1.7 to 4.1% (Durand 1981; Philouze 1974). The *ms10*^{35} gene is also linked to the *wo* gene for strong pubescence in leaves and stems with a recombination frequency of 1.1% (Durand 1981). Thus, we used the presence or absence of the *ms10*^{35}, *aa* and *wo* phenotypes as morphological markers in a segregating progeny derived from donor plants heterozygous for the three genes.

Our results shed light on the remarkable impact of somatic cell proliferation in the production of calli from specific cell types of the locular tissues of anthers, as well as on the fusion of genetically different haploid nuclei within the meiocyte. Together, these results explain why it is so difficult to

obtain doubled haploids in tomato via anther culture. These results are also relevant for a better understanding of androgenesis induction in tomato, but also for future designs to improve its efficiency.

Materials and methods

Plant material

Tomato donor plants of the 'Resaplus' cultivar (purple, moderately pubescent and fertile) and a 'Resaplus' male sterile mutant line carrying the *ms10*^{35} gene (green and hairy, recessive for the *wo* and *aa* genes) were obtained from the COMAV germplasm collection and used as donor plants. Hybrids between these two genotypes (and therefore heterozygous for the three linked genes) were also obtained, to be used as donors as described in Results for the morphological and molecular characterization. From now on, these three genotypes will be abbreviated as +/+, *ms10*^{35}/*ms10*^{35}, and *ms10*^{35} /+, respectively. Plants were grown in the greenhouses of the COMAV, at the Universidad Politécnica de Valencia, at 18ºC under natural light during the months of September to February for three consecutive years.

In vitro anther cultures and plant regeneration

Buds ranging in length from 4 to 5 mm were collected from flowering plants and their anthers were dissected and plated as described in Seguí-Simarro and Nuez (2007). As a control, one out of the six anthers in a single bud was live squashed on a microscopic slide, stained with DAPI and observed under the microscope. Only anthers containing meiocytes before tetrad cellularization (between metaphase I and telophase II) were inoculated in plates with induction medium. Induction medium consisted on Murashige and Skoog (MS) basal medium + vitamins (Murashige and Skoog 1962), pH 5.7, supplemented with 2.5 g L^{-1} Phytagel, 20 g L^{-1} sucrose, 1 mg L^{-1} 2ip and 2 mg L^{-1} IAA). Dishes were kept in a growth cabinet at 25ºC, in darkness for 1 month, and then under a 16/8 photoperiod. Anthers and developing calli were transferred to fresh medium on a monthly basis, discarding anthers either necrotic or not responding to the induction treatment. Green or partially green, proliferating calli were transferred to regeneration medium (4.4 g L^{-1} MS medium +

vitamins pH 5.7, 2.5 g L^{-1} Phytagel, 20 g L^{-1} sucrose and 0.25 mg L^{-1} zeatin riboside). Developing shoots were excised from the callus and transferred to glass tubes or magenta pots containing regeneration medium. Non-rooting, developed shoots were transferred to rooting medium (2.2 g L^{-1} MS + vitamins, 2.5 g L^{-1} Phytagel and 10 g L^{-1} sucrose, pH 5.7). Complete, rooted plantlets were transferred to pots with soil and acclimated for one week under greenhouse conditions.

Flow cytometry

Small pieces of cultured young calli and leaves from regenerated plants were processed using the CyStain UV Precise P kit (Partec GmbH, Münster, Germany). Additionally, young leaf samples from donor plants were analyzed and used as standards for 2C DNA content. Samples were chopped at 4ºC with a razor blade in 400 µl of nuclear extraction buffer (NEB). After 1 minute incubation in NEB, 1.6 ml of DAPI-based staining buffer was added and incubated for 2 minutes. 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

To determine the origin of the regenerants, donor plants of the *ms10*^{35}/+ genotype were screened using microsatellite markers (SSR). Young leaf tissue was sampled from 5 different 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 plant was analyzed using the following 21 SSR markers known to be polymorphic in other tomato cultivars: LE21085, LEATPACAb, LEMDDNb, LEWIPIG, LPHFS24, LEGAST1, LELEUZIP, LE20592, LECAB9, LEGTOM5, LEATPACAa and LEILV1B selected from Smulders et al. (1997); TMS9 and TMS33 selected from Areshchenkova and Ganal (1999) and SSR287, SSR63, SSR344, SSR70, SSR20, SSR111 and SSR248 selected from http://www.sgn.cornell.edu/). The forward primers were labeled with different fluorescent dyes and six loci were simultaneously detected using an ABI PRISM 310 Genetic Analyzer. Among the 21 markers tested, 15 did not reveal any polymorphism in the donor plants, and two (TMS9 and SSR20) appeared polymorphic for some individuals and monomorphic for others. Only four markers (TMS33, SSR63, SSR356 and SSR248)

were clearly polymorphic for all donor plants tested. These markers were used to verify the genetic status of regenerants.

Light and electron microscopy

We processed samples of anthers of the *ms10*^{35}/+ genotype soon after anther culture initiation and of the *ms10*^{35}/*ms10*^{35} and +/+ (Resaplus) genotypes at different stages during microsporogenesis. For light microscopy, samples were processed as in Seguí-Simarro and Nuez (2005). Thin (2 µm) sections were produced with a Leica UC6 ultratome and observed under bright field and phase contrast in a Nikon Eclipse E1000 microscope. For electron microscopy, samples were fixed in the Karnovsky´s fixative, post fixed in 2% OsO4, dehydrated in ethanol series, embedded and polymerized in Epon resin according to Seguí-Simarro and Nuez (2007). Ultrathin (~80 nm) sections were produced with a Leica UC6 ultratome, mounted onto formvar and carbon-coated copper grids, counterstained with uranyl acetate and lead citrate, and observed in a Philips CM10 transmission electron microscope operating at 100 kV.

Results

Callus production and plant regeneration from tomato anther cultures of the ms10^{35}/ms10^{35}, ms10^{35}/+ and +/+ genotypes.

A total of 1842 anthers (744 from *ms10*^{35}/*ms10*^{35} donor plants, 720 from *ms10*^{35}/+ plants and 378 from +/+ plants) were excised at stages corresponding to meiotic metaphase I through telophase II (Seguí-Simarro and Nuez 2005). Upon culture, callus masses were observed to emerge out of the locules of swollen anthers (Fig. 1A). A total of 965 calli were obtained from the three genotypes. As long as they were maintained in darkness on the induction medium, most calli proliferated quickly and considerably increased in size. Upon exposure to light, green regions appeared at the callus surface (Fig. 1B). In viable calli transferred to regeneration medium, shoots were observed to grow from the green regions (Fig. 1C) and rapidly regenerated aerial organs (Fig. 1D). Upon excision, they soon rooted and became plantlets (Fig. 1E). A total of 93 plants were regenerated: 62 from the *ms10*^{35}/*ms10*^{35}

donors, 21 from the *ms10*^{35}/+ donors and 10 from the +/+ donors. After acclimatization, plantlets were transferred to a greenhouse. In a few cases these plants had reduced habitus and leaf size (Fig. 1F). However, most of the plants (Fig. 1G) were comparable to the plants used as donors (Fig. 1H). Flow cytometric and molecular marker analyses indicated that among regenerants were haploids (Fig. 1F) and doubled haploids (Fig. 1G).

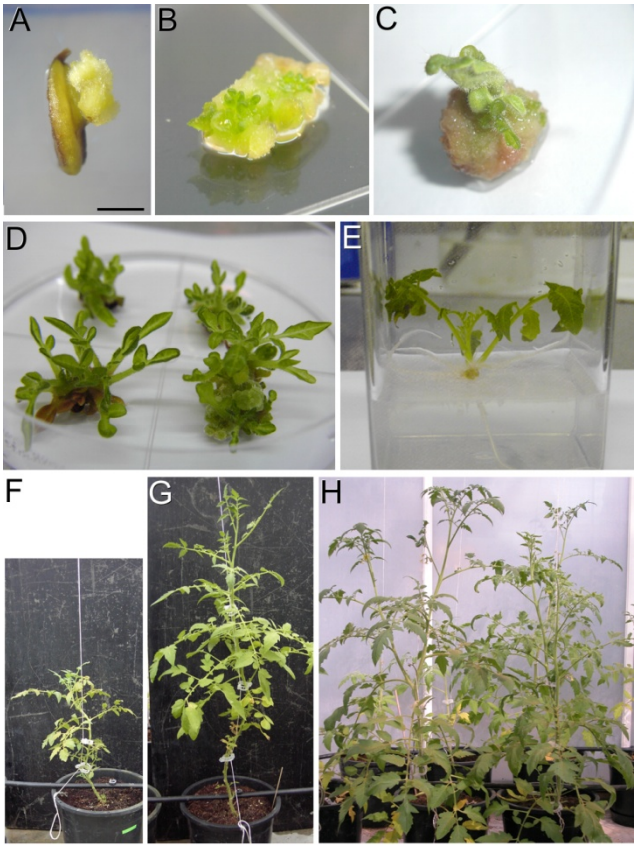

Fig. 1 Anther culture and plant regeneration in tomato. **A**: Anther with a young callus emerging from the anther locule. **B**: Callus with shoot initials at its surface. **C**: Regenerating shoot over the surface of the callus. **D**: Developing shoots and leaves over old, necrosing calli. **E**: *In vitro*, regenerated and rooted tomato plantlet. **F-G**: Tomato plants regenerated from anther cultures, acclimated and grown at the greenhouse. **F**: Haploid regenerant. Note the reduced habitus. **G**: DH regenerant. **H**: Normal tomato *ms10*^{35}/+ plants, used as donors of anthers. Bars in A: 1 mm.

There were important differences in the regeneration efficiency among the three genotypes used, expressed as a number of calli and regenerated plans per anther. The *ms10*^{35}/*ms10*^{35} donor plants produced significantly more calli and regenerants per anther than the *ms10*^{35}/+ and the +/+ plants (Fig. 2). This implies that homozygosity for the *ms10*^{35} allele increased both callus production and plant regeneration. Heterozygosity for this allele did not have any positive effect as the efficiency was not different from homozygotes null.

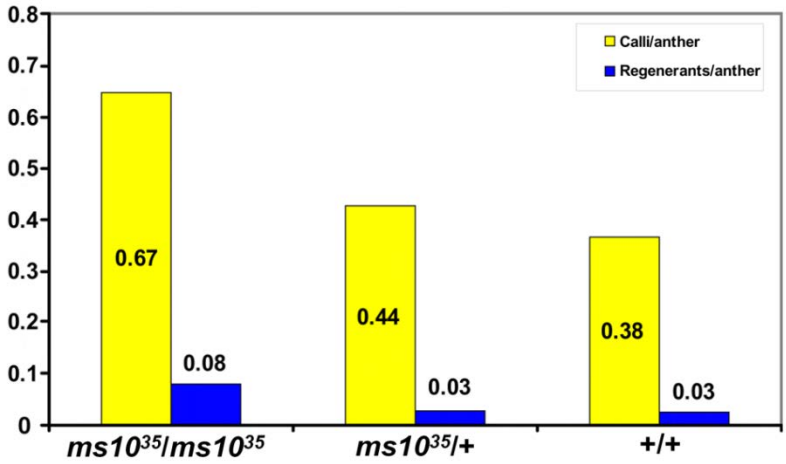

Fig. 2 Analysis of the number of calli (light bars) and regenerated plants (dark bars) derived from anther cultures of the *ms10*^{35}/*ms10*^{35}, *ms10*^{35}/+ and +/+ genotypes. In order to be able to compare between different experiments, measurements are given as number of calli or regenerants per excised and cultured anther.

Flow cytometric analysis of calli and regenerated plants.

Once the effect of the *ms10*^{35} allele was established, we attempted to determine the origin of the anther-derived calli and regenerants. For this, we focused our study on the *ms10*^{35}/+ genotype, heterozygous for the morphological markers listed above. Additional anther cultures from *ms10*^{35}/+ hybrid donors were performed and a total of 66 different viable calli and 83 regenerated plants were produced. Some calli did not respond to organogenesis, whereas others gave rise to more than one regenerant. Despite the fact that

regenerants originating from the same callus may be clones, we decided to study the entire set of plants obtained, for the following reasons: (1) frequent mixoploidy in anther-derived calli is well-documented (Seguí-Simarro and Nuez 2005; 2007; Shtereva et al. 1998; Zagorska et al. 1998; Zagorska et al. 2004) and it implies the possibility of genetically different individuals originating from different regions of the same mixoploid callus; (2) all plants regenerated from a single callus may provide information about the regenerative capacity of the different tissues involved in the callus and plant production.

The ploidy levels of the *ms10*^{35}/+ calli and regenerants were analyzed by flow cytometry. Leaves from the donor plants presented a G1 DNA peak at channel ~100, which was set as the 2C standard for diploid cells, together with a small peak at channel ~200, characteristic of diploid G2 cells. Based on this, 58 of the 66 calli were mixoploid. Among them, 24 presented a 2C+4C DNA content (Table 1) and 34 presented a 1C+2C DNA content (Table 1). Seven calli had 2C DNA content, and only one was clearly identified as haploid (1C DNA content). This callus necrosed and died before showing any sign of organogenesis on its surface.

Table 1: Flow cytometric analysis of callus and regenerants derived from anther cultures of the *ms10*^{35}/+ genotype.

Calli		Regenerants		
Ploidy	Number	Ploidy	Number	Origin (SSR)
2C+4C	24	2C	23	Somatic
		4C	5	Somatic
2C	7	2C	21	Somatic
C+2C	34	C	3	Androgenic
		2C	31	*See Table 2*
C	1	---	---	---
Total	66	Total	83	

Among the 83 regenerated plants that were analyzed by flow cytometry, no mixoploidy was observed (Table 1). From the 24 2C+4C calli, 28 plants were obtained of which 23 had a 2C DNA content and five a 4C content. From

the seven identified 2C calli, 21 2C plants were obtained, with an average of three plants per callus. From the 34 C+2C calli, three 1C plants and 31 2C plants were obtained.

The 83 regenerated plants were screened for the three morphological markers present. All 28 plants derived from the 2C+4C calli and the 21 plants derived from the 2C calli (see Table 1) had the same phenotype: purple stems, moderate pubescence (Fig. 3A) and normal microspores and pollen (data not shown). The phenotypical uniformity of these 49 plants and an absence of haploid cells in their original calli are indicative of somatic origin.

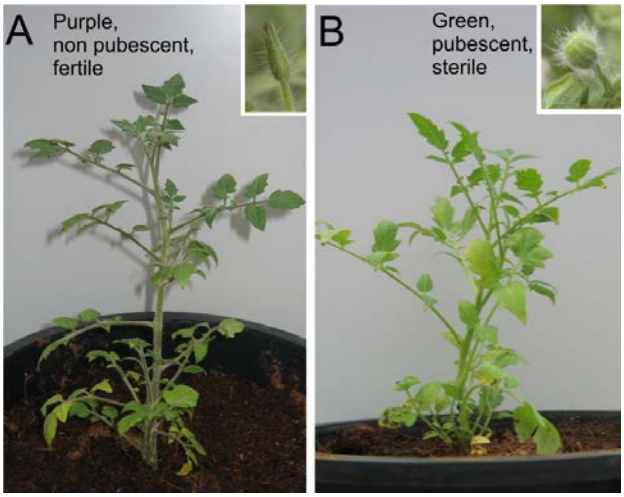

Fig. 3 Phenotypic analysis of regenerants from *ms10*^{35}/+ plants. **A**: purple, moderately pubescent, fertile regenerant. **B**: Green, strongly pubescent and sterile regenerant. Insets in A and B show a detail of a typical bud of each phenotype.

Among 34 plants regenerated from the C+2C calli (Table 1) there were:

- Three haploids (1C as revealed by flow cytometry) that were green, highly pubescent and male sterile. As expected, they had a reduced plant stature (Fig. 1F) typical of haploids.

- Among the remaining 31 diploid (2C) plants, three were green, highly pubescent and sterile (Fig. 3B), and 28 were purple, moderately pubescent and fertile (Fig. 3A).

Segregation for morphological markers among the 31 diploid plants derived from mixoploid C+2C calli raised concerns about their origin. Thus, the analysis based on morphological markers was combined with an analysis by microsatellite (SSR) markers. Based on the four polymorphic SSR (Table 2), the following genotypes were observed:

Table 2: Molecular marker (SSR) analysis of *ms10*^{35}/+ callus-derived regenerants.

	Number of individuals	Individuals homozygous for 4 SSR	Individuals heterozygous for:			
			4 SSR	3 SSR	2 SSR	1 SSR
Regenerants from donor 1	8	---	7	---	---	1
Regenerants from donor 2	8	1	6	---	---	1
Regenerants from donor 3	7	1	4	---	1	1
Regenerants from donor 4	8	1	3	---	2	2
Total Regenerants	31	3	20	---	3	5
Total Regenerants (%)	100	9.7	64.5	---	9.7	16.1

- Three individuals were homozygous for all four SSR markers. Since they were also recessive for morphoplogical markers (green, highly pubescent and sterile), we considered them doubled haploids, therefore, originating from haploid nuclei.

- 20 individuals were heterozygous for all four SSR markers. They were also purple, moderately pubescent and fertile, i.e. non-recessive for the *aa, wo* and *ms10*^{35} genes. Thus, we attributed them a somatic origin.

- Eight individuals were heterozygous, three being heterozygous for two SSR markers and five being heterozygous for one SSR marker. In addition, seven out of the eight plants were purple, moderately pubescent and fertile and one was green, strongly pubescent and sterile. The heterozygosis for some markers observed in these these individuals excludes both a somatic and haploid origin. Thus, we analyzed the locular contents of anthers induced to androgenesis in search for a possible explanation for these genetic combinations.

Electron microscopic reconstruction of the first stages of meiocytic induction.

We used transmission electron microscopy to analyze the locules of plated anthers few hours after the culture initiation (Fig. 4). By the end of meiosis II, in normally-looking meiocytes the first signs of the post-meiotic cytokinesis were already evident (Fig. 4A). However, as cytokinesis proceeded, clear signs of deviations from the typical syncytial-type pattern of post-meiotic cytokinesis (Seguí-Simarro et al. 2008) were observed in some meiocytes. As seen in Fig. 4B, some cell walls failed to separate the corresponding two neighboring haploid nuclei, giving rise to cytoplasmic bridges between adjacent cells. Other meiocytes showed a total absence of cell walls separating adjacent nuclei (Fig. 4C). The conspicuous presence of a nucleolus was indicative of well formed nuclei, presumably at the G1 stage of the cell cycle, when cell walls are supposed to be completed in intact meiocytes. In meiocytes with defective or absent cell walls, nuclei coexisting in the same cytoplasm were observed at a very close distance, having their respective envelopes closely apposed (Fig. 4D) or even in tight physical contact (Fig. 4E). Such close contact was highly indicative of subsequent nuclear fusion. Indeed, some of the defective meiocytes showed enlarged nuclei with more than one visible nucleolus (Fig. 4F). Normal meiotic haploid nuclei should exhibit only one nucleolus after nuclear reconstitution (Figs. 4C-E), since in *Solanum* only one nucleolar organizing region (NOR) is present, located at chromosome 2 (Ivanova et al. 2000). Thus, the presence of two nucleoli was an indication of possible of nuclear fusion.

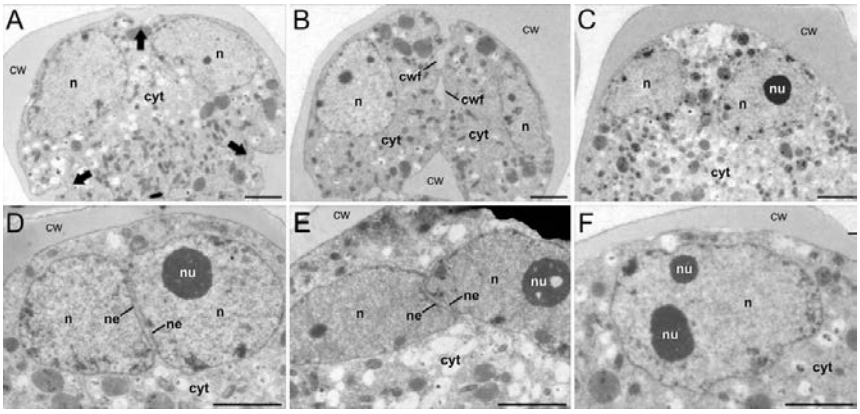

Fig. 4 Electron microscopical reconstruction of the process of meiocytic nuclear fusion. **A:** Normally-looking meiocyte at the onset of post-meiotic cytokinesis, as revealed by the appearance of new cell wall protrusions (arrows) at defined regions of the original cell wall (cw). **B:** Incomplete walling of an induced meiocyte, allowing for the appearance of cytoplasmic bridges between cell wall fragments (cwf). **C:** Coexistence of two nuclei (n) in the common cytoplasm (cyt) of a meiocyte with absent cell walls. **D:** Two nuclei of a non-walled meiocyte with their nuclear envelopes (ne) in close apposition. **E:** Two nuclei of a non-walled meiocyte with their envelopes in physical contact. **F:** Single, enlarged nucleus presumably coming from the fusion of two haploid nuclei, as revealed by the presence of two nucleoli (nu). Bars: 2 µm.

Light and electron microscopic reconstruction of microsporogenesis in +/+ and ms10^{35}/ms10^{35} genotypes.

In view of the high percentage of somatic regenerants, we next analyzed the development of meiocytes and microspores in both fertile (+/+) and male sterile (*ms10^{35}*/*ms10^{35}*) lines by means of light and transmission electron microscopy. In the fertile genotype, meiocytes occupied most of the anther locule, surrounded by a well developed tapetum with normally-looking cells and no signs of degeneration (Fig. 5A). Meiocytes at this stage were actively dividing, either at late stages of meiosis I or at early stages of meiosis II (Fig. 6A), the sensitive timeframe for in vitro induction of callus proliferation (Seguí-Simarro and Nuez 2005). Immediately after the inducible timeframe, anthers presented a wider anther locule, surrounded by a thinner tapetal layer with signs of cell death (Fig. 5B). In the locule, most of the cell types were tetrads, with only few young microspores or cellularizing meiocytes present. All tetrads had a normal architecture (Fig. 6B), with four independent cells separated by thick cell walls. At the stage when young microspores are released from the tetrad, microspores appeared well formed (Fig. 6C). At this stage, the anther locule was surrounded by a thin layer of degraded tapetum. The anther wall began to thin along the dehiscence line (Fig. 5C). Interestingly, in transversal sections of all anthers analyzed at this stage, a tissue stub (asterisk in Fig. 5C) could be observed to emerge from the connective tissue separating both pollen sacs of the same theca, partially invading the anther locule. In anthers containing late, vacuolate microspores (Fig. 6D), the stubs appeared swollen, occupying a significant part of the pollen sac (Fig. 5D). Light microscopical sections demonstrated that these stubs had a tissular architecture identical to that of the adjacent connective tissues, being both clearly connected.

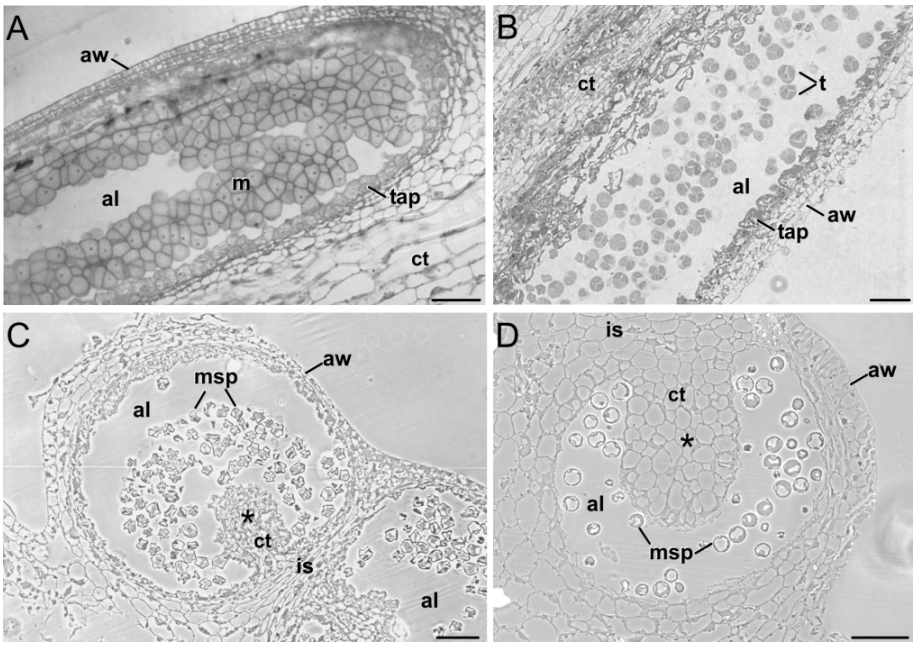

Fig. 5 Light microscopical analysis of meiocyte and microspore-carrying anthers of the +/+ donor plants. **A**: Longitudinal section of an anther carrying early meiocytes (m), at a stage before meiosis II. **B**: Longitudinal section of an anther carrying completely walled tetrads (t), prior to microspore release. **C**: Transversal section of an anther carrying young microspores (msp), just released from the tetrad. Note the emergence of a tissue stub (asterisk) from the connective tissue (ct) of the interlocular septum (is) between both adjacent anther locules (al). **D**: Transversal section of an anther carrying vacuolated microspores (msp). Note the growth of the stub (asterisk) originated from the layers of connective tissue (ct) of the interlocular septum (is). aw: anther wall; tap: tapetum. Bars: 50 µm.

In plants of the male-sterile, *ms10*^{35}/ *ms10*^{35} genotype, anthers at the inducible interval (Fig. 7A) carried 100% of actively dividing meiocytes, as in the fertile genotype (compare Figs. 6A and 8A). However, the next stage in anther development deviated from the fertile line. In anthers of the size equivalent to that of Fig. 5B, the locular width was dramatically reduced (Fig. 7B). Signs of tapetal degradation were more evident than in the fertile counterpart. Most importantly, the locular structures, presumably tetrads, showed clear signs of death, as revealed by their intense dark staining and the irregular, collapsed shapes (Fig. 7B). Fig. 8B shows a tetrad corresponding to an anther of this size, where one of the three cells included in the section (arrow) presented an intensely stained cytoplasm, abundant vacuolation and a

general collapse, all signs of cell death. Anthers of a size equivalent to that of Fig 5C were characterized by the presence of degenerating or completely degenerated tetrads (Fig. 8C) within a collapsed anther locule (Fig. 7C).

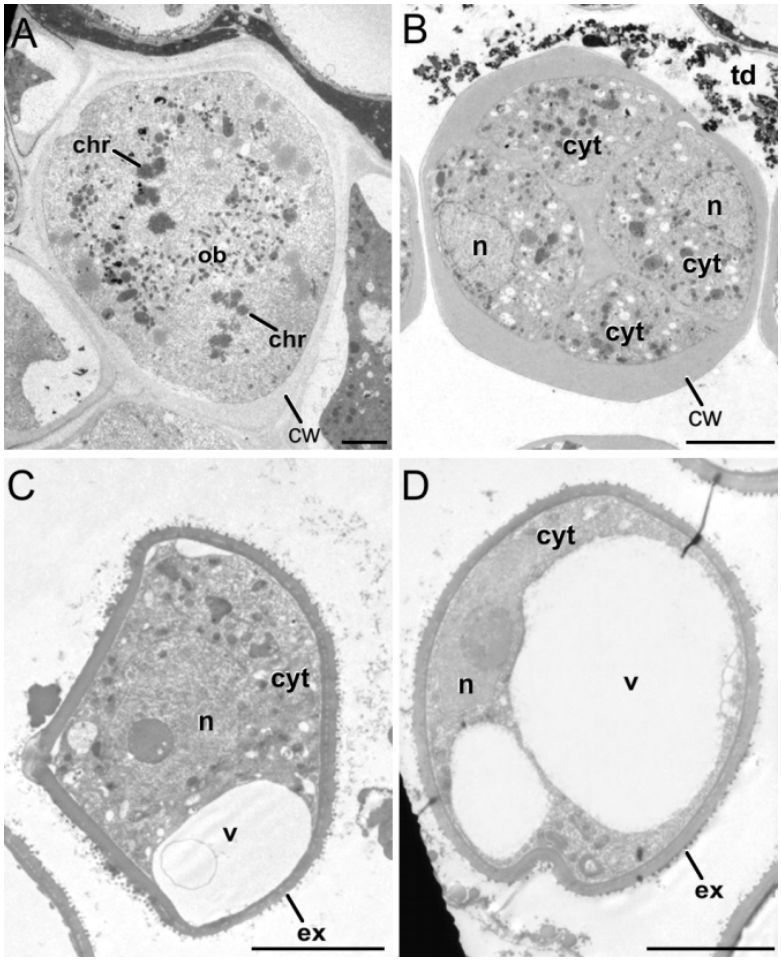

Fig. 6 Transmission electron microscopical analysis of meiocytes and microspores of the +/+ donor plants. **A**: Meiocyte at metaphase II, where two metaphasic plates of aligned chromosomes (chr) are observed at both sides of the equatorial organellar band (ob). **B**: Tetrad just before microspore release, still enclosed within the callosic wall (cw). Note the presence of tapetal debris (td) in the pollen sac. **C**: Young microspore released from the tetrad, with a centrally located nucleus, slight signs of vacuolation, a polygonal shape derived from their previous position within the tetrad, and a sculptured exine coat (ex) at the outermost surface. **D**: Late, vacuo-

late microspore showing its typical off-centered nucleus, slightly rounded shape and massive vacuole. cyt: cytoplasm; n: nucleus; v: vacuole. Bars: 5 µm.

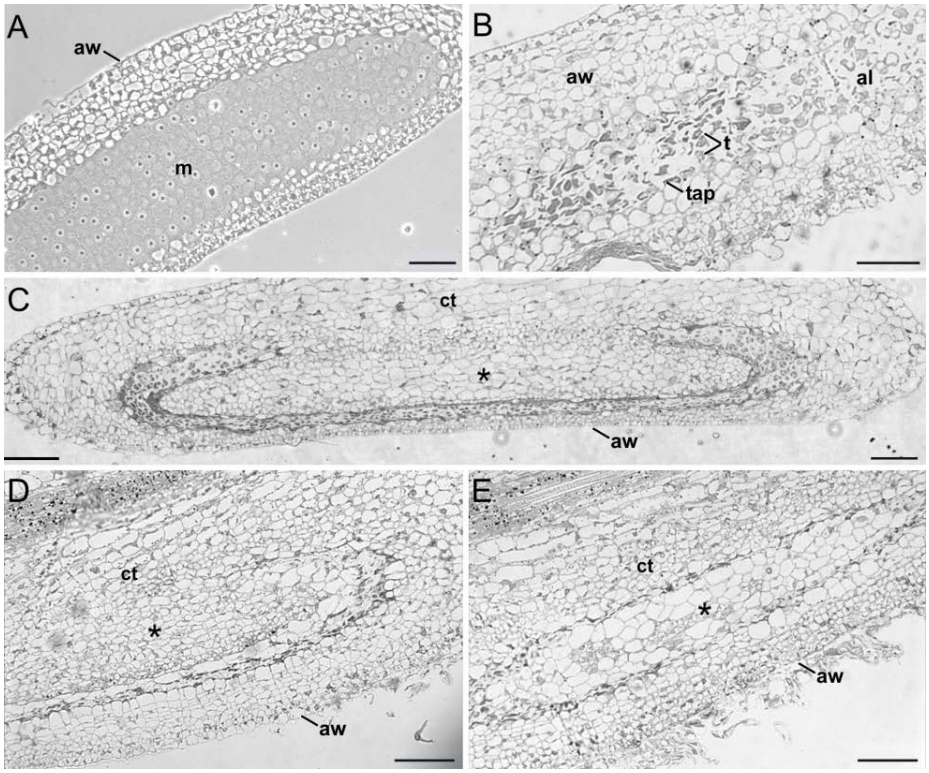

Fig. 7 Light microscopical analysis of meiocyte-carrying anthers of the *ms10*^{35}/*ms10*^{35} donor plants. **A**: Longitudinal section of an anther carrying early meiocytes (m), just entering meiosis and still at the uninucleate stage. **B**: Longitudinal section of an anther at a stage equivalent to that of fig. 5B (tetrads). These anthers carry degenerated and/or dying tetrads (t) within the anther locule (al). **C**: Longitudinal section of an anther at a stage equivalent to that of fig. 5C (young microspores). The locular volume is dramatically reduced by the emergence of a callus-like protrusion (asterisk) from the connective layers of the anther (ct). Within the locule, degenerating tetrads are present. **D**: Longitudinal section of an anther at a stage equivalent to that of fig. 5D (vacuolate microspores). Nearly all of the locular volume is occupied by the callus (asterisk) connected to the connective tissue (ct), with only a narrow band of cell remnants present at the periphery of the callus. **E:** Longitudinal section of an anther at a stage equivalent to that of fig. 5D, where a callus (asterisk) with no evident link with the connective tissue (ct) occupies the locular volume. aw: anther wall; tap: tapetum. Bars: 50 µm.

A callus-like stub linked to the connective tissue, similar to that of fertile anthers but significantly larger, invaded most of the locular volume. Further developmental stages had a similar architecture, but the growing callus-like stub occupied nearly all of the locular space, displacing the tetrad remnants to a thin, peripheral layer (Fig. 7D). No other cell types of gametophytic nature (microspores or pollen) were observed within the locular space. Occasionally, the link of the callus-like stub with the connective tissue was not evident through a series of serial sections covering all of the anther locule depth (Fig. 7E). This could likely be indicative of the presence of a meiocyte-derived callus, as previously demonstrated (Seguí-Simarro and Nuez 2007).

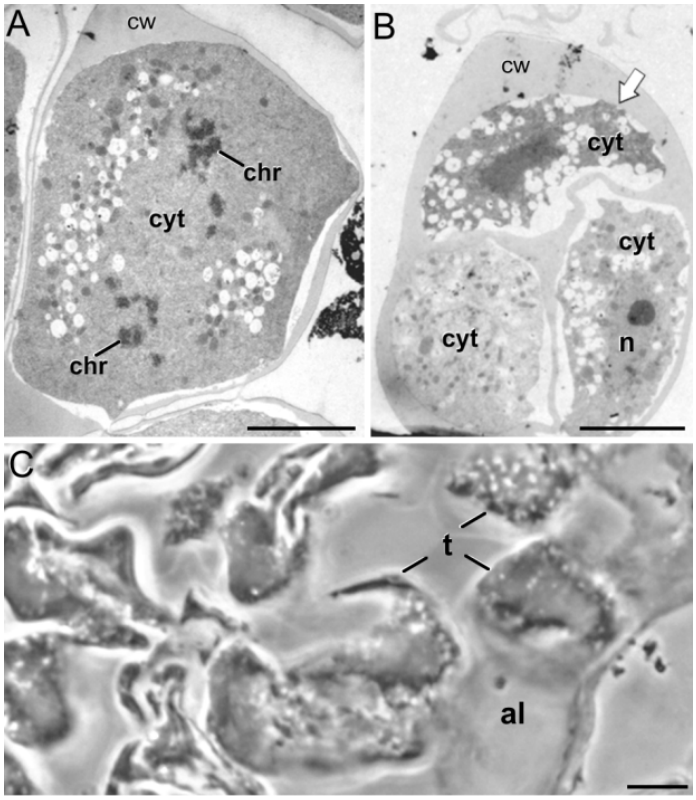

Fig. 8 Transmission electron microscopical analysis of meiocytes of the *ms10*^{35}/*ms10*^{35} donor plants. **A**: Meiocyte at late anaphase I, where each haploid set of chromosomes (chr) are reaching the cell poles. **B**: Tetrad coming from an anther at a developmental stage equivalent to that of fig. 6B. In one of the three visible cells (arrow), clear signs of cell death can be observed. The

other two cells do not show such a dramatic degeneration, but signs of incipient death such as massive vacuolation and detachment of the plasma membrane from the callosic wall (cw) can be seen. **C**: Tetrad (t) remnants from an anther at a developmental stage equivalent to that of figs. 6C and beyond. Tetrads are dead and collapsed. Not a single viable cell is observed. al: anther locule; cyt: cytoplasm; n: nucleus. Bars: 5 µm.

Discussion

In ms10^{35} mutants, meiosis is delayed and the inducible stage is extended in time.

In this work we have shown that homozygous *ms10^{35}* mutant gene promotes the degradation of meiotic cells still enclosed within the tetrad, and eventually the collapse of the entire tetrad (Fig. 8). Together with an enhanced rate of callus production, *ms10^{35}* mutants has delayed meiosis, as revealed by the different locular contents of male-sterile anthers equivalent in size to those of the fertile line (compare Figs. 5 and 7). This suggests that the enhanced rate of meiocyte-derived callus production is due to an extension of the inductive window. In other words, the mutation allows for more meiocytes at the optimal stages to be induced. However, this may not be a general feature of all tomato male sterile mutants with disrupted meiosis. As pointed out by Zamir et al. (1980), other meiosis-affecting, male sterile mutants such as the *ms12*, *ms5* and *ms33* fail to show a callogenic response, perhaps due to the presence of additional defects in the tapetal development (Rick 1948).

Three different callus types are produced, but only one is interesting for breeding purposes

Based on the ploidy data, the morphological characterization and the molecular analysis, we conclude that from our 83 anther-derived regenerants, only three (3.6%) were haploid and three were doubled haploid. For the DHs, we cannot entirely exclude a theoretical (although unlikely in practice) possibility that some of these three plants are not in fact true true DHs. The fact that some other individuals were homozygous for all but one of the seven markers establishes a precariously thin line between what is considered a doubled haploid and what is not. This reduced theoretical possibility could further be reduced by making use of more SSR markers, but this was not fea-

sible in the current study. In conclusion, the total number of haploid-derived regenerants here was six, or 7.3% of all regenerated plants.

Eight (9.6%) diploid regenerants, all of them derived from mixoploid calli, possed degrees of heterozygosity not observed in the donor plants. This excludes both haploid and somatic origins of these plants. It could be reasonably argued that these calli may originate from a mixture of haploid and somatic tissue, forming a chimeric tissue from which haploids, DHs and somatic diploids can arise. The presence of diploid regenerants polymorphic for some of the genetic markers used may also be explained by regeneration from the products of the first meiotic division. A nucleus of a binucleated meiotic intermediate has a haploid number of chromosomes, but with sister chromatids attached. As a consequence of crossing over, some of these sisters may be non-identical. This attractive hypothesis would explain the observed heterozygosity for a very low number of markers (such as one marker out of all tested), as a direct consequence of crossing over, with no need for nuclear fusion. While this may explain a part of the observed results, we are inclined to believe that most mixoploid, partially heterozygous calli and their corresponding regenerants come from a process of nuclear fusion within the meiocyte, based on the following rationale. We have shown (Fig. 4) that after induction, some meiocytes present nuclear profiles highly indicative of the occurrence of nuclear fusions between some of the haploid nuclei of the induced meiocytes. This fusion would be favored by the absence of, or incomplete, cell walls that fail to separate haploid nuclei. Although fusing membrane intermediates are difficult to identify in electron micrographs due the instantaneous nature of the process of membrane fusion, the observation of nuclei closely apposed or even in physical contact and the presence of enlarged nuclei with two nucleoli are good indications of nuclear fusion events. It was previously proposed that the last steps of meiosis are disturbed by the culture conditions, starting from serious alterations during post-meiotic cytokinesis, including the formation of incomplete or absent cell walls (Seguí-Simarro and Nuez 2007). This, in turn, would allow for the apposition and eventual fusion of two genetically different, haploid nuclei. According to this, the most reasonable origin for these 8 partially heterozygous plants would be a diploid region of the C+2C mixoploid callus. Whereas the three DHs mentioned above would originate from a haploid region, the eight partial heterozygotes would come from a diploid region formed by cells coming from the fusion of two genetically different meiocytic nuclei. Presence of these plants provides additional support from the genetic

side to the model previously proposed (Seguí-Simarro and Nuez 2007) to explain the low efficiency of androgenic DH production in tomato.

However, fusion of meiotic nuclei is neither the only nor the main drawback for the DH production in tomato. Indeed, based on the same ploidy level and the presence of genetic/DNA markers as in the donor plants, we postulate somatic origin for 69 out of the 83 regenerated plants (83% of the total). Such a rate of recovery of non-haploid regenerants is not a trivial issue. Moreover, for all practical purposes this percentage should be increased by the 9.6% of plants derived from meiocyte nuclear fusions, for a total of 92.7% of undesirable individuals. This means that only one in each 13 regenerants will be haploid or DH, and this explains why it is so difficult to obtain androgenic tomato haploids or DHs in quantities sufficient for breeding purposes.

The increased production of callus in the ms10^{35} male-sterile plants is due to a massive growth of the locular stubs of the connective tissue.

From the results shown in Fig. 2, it is clear that the *ms10*^{35}/*ms10*^{35} genotype outperforms the other two in terms of callus and plant production. Thus, the control of the *ms10*^{35} mutation over callus production is clearly recessive. This is in agreement with its effect in male sterility, which in turn constitutes an additional proof of the link between these two phenotypic expressions of the mutation. At issue is, why do these male sterile plants produce more calli and regenerants?

In tomato, normal anther development at the microsporogenic stage is characterized by the growth of a stub from the connective tissue at both sides of the interlocular septum (Figs. 5C, 5D; Goldberg et al. 1993; Senatore et al. 2009). As the anther matures and dehisces during pollen development, the stubs and the interlocular septum undergo a progressive loss of cell layers, becoming thinner and allowing for the aperture of the anther for pollen dispersal through the line of dehiscence (Senatore et al. 2009). This normal succession of events is disrupted in the *ms10*^{35}/*ms10*^{35} genotype, where microspores are not released from the tetrad. In parallel to the meiocyte degeneration within the anther locule, we show overproliferation of the stubs (Figs. 7C, 7D). Subsequently, the cell mass growing from the stub occupies most of the locular volume, at stages where no stub growth is described in the

fertile counterpart. Later stages of anther development are characterized by the total locular invasion of the stub-derived cellular mass. In summary, stubs in the *ms10*^{35}/*ms10*^{35} genotype resume a proliferative growth that ends up with the production of an intralocular, callus-like cellular mass. When anthers are excised and cultured in the presence of growth regulators, these masses eventually break the anther walls and emerge, giving rise to somatic calli from which most of the tomato regenerants are obtained.

These observations indicate that (1) the stubs from the interlocular septum may be the origin of the somatic callus and (2) the *ms10*^{35} mutation is not only defined by the degeneration and death of the meiocytes, but also by the induction of proliferation in the connective tissue of the anther. Thus, when we inoculate *ms10*^{35}/*ms10*^{35} anthers for *in vitro* culture, there is already a callus-like, proliferating mass inside, which makes generation of an extralocular callus easier. In fertile lines, where stubs do not proliferate, the growth of the stubs would only be induced *in vitro* and upon exposure to growth regulators, and therefore, by the same time in culture, their callus would be smaller and less numerous. Most likely, this would be the main reason why significantly lower amounts of calli and regenerants are obtained from fertile genotypes (Seguí-Simarro and Nuez 2007; Zagorska et al. 1998; Zamir et al. 1980).

Haploid or DH calli may have to compete for resources with the massive somatic callus

As seen hereby, somatic calli are predominant but not the only possible. Soon upon culturing some meiocytes are induced to proliferate, becoming haploid or mixoploid calli (this work; Seguí-Simarro and Nuez 2007). Conceivably, meiocyte-derived calli and calli derived from somatic tissues coexist in some anthers. Both callus types compete for the limited resources available within the anther locule. However, the outcome may be biased towards somatic (diploid) tissues, that are genetically more stable and with more regenerative capacity. As seen in Table 1, diploid calli coming from somatic tissue yielded three plants per callus, significantly higher than the rest, with different origins or ploidy levels.

The fact that the somatic-type callus is only one, proliferates at a faster rate, and is connected to the rest of the anther, will make it a candidate to succeed, displacing and collapsing the growth of the independent

meiocyte-derived calli. Perhaps, the actual rate of induction of meiocyte proliferation is higher than that reflected in the number of haploid or DH calli produced, but competition with the somatic cellular mass may make many meiocyte-derived calli abort at early stages of development. This could be an additional argument explaining low percentage of haploid or DH calli. On the other hand, it is clear that some of them are capable to survive and emerge from the anther locule. Following this line of speculation, since meiocytes are formed in the anther locule prior to the growth of the interlocular stub, it might well be possible that once a meiocyte-derived callus is established, the proliferative growth of the stub is somehow inhibited.

Concluding remarks

In this work, we showed that male sterility in *ms10*^{35} mutants is due to the collapse and death of the tetrads, prior to the release of the microspores to the anther locule. This allows for an extended window for callus induction. We also demonstrated that proliferation of cells from a protruding appendage of the interlocular septum is the main source of anther-derived calli in our tomato genotypes. The callus-like structure formed may likely account for the frequent presence of calli of somatic origin in cultured *ms10*^{35} tomato anthers. In addition, we provided ultrastructural and genetic evidence for the appearance of calli and regenerants derived from the fusion of two different haploid meiotic nuclei. As seen, although possible, there are many collateral, non desirable processes that make difficult to induce DHs from tomato meiocytes.

These collateral processes carry important practical implications. First, only one plant for every thirteen regenerated is haploid or DH. Second, time and resources must be spent to identify the haploid or DH plants among the total population. Third, the similar percentage of haploids and DHs (50:50) would make mandatory an additional step of genome doubling for haploid regenerants. In conclusion, the work presented hereby provides a reasonable explanation as to why *in vitro* anther culture in tomato has been highly unsuccessful if not impossible during four decades, and stresses the need for a search of alternative ways to overcome these limitations. Alternatives such as the culture of isolated meiocytes have not been approached yet, possibly due to the discouraging previous reports about the culture of this extremely delicate cell type (Shivanna and Johri 1985). Other more promising alternative, the culture of isolated microspores, has already been addressed.

However, results are still very preliminary (Bal and Abak 2005; Seguí-Simarro and Nuez 2007), and more time and efforts will be needed to refine this method.

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Capítulo 2

Efficient production of callus-derived doubled haploids through isolated microspore culture in eggplant (*Solanum melongena* L.)

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Abstract

Production of doubled haploids (DHs) through androgenesis induction is an important biotechnological tool for plant breeding. In some species, DHs are efficiently obtained through embryogenesis from isolated microspore cultures. In eggplant, however, this process is still at its infancy, despite the economic relevance of this important agricultural crop. To date, only two studies have focused previously on this process, suggesting that in eggplant microspore cultures, the only morphogenic response is callus formation. Given the notable lack of studies on eggplant microspore cultures, in this work we explored this process with different experimental approaches. We studied the response of different cultivars and characterized the development of microspores induced to divide and proliferate. We demonstrated that microspore-derived embryos (MDEs) can be produced in eggplant; however, MDEs stopped at the globular stage, to turn into euploid and principally mixoploid calli. From these calli, 60% of DH plants could be regenerated. In order to promote microspore induction we evaluated the effect of polyethylene glycol (PEG) and mannitol. PEG, but not mannitol, significantly increased induction of microspore embryogenesis. We also tested the ability of eight different media compositions to promote efficient plant regeneration from calli. In order to test it in a genotype-independent manner, we previously developed a method to generate clonal callus populations derived from single microspore-derived calli. Together, the results presented hereby constitute an efficient way to produce eggplant DHs through microspore culture. In addition, they contribute significant insights into the knowledge of the particularities of androgenesis induction in this species.

Introduction

Doubled haploid (DH) technology is a fast and convenient alternative to classical breeding for the generation of pure lines, essential for hybrid seed production (Germanà 2011). One of the most efficient ways to obtain DHs is to induce androgenesis in microspores. Through androgenesis, microspores are *in vitro* deviated from their original gametophytic pathway either after isolation from the anthers (isolated microspore culture), or within the anthers (anther culture). Plants can be directly regenerated by microspore-derived, haploid

embryogenesis, or indirectly through organogenesis from microspore-derived calli (Seguí-Simarro 2010). After chromosome doubling of haploid individuals, the result will be a 100% homozygous plant (a pure line) with a genetic background exclusively coming from the donor (male) microspores. This biotechnological approach to doubled haploidy can be induced in a number of angiosperm species. As of 2003, protocols to induce androgenesis were described for more than 250 species (Maluszynski et al. 2003). However, the efficiency of DH production is still very low in many crops of high agronomic interest. Good examples are those belonging to the Solanaceae family (Seguí-Simarro et al. 2011). Among the five major solanaceous crops (tobacco, pepper, potato, tomato and eggplant), at present in only one (tobacco) is DH technology sufficiently developed to produce DHs efficiently from anther and isolated microspore cultures. Others, like eggplant, may still be considered recalcitrant species.

Eggplant (*Solanum melongena* L.) is one of the most important vegetables worldwide, with a global production in 2009 of 35,326,379 t and 1,974,920 ha harvested, ranking sixth and seventh among vegetable crops in terms of production and area harvested, respectively (FAOSTAT 2010). With respect to androgenesis, positive results have been obtained in some genotypes through anther culture. The first report of plant regeneration from anther cultures dates from 1973 (Raina and Iyer 1973). Later studies reported the production of haploid or DH individuals (Chambonnet 1988; Dumas de Vaulx and Chambonnet 1982; Isouard et al. 1979; Misra et al. 1983). Nearly 40 years after the first report, eggplant anther cultures have been adopted by several laboratories and private companies, and at present, pure DH and dihaploid lines of some varieties and somatic hybrids have already been developed (Rotino 1996; Rotino et al. 2005; Salas et al. 2011) based principally on modified versions of the protocol published by (Dumas de Vaulx and Chambonnet 1982) for anther culture. Despite the wide adoption of eggplant anther cultures, this method presents a number of drawbacks that limit their practical usefulness. It does not prevent the occasional appearance of somatic calli and/or embryos from anther tissues, a limited efficiency restricted to just a few microspore-derived embryos (MDEs) per anther, or the uncontrollable secretory effect of the tapetal layer that surrounds the pollen sac, which prevents us from having a strict control of culture conditions (Seguí-Simarro et al. 2011). We also found recently that the thick anther walls of eggplant flowers appear to prevent media components, including growth factors, from accessing the anther locule, reducing their effect over inducible microspores (Salas

et al. 2012). Furthermore, anther culture precludes the study of the first stages of microspore embryogenesis, which takes place within the pollen sac, surrounded by anther walls. All of these limitations can be surpassed by the direct isolation and culture of microspores. In species like rapeseed, tobacco or barley, where isolated microspore cultures are well set up, it is possible to produce several hundreds of embryos from the microspores isolated from a single anther.

Despite the advantages this technique would allow, it is surprising that very few studies have dealt with isolated microspore cultures in eggplant. Among them, only two reported on the regeneration of entire plants, suggesting in both cases that microspores or pollen grains are induced to proliferate as callus masses (Gu 1979; Miyoshi 1996). The third study only documented the formation of microspore-derived, undetermined multicellular structures (Bal et al. 2009). None of them reported on the production of MDEs. Given the lack of studies on eggplant microspore cultures, and the relevance of such an improved biotechnology for DH production, in this work we explored the process of microspore reprogramming in eggplant microspore cultures through different experimental approaches. We studied the response of different cultivars and characterized the development of the induced, dividing microspore up to the final step of plant regeneration. We also studied the origin of the different structures formed during *in vitro* development, including the putative DH plants, with light microscopy, flow cytometry and molecular marker analysis. Then, we evaluated the effect of different osmotics in the promotion of microspore induction. Osmotic, non-metabolizable agents such as PEG or mannitol have been described as effective for improving embryogenesis induction (Ferrie and Keller 2007; Ilic-Grubor et al. 1998). Mannitol has also been reported as beneficial for microspore induction and embryo survival in several species, including cereals (Cistué et al. 1994; Huang et al. 1991; Kasha et al. 2001; Labbani et al. 2007) and solanaceae like pepper (Gémes Juhász et al. 2009), eggplant (Bal et al. 2009) or tobacco (Imamura and Harada 1980). However, no data on this aspect have been published for eggplant. Next, we evaluated the effect of eight different culture media in the regeneration of entire plants. Given the critical role that the genotype plays in all *in vitro* developmental processes, it would be desirable to have a genotype-independent way to test the effect of these different media compositions. For this, we developed a method to produce clonal microcalli from single microspore-derived callus. Together, our results constitute an efficient protocol for DH production from eggplant isolated microspores. In

addition, we provide significant insights into the details of androgenesis in this species, suggesting that embryogenesis and not callogenesis is induced, the occurrence of a blockage in embryogenesis at the globular stage that gives rise to callus, mostly mixoploid, and the regeneration of a majority of DH plants from these calli.

Materials and methods

Plant materials and culture conditions

We used as donor plants the following four eggplant genotypes: Bandera (an F1 hybrid from Seminis Vegetable Seeds Iberica, S.A., Spain), Ecavi (an F1 hybrid from Rijk Zwaan Ibérica S.A., Spain), Cristal (an F1 hybrid from Semillas Fitó S.A., Spain), and ANS-3 (a Valencian local variety from the germplasm collection of our Institute, COMAV). Different batches of plants were grown in 30 cm pots in greenhouses, at 18ºC under natural light during two consecutive years.

Microspore culture and plant regeneration

Flower buds at the appropriate stage of development (containing mostly vacuolate microspores and young bicellular pollen) were selected and immediately transported to the laminar flowhood on melting ice. Anthers were excised and surface sterilized by submerging them in 70% ethanol for 30 seconds and then in a 10% solution of commercial bleach (40 g/l of NaClO) for 5 minutes, prior to three washes with sterile water. Then, anthers were crushed under sterile distilled water. Their locular content was isolated by filtration through 40 µm nylon meshes (Millipore). Microspore suspensions were plated in 6-cm culture dishes and incubated at 35ºC for 3 days. After heat treatment, microspores were transferred to liquid NLN culture medium (Lichter 1982) supplemented with 2% sucrose, 0.5 mg/l naphthaleneacetic acid (NAA), and 0.5 mg/l 6-benzylaminopurine (BAP), pH 5.9, according to (Miyoshi 1996). Dishes were incubated in a Sanyo Growth Cabinet for a month at 25ºC in darkness. After a month of culture, induced calli exceeding 1 mm were isolated and individually transferred to dishes with solid MS medium (Murashige and Skoog 1962) supplemented with 4% Bacto-agar, 2% sucrose, 0.2

mg/l indoleacetic acid, and 4 mg/l zeatin. Dishes were incubated at 25ºC under a 16/8 h photoperiod (300 µE m^{-2} s^{-1}), subculturing every 15 days, until they regenerated full plantlets or apical shoots. Apical shoots were induced to root by transferring them to V3 medium (Dumas de Vaulx and Chambonnet 1982) supplemented with 0.8% Bacto-agar, 3% sucrose, 0.1 mg/l NAA, and 0.2 mg/l BAP. Rooted plantlets were individualized and grown *in vitro* in pots and when appropriate, transferred to the greenhouse and acclimated within plastic bags. These procedures were repeated eight times with different plant batches and during different seasons during a period of two consecutive years, with a minimum of 15 replicates each time. For each repetition, all replicates came from the same batch of microspores, isolated from buds of different individuals, and subjected to the same experimental conditions.

Study of the effect of osmotics

To study the effect of osmotics, sucrose, PEG 4000 (Fluka BioChemika) and mannitol (Duchefa Biochemie) were added to culture media after the 3-day heat treatment at different concentrations as explained in Results. For each experiment, a minimum of five replicates of each concentration were performed, with all replicates and concentrations under the same experimental conditions and coming from the same batch of isolated microspores. Each experiment was repeated a minimum of three times. In all cases, controls without osmotica were also done. Effects were evaluated in 1-month old cultures. The following parameters were measured per individual dish: number of total calli per bud plated, total callus weight per bud plated, and number of calli larger than 1 and 2 mm per bud plated, as an estimation of callus size. For each factor, data were compared by a one-way ANOVA test using the Statgraphics software.

Light microscopy

The first stages of microspore development were observed with DIC optics in a Nikon Eclipse E1000 light microscope and a Carl Zeiss inverted light microscope, directly in the culture dish. Microcalli were observed in bright field through the inverted microscope and a dissection microscope, both equipped with digital image capture systems. Samples of microspore-derived embryo-like globular structures as well as callus-like structures were fixed in Karnovsky fixative solution (4% formaldehyde + 5% glutaraldehyde in 0.025 M

cacodylate buffer, pH 7), dehydrated in ethanol series, embedded and polymerized in EM-bed 812 resin (Electron Microcopy Sciences). Thin (1 µm) sections were produced with a Leica UC6 ultramicrotome, mounted and observed under phase contrast in a Nikon Eclipse E1000 microscope.

Flow cytometry

Small pieces of young leaves from regenerants were analyzed using the CyStain UV Precise P kit (Partec). Young leaf samples from donor plants were used as standards for 2C DNA content. Samples remained on ice throughout the process. Leaf samples were cut into small pieces with a razor blade in 400 µl of nuclei extraction buffer (NEB).After 1 minute incubation, 1.6 ml of DAPI-based staining buffer was added and incubated for 5 minutes. The extracted nuclei preparation was filtered through 30 µm Cell Tricks filters (Partec) and analyzed in a Partec PA-I Ploidy Analyzer.

Genetic analysis with microsatellite molecular markers

Prior to their use to determine the origin of calli and regenerants, Bandera donor plants were screened using microsatellite markers (SSR). Young leaf tissue was sampled from 10 different, randomly chosen donor plants, and genomic DNA was isolated from 50 mg of tissue using the modified CTAB (hexadecyl trimethylammonium bromide) method as described in (Ferriol et al. 2003). Each donor plant was analyzed using the following 27 SSR markers known to be polymorphic in other eggplant materials (Nunome et al. 2003; Salas et al. 2011; Stagel et al. 2008; Vilanova et al. 2012): CSM1, CSM8, CSM13, CSM15, CSM18, CSM19, CSM23, CSM25, CSM26, CSM27, CSM36, CSM33, CSM40, CSM41, CSM42, CSM44, CSM57, CSM58, CSM59, CSM63, CSM65, CSM68, EEMS37, EEMS49, EM107, EM126, and EM145. The forward primers were labeled with different fluorescent dyes following the M13-tail method described by (Schuelke 2000) to facilitate the incorporation of a dye label during PCR amplification, which was performed as described in (Vilanova et al. 2012). Six loci were simultaneously detected using an ABI PRISM 310 Genetic Analyzer. Heterozygous loci were consistently found for seven SSR markers (CSM19, CSM33 *locus 1*, CSM36, CSM40, CSM58 and CSM63) in all Bandera donor plants. These markers were used as described above in microspore-derived calli and regenerants to verify their haploid origin.

Clonal callus propagation

In order to multiply single microspore-derived calli into clonal microcalli, four different mechanical methods were assessed. One month-old microspore-derived calli were isolated in individual flasks with liquid MS medium supplemented as described above, and submitted to (1) 2 days in constant agitation in a orbital shaker at 300 rpm; (2) 7 days in constant agitation in a orbital shaker at 100 rpm; (3) mechanical disaggregation by hand by crushing them with a cylindrical piston; and (4) three-minute agitation with a vortex at full speed. In order to determine which was the most effective method, the following parameters were measured: (1) microcallus viability, measured as the number of microcalli with a normal, whitish and friable appearance one month after disaggregation; (2) total average number of microcalli obtained per dish; (3) average number of microcalli larger than 1 mm obtained per dish; (4) average number of microcalli larger than 2 mm obtained per dish; and (5) average weight of microcalli obtained per dish. For each parameter, data from different methods were compared by a one-way ANOVA test using the Statgraphics software.

Plant regeneration from calli

We tested the effect on plant regeneration from microspore-derived calli of eight different media previously described in the literature as successful in regenerating plants from eggplant calli. Clonal calli were obtained as described above from different single microspore-derived calli. Clonal calli of each genotype were equally distributed in 9 mm, deep culture dishes with each of the different media at 25ºC in a growth cabinet with a 16/8 photoperiod. Culture media was refreshed every two weeks by subculturing calli into dishes with freshly-prepared medium. A total of 60 calli were used to test each medium. The eight media used, their main constituents and their bibliographic references are included in Table 1. Briefly, M1, M4, M5 and M6 are variants of the MS medium originally described by Murashige and Skoog (1962), supplemented with different combinations of growth factors as described in Table 1. M2 is a modification with activated charcoal of M3, which is the original R medium described by Dumas de Vaulx and Chambonnet (1982). M7 is based on the B5 medium original from Gamborg et al. (1968). M8 is the V3 medium described in Rotino (1996) and originally attributed to Chambonnet (1988). After six weeks in culture, callus viability was estimated

by calculating the following parameters: (1) percentage of growing calli, (2) percentage of shoot-producing calli, and (3) percentage of root-producing calli. For each parameter, data from different media were compared by a one-way ANOVA test using the Statgraphics software. Means were separated using a Tukey's test with $p \leq 0.05$.

Table 1: Different media used for plant regeneration from microspore-derived calli.

Medium	Macronutrients, micronutrients and vitamins	Carbon source	Gelifying agent	Growth regulators	Other compounds	Reference
M1	MS	2% sucrose	0,8% plant agar	0,2 mg/l IAA 4 mg/l zeatin	---	(Miyoshi 1996)
M2	R	3% sucrose	0,8% bacto agar	0,1 mg/l kinetin	1g activated charcoal	(Gémes Juhasz et al. 2006)
M3	R	3% sucrose	0,8% bacto agar	0,1 mg/l kinetin		(Dumas de Vaulx and Chambonnet 1982)
M4	MS	3% sucrose	0,4% Phytagel	0,1 mg/l IAA 2 mg/l zeatin		(Borgato et al. 2007)
M5	MS	3% sucrose	0,8% plant agar	4 mg/l IAA 2.56 mg/l kinetin	2 mg/l putrescine	(Misra et al. 1983)
M6	MS	3% sucrose	0,8% plant agar	0,2 mg/l 2,4-D	400 mg/l inositol	(Misra et al. 1983)
M7	B5	3% sucrose	0,8% plant agar	0.1 mg/l NAA 2 mg/l kinetin		(Rotino and Gleddie 1990)
M8	V3	3% sucrose	0,8% bacto agar	0.1 mg/l NAA 0.2 mg/l 6-BA		(Dumas de Vaulx and Chambonnet 1982)

Results

Identification of the most responsive eggplant genotype

To identify a good genotype to work with, we studied, as a preliminary step, the response to microspore culture of four eggplant accessions previously used by our group for anther culture (Salas et al. 2011). These included the two best performing genotypes (cvs. Bandera and Ecavi) and two with a low response (cvs. ANS-3 and Cristal). We determined the efficiency of

each cultivar defined as the number of proliferating structures produced per bud used. As seen in Figure 1, the highest efficiency was obtained with Bandera, followed by Ecavi. Thus, cv. Bandera was selected as a reference for further analyses.

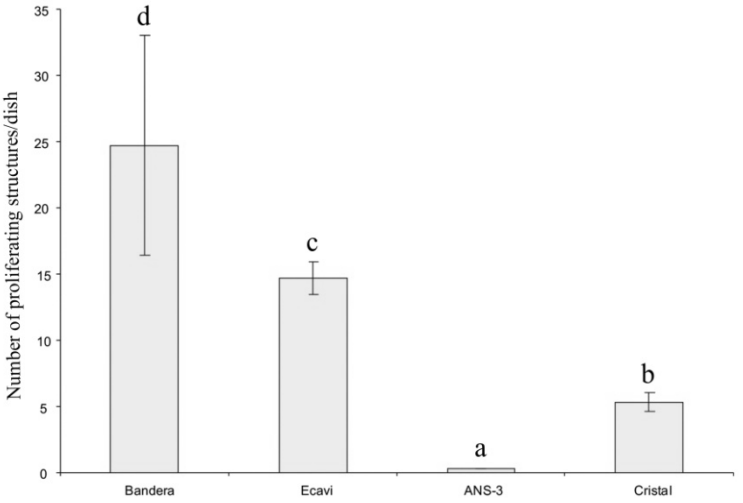

Figure 1: Comparison of the efficiency of androgenic response of four eggplant cultivars. Efficiency was measured as number of proliferating structures per dish. Different letters indicate statistically significant according to a Tukey's test with $p \leq 0.05$.

Analysis of microspore culture and plant regeneration

We monitored the entire process of plant production from isolated microspores through light microscopy (Figures 2, 3). This process was similar for the four genotypes used. For this reason, only images from a representative one (Bandera) will be shown. At the moment of microspore isolation and inoculation, liquid cultures consisted mainly of vacuolate microspores and young bicellular pollen (Figure 2A). Five days after application of the inductive treatment, the first indications of induction could be detected by the appearance of star-like microspores (arrowhead in Figure 2B) and of the subsequent first symmetric division in some microspores (arrow in Figure 2B), indicating the onset of the sporophytic route. Other microspores continued a gametophytic-like development, becoming mature pollen-like structures (Fig-

ure 2B'). Most of the microspores, however, did not show any morphological change, remaining similar to those initially plated (asterisk in Figure 2B). Most likely, they were dead or developmentally arrested. Within the first five days of culture, multicellular microspores composed of up to six cells could be identified (Figure 2C). Between days 15-20, these multicellular structures developed into globular embryo-like structures, clearly distinguishable by their round and compact appearance, and the presence of a differentiated protoderm-like layer (Figure 2D). Internally, these structures presented a distinct protodermal layer at the periphery (Figure 3A). At the central region, small, polygonal and compact cells were clearly different from the surrounding, parenchymatic, larger and vacuolated cells (Figure 3B). The frequency of these structures was variable among experiments, most likely due to seasonal effects on greenhouse-grown donor plants.

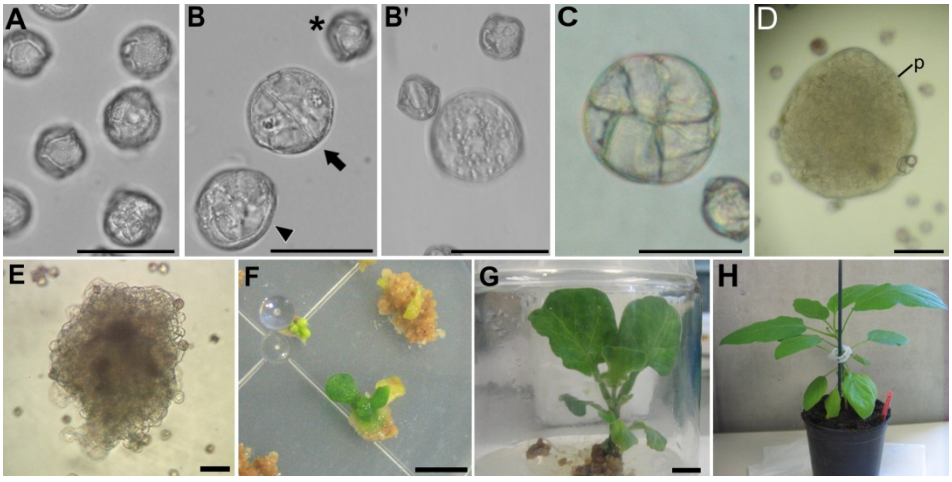

Figure 2: Androgenic development of eggplant microspores. A: Freshly isolated microspores. B: Induced microspores just before (arrowhead) and after the first embryogenic division (arrow), together with arrested or dead microspores (asterisk). B': Non-induced microspores that enter a gametophytic-like developmental pathway. Note the presence of abundant starch granules, mostly surrounding the two sperm-like cells of the central pollen-like structure. C: Six-celled multicellular microspore. D: Globular embryo with the protoderm (p) layer. E: Microscopic callus-like structure. F: Macroscopic calli with organogenic shoots at their surface. G: *In vitro* microspore-derived plantlet. H: Acclimated eggplant DH plant. Bars: A-E: 50 µm; F-G: 1 cm.

After day 20, the presence of globular embryo-like structures gradually declined. No embryos were observed to progress further in embryogenic

development. Instead, the presence of microscopic callus-like, irregular structures (Figure 2E) dramatically increased. When sectioned and observed under the light microscope, these structures presented a disorganized tissue architecture, markedly different from embryo-like structures. At the periphery (Figure 3C), no clear protoderm could be observed, and all cells appeared parenchymatic, round and with an enormous vacuole occupying most of the cell volume. The presence of air spaces between cells was also significant. Many cells presented large starch deposits, likely amyloplasts. Central regions (Figure 3D) presented similar cell types, with large vacuoles, air spaces and massive starch deposits. As in the periphery, this disorganized tissue architecture was notably different from embryo-like structures (compare Figures 3B and 3D).

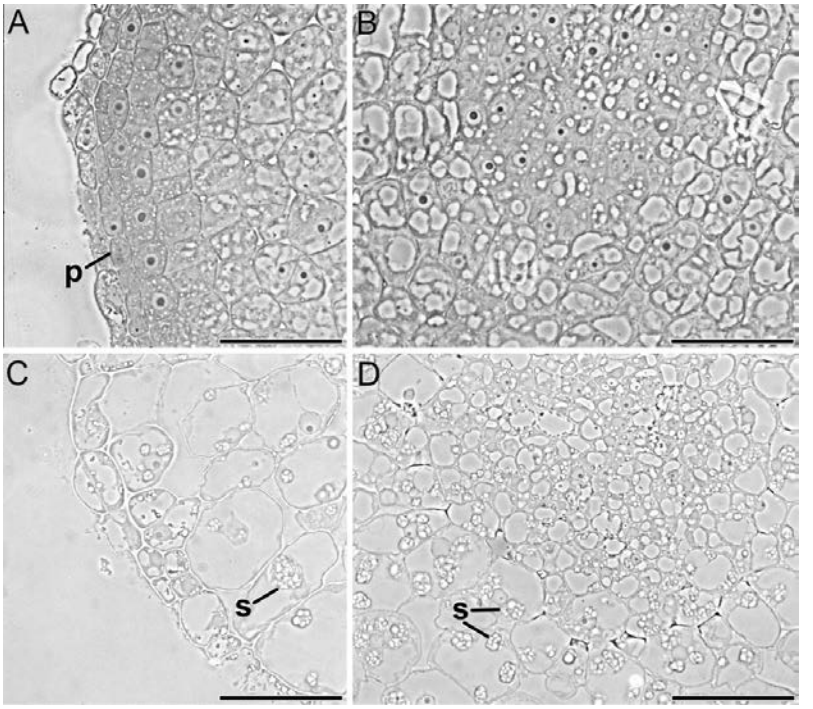

Figure 3: Internal architecture of MDEs and embryo-derived calli. A: peripheral region of a globular MDE showing the differentiation of an outer protoderm-like layer (p). B: central region of a MDE. C: peripheral region of an embryo-derived callus showing the absence of clear tissue differentiation, and the abundant presence of starch granules (s). D: central region of an embryo-derived callus. Bars: 100 µm.

After a month, all of the developing structures were calli. An average of 76.25 calli/dish were obtained in the eight different experiments performed. Since the number of buds used for each experiment equaled the number of dishes obtained, we could assume a rounded average of 75 calli/bud. One month-old calli with a diameter of 1 mm or greater were isolated and transferred to solid medium to stimulate their growth. After two weeks on solid medium, most calli increased in size, becoming now visible with the naked eye. Some of them became hard and non friable. They eventually necrosed and died. Other calli became pale green and friable. After a month on solid medium, the first buds were observed to arise from their surface (Figure 2F). Buds gave rise to shoots (Figure 2G) that rooted either spontaneously or after transfer to V3 medium, giving rise to entire plantlets. After acclimation, they became fully functional regenerated plants (Figure 2H), approximately 5 to 6 months after microspore *in vitro* culture. An average of 5 plants every 100 calli produced was calculated. In terms of buds, these numbers would imply 3.75 plants per bud used to isolate microspores. Two months later, regenerants entered the reproductive phase, producing fertile flowers. In general, 8 months from the onset of microspore culture were needed to obtain mature, flowering microspore-derived regenerants.

Analysis of the origin of calli and regenerated plants

In order to shed light into their origin, the ploidy levels of macroscopic calli and regenerants were analyzed by flow cytometry. From a total of 41 different calli analyzed (Table 2), no haploid calli were detected. Two calli (5% of the total) presented a 2C DNA content, equivalent to that of diploid donor plants, whereas 3 (7%) were tetraploid. However, most of the calli (36, 88% of the total) were mixoploid, presenting three different DNA contents: 1 presented a C+2C content, 8 presented a C+2C+4C content, and 27 presented a 2C+4C content. This situation clearly contrasted with that observed for regenerated plants. From 20 plants analyzed, coming from clonally different calli, 4 (20%) were haploid, 1 (5%) was tetraploid and 3 (15%) were mixoploid, all of them presenting a 2C+4C DNA content. The majority of plants (12, 60%) showed a diploid (2C) DNA content, being putative DH individuals.

Considering the nature of this *in vitro* technique, based on the isolation and culture of haploid microspores, the most reasonable origin for all of the calli and plants obtained are microspores. This origin was evident for all

haploid plants and calli, as well as for those mixoploid calli containing haploid cells. However, a possibility of somatic origin exists, at least theoretically, for diploid calli and plants. In order to exclude or confirm this possibility, we used molecular microsatellite markers (SSRs) on diploid regenerants. We used six SSR markers (CSM19, CSM33 *locus 1,* CSM36, CSM40, CSM58 and CSM63) previously characterized as heterozygous in Bandera individuals (Salas et al. 2011). We first tested them in 12 randomly chosen donor plants of our Bandera population. As expected, the six SSR loci appeared heterozygous for all 10 individuals. Then, we analyzed eight regenerants, including four randomly chosen diploids and four randomly chosen mixoploids. The 8 plants were homozygous for all 6 SSR markers. Thus, a gametophytic, initially haploid origin was confirmed for the plants obtained both euploids (DHs) and mixoploids.

Table 2: Ploidies of microspore-derived calli and callus-derived plants

	Calli	Plants
Haploids	--	20%
DHs	5%	60%
Mixoploids	88%	15%
n+2n	3%	--
n+2n+4n	23%	--
2n+4n	73%	100%
Tetraploids	7%	5%

Analysis of the effect of osmotic agents

Our next goal was to determine whether non-metabolizable osmotic agents could exert an effect over the number of calli produced. As non-metabolizable osmotics we used PEG and mannitol, and evaluated their effect after a month of culture either combined with sucrose (the usual carbon source) or in sucrose-free media (Figure 4). When 2% sucrose was replaced by

2% PEG, callus production was dramatically reduced with respect to controls, as reflected in all of the measured parameters. When 2% sucrose was replaced by 2% mannitol, no calli were produced. Addition of 1% PEG to 1% sucrose-containing medium had a strongly positive effect, yielding nearly 2.5 times more calli than with sucrose alone (Figure 4A). The positive effect was also reflected in total callus weight (Figure 4B) and size (Figures 4C, D). In contrast, addition of 1% mannitol to 1% sucrose-containing medium significantly reduced callus number, weight and size. Thus, increasing the initial osmotic pressure of sucrose by the addition of PEG significantly improved callus induction and proliferation, whereas mannitol had negative effects. Replacing sucrose by either PEG or mannitol had negative effects in all cases.

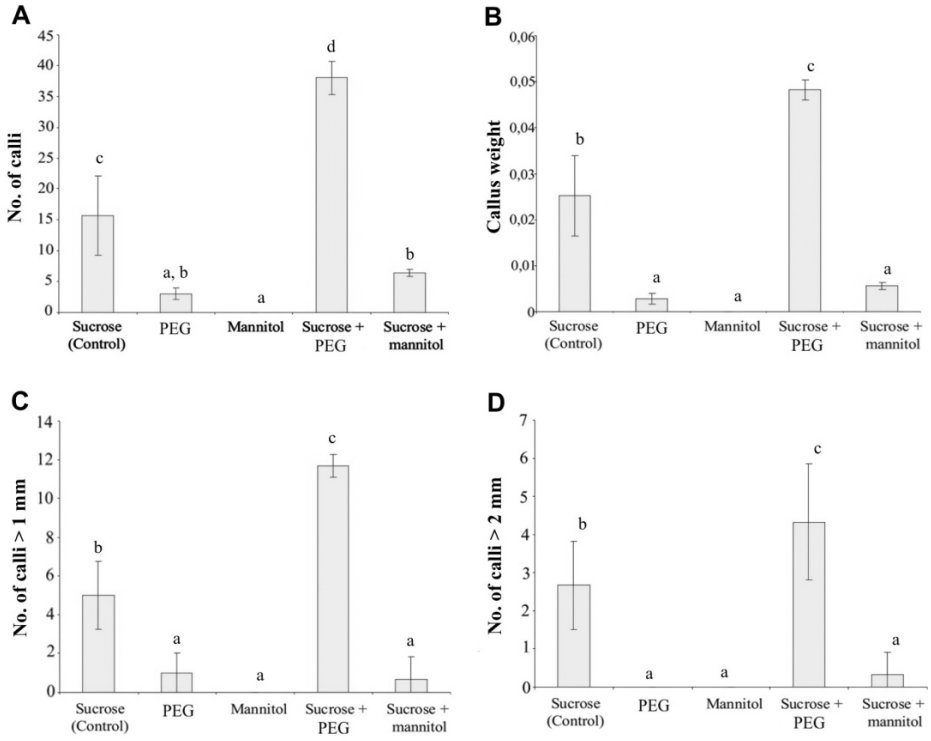

Figure 4: Effect of osmotic agents. Quantitative effects of the addition of sucrose (control), PEG, mannitol, and combinations of PEG+sucrose and mannitol+sucrose, expressed as total number of calli/dish (A), total callus weight (B), number of calli/dish larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences according to a Tukey's test with $p \leq 0.05$.

Clonal multiplication of microspore-derived calli

We tried four mechanical ways to disaggregate calli into microcalli. Results are represented in Figure 5. Among the four procedures, mechanical crushing provided the best results in terms of number, size and weight (Figures 5A-D), but viability of calli was the lowest (Figure 5E). In turn, vortexing for 3 minutes was, together with 2-day agitation, the less effective in terms of number, size and weight of the microcalli obtained (Figures 5A-D), but the best in terms of viability (around 80%; Figure 5E). Seven-day agitation yielded intermediate results for all measured parameters. In order to decide which was the most suitable method, we combined the results of viability for each method with those of the other four parameters. As seen in Figure 5F, by combining viability with the average of calli we found that vortexing provided the highest number of viable calli per dish, and was thus superior to any other method. By combining viability with the other three parameters, similar results were observed (data not shown). Thus, we concluded that 3-minute vortexing was the most effective method in order to disaggregate single microspore-derived calli into multiple clonal microcalli.

Evaluation of different plant regeneration media

In order to increase the rate of plant regeneration from our microspore-derived calli, we assessed eight culture media previously described as successful for plant regeneration from eggplant calli or embryos derived from microspores, anthers or protoplasts. Clonic calli obtained by vortexing single microcalli as described above were subjected to each different culture media. Two weeks after culturing, the first differences in callus growth and organogenesis induction could be observed (data not shown), but it was at week 5 when clear differences could be attributed to the different culture conditions (Figure 6). Callus growth was observed in all media tested, although great differences were evident among them. M1 and M4 were the most effective, with growth in more than 75% of the cultured calli. Shoot growth, however, was restricted to M1, M2, M4 and M5, being also M1 and M4 the most effective with percentages of around 50% of the cultured calli. With respect to rooting, all media but M6 promoted rooting, with discrete percentages never exceeding the 19% (the highest rooting rate) observed in M5.

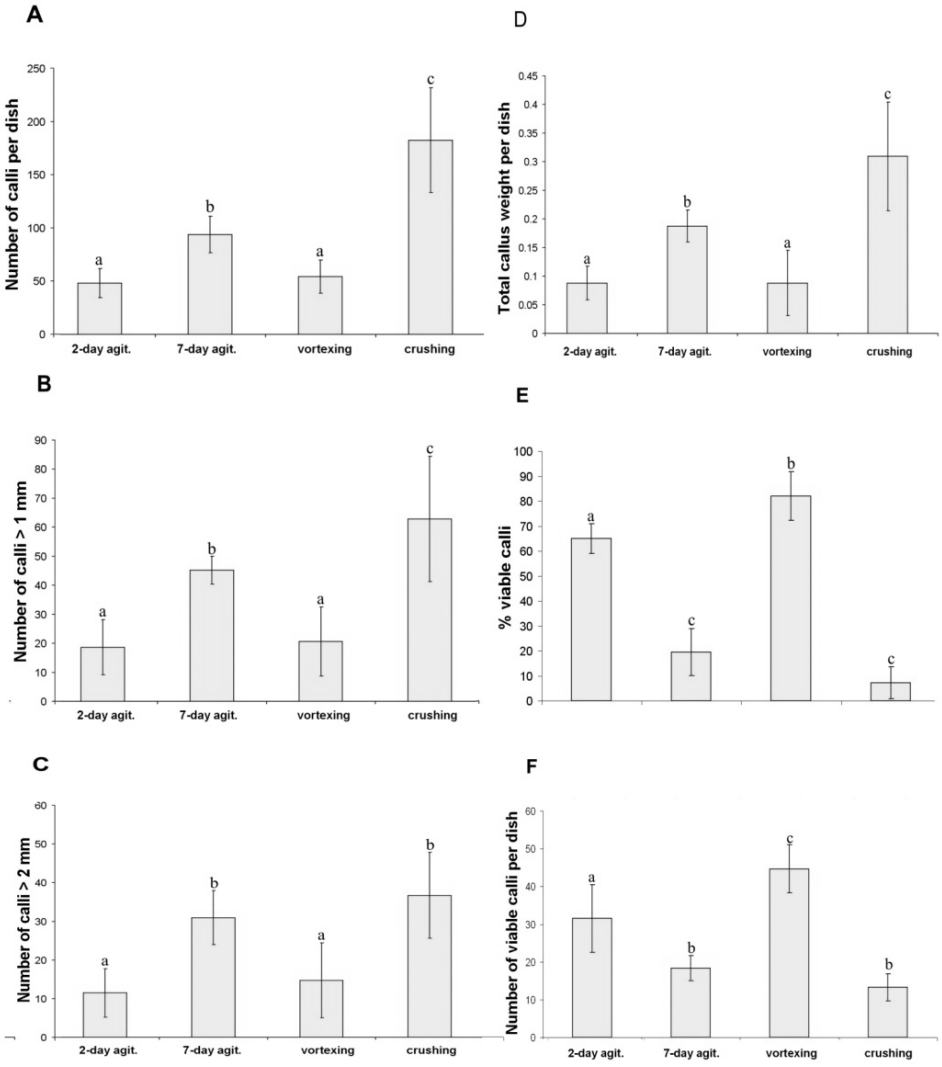

Figure 5: Methods for clonal multiplication of microspore-derived calli. Comparison of four methods to disaggregate single calli into microcalli, expressed as total number of microcalli/dish (A), number of microcalli/dish larger than 1 mm (B) and 2 mm (C), total microcallus weight (D) and number of viable microcalli/dish (E). Figure F represents a combination of number and viability data. Different letters indicate statistically significant differences (Tukey's test with $p \leq 0.05$).

According to their composition (Table 1), the best results in terms of callus growth and shoot production were obtained with media including IAA and zeatin as growth factors (M1 and M4), with a auxin/cytokinin ratio clearly below 1. In turn, the best medium for rooting was M5, characterized by a auxin/cytokinin ratio clearly higher than 1 and the addition of putrescine. Other factors like the type of inorganic salts and vitamins, the differences in sucrose concentration, the type of gelling agent or the addition of activated charcoal did not seem to provide any significant benefit. Interestingly, all the roots in M4 arose in shoot-producing calli, and all the shoots in M5 arose in rooted calli, whereas in M1 there were some rootless, shoot-producing calli and very few shootless, rooted calli. This meant that for M4, the percentage of rooting calli (6.9%) corresponded to entire, regenerating plantlets, the same as for M5 with the percentage of shoot-producing calli (3.45%). Considering all these data together, we concluded that the best medium was M4, followed by M5.

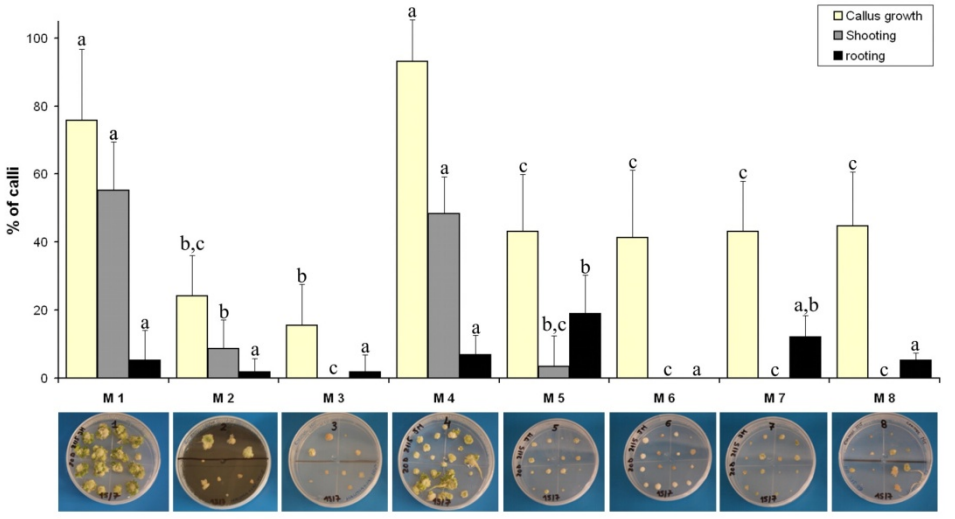

Figure 6: Media for plant regeneration. Comparison of eight regeneration media, expressed in terms of percentage of growing, shoot-producing calli and rooting calli in each medium. Images below each set of bars correspond to representative one-month old dishes containing calli cultured with the corresponding medium. Different letters indicate statistically significant differences (Tukey's test with $p \leq 0.05$) for each of the three parameters.

Discussion

In eggplant microspore culture, embryogenesis is induced and arrested later on

It is widely accepted that microspore-derived DHs are originated through the trigger of embryogenesis during the inductive phase. In some species, however, triggering of an alternative way to DHs is also possible. This alternative involves proliferation of microspore-derived cells to generate an undifferentiated, callus-like structure. DH plants are then obtained through organogenesis. Examples of species where DHs are obtained from callus include coffee, loquat, poplar, cereals such as rye, oat or wild barley relatives, and ornamentals such as lily, narcissus, coneflower, *Anemone*, *Dianthus* or chrysanthemum (reviewed in Seguí-Simarro 2010). In the particular case of eggplant, embryo-like structures have been consistently reported through anther culture (see Rotino 1996; Salas et al. 2011; Salas et al. 2012; Seguí-Simarro et al. 2011 as some examples). However, the only two previous studies demonstrating plant production through isolated microspore culture described callus formation as the exclusive response of cultured microspores (Gu 1979; Miyoshi 1996). It was believed that eggplant microspores could respond differently to different culture conditions and/or technical procedures. In this work we demonstrated that when induced, eggplant microspores are deviated towards an embryogenic pathway similar to that described for many other species (Dunwell 2010).

We showed that in all cases, the first stages of microspore development produce globular structures equivalent in external shape and internal architecture to those observed in model systems like rapeseed. In this species, a very refined temperature control may exceptionally lead to the reproduction of the whole embryogenic process, including the presence of suspensor-bearing embryos at 2, 4, 8-celled and dermatogen stages (Dubas et al. 2011; Supena et al. 2008). However, most of the induction and culture conditions used in rapeseed, as well as in many other species, lead to suspensor less globular masses where the first signs of embryo differentiation appear at the globular stage (Seguí-Simarro and Nuez 2008a), exactly as we observed in our cultures. As an additional proof for the embryonic origin of our callus, other experiments conducted in our group showed that under certain experimental conditions, it is possible for some embryos to progress through the

radial-to-bilateral transition, transforming into elongated torpedo-like embryos which can even develop radicle-like structures, although with abnormal shoot apices (Salas et al. 2012; Seguí-Simarro et al. 2011). Thus, we could conclude that in eggplant microspore cultures, callus proliferation is not an alternative to embryogenesis, but a consequence of embryo transformation into callus, likely due to culture conditions not well adjusted to embryo demands.

PEG addition promotes embryo induction and callus development

When sucrose was replaced by either PEG or mannitol, results did not improve those obtained with sucrose. However, by combining PEG with a sucrose concentration half of the usual, results were highly positive for callus growth in terms of number, weight and size. Thus, the first conclusion is that a carbon source (sucrose in our case) is essential during at least the first developmental stages. In microspore culture of other species it was shown that sucrose may be replaced by a non-metabolizable osmotic, and results were still satisfactory or even better than with sucrose (Ilic-Grubor et al. 1998). In eggplant, it appears evident that a minimum level of a conventional, easily convertible carbon source must be always available. The occurrence of induction and growth (although at a very low level) in PEG-only dishes could be explained by the presence of residual low molecular weight PEG which embryos might use as an alternative carbon source in the absence of sucrose (Ilic-Grubor et al. 1998). A second conclusion is that the additional osmotic pressure provided by PEG is beneficial for early eggplant embryo induction and further callus development, after the embryo-to-callus conversion. In our system, this effect is translated in a 2.5-fold increase in the number of calli produced. These results would be consistent with the previous knowledge on PEG effects on MDEs. It is interesting to note that the mannitol-mediated increase in osmotic pressure produced negative results. These negative results are not striking, since besides the positive effects, negative or null effects have also been attributed to mannitol (Gustafson et al. 1995; Tiainen 1992). A possible explanation for these inhibitory effects could be the presence of traces of substances inhibitory of embryo growth, as reported for other common components of *in vitro* culture media such as agar (Dunwell 2010; Kohlenbach and Wernicke 1978).

Generation of clonal microcalli is a convenient approach to assess environmental factors in a genotype-independent manner

It is widely known that the genotype is a crucial, if not the most important factor in nearly all *in vitro* developmental processes, including not only androgenesis induction but also organogenesis and somatic embryogenesis. Thus, when assessing the *in vitro* role of a given factor, it would be ideal to exclude such a variable from the experimental analysis by generating, for example, a clonal population of individuals. For this, one useful approach may be to disaggregate a single callus into multiple microcalli, and then regenerate *in vitro* plants from them. We explored this option to produce clonal calli, and concluded that by a three minute-vortexing up to 45 viable, growing calli can be produced from a single microspore-derived calli. Other more aggressive methods provided higher numbers of disaggregated microcalli, but at the expense of a reduced viability. It was evident that aggressive methods were more effective, but also more damaging for cells, thus reducing the number of useful calli.

This approach can be very helpful as a tool for the experimental evaluation not only of the effect of different regeneration media, but of any other environmental factor over *in vitro* populations. Individuals were calli in our case, but they could also be callus-derived haploid or DH clonal plant populations, if need be. Therefore, the method described hereby can be used as a convenient tool for this type of *in vitro* studies, and potentially even for any other type of *ex vitro* design needing eggplant haploid or DH clonal populations.

Plant regeneration with M4 and M5 would make microspore-derived calli an efficient source of eggplant DHs

In order to improve regeneration efficiency in our microspore-derived calli, we used clonal callus populations to assay eight previously used regeneration media in a genotype-independent manner. The principal difference between shoot-promoting and root-promoting media were their auxin/cytokinin ratio (Table 1), being below 1 for the former (M1 and M4) and above 1 for the latter (M5). The slight differences in efficiency between M1 and M4 could be attributable to the lower concentration of zeatin in M4. The best rates of regeneration of entire plantlets were obtained with M4 (6.9%),

followed by M5 (3.45%). According to this, we could think that in principle, the best conditions for plant regeneration would be those provided by M4. However, regeneration in M4 alone was slightly better than that obtained with the initial method (5%), which would mean little improvement with respect to the starting point. In contrast, a sequential use of M4 and M5 would make an improvement up to three-times higher. The rationale is as follows. For every 100 calli in M4, 6.9 developed into plantlets with shoot and root, and 41.4 developed only shoots. By transferring the 41.4 shoot-producing calli to M5, 19% of them would develop roots, which would make 7.9 additional plantlets, for a total of 14.8 plantlets every 100 microspore-derived calli. These calculations suggest that the best conditions for plant regeneration through organogenesis from eggplant microspore-derived calli would be the use of M4 for callus growth, shoot production and initial rooting, and then culturing shoot-producing, rootless calli in M5 for additional rooting. This way, the number of entire plantlets might be increased up to three fold.

An efficient protocol to obtain DHs through isolated microspore culture

We present an improved method to obtain eggplant DHs from isolated microspore cultures, which conceivably presents all of the advantages of microspore culture over anther culture: increased efficiency (compare results presented hereby for Bandera with those reported in Salas et al. 2011, 2012), absence of somatic tissues, absolute control over media composition and the possibility for direct isolation of microspores at their right developmental stage. Once induced, microspores develop as embryos up to the globular stage, when they transform into callus. Most calli are mixoploid, with variable ploidy types ranging from haploid to tetraploid. Nevertheless, mixoploidy does not appear to be a significant problem, since up to 60% of the plants regenerated from them were DH, as confirmed by molecular marker analysis. It seems that plant regeneration is favored from the diploid regions of mixoploid calli, as also described for tomato anther-derived mixoploid calli (Corral-Martínez et al. 2011). This percentage of 60% direct DH plants, obtained with no additional treatments for genome doubling, is high compared to other species (Seguí-Simarro and Nuez 2008b) and may be sufficient for some breeding purposes. Anyway, additional treatments (colchicine for example) may be applied to make this percentage even higher. Indeed, previous experiments in our group applying lanolin paste with 0.5% colchicine to axillary buds of plantlets

derived from anther cultures yielded an additional 25% of DHs (our unpublished results).

Important differences in terms of embryo induction were observed among the four genotypes tested. This is not surprising, since it is widely known that even within a species, the genotype is the most important factor for a cultivar, population or individual to respond (Dunwell 2010; Seguí-Simarro and Nuez 2008a). Although only one (Bandera) was used for further improvement of culture conditions, according to our previous experience in eggplant anther cultures (Salas et al. 2011) it is likely that many other eggplant cultivars will increase their response with the improved protocol for microspore culture. Furthermore, specific adaptations for each particular genotype would further improve the protocol.

Usually, microspore cultures are defined by microspore embryogenesis and embryo germination, which implies the production of one embryo per induced microspore. In the process described hereby, different shoots may arise at the surface of a callus derived from a single microspore, being potentially regenerable through organogenesis into several entire plants. Since the goal of DH production for plant breeding is to obtain a DH population of genetically different individuals, only one plant should be regenerated from a single callus, discarding the rest of shoots. This should not be a problem, provided that a sufficient number of independent calli is obtained. In the method presented hereby, we start from 75 embryos (transformed into calli) per bud. By replacing 2% sucrose by 1% sucrose + 1% PEG, an additional 2.5x increase can be achieved, for a total of 187.5 calli/bud. This number is considerably higher compared to anther cultures. As a reference, anthers of this same cultivar (Bandera) yielded a maximum of 3.75 embryos/bud when cultured at the optimal stage (Salas et al. 2012). For plant regeneration, the proposed combination of M4 and M5 would provide 14.8 plantlets/100 calli. In terms of buds, this would be translated into 27.8 plantlets/bud. Compared to anther cultures, the increase in efficiency is striking, since only 0.17 plants/bud could be obtained from Bandera through anther culture (Salas et al. 2011).

In conclusion, the process presented hereby constitutes by itself an easy and efficient way to obtain eggplant microspore-derived DHs through organogenesis. It is evident that it would be desirable to obtain them through direct embryogenesis, as it occurs for many other species. Indeed, our current efforts are focused on the design of proper conditions to allow for the embryo to progress beyond the transitional stage, while keeping such high induction

efficiency. Nevertheless, the biotechnological approach to doubled haploidy presented hereby is efficiently enough to be potentially applicable for breeding purposes.

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Capítulo 3

Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins

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Enviado a *Euphytica*.

Abstract

Microspore embryogenesis is an inducible experimental pathway interesting from basic and applied perspectives. For plant breeding, it is a powerful tool to produce doubled haploids, useful as pure lines. The most efficient way to produce them is through isolated microspore culture. In eggplant, one of the most important vegetable crops, this method is still poorly explored. So far, it is possible to produce doubled haploids, but not directly from embryos, because they transform into calli early during their development. In this work we evaluated the effect of abscisic acid, epibrassinolide, polyethylene glycol, and arabinogalactans and arabinogalactan proteins, previously described as promoters of embryo induction and development in other species. When added individually to the standard protocol, all of them significantly increased induction of microspore embryogenesis and callus cell proliferation, producing more and larger calli. Particular combinations of them further improved the efficiency of the method. In particular, gum arabic containing arabinogalactans and arabinogalactan proteins allowed embryos to progress beyond the globular stage, constituting a significant improvement in order to achieve the desired direct induction of viable, germinating embryos. We also evaluated the effect of altering the concentration and relative ratio of naphthaleneacetic acid and 6-benzylaminopurine, used in the standard protocol. Significantly better results were obtained by reducing their concentration. Together, our results shed light on the morphogenic and regulatory roles of these substances on microspore embryogenesis, opening ways to further increase the efficiency of production of androgenic doubled haploids through microspore culture in eggplant.

Introduction

The deviation of the microspore towards embryogenesis is a powerful biotechnological tool to produce doubled haploids (DHs) for breeding purposes. Androgenic DH technology represents a shortcut to classical breeding for the generation of pure lines, essential for hybrid seed production (Dunwell 2010; Seguí-Simarro 2010; Germanà 2011). This technology consists on the *in vitro* deviation of microspores from their original gametophytic fate to develop a haploid or DH plant. To be triggered, this biotechnological pathway re-

quires a source of abiotic stress (heat, cold, starvation, osmotica, etc; Shariatpanahi et al. 2006) to be applied to the micropores. After this initial inductive stage, microspores transform into embryos. Technically, this can be achieved by directly culturing anthers (anther culture), or through the isolation of microspores from the anthers and subsequent culture in liquid medium (isolated microspore culture). Microspores usually develop into microspore-derived embryos (MDEs) through haploid embryogenesis, but in some species they tend to proliferate as calli. From them, DH plants can be obtained through organogenesis. Examples of MDE-producing species include well-known model systems such as rapeseed, barley, tobacco or maize, among many others (Maluszynski et al. 2003). Examples of species where DHs have been obtained from callus include coffee, loquat, poplar, cereals such as rye, oat or wild barley relatives, ornamentals such as lily, narcissus, coneflower, *Anemone*, *Dianthus* or chrysanthemum (reviewed in Seguí-Simarro 2010), and recalcitrant solanaceae as tomato or eggplant (Seguí-Simarro et al. 2011).

Eggplant (*Solanum melongena* L.) is one of the most important vegetables worldwide. In eggplant, embryos can be successfully induced from microspores through anther culture (Salas et al. 2011; Salas et al. 2012). However, previous research has evidenced several limitations of this practical approach, including the occasional appearance of somatic embryos from anther tissues, the uncontrollable secretory effect of the tapetum, which precludes from a strict control of culture conditions, and an efficiency limited to only a few embryos per anther cultivated. All of these limitations can be overcome by the direct isolation and culture of microspores. However, the number of studies published on the successful production of DH plants from isolated microspores is still very limited. Apart from the pioneering studies of Gu (1979) and the work of Miyoshi (1996), only a very recent paper dealt with this topic (Corral-Martínez and Seguí-Simarro 2012). These three studies demonstrated that DH plants can be obtained through organogenesis from the calli formed upon culturing eggplant microspores in liquid medium. However, the latter study also revealed that actually, microspores are not directly transformed into calli. A detailed study of the process of microspore proliferation showed that immediately after induction, eggplant microspores enter an initial stage of embryogenesis that arrests at the globular embryo stage. Instead of experiencing the radial-to-bilateral symmetry transition typical of zygotic embryos, eggplant MDEs enter a proliferative, undifferentiated development transforming into callus-like structures (Corral-Martínez and Seguí-

Simarro 2012). Haploid and DH plants can be regenerated from these organogenic calli.

As deduced from this study, eggplant microspore cultures were characterized by a blockage of embryogenesis and by a good but still improvable efficiency. Thus our next efforts were devoted to identify factors that could help to overcome this arrest, as well as to improve the efficiency of embryogenesis induction. For this goal, we evaluated in this work the effect of different substances on the initial stages of eggplant microspore embryogenesis, i.e. the promotion of microspore induction, embryo development and transformation into callus. These substances included abscisic acid (ABA), epibrassinolide, polyethylene glycol (PEG), and gum arabic. These compounds have been previously described in other species as promoters of microspore induction to embryogenesis, and as regulators of MDE development. For example, a number of reports have clearly shown that ABA plays a role during both zygotic and microspore-derived embryogenesis in several species including *Brassica napus* (Hays et al. 2001), barley (van Bergen et al. 1999), and tobacco (Imamura and Harada 1980; Kyo and Harada 1986). In addition to this role, it was also proposed that early ABA accumulation would be necessary for embryo progression, and principally for a correct establishment of the shoot apical meristem (Ramesar-Fortner and Yeung 2006). Thus, it was suggested that soon after induction, MDEs should be permanently exposed to exogenous ABA in order to avoid morphological disorders. Such a relevant morphogenic role in early (globular) embryos was also attributed to ABA in other *in vitro* processes such as somatic embryogenesis in *Daucus carota* (Nickle and Yeung 1994). In addition to its role in growth and developmental processes, ABA has been proposed to have a protective effect against different types of stress, including oxidative (Xiong et al. 2006), osmotic (Zhu 2002; Ozfidan et al. 2012) and heat stress (Larkindale et al. 2005).

Brassinosteroids are a group of growth factors widely present in plant organs, but mostly in reproductive tissues such as pollen or immature seeds (Moore 1989). Even at very low concentrations, brassinosteroids promote cell growth, differentiation and elongation (Brosa 1999) as well as adaptation to abiotic and biotic environmental stresses including heat, cold, drought and salinity (Divi and Krishna 2009). Of the nearly 60 different types of brassinosteroids identified at present, brassinolide and 24-epibrassinolide (EBr) are the most active when applied exogenously (Fujioka and Yokota 2003). In fact, this exogenous application has yielded very positive results in terms of in-

creased frequency of induction of somatic embryogenesis in various species of conifers and rice (Pullman et al. 2003), as well as in coconut (Azpeitia et al. 2003). Enhanced induction was also described for microspore embryogenesis in several *B. napus* and *B. juncea* cultivars (Ferrie et al. 2005), including highly recalcitrant genotypes, in a stable and inheritable way (Malik et al. 2008). Also in rapeseed, the use of brassinosteroids has been associated to the acquisition of a proper shoot apical meristem identity and its further development and function, through the control over the redox potential and the expression of genes involved in shoot meristem development and maintenance, such as *SHOOTMERISTEMLESS*, *CLAVATA1* and *ZWILLE* (Belmonte et al. 2010).

Osmotic, non-metabolizable agents such as PEG or mannitol have been described as effective for improving embryogenesis induction (Ilic-Grubor et al. 1998; Shariatpanahi et al. 2006; Ferrie and Keller 2007), due to the additional stress provided by the change in osmotic potential. However, their effect may not always be positive, causing excessive detrimental effects. A good example can be found in eggplant microspore cultures, where it was shown that addition of 1% PEG increased the frequency of embryogenesis induction, but mannitol dramatically reduced it (Corral-Martínez and Seguí-Simarro 2012). Arabinogalactan proteins (AGPs) are extracellular matrix glycoproteins, highly glycosylated with arabinose and galactose (arabinogalactan) residues. AGPs and arabinogalactans have been related to different aspects of plant growth and development, including embryogenesis and cell proliferation (Cheung and Wu 1999; Majewska-Sawka and Nothnagel 2000; Hernández-Sánchez et al. 2009). In the context of microspore embryogenesis, a decisive role during the inductive phase, as well as for MDE development, has been attributed for them in wheat (Letarte et al. 2006), maize (Paire et al. 2003) or rapeseed, where they have been specifically involved in the formation of early embryogenic patterns, or in the subsequent bipolar growth along the apical-basal axis of the embryo (Tang et al. 2006).

Based on these evidences, these substances were postulated as candidates to improve the efficiency of microspore culture in eggplant as well as in other species where they have not been tested so far (Seguí-Simarro et al. 2011). Thus, in this work we tested the effect of adding them, either alone or combined, to the standard protocol for eggplant microspore culture previously published by us (Corral-Martínez and Seguí-Simarro 2012). In addition, we evaluated the effect of altering the concentration and relative ratio of the auxin and cytokinin used in the mentioned standard protocol, naphtha-

leneacetic acid (NAA) and 6-benzylaminopurine (BAP), respectively. Our results demonstrate that when added individually, all of the substances tested are beneficial for embryogenesis induction and/or further callus growth. When combined, some of the combinations provide additional increases of the amount induced microspores, thus increasing the efficiency of this method. In particular, one of these substances (gum arabic containing arabinogalactans and AGPs) was capable of overcoming the embryo arrest, allowing for the progression of embryos beyond the globular stage. These results have the potential to be applied for the efficient production of eggplant DHs through microspore culture. In addition, they provide new insights on the morphogenic and regulatory roles of these substances.

Materials and methods

Plant materials and culture conditions

As donors of microspores, we used Bandera plants (a F1 hybrid from Seminis Vegetable Seeds Iberica, S.A., Spain). Plants were grown in 30 cm pots at COMAV greenhouses (Universitat Politècnica de València), at 18ºC under natural light at autumn, winter, and spring months during two consecutive years.

Microspore culture and plant regeneration

Flower buds at the appropriate stage of development (containing anthers with a majority of vacuolate microspores and young bicellular pollen) were selected according to Salas et al. (2012), immediately transported to the laminar flowhood under melting ice, and processed as previously described (Corral-Martínez and Seguí-Simarro 2012). Basically, anthers were excised, surface sterilized with diluted commercial bleach and crushed under sterile distilled water. The locular content was filtrated, spun down and washed thrice. Microspore pellets were suspended in sterile distilled water, plated in 6-cm petri dishes and incubated at 35ºC for 3 days. Then, microspores were transferred to liquid NLN culture medium supplemented with 2% sucrose, 0.5 mg/L NAA, and 0.5 mg/L BAP, pH 5.9. Dishes were incubated at 25ºC in darkness during one month, after which they were analyzed as explained below.

For the analysis of the percentage of organogenic calli, one-month old induced microcalli exceeding 1 mm were isolated and individually transferred to dishes (10 calli per dish) with solid MS medium supplemented with 0.4% phytagel, 3% sucrose, 0.1 mg/L indoleacetic acid, and 2 mg/L zeatin. Dishes were incubated at 25ºC under a 16/8 h photoperiod, subculturing every 15 days for a total of 1.5 months (2.5 months from microspore isolation), after which calli were analyzed as described below.

Study of the effect of BAP, NAA, ABA, EBr, PEG, and gum arabic

For the experiments of BAP and NAA concentration and ratio, the concentrations of BAP and NAA mentioned above were modified as described in Results. For the rest of the factors studied, experiments consisted on the addition, either individually or combined, of ABA (Duchefa Biochemie), EBr (OlChemIm Ltd), PEG 4000 (Fluka BioChemika), and gum arabic (Fluka BioChemika) at different concentrations as explained in Results. Gum arabic is a mixture of arabinogalactans and AGPs traditionally obtained from exudates of *Acacia* trees. For this factor, additional experiments adding 20μM ß-D-glucosyl Yariv reagent (ßGlcY) were performed. ßGlcY is a synthetic phenylglycoside that specifically binds to AGPs (Yariv et al. 1967), preventing their roles during embryogenesis in a concentration-dependent manner (Tang et al. 2006). For all experiments, a minimum of five replicas of each concentration were performed, being all replicas and concentrations performed under the same experimental conditions and at the same time, all of them coming from the same batch of isolated microspores. Each experiment was repeated a minimum of three times (sessions). In all cases, controls excluding the factor tested were included. Effects were evaluated in 1-month old cultures, unless otherwise specified in Results. For quantitative studies, the following parameters were measured per individual dish: number of total calli per bud plated (approximately one bud per dish), total callus fresh weight per bud plated, and number of calli larger than 1 and 2 mm per bud plated, as an estimation of callus size. Numbers of calli were measured by hand counting under a dissecting microscope. Callus fresh weight was measured by isolating them from the culture medium and weighing in a precision weighing scale. For the experiments of combined factors, callus weight was not measured since they were needed to evaluate the percentage of organogenic calli 1.5 months after transfer to solid medium. This percentage was calculated by counting the number of calli developing organogenic buds or shoots, divided by the total of

calli per dish (10). To analyze all factors studied, a General Linear Model (GLM) of a commercially available statistics package (Statgraphics Plus, Version 4.1, STSC Inc., Rockville, MD, USA) was performed for each experiment, including as fixed effects the treatment and the session factors. Statistical significance was indicated by a P value < 0.05.

Results

Analysis of the effect of the exogenous addition of ABA

We studied the effect of adding ABA to the induction and culture medium at different concentrations, collecting data one month after application. As a preliminary trial, we first applied a wide range of ABA concentrations, including 2.10^{-6}M, 5.10^{-6}M, 10.10^{-6}M and 15.10^{-6}M (data not shown). From them, only 2.10^{-6}M produced acceptable results, with higher numbers of calli larger than 1 and 2 mm, but with no differences in terms of number of calli. Higher concentrations consistently produced remarkably less calli than controls. According to this, we decided to perform a second test with a lower range of ABA concentrations, ranging from $0.5.10^{-6}$M to 2.10^{-6}M (Figure 1). Among them, the best concentration was $0.5.10^{-6}$M, not only in terms of callus number (Figure 1A), but also of callus weight (Figure 1A') and size (Figures 1B, B').

Microscopic observation of control dishes with no ABA revealed irregular, disorganized and highly proliferative callus-like structures (Figure 1C). The use of $0.5.10^{-6}$M ABA (Figure 1D) gave rise to similar structures, although slightly smaller and more rounded. 1.10^{-6}M (Figure 1E) and 2.10^{-6}M ABA (Figure 1F) generated round, globular embryo-like structures instead of calli. However, 5.10^{-6}M (Figure 1G) and higher concentrations generated again irregular, callus-like structures, but significantly smaller and less numerous that in controls, as also revealed in quantitative analyses (data not shown). Thus, application of ABA at concentrations ranging from 1.10^{-6}M to 2.10^{-6}M appeared to have a positive effect preventing embryo disorganization into callus. However, all of the structures observed after 30 days of culture were calli. No embryo progression beyond the globular stage was observed at any ABA concentration. Together, the quantitative and qualitative results obtained from ABA application showed that $0.5.10^{-6}$M ABA was the most beneficial concentration

in terms of callus number, size and weight. However, it was unable to promote embryo progression.

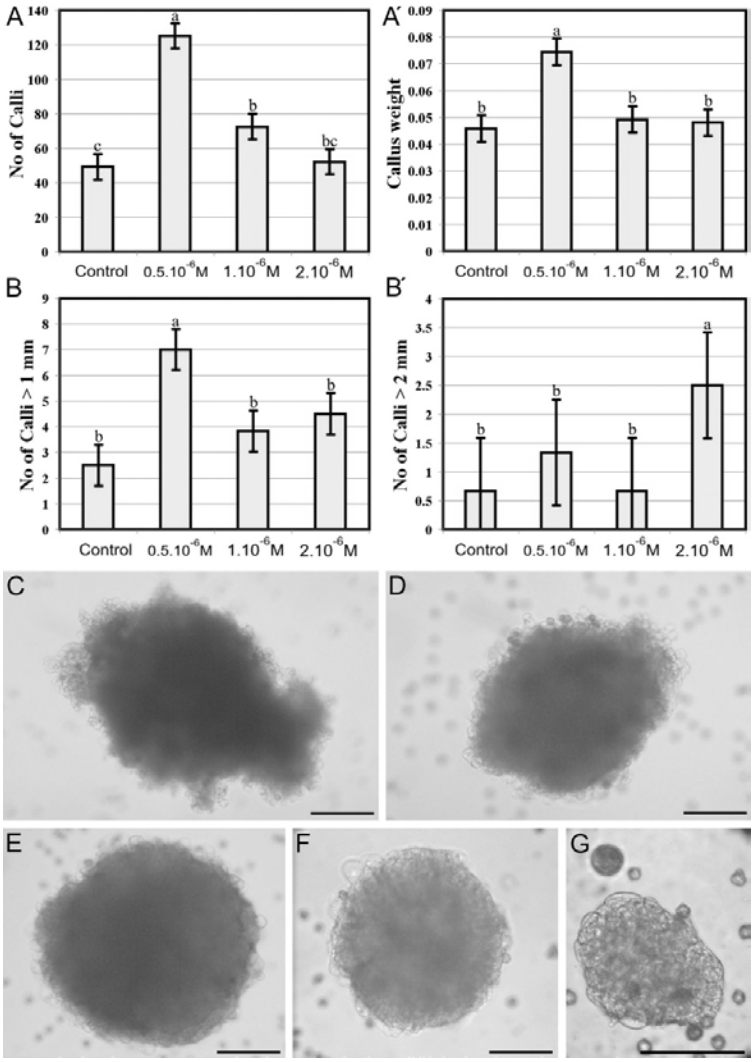


Figure 1: Effects of the addition of ABA. A-B": Quantitative effects expressed as total number of calli/bud (A), total callus weight/bud (A'), and number of calli/bud larger than 1 mm (B), and 2 mm (B'). Different letters indicate statistically significant differences ($p<0.05$). C-G: Callus-like structures after 15 days in culture with different ABA concentrations. A: Control without ABA. B: $0.5.10^{-6}$M. C: 1.10^{-6}M. D: 2.10^{-6}M. E: 5.10^{-6}M. Bars: 200 µm.

Analysis of the effect of the exogenous addition of EBr

In parallel, we studied the effect of the application of EBr at different concentrations from 10^{-9} M to 10^{-5} M. After one month of culture, the microspore-derived structures presented important qualitative differences. As seen in Online Resource 1, increasing concentrations of EBr had negative effects in the total amount of mass produced per plate, and also in the morphology of calli. Lower EBr concentrations produced calli with a disorganized architecture similar to those observed in control dishes, and higher EBr concentrations produced more compact, rounded and opaque calli. However, we could not identify any structure similar to late globular embryos at any of the concentrations used. Contrasting with these results, a quantitative analysis (Figure 2) revealed that EBr concentrations ranging from 10^{-9}M to 10^{-8}M had a positive effect in the number (Figure 2A) and weight (Figure 2B) of the calli produced, being 10^{-8}M the best to increase the number of callus produced. Higher concentrations had null or negative effects. With respect to callus size (Figures 2C, D), application of EBr at any concentration was clearly detrimental.

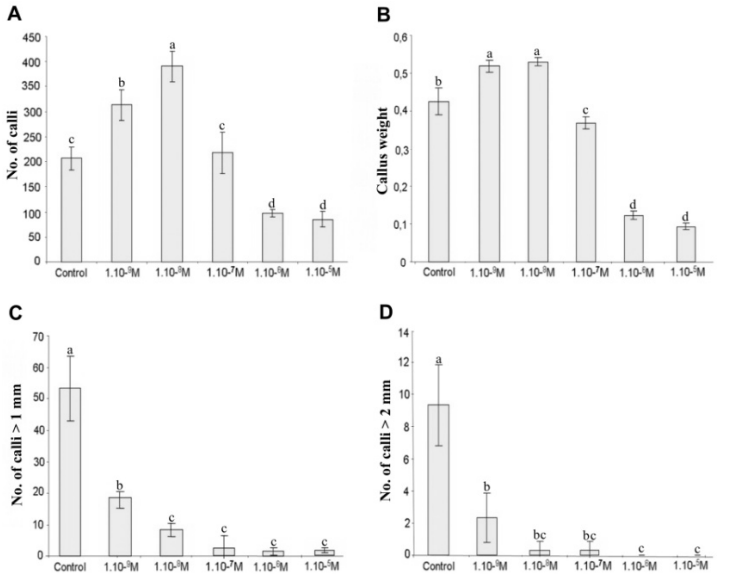


Figure 2: Quantitative effects of the addition of EBr, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences ($p<0.05$).

In summary, the application of EBr at a concentration of 10^{-8}M increased the number of microspore-derived structures, whereas higher concentrations reduced this number but favored the appearance of smaller and more compact structures (Figure 3).

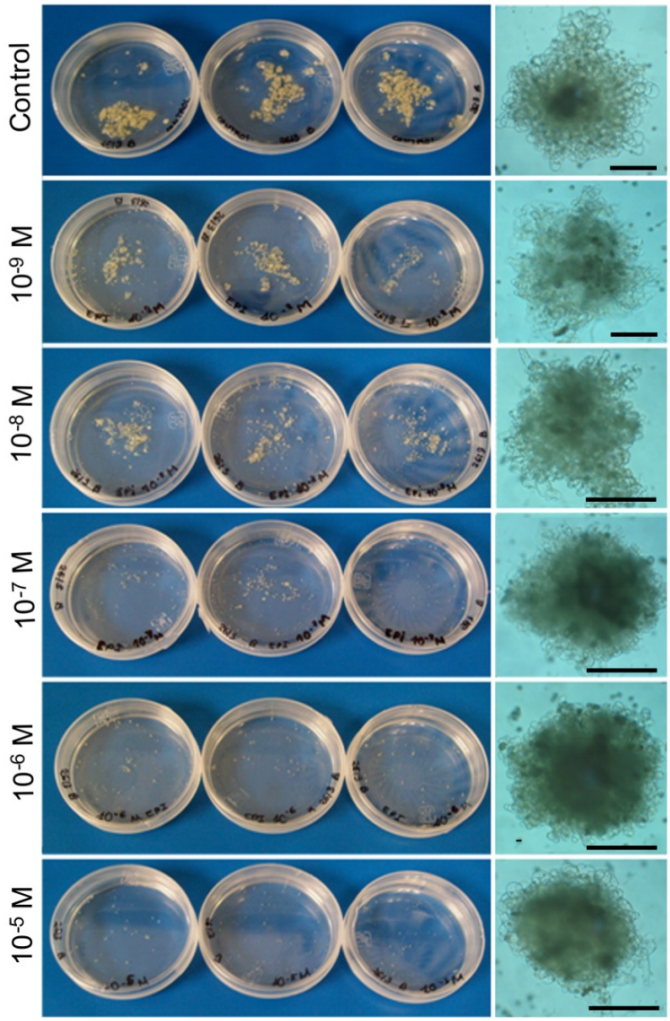


Figure3: Callus-like structures after 30 days in culture with different EBr concentrations. Left column shows three replicas of each concentration, where differences in callus number and size can be observed. The B column shows microscopic images of the calli produced at each concentration. Bars: 200 µm.

Analysis of the effect of the exogenous addition of gum arabic

Our third set of experiments aimed to the evaluation of the qualitative and quantitative effects of gum arabic (a source of mixed arabinogalactans and AGPs), applied to the cultures at different concentrations. In a first experiment, we tested concentrations ranging from 1 to 100 mg/L. No effect in callus morphology was observed after a month of culture at any of the concentrations of this range (data not shown). However, a positive correlation was found between concentration and callus number, size and weight (Figure 4), being 100 mg/L the best of the range. In order to find the optimal concentration of gum arabic, we designed a second experiment using a higher range (100 to 400 mg/L). As with the first range, we observed no changes in callus morphology. However, progressive increases in callus number and total weight were found (Figures 5A, B). Changes in callus size were less consistent, with no clear ascending or descending trends (Figures 5C, D). Then, we tested a third interval of gum arabic concentrations, ranging from 400 to 1600 mg/L.

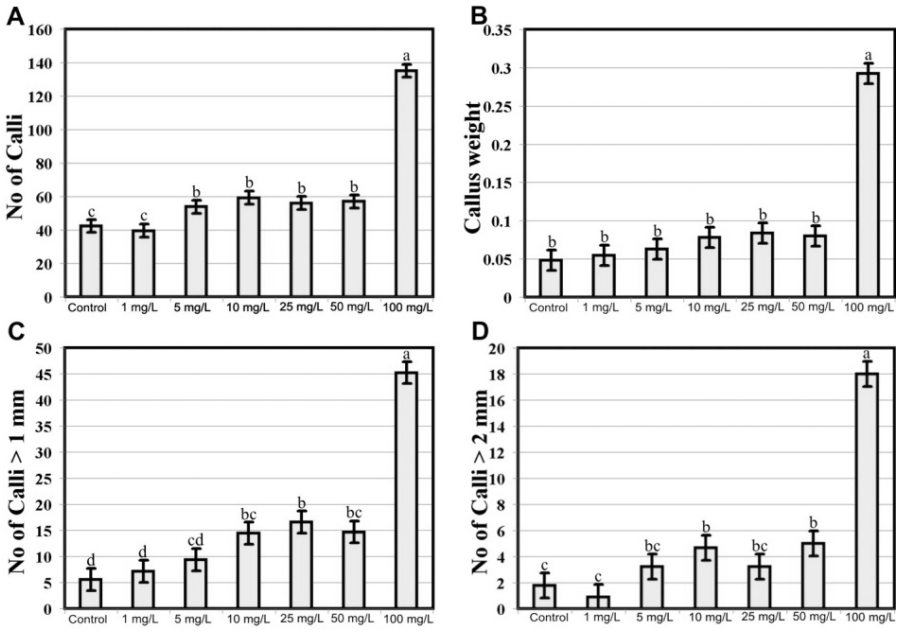
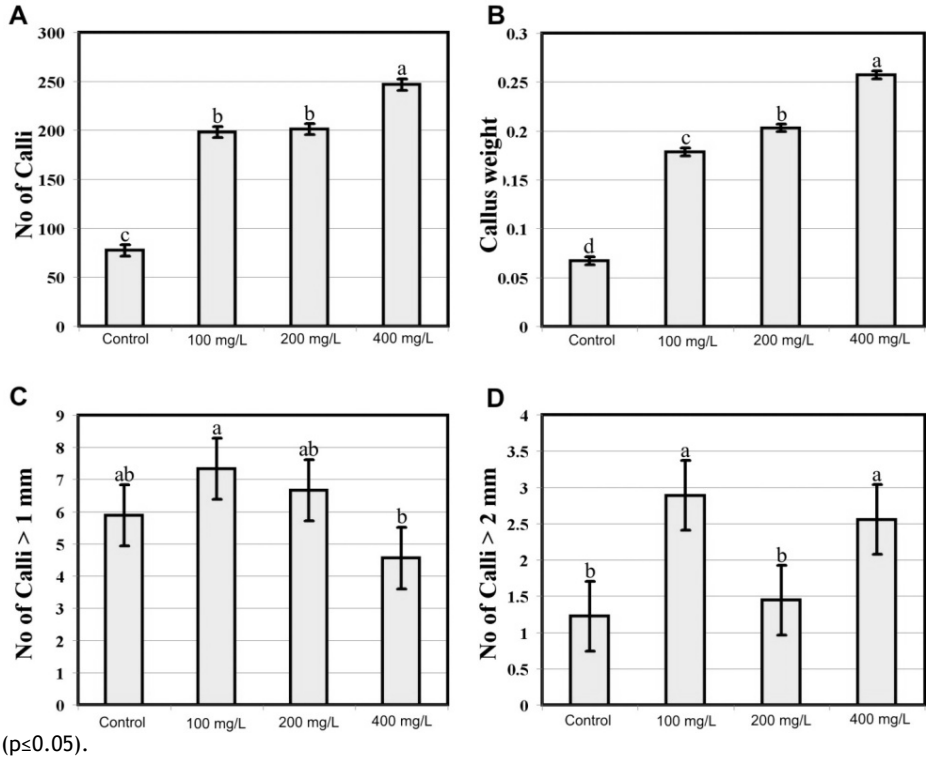


Figure 4: Quantitative effects of the addition of gum arabic at concentrations ranging from 1 to 100 mg/L, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences



(p≤0.05).

Figure 7: Quantitative effects of the addition of gum arabic at concentrations ranging from 100 to 400 mg/L, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences (p≤0.05).

Again, a positive correlation was found between concentrations of gum arabic and number of calli and total weight (Figures 6A, B), with an optimum at 1600 mg/L. In addition, this concentration produced the highest number of calli larger than 1 mm (Figure 6C), but not than 2 mm (Figure 6D). A fourth and final experiment was performed using concentrations higher than 1600 mg/L (3000, 5000, 7500 and 10000 mg/L). However, this experiment using extremely high concentrations gave very inconsistent results, with extreme differences between repetitions, and always below or similar to controls (data not shown). Therefore, we concluded that the use of concentrations higher than 1600 mg/L was not advisable. Once established the positive effect of gum arabic in androgenesis induction, we performed an additional

experiment to confirm the role of arabinogalactan and AGPs in such an effect. We cultured microspores with the standard protocol without gum arabic (control), with gum arabic at 1600 mg/L, and combining gum arabic (1600 mg/L) with βGlcY in order to precipitate the AGPs present in gum arabic. As shown in Figure 7A, addition of gum arabic was beneficial as expected, increasing the number and size of callus produced after one month of culture. However, the addition of βGlcY reduced dramatically the efficiency of the method, producing very few calli, and being only one per plate larger than 1 mm. Together, these results pointed to AGPs as the main responsible of the observed increases, suggesting a specific, positive and dose-dependent effect in the production of microspore-derived structures.

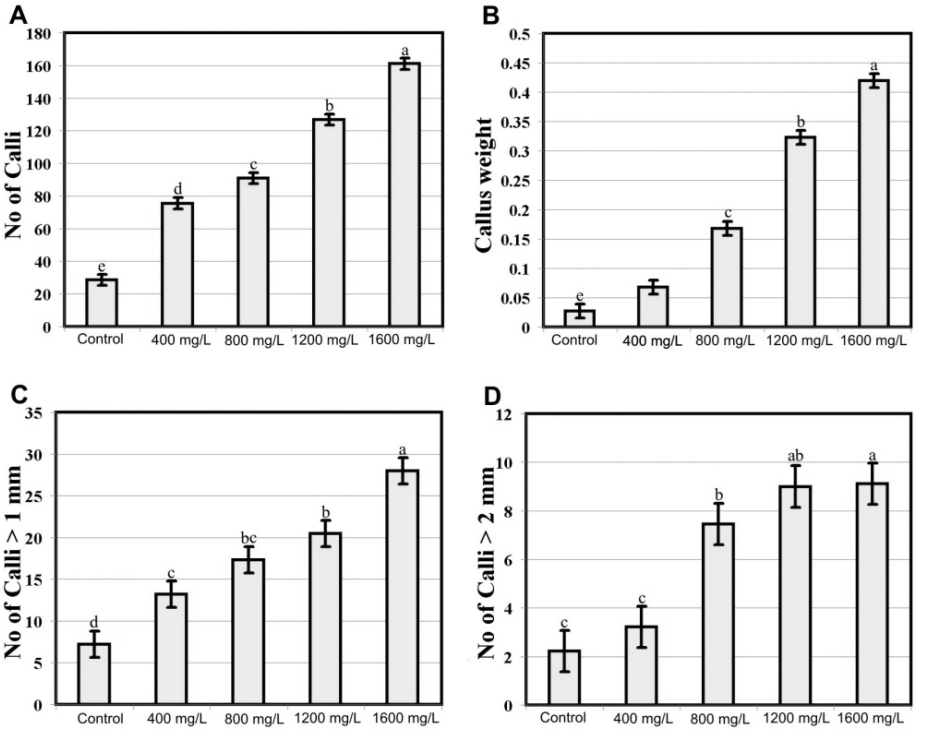


Figure 6: Quantitative effects of the addition of gum arabic at concentrations ranging from 400 to 1600 mg/L, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences ($p<0.05$).

Nevertheless, the most important effect of the use of 1600 mg/L of gum arabic was observed at the morphological level. In addition to callus-like structures, we observed embryo-like structures at stages beyond the globular stage (Figure 7B). Some of them were round, whitish and with a defined, smooth surface, typical of globular embryos (arrow in Figure 7B). Others presented a bilateral pattern (asterisk in Figure 7B) with a clearly defined, elongated hypocotyl and in some examples, elongating roots (arrowhead in Figure 7B). When observed under the inverted microscope (Figure 7C), elongated embryos presented a differentiated vascular cylinder and a distinct radicle at the basal end of the hypocotyl. However, in all cases embryos presented absent or defective shoot apical meristems, with blunt or flat apical ends, and no traces of cotyledons. In conclusion, the use of gum arabic at 1600 mg/L not only promoted the induction of significantly more microspore-derived structures, but also prevented their transformation into calli, giving rise to embryo-like structures with defective shoot apices.

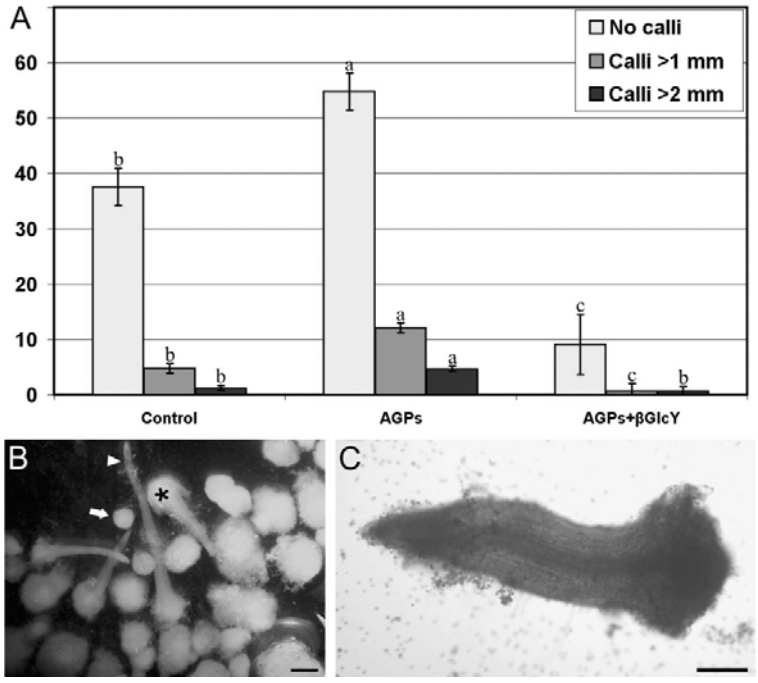


Figure 7: Effects of the addition of gum arabic. A: Quantitative effects of adding gum arabic alone (AGPs) and together with βGlcY (AGPs+ βGlcY), expressed as total number of calli/bud

(light bars), number of calli/bud larger than 1 mm (grey bars) and 2 mm (dark bars). Different letters indicate statistically significant differences ($p<0.05$). B: MDEs and calli obtained with 1600 mg/L gum arabic. Arrow points to a globular embryo, asterisk marks an elongating embryo, and arrowhead points to an embryo with a germinated root. C: Eggplant MDE showing a normal, zygotic-like hypocotyl and root apex, and a defective shoot apex. Bars: B: 500 µm; C: 200 µm.

Combined effects of ABA, EBr, PEG and AGPs

Once determined the optimal concentrations of ABA, EBr and AGPs when added alone to the standard protocol, we next evaluated their combined effect when used together with AGPs and at the optimal concentration (Figure 8A). We decided to include AGPs in all combinations because this was the factor that yielded better results, in both quantitative and qualitative terms. We also considered for this experiment the use of 1% PEG, previously demonstrated to be beneficial when used together with a parallel reduction of sucrose concentration from 2% to 1% (Corral-Martínez and Seguí-Simarro 2012). First, we tested the inductive effect of combinations of two, three and the four factors. A quantitative analysis of the results revealed that despite its positive effect on androgenesis induction when applied alone, the use of ABA combined with any other factor was either not or just slightly beneficial for callus production. On the other hand, all combinations excluding ABA gave rise to callus numbers remarkably higher than controls. However it must be noted that most of the combinations, both including and excluding ABA, produced calli of larger size, as revealed by the increase in the number of calli larger than 1 and 2 mm. Nevertheless, the best overall results were obtained with ABA-excluding combinations. Among them, the best performing included the combined use of PEG+AGPs, followed by EBr+AGPs and EBR+PEG+AGPs. Thus, it could be concluded that ABA would somehow inhibit the inductive effect of the other factors. Among these other factors, the best results were always related to the use of AGPs combined with PEG, EBr or both.

Microscopical observations of microspore-derived structures in two-week old cultures revealed that almost all treatments produced large, disorganized callus-like structures (Figure 8B), similar to those observed for low ABA concentrations (Figures 1C-E) and for EBr (Online Resource 1). The only two exceptions were the PEG+AGPs and ABA+PEG+AGPs combinations, which produced smaller, rounded globular embryo-like structures (Figure 8C), very similar to those shown in Figure 4B (arrow). Observations in one-month old cultures revealed that all treatments, including the PEG+AGPs and

ABA+PEG+AGPs combinations, gave eventually rise to callus-like structures, with no traces of embryo progression beyond the globular stage. Thus, it appeared that PEG+AGPs, with or without ABA, had a slightly beneficial effect in preserving embryo identity for longer time. This effect, however, would be insufficient to allow for embryo progression.

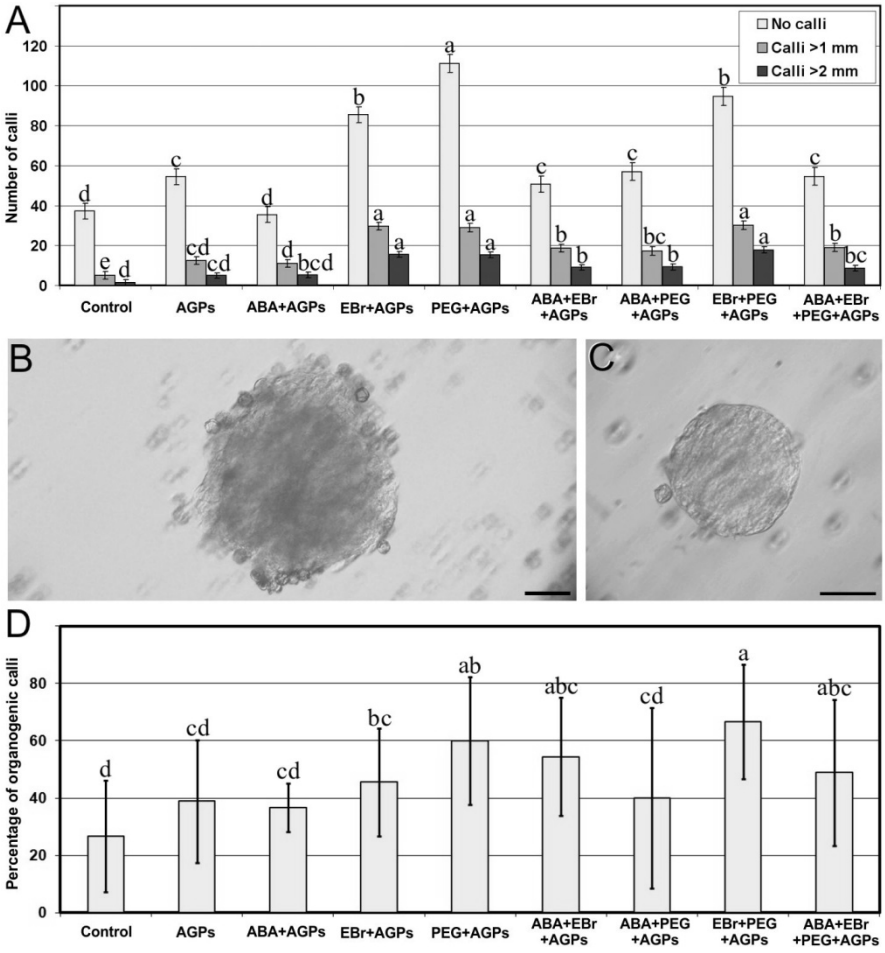


Figure 8: Effects of the combined addition of AGPs, ABA, EBr and PEG. A: Quantitative effects on microspore induction, expressed as total number of calli/bud (light bars), number of calli/bud larger than 1 mm (grey bars) and 2 mm (dark bars). B: Examples of structures obtained with all factor combinations except for PEG+AGPs amd ABA+PEG+AGPs, which are illustrated in C. Bars: 50 µm. D: Quantitative effects on regeneration from microspore-derived calli, expressed as per-

centage of organogenic calli from total number of calli per plate. Different letters indicate statistically significant differences ($p<0.05$).

Then, we evaluated the effect of the initial exposure to these factor combinations for one month, in promoting regeneration from the calli obtained. Upon transfer of calli to solid medium at month 1 of culture, we cultured them during 1.5 additional months and then evaluated the number of calli undergoing organogenesis and developing organogenic buds and then shoots at their surface. As seen in Figure 8D, results for regeneration were in general consistent with those obtained for induction, but with two principal differences: the combined use of EBr+AGPs was demonstrated not as beneficial as for induction, whereas the use of ABA+EBr+AGPs was remarkably better than control. In other words, the clearly positive effect of EBr for induction was found moderate for regeneration, whereas ABA had a positive effect on organogenesis promotion when combined with EBr and AGPs. Nevertheless, the best results were obtained combining PEG+AGPs, with or without EBr, as for induction.

Effects of BAP and NAA concentration and ratio

In the last experiment, we evaluated the effect of altering the concentrations and relative proportions of BAP and NAA, the two growth factors used in the standard protocol (Figure 6). We tested the effect of changing BAP and NAA concentrations to a half (0.5x), one fifth (0.2x), twice (2x) and fivefold (5x) the initial concentration of the standard protocol (1x, control). In parallel, we altered the BAP:NAA ratio by changing BAP concentration to a half (0.5 BAP) and twice (2x BAP) the initial, and also by changing NAA concentration to a half (0.5 NAA) and twice (2x NAA) the initial. Figure 9 shows that the inductive response of eggplant microspore cultures was inversely proportional to the concentration of BAP and NAA used. Moreover, we found that the 1x concentration initially used was not the best. Instead, a 0.2x reduction of both regulators yielded the best results in terms of callus number and size. The individual reduction of BAP only was found to be beneficial, whereas an individual decrease of NAA, or the individual increase of either BAP or NAA did not produce significant differences with respect to control dishes. In conclusion, a proportional reduction of both regulators to one fifth of the initial concentration was found better than any other alteration of the BAP and NAA concentration or ratio.

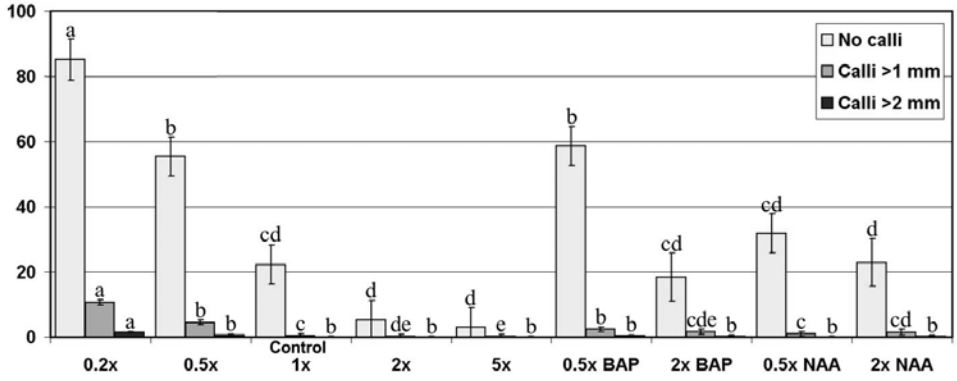


Figure 9: Quantitative effects of altering concentration and ratio of BAP and NAA, expressed as total number of calli/bud (light bars), number of calli/bud larger than 1 mm (grey bars) and 2 mm (dark bars). Different letters indicate statistically significant differences ($p<0.05$).

Discussion

ABA slightly promotes embryo induction and growth, but not embryo progression

In this work we showed that addition of $0.5.10^{-6}$M ABA had the most markedly beneficial effect for callus production and growth. With respect to controls, more microspores were induced to divide, and they divided faster, as revealed by the increase in callus number, weight and size. This is in accordance with previous results in tobacco or barley. In tobacco, Kyo and Harada (1986) demonstrated that ABA application stimulated embryo production, and Imamura and Harada (1980) proposed that a specific level of ABA was required for induction of androgenesis. In barley anther cultures, a peak of endogenous ABA after the first 24 h of pretreatment was proposed to prevent microspore death, increasing the amount of viable microspores (van Bergen et al. 1999). In our eggplant system, low ABA concentrations promoted MDE induction, perhaps by increasing microspore viability as suggested by van Bergen et al. (1999) or by limiting the extent of osmotic stress-induced oxidative damage. Indeed, ABA was considered a key blocker of H_2O_2 production due to osmotic stress (Ozfidan et al. 2012). According to the morphology of the structures observed (Figures 1C-G), low ABA concentrations had also a

role in the maintenance of the identity of globular embryos, preventing embryo arrest and callus growth but only up to a certain extent, since all embryos eventually transformed into calli. Therefore, low ABA doses would promote embryo induction but would not be able to maintain embryo identity during the transition from globular to heart-shaped embryos. In other words, low exogenous ABA doses would have a slightly positive effect during the initial stages of embryogenesis, but not at later stages. In the context of the protective role of ABA against abiotic stress, this is not surprising, since it was previously shown that exogenous ABA played a dual role in the regulation of stress-protective defense strategies, by which a beneficial role in the initial stages shifted to a detrimental one under prolonged treatments (Xiong et al. 2006).

EBr has a beneficial role limited to microspore induction towards embryogenesis

As to the effect of EBr, our results showed a positive effect on microspore induction when added at 10^{-8}M. However, these microspores soon diverted from the embryogenic pathway and transformed into small, disorganized microcalli. As discussed for ABA, the role of EBr may be related to the protection against abiotic stresses provided by EBr. It is known that EBr has a positive impact in the acquisition of thermotolerance (Divi et al. 2010). In the context of the inductive protocol used hereby, characterized by the exposure to sublethal temperatures (35ºC) during 3 days, this protection during the initial stages appears essential to increase the survival rate. These results are consistent with the role as embryogenesis enhancers reported for several *B. napus* and *B. juncea* cultivars (Ferrie et al. 2005). EBr, however, was unable to promote embryo progression and further differentiation. Thus, EBr could possibly be considered as part of a practical protocol aimed to produce callus-derived eggplant DH plants, but not to produce them through embryogenesis.

AGPs promote microspore induction and proliferation, and are sufficient to promote the globular-to-bilateral embryo transition in eggplant MDEs

Among the different substances tested in this work, the most effective to promote microspore induction and callus growth was gum arabic. Chemical-

ly, gum arabic is a complex mixture of arabinogalactans (~90%) and AGPs (~10%), together with a residual ~2% of glycoproteins (Phillips 2009). Both arabinogalactans and AGPs have been related with beneficial effects in microspore embryogenesis. For example, Larcoll (an arabinogalactan extracted from *Larix occidentalis*) was shown to decrease mortality rate and to stimulate rapid cell division of cultured wheat microspores (Letarte et al. 2006). In maize, AGPs were found essential for the viability and development of both MDEs and zygotic embryos (Paire et al. 2003). Even in recalcitrant maize genotypes, the androgenic response could be augmented through the use of AGPs (Borderies et al. 2004). Our results were consistent with these and other studies reporting beneficial effects of AGPs on embryogenesis induction. However, our experiments with βGlcY indicated that at least in our system, the principal responsible of the increases in callus number, size and weight was the ~10% of AGPs present in gum arabic, and not the major fraction of arabinogalactans. In addition, we could speculate that βGlcY would also be interfering with endogenous AGPs synthesized by microspores and MDEs, since addition of βGlcY produced a remarkable decrease of the induction rate, notably lower than controls with neither AGPs nor βGlcY.

Considering the ranges of concentration tested by us, it can be deduced that up to a limit of 1600 mg/L, increasing concentrations of AGPs are directly related to the induction of more microspores to divide (nearly 8x with 1600 mg/L), and to the production of larger calli, as revealed by the increases in number, size and weight. From 3000 mg/L and beyond, it is possible that these very high concentrations could exert some kind of deleterious effects, perhaps due to the high osmotic pressure created, or maybe due to high levels of toxic or inhibitory compounds also present in gum arabic. This would not be surprising, since it has been reported for other common components of *in vitro* culture media made from plant extracts, such as agar (Kohlenbach and Wernicke 1978; Dunwell 2010). Nevertheless, it is evident from our results that AGPs exert in eggplant a strong stimulatory effect over microspore viability and division of microspore-derived cells, as also reported in other species. Additionally, the use of AGPs allowed us to overcome the blockage in embryo progression initially observed at the transition between globular and heart-shaped stages (this study; Corral-Martínez and Seguí-Simarro 2012), obtaining MDEs of a morphological quality at least similar to those obtained by anther culture (Salas et al. 2011; Seguí-Simarro et al. 2011). Indeed, we showed eggplant bilateral, elongating MDEs that progressed through the radial-to-bilateral transition, and finally transformed into torpedo-like embryos. These

embryos were characterized by the unambiguous presence of a root apex and an elongated hypocotyl composed of structured cell layers surrounding a clearly distinguishable provascular cylinder. However, they also presented many of the severe morphological abnormalities and functional problems observed in anther-derived eggplant MDEs (Salas et al. 2011; Seguí-Simarro et al. 2011), principally at the shoot apex. It is known that AGPs are necessary to modulate the developmental fate of the early embryo, in particular during the globular to heart-shaped embryo transition (this study; Tang et al. 2006). Many plant growth and differentiation events are also influenced by AGPs, including the establishment of the shoot and root apical meristems (Tang et al. 2006), the formation and vascularization of cotyledons, and embryo germination (Zhong et al. 2011). However, we showed that in eggplant, addition of AGPs was not sufficient to promote adequate shoot apical development, which suggest that other external factors would be needed to promote, in conjunction with AGPs, shoot apex establishment and proper development.

In summary, AGPs are capable to promote direct embryogenesis from eggplant isolated microspores more effectively than any other substance tested to date. Although more work needs to be done to overcome the functional limitations observed, this is an important step towards an efficient protocol for DH production through direct microspore embryogenesis, still absent.

Low BAP and NAA concentrations increase the efficiency of microspore induction

The main goal of this work was to optimize the previously existing method to obtain DHs from isolated eggplant microspores, evaluating the effect of new factors, but also revising its previous composition. Since growth regulators play an essential role in most *in vitro* proliferation and differentiation processes, we previously checked the role of different growth regulators in the process of plant regeneration from microspore-derived calli (Corral-Martínez and Seguí-Simarro 2012). In this work, we re-evaluated the role of growth regulators, but in the process of microspore switch towards embryogenesis. We found that microspore induction could be increased by reducing the individual concentration of either BAP or NAA. However, the highest increase (four fold) was achieved by proportionally reducing both BAP and NAA to one fifth of their original concentrations. Although it is widely known that the auxin:cytokinin ratio is key to induce/inhibit most *in vitro* responses in-

cluding proliferation, differentiation, rooting or shoot formation (Skoog and Miller 1957), it seems that eggplant microspores are considerably more sensitive to the absolute concentrations of BAP and NAA than to their relative proportions. This is consistent with the notion that true microspore embryogenesis should be induced regardless of growth regulators. In the model species where microspore embryogenesis is consistently induced and high amount of quality embryos are obtained, growth regulators are not used, or used in very low amounts (Seguí-Simarro 2010), suggesting that hormonal autotrophy defines true embryogenesis, either zygotic or androgenic (Aionesei et al. 2005). According to this, hormones are used in relatively recalcitrant systems where hormone-free inductive treatments are still insufficient to promote a sustained proliferation of microspore-derived cells.

The combined use of ABA, EBr, PEG, and AGPs leads to positive and negative interactions between them

Despite its positive effect on embryo induction when used alone, we found that when ABA was combined with EBr, PEG or AGPs, results were not satisfactory enough. Although clearly positive differences with respect to control experiments were found in terms of callus size or in regeneration, the effects in the number of induced calli were not so evident. In some cases, it seemed that the addition of ABA masked the positive effects of other factors. This was the case of the EBr+AGPs and PEG+AGPs combinations. Without ABA, the number of embryos produced was nearly twice and thrice higher than controls, respectively, whereas with ABA they were just slightly higher than controls. Somehow, ABA could be interacting with the signaling pathways or with the effects triggered by EBr, PEG and/or AGPs, thus reducing their stressing and therefore their inductive role during microspore embryogenesis. For the cases of EBr and PEG, this speculation would be reasonable, since it is known that ABA may mask or even inhibit EBr effects in plant responses against stresses such as salt and heat (Divi et al. 2010), and PEG-derived osmotic stress may be compensated by exogenous application of ABA (Ozfidan et al. 2012). This way, ABA would be preventing the stressing (inductive) effects of PEG. Considering the limited effect of ABA in the promotion of microspore embryogenesis and its null effect in MDE progression, together with its negative interaction with other, more beneficial factors, ABA should be discarded as part of an efficient protocol for androgenic eggplant DH production.

This study also demonstrated a limited effect of EBr when used alone. However, Figure 5A showed that when combined with AGPs (with or without PEG), the difference with controls is higher than when used alone. When combined with AGPs and PEG, the percentage of regenerating calli was the highest (Figure 8B). In other words, EBr seems to act synergistically with AGPs and PEG, adding its protective effects to those derived from AGPs and from PEG-derived osmotic stress. It is known that EBr exerts anti-stress effects both independently as well as through interactions with other growth factors (Divi et al. 2010). In addition to the negative interaction described above for EBr and ABA, positive interactions EBR-PEG, and EBr-AGPs might well be possible. In the case of PEG, EBr would be protecting against the deleterious effects of osmotic stress while not affecting its inductive potential. With respect to the possible EBr-AGP interaction, our results suggest that this would be more than a possibility, since the combination of EBr+AGPs gave the second highest number of calli. In addition, the size of the calli produced with this combination was the highest. This observation is important, because the use of EBr alone gave rise to more calli, but significantly smaller than controls. It seems that AGPs somehow "compensate" for this, allowing for the production not only of more calli, but also of larger calli.

Refining the protocol to obtain eggplant androgenic DHs

As explained above, one of the principal conclusions of this study is that AGPs should be routinely used to increase the efficiency of microspore embryogenesis, to reduce embryo-to-callus transformation, and to increase the rate of organogenic calli. According to our combinative experiments, AGPs could be used for callus production either alone or together with EBr and/or PEG. According to their efficiency in microspore induction, the combined use of PEG+AGPs would be advised. According to the percentage of organogenic calli, however, the addition of EBr to PEG+AGPs would not have a significant impact. Therefore, considering the high price of commercial EBr, we would not recommend to include it in a practical protocol for routine production of eggplant DHs. In conclusion, according to our results such a protocol should be optimized by reducing BAP and NAA concentrations to one fifth, by replacing 2% sucrose by 1% sucrose + 1% PEG, and by adding 1600 mg/l of gum arabic.

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Bloque II

COLZA

Brassica napus (colza) es una planta de la familia de las Brasicáceas. Tiene una gran utilidad desde el punto de vista económico, ya que sus semillas son utilizadas para la obtención de aceite vegetal para el consumo humano y como biodiésel. Además se utiliza como forraje para el ganado y como planta de cubierta de suelo. La colza es cultivada en todo el mundo, siendo su producción mundial y el área cosechada de 59.007.753 Tm y 31.640.750 Ha en 2010 respectivamente. Como cultivo oleaginoso en 2010 ocupaba el quinto puesto en producción a nivel mundial y el tercero en cuanto al área cosechada. Desde un punto de vista científico, el interés de la colza radica en su utilización como modelo para el estudio de diferentes procesos biológicos, y en el contexto de la presente Tesis Doctoral para el estudio de la embriogénesis de la microspora. Por su elevada respuesta a los tratamientos inductores, constituye un modelo para el estudio de la inducción y desarrollo de los embriones androgénicos (Chupeau et al. 1998; Friedt and Zarhloul 2005; Seguí-Simarro and Nuez 2008), proporcionando una herramienta muy valiosa para el estudio de los cambios celulares y moleculares que suceden como consecuencia de este proceso. Desde hace años se dispone de un protocolo eficaz que permite obtener de forma rutinaria un elevado número de embriones, lo cual ha permitido que se use esta especie como modelo para la realización de numerosos estudios a nivel celular, molecular, genético, genómico, transcriptómico, o metabolómico, entre otros (revisado en (Seguí-Simarro and Nuez 2008)). Desde una perspectiva de investigación básica, los embriones androgénicos se pueden utilizar, entre otras cosas, para:

- El estudio de cambios a distintos niveles, asociados al cambio de programación en el desarrollo.
- Transformación genética, debido a su utilidad para evitar hemicigosis. Esto puede conseguirse transformando directamente microsporas antes de la inducción, o transformando embriones androgénicos y obteniendo después embriones secundarios a partir de éstos para evitar la posible aparición de quimeras.

- El estudio del proceso de embriogénesis en angiospermas, por ser un sistema que reproduce fielmente la embriogénesis cigótica, pero *in vitro*, en una placa de cultivo accesible al observador sin la dificultad para el estudio que imponen las distintas capas del endospermo y tejido materno que rodean al embrión cigótico en desarrollo.

Capítulo 4

Novel features of *Brassica napus* embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning

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This manuscript corresponds to the article entitled: "*Novel features of Brassica napus embryogenic microspores revealed by high pressure freezing and freeze substitution: evidence for massive autophagy and excretion-based cytoplasmic cleaning*", by Patricia Corral-Martínez and Jose M. Seguí-Simarro, submitted to *Journal Experimental Botany.*

Abstract

Induction of embryogenesis from isolated microspore cultures is a complex experimental system where dramatic changes in developmental fate coexist with severe abiotic stresses. After approximately 40 years of application of electron microscopy to the study of the ultrastructural changes undergone by the induced microspore, there is still room for new discoveries. In this work we used High Pressure Freezing and Freeze Substitution (HPF/FS), the best procedures known to date for ultrastructural preservation, to process Brassica napus isolated microspore cultures covering all the stages of microspore embryogenesis. Analysis of these cultures by transmission electron microscopy revealed massive processes of autophagy exclusively in just induced, embryogenic microspores, but not in any other cell type before, during or after the androgenic switch. However, a significant part of the autophagosomal cargo was not recycled. Instead, it was excreted out of the cell, producing numerous deposits of extracytoplasmic fibrillar and membranous material. Our observations suggest that induction of embryogenesis implies both a massive autophagic response for a profound cytoplasmic cleaning, and a excretion of the removed material in order to avoid excessive growth of the vacuolar system. Together, our results demonstrate that the application of HPF/FS to the study of the androgenic switch is the best option currently available to identify the complex and dramatic changes in subcellular architecture undergone by induced microspores. In addition, they provide significant insights to understand the cellular basis of induction of microspore embryogenesis, and open a new door for the investigation of this intriguing developmental pathway.

Introduction

Microspore embryogenesis is a complex experimental process whereby a haploid microspore is reprogrammed to become a haploid or doubled haploid (DH) embryo. DHs are valuable tools for genetic and developmental research, but their principal application relates to plant breeding, where they may serve as pure lines for hybrid seed production. This androgenic switch is induced *in vitro* by the application of different types of abiotic stresses, including heat shock, cold, and starvation, among others (Shariatpanahi *et al.*,

2006). Therefore, the embryogenic microspore is an extremely complex biological system where cellular responses to *in vitro* exposure and to abiotic stresses coexist with the reprogramming towards a new developmental fate and the cessation of the old program. It is easy to conceive that all these simultaneous changes must be reflected in dramatic remodeling of cell architecture. Indeed, this topic has attracted the attention of cell biologists since the discovery of this experimental phenomenon. As early as in 1974 Sunderland and Dunwell initiated the publication of their pioneering transmission electron microscopy (TEM) studies on the ultrastructural changes undergone by embryogenic microspores of *Nicotiana tabacum* (Dunwell and Sunderland, 1974a, b, 1975; Sunderland and Dunwell, 1974) and *Datura innoxia* (Dunwell and Sunderland, 1976a, b, c). Then, the identification of *Brassica napus* as a highly responding, model system extended ultrastructural research to this species (Hause *et al.*, 1992; Seguí-Simarro *et al.*, 2003; Simmonds and Keller, 1999; Telmer *et al.*, 1993, 1995; Zaki and Dickinson, 1990; Zaki and Dickinson, 1991, among others). For example, it was found that embryogenesis induction gives rise to a profound cytoplasm remodeling, including the clearing of large cytoplasmic regions devoid of organelles and ribosomes, the synthesis of different types of heat shock proteins or the occurrence of somatic-type cytokinesis producing two equivalent nuclei and cells, as opposed to the asymmetric pollen mitosis where two clearly different generative and vegetative cells are formed. This approach continued providing useful insights in these and other species (for reviews, see Barany *et al*., 2005; Coronado *et al*., 2002; Dunwell, 2010; González-Melendi et al., 1995, 200 Kasha, 2005; Maraschin *et al.*, 2005; Pauls *et al.*, 2006; Seguí-Simarro and Nuez, 2008a, b; Seguí-Simarro, 2010; Testillano *et al*., 2000; Testillano *et al*., 2005), for a total of nearly 40 years of application of TEM to the study of microspore embryogenesis.

All the studies mentioned above had in common the use of the most popular procedures available at that time to preserve cell ultrastructure. In other words, they used aldehyde-based chemical fixatives (principally paraformaldehyde and glutaraldehyde) to preserve cells prior to TEM observation. These multifunctional fixatives are widely used, due principally to their relatively low cost, availability and ease to use. They preserve cell ultrastructure by crosslinking subsets of cellular molecules, thereby preserving their spatial organization. However, it is important to note that chemical fixation takes seconds to minutes to immobilize cellular processes, and different cellular components are fixed at different rates (reviewed in Gilkey and Staehelin, 1986). Considering that subcellular elements such as phragmoplasts, com-

prising microtubules and microfilaments, growing cell plates, vacuolar systems, Golgi stacks or secretory vesicles undergo structural changes in the time range of seconds and even fractions of seconds, it is easy to deduce that most short-lived structural intermediates, in particular those of membranous nature, will be created and dismantled before chemical fixatives have enough time to fix them. Further, penetration of chemical fixatives impose cells severe osmotic stresses known to cause swelling or shrinkage of cells and organelles, whose main ultrastructural consequence is the presence of wavy membrane contours (Lee *et al.*, 1982). In addition to this, it is well known that glutaraldehyde may cause vesiculation of the endoplasmic reticulum (ER), plasma membrane or chloroplast membranes and in parallel, artifactual fusion of vesicles with the plasma membrane (Gilkey and Staehelin, 1986). In other words, chemical fixatives may potentially create artifactual vesicles in regions where they should be absent, or eliminate them from regions where they are actually present. Since these subcellular elements are below the resolution of conventional light and confocal microscopes, their deleterious effects are not detected with these microscopical approaches. However, all of these facts count against the notion of chemical fixatives as ideal tools for preserving cell ultrastucture to be observed by TEM.

Today, chemical fixation methods are being progressively replaced by ultra-rapid, low temperature-based fixation technologies. Among them, the combination of high pressure freezing (HPF) with freeze substitution (FS) is nowadays the best option to avoid the limitations of chemical fixatives. HPF has the potential to instantly stabilize the cells, due to their ability to immobilize all cellular molecules within milliseconds (Gilkey and Staehelin, 1986). Samples preserved in this manner can then be freeze-substituted at –80 to -90ºC, which increases the probability of viewing even the most labile cellular structures. HPF/FS presents only one disadvantage for a routine use: its high cost and therefore its reduced availability. Perhaps, this is one of the reasons why these methodologies have not been used to study the changes undergone by microspores as a consequence of induction of embryogenesis.

In this work we applied HPF/FS to process samples of *B. napus* isolated microspore cultures at different stages before, during and after induction towards embryogenesis. We revisited the ultrastructural changes undergone by the different cell types present in cultures, as potential consequences of the androgenic switch, but also to the heat shock treatment used to induce microspores. Thanks to the application of this improved methodology, we

were able not only to confirm previous observations, but also to discover new subcellular structures involved in processes considered critical for a successful switch. In addition, we could attribute these processes specifically to the developmental switch, and not to the inductive heat shock.

Materials and methods

Plant materials

B. napus L. donor plants of the highly embryogenic cv. Topas were grown as previously described (Seguí-Simarro *et al.*, 2003). Plants were grown in the greenhouses of the COMAV Institute (Universitat Politècnica de València, 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 vacuolated microspores were selected as previously described (Seguí-Simarro *et al.*, 2003), surface sterilized with 5.25 g/l sodium hypochlorite for 5 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 41 µm nylon cloths. The filtrate was transferred to 50 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.

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.

Results

We processed by HPF/FS *B. napus* isolated microspore cultures, including the gametophytic-like structures and the induced microspores and microspore-derived embryos (MDEs) present in advanced culture stages. In addition to this, we also processed by HPF/FS other stages during *in vivo B. napus* microsporogenesis and microgametogenesis, in order to check whether the changes observed were due to really different internal architectures or to artifactual phenomena derived from processing procedures. Once processed,

we analyzed our collection of TEM images covering all these stages and cell types, paying special attention to the first moments of the androgenic switch and to the changes produced in cells as a consequence of the inductive treatment. The results of this analysis are presented in the following sections.

Induced multicellular microspores show specific ultrastructural features, not present in other cell types

Freshly isolated microspores at the optimal stage for induction (the vacuolated stage; Figs. 1A, A') presented a ribosome-rich cytoplasm, with abundant rough ER cisternae, and some Golgi stacks and mitochondria. The quality of the HPF/FS processing could be verified by the visibility of membranous elements such as Golgi cisternae, tonoplast or plasma membranes (Fig. 1A'). These membranes presented smooth profiles, without the undulations typically present in many chemically-fixed samples (Gilkey and Staehelin, 1986). Gametophytic, pollen-like structures (Fig. 1B), derived from microspores exposed to the same inductive conditions but not switching to embryogenesis, presented a dense cytoplasm, with abundant starch deposits and lipid bodies (Fig. 1B'). Mitochondria and long rough ER profiles were also characteristic of these cells, indicating a high biosynthetic activity. In contrast, multicellular microspores, just committed to embryogenesis (Fig. 1C) presented a remarkably different ultrastructure. Two to four-celled microspores showed a cytoplasm with a considerably lower density of ribosomes, and some regions devoid of organelles. The nucleus was centrally positioned and surrounded by numerous, round or slightly oval vacuoles of up to 3 µm diameter (Fig. 1C'). These vacuoles, likely generated by fragmentation of the large vacuole of microspores, frequently presented electron dense luminal contents, notably different from the light lumen of vacuolated microspores (Fig. 1A). Whereas mitochondria were present as in previous stages, starch deposits were almost absent, lipid bodies were scarce, and in general the cytoplasm was less densely filled than in other stages. Irregular cell walls and growing cell plates were observed, occasionally presenting holes and incomplete regions (arrow in Fig. 1C'') never observed in previous stages. However, the most striking feature of these cell walls was the presence of swollen and electron dense regions (arrowheads in Figs. 1C', C''). In addition to this, unusual membranous profiles engulfing entire organelles and cytoplasm regions were frequently observed in dividing cells (see next section). Once more,

these profiles were absent from previous stages, and also from cells following a pollen-like development.

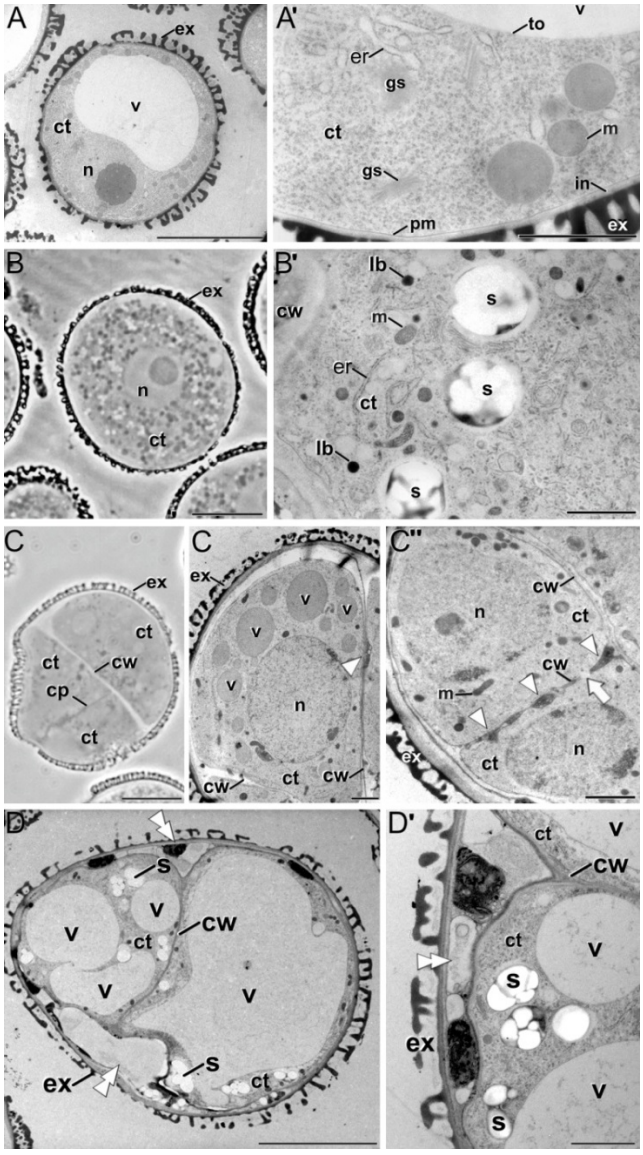

Fig. 1: Ultrastructure of *B. napus in vitro* cultured microspores. A,A': Freshly isolated microspores, immediately before induction. B, B': Pollen-like microspores following a gametophytic-

like developmental pathway. Note the presence of a dense cytoplasm (ct) with lipid bodies (lb) and large starch deposits (s) in amiloplasts. C, C', C'': Embryogenic microspores. Note the presence of several dense vacuoles (v), and of irregular cell walls (cw) with small fenestrae (arrow) and deposits of dense material (arrowheads). D: Non-embryogenic, few-celled microspore. Note the massive vacuolation (v), the abundant starch deposits (s), and the presence of dead lateral cells (double arrowheads). D': Detail of a non-embryogenic microspore. Note the presence of necrotic remnants and cell debris in the dead lateral cell (double arrowhead). er: endoplasmic reticulum; ex: exine; gs: Golgi stack; in: intine; m: mitochondrion; n: nucleus; pm: plasma membrane; to: tonoplast. Bars: A-D: 10 µm; A'-D' and C'': 2 µm.

A third type of microspores was found in cultures exposed to the inductive heat shock. They usually presented a combination of some of the features described for pollen-like and embryogenic microspores. As seen in Fig. 1D, this third type presented one or few sporophytic divisions, as revealed by the presence of conventional cell walls, and abundant starch deposits, similar to those observed in pollen-like microspores. Nevertheless, the most prominent trait of these cells was their enormous vacuolation. Vacuoles occupied most of the cellular volume, in the form of several vacuoles of different sizes, or of a single large, central vacuole. Accordingly, the cytoplasm was reduced to thin layers between vacuoles or between the vacuole and the plasma membrane. It was frequent to find some of their cells dying or dead, as revealed by the presence of necrotic remnants and extracellular debris (Fig. 1D'). These few-celled microspores were observed only during the first days of culture. At later stages this microspore type, as well as pollen-like microspores, always showed clear signs of general degeneration and death, indicating that they never proceeded further in embryogenesis, and eventually died and degenerated (data not shown).

In summary, the ultrastructural analysis of the early stages of microspore embryogenesis revealed two main cell architectures: (1) that present only in early multicellular microspores, characterized by cell walls with swollen, electron dense regions, and by membranous structures engulfing entire cytoplasmic regions; and (2) that present in other microspore types, not including any of these features, and never progressing through embryogenesis. Due to the significance of the features described for early embryogenic microspores, we performed a detailed analysis of them, as described below.

Embryogenic microspores present abundant autophagy-related structures

One of the principal features observed in embryogenic microspores was the presence of membranous elements engulfing large cytoplasmic regions (Fig. 2). We frequently observed cup-shaped membranous elements, likely phagophores (Fig. 2A), as well as closed, double membrane-bound structures enclosing small portions of cytoplasm (Fig. 2B), equivalent to those previously documented in autophagic processes and described as autophagosomes (Aubert *et al.*, 1996; Otegui *et al.*, 2005; Reyes *et al.*, 2011; Rose *et al.*, 2006). Occasionally, phagophore-like structures (Fig. 2C) and autophagosomes (Fig. 2D) were observed enwrapping larger cytoplasmic domains containing different types of organelles. In addition to phagophores and autophagosomes, we observed different types of vacuoles with luminal contents of different levels of electron density (Figs. 2E-G), ranging from high, densely stained, to light. Light vacuoles used to present membranous remnants and masses of fibrillar material dispersed throughout the lumen. Sometimes vacuoles of different types were observed tightly apposed or physically contacting (Fig. 2G), suggesting fusion between them. Since these different vacuoles usually had a size similar to autophagosomes (0.5-3 μm), we concluded that they could be representing different stages in the process of digestion of their contents. We also observed larger autophagosomes, sometimes fusing with dark vacuoles (Fig. 2H), and also with large, light vacuoles derived from the fragmentation of the large vacuole of the original microspore (data not shown). Considering all these observations together, we concluded that the structures observed corresponded to different steps of a typical process of macroautophagy, whereby cytoplasmic regions and even organelles were introduced into lytic vacuoles to be digested and recycled. Although sporadically, we also observed the presence of large (up to 5 μm) and complex membranous structures, either single or double-membrane bounded, with invaginations of their external membrane, similar to those described for microautophagic vacuoles (Fig. 2I).

These structures, similar to the prevacuolar compartments described in other plant systems (Reyes *et al.*, 2011), typically included small, single membrane-bound autophagic bodies, occasionally containing entire organelles. In contrast to embryogenic microspores, pollen-like microspores (Figs. 1B, B') and highly vacuolated, non-embryogenic microspores (Fig. 1D) did not show such a massive presence of autophagic profiles. Only scarce, isolated

examples of such profiles could be sporadically identified in non-embryogenic microspores (data not shown). In summary we observed exclusively in embryogenic microspores an abundant set of membranous structures that strongly pointed to the massive occurrence of macroautophagy, and to a lower extent, microautophagy.

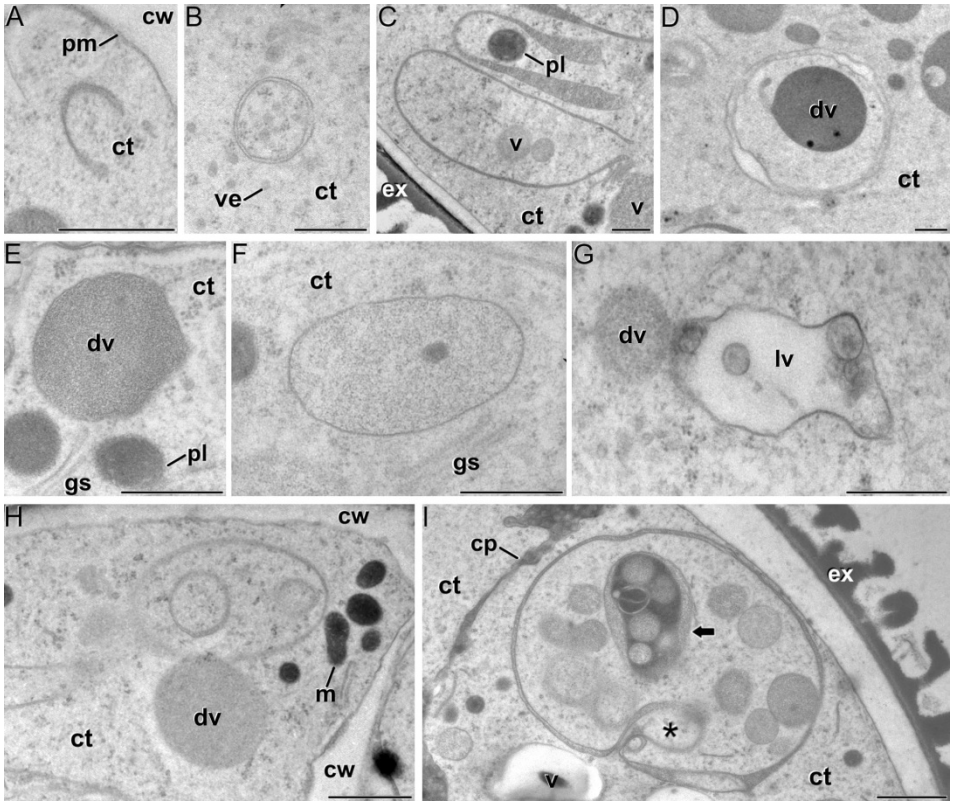

Fig. 2: Autophagy in embryogenic microspores. A: Small phagophore. B: Small autophagosome. C: Large phagophore surrounding a small vacuole (v). D: Large autophagosome containing a dense vacuole. E-G: Dense (E), medium (F) and light (G) vacuoles, representing different stages during the digestion of their content. Note in G the close proximity of a dense vacuole (dv) and a light vacuole (lv), suggesting imminent fusion between them. H: Large autophagosome contacting a dense vacuole (dv), suggesting fusion between them. I: Large prevacuolar compartment containing autophagic bodies, one of them with an organelle, apparently a proplastid being digested (arrow). Note the presence of an invagination (asterisk), suggesting a process of microautophagy. ct: cytoplasm; cw: cell wall; ex: exine; gs: Golgi stack; m: mitochondrion; pl: plastid; pm: plasma membrane; ve: vesicle. Bars: A-G: 500 nm; H-I: 1 µm.

Embryogenic microspores undergo massive excretion of cytoplasmic material

In addition to the mentioned evidences of autophagy, we observed the presence of abundant material unusually deposited in the cell walls of embryogenic microspores (Fig. 3A). When observed at higher magnification, we could distinguish two types of material in cell walls. The first type presented a membranous nature (Fig. 3B). In general, these vesicle-like structures showed an enormous dispersion with diameters ranging from 23 to 246 nm, and a majority around 30-40 nm (Fig. 4A), indicating that they do not corresponded to conventional, Golgi derived secretory vesicles (average diameter of 80-90 nm; (Fig. 4B). The second type was an electron dense, homogeneously fibrillar material (asterisk in Fig. 3C), identical to that observed in the lumen of dense cytoplasmic vacuoles (asterisks in Fig. 3A). As seen in Fig. 3A, dense cytoplasmic vacuoles were frequently observed in the vicinity of the cell wall. Interestingly, cell wall depositions were frequently observed at the side of the middle lamella corresponding to the cell with abundant dense vacuoles close to the cell wall (Fig. 3A). Very frequently, the extracellular fibrillar depositions (Fig. 3B) occupied an area equivalent to that of dense vacuoles (Fig. 3A). All these facts strongly suggested that the dense vacuoles and extracellular fibrillar depositions contained the same dense, fibrillar material. In other words, it appeared as if at least some of the dense vacuoles were directed to the plasma membrane, fused with it, and excreted their material to the cell wall. Occasionally, we also observed vacuoles containing a mixture of dense, fibrillar material and vesicle-like, membranous structures (Fig. 3D). Consistent with the observations described for dense vacuoles and fibrillar deposits, extracellular deposits of fibrillo-vesicular material were also observed, with a size equivalent to that of fibrillo-vesicular vacuoles (Fig. 3E). Thus, we deduced that the material illustrated in Figs. 3D and 3E was essentially the same, but observed at different moments during the process of excretion. We also identified vacuoles containing membranous multilamellar structures fusing with the plasma membrane (Fig. 3F). This material was remarkably similar to that previously described as remnants from the digestion of cytoplasmic organelles (Aubert *et al.*, 1996; Wu *et al.*, 2009). All the deposits described hereby were visible in newly formed cell walls (Figs. 3A-D), as well as in the microspore coat (Figs. 3E-F). In either case, the presence of these deposits was usually associated to swelling, irregularities and in some cases dramatic deformations of the cell wall, most likely caused by the mas-

sive accumulation of this material. None of the structures described in this section was observed neither in pollen-like microspores (Figs. 1B-B') nor in non-embryogenic microspores (Fig. 1D).

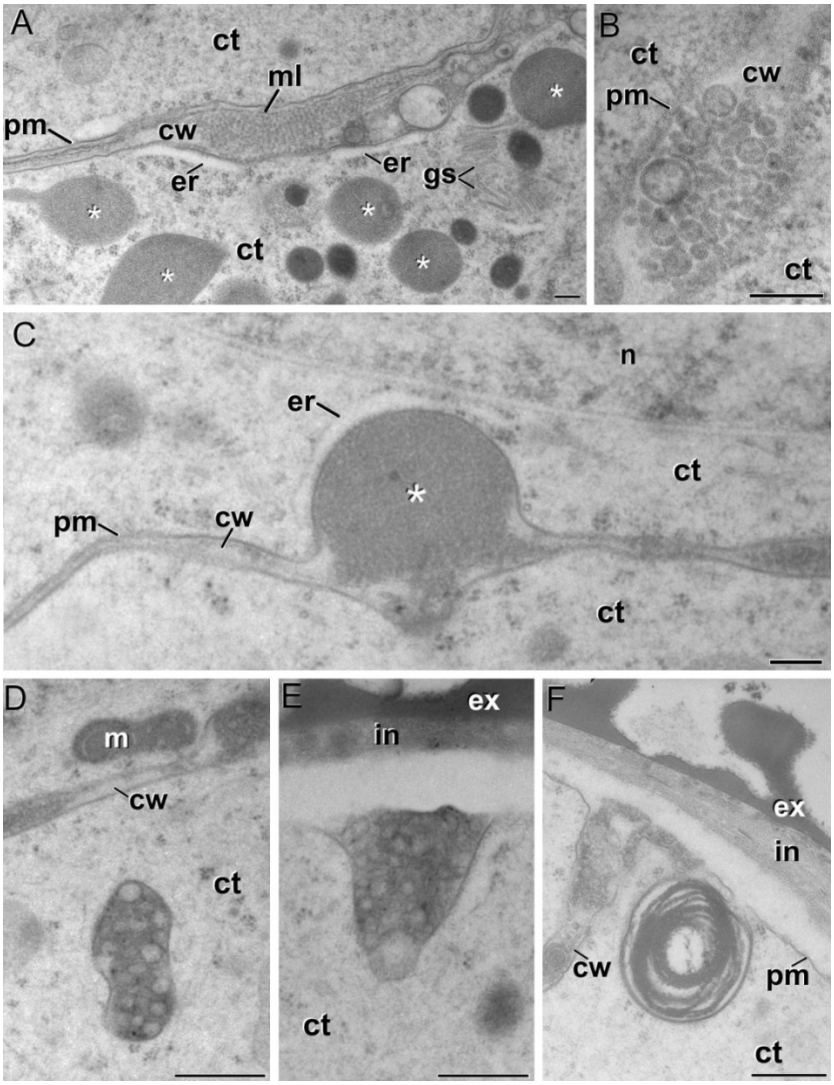

Fig. 3: Extracytoplasmic excretion in embryogenic microspores. A: Newly formed cell wall (cw) with abundant vesicular material. Note that all the vesicles are at the side of the middle lamella (ml) where numerous dense vacuoles (asterisks) are observed close to the cell wall. B: Enlarged

view of a vesicular deposit, showing their membranous nature and their size heterogeneity. C: Deposit of fibrillar, dense material (asterisk), similar in size to the dense vacuoles shown in A (asterisks). D: Dense vacuole with fibrillo-vesicular material, near to the cell wall. E: Fibrillo-vesicular cell wall deposit. Note the similarity between the deposited material and that of the vacuole shown in D. F: Multilamellar deposit fusing with the plasma membrane (pm). ct: cytoplasm; er: endoplasmic reticulum; ex: exine; gs: Golgi stack; in: intine; m: mitochondrion; n: nucleus. Bars: 200 nm.

In summary, we identified a number of ultrastructural evidences which strongly suggested that embryogenic microspores (but not those following a pollen-like or a non-embryogenic pathway) undergo a series of processes destined to excrete considerable amounts of intracellular, partially digested fibrillar and membranous material.

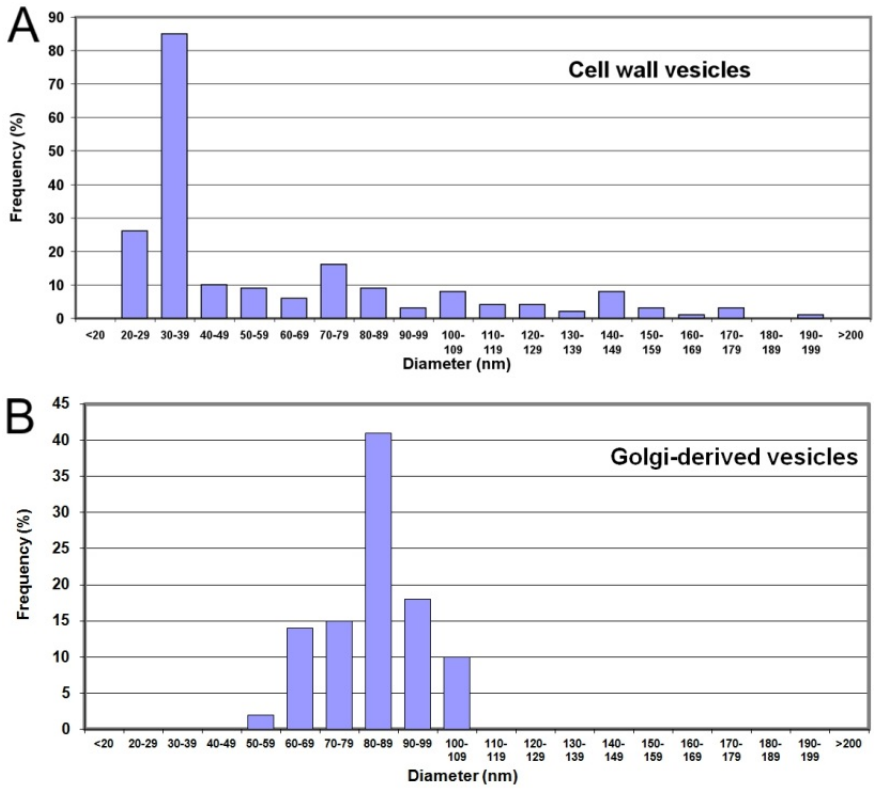

Fig. 4: Distribution of diameter frequencies for vesicles of cell wall depositions (A) and for Golgi-derived, cytoplasmic vesicles. Frequency results are expressed as percentages.

Young microspores, pollen grains and MDEs do not show autophagic profiles or massive excretion

As an additional control to validate the unique ultrastructural features observed in embryogenic microspores, we processed by HPF/FS *in vivo B. napus* young microspores (Figs. 5A, A'), pollen grains (Figs. 5B-B'') and *in vitro* cultured MDEs (Figs. 5C-D'). These stages are before (microspores) and after (pollen) the developmental window when microspores are responsive to the androgenic switch, and after *in vitro* embryogenesis induction (MDEs). Young microspores, prior to the formation of the large vacuole that displaces the nucleus close to the plasma membrane, presented a slightly lobed shape and a centered nucleus surrounded by organelles (Fig. 5A). The cytoplasm was dense, with abundant ribosomes, ER, Golgi stacks, mitochondria and plastids initiating slight starch accumulation (Fig. 5A'). Only small, light vacuoles (less than 100 nm in diameter) were identified. No signs of autophagy were observed. The microspore coat consisted on a thin intine layer covered by a sculptured exine coat. No signs of any fibrillar or membranous deposit were observed in the microspore coat of any of the cells studied. Bicellular pollen grains presented the typical vegetative and generative cells, including their respective vegetative and generative nuclei (Fig. 5B). Whereas the vegetative nucleus was larger and presented a typical pattern of decondensed chromatin, the smaller generative nucleus showed a condensed chromatin pattern, with most chromatin masses close to the nuclear envelope (Fig. 5B'). The cytoplasm was densely filled with ribosomes, rough ER cisternae, lipid bodies, starch-containing plastids and mitochondria (Fig. 5B''), typical of *B. napus* pollen grains. Only light vacuoles were observed, as expected for maturing pollen where the large microspore vacuole is fragmented and progressively reabsorbed. The pollen coat showed a thin intine layer, slightly thickened at the region of the apertures, and a more mature exine layer, with clear signs of pollenkitt deposition (Fig. 5B). Again, no indications of autophagy or massive excretion could be observed in these cells. In young developing MDEs including globular, transitional and heart-shaped embryos (Fig. 5C), the unusual ultrastructural features described for embryogenic microspores were no longer observed. All cells presented smooth plasma membranes and straight cell walls with no gaps or dense deposits (Fig. 5C'). In other words, these cells presented conventional, somatic-type cell walls (Seguí-Simarro *et al.*, 2008), similar to their zygotic counterparts. The cytoplasm presented a conventional appearance as well. In torpedo MDEs (Fig. 5D), cells also showed the typical

ultrastructure of *B. napus* zygotic embryos, characterized, among other features, by a dramatic increase of starch and lipid deposits (Fig. 5D').

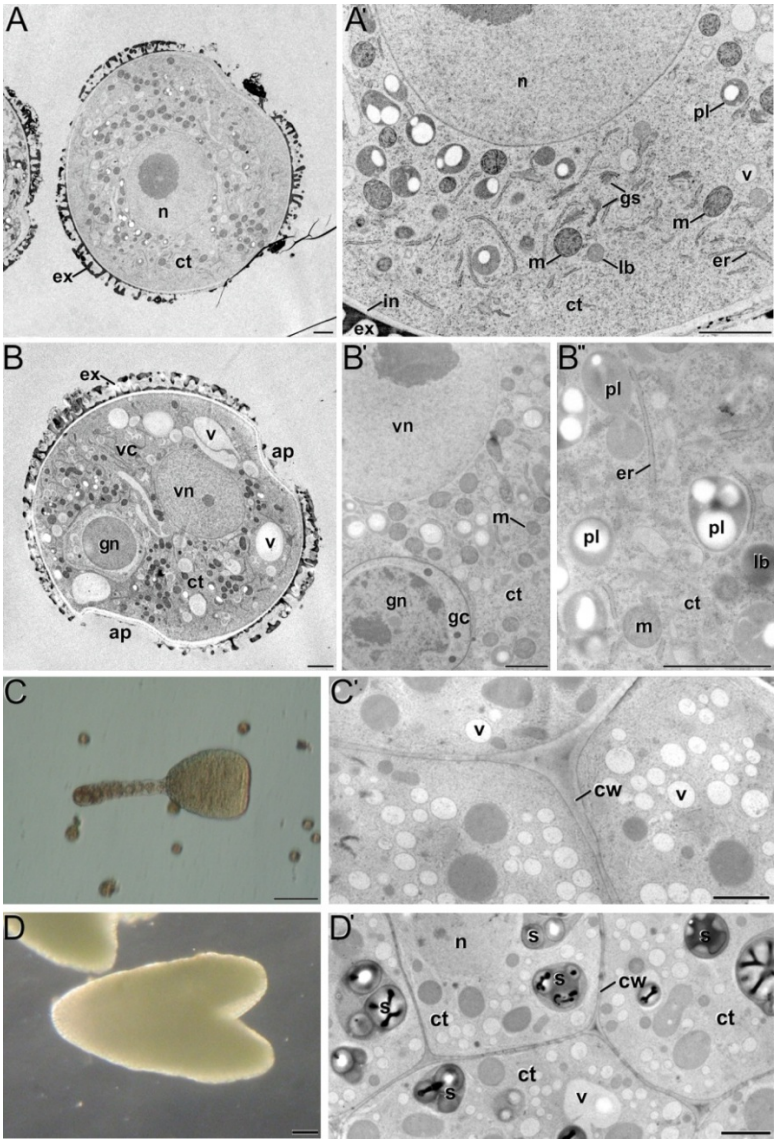

Fig. 5: Ultrastructure of *B. napus in vivo* nicrospores and pollen grains. A: Overview of a young microspore within the anther. A': Detail of the cytoplasm (ct) of a young microspore, showing a

high ribosome density, abundant ER (er), Golgi stacks (gs), and to a lower extent, lipid bodies (lb) and small plastids (pl) beginning to accumulate starch. Very few, small vacuoles (v) are observed. B: Overview of a mid, bicellular pollen grain within the anther. Note the presence of several, medium-sized light vacuoles, likely produced from fragmentation of the large vacuole of the microspore. B': Enlarged view showing the striking morphological differences between the vegetative (vn) and the generative nucleus (gn) of the generative cell (gc), embedded in the vegetative cell (vc). B'': Detail of the ribosome-rich cytoplasm of a vegetative cell, showing large starch-containing plastids (pl), mitochondria (m) and lipid bodies (lb). C, C': MDE at the transition between globular and heart-shaped. A vacuolated cytoplasm, enriched in lipid bodies and with straight, conventional cell walls can be seen. D, D': Torpedo MDE, with the typical cytoplasm of *B. napus* embryo cells, characterized by massive accumulation of starch and lipid bodies. ap: aperture; ex: exine; in: intine; n: nucleus. Bars: 200 nm except for A, B (2 μm), and D, E (50 μm).

All these observations were in perfect agreement with the subcellular architecture widely reported for these stages of *in vivo* microspore development and in vitro MDE growth in *B. napus* as well as in many other species. These cells were subjected to the same processing techniques than embryogenic microspores, but no signs of massive excretion or autophagy were observed. Thus, we concluded that the remarkable differences of embryogenic microspores should not be attributable to artifacts produced by processing techniques, but to a series of real structural and physiological changes.

Discussion

Induction of microspore embryogenesis implies a profound cytoplasmic cleaning based on autophagy mechanisms

We have described two principal autophagic mechanisms to operate in induced microspores. These two mechanisms would be essentially comparable to the known plant microautophagic and macroautophagic pathways (Bassham, 2007; Li and Vierstra, 2012). Direct evidences of the occurrence of such events during microspore embryogenesis are nearly absent to date. In 1974, Sunderland and Dunwell described the lysosome-mediated destruction of some organelles in induced tobacco microspores. Aside of this, other previous observations like reductions in the number of ribosomes, starch granules and lipid bodies, and presence of organelle-free regions (reviewed in Maraschin *et al*., 2005) have been considered as indirect proofs for some sort of large-scale cell cleaning. Based on this, several reviews speculated about autophagy as a way to remove gametogenesis-related molecules and stress-

damaged cellular components from the cytoplasm of induced microspores (Forster *et al.*, 2007; Hosp *et al.*, 2007; Maraschin *et al.*, 2005). However, to our knowledge this is the first time that microautophagy and macroautophagy are specifically documented during microspore embryogenesis. With respect to microautophagy, we showed evidences of invaginating membranes in vacuoles containing single membrane-bound autophagic bodies similar to those previously described as produced by microautophagy events (Saito *et al.*, 2002; Van der Wilden *et al.*, 1980). As for macroautophagy, we identified the presence of cup-shaped phagophores engulfing cytoplasm portions. These structures are necessary intermediates in the process of autophagosome generation (Li and Vierstra, 2012). We also showed the massive production of autophagosomes only in the cytoplasm of embryogenic microspores, and not in any other cell type neither before, nor during nor after embryogenesis induction. These bodies were double membrane-bound, and remarkably similar in morphology and size to the autophagosomes previously described in other plant cell types (Aubert *et al.*, 1996; Otegui *et al.*, 2005; Reyes *et al.*, 2011; Rose *et al.*, 2006). Based on these evidences, it is reasonable to assume that we are observing autophagosomes and autophagic vacuoles involved on the massive removal of useless cytoplasmic material. Thus, both microautophagy and macroautophagy coexist in embryogenic microspores, although according to the relative abundance of microautophagic and macroautophagic profiles, macroautophagy would be the preferred pathway for cytoplasm cleaning in *B. napus* induced microspores.

Plants use two principal ways to recycle/remove useless cell material, the ubiquitin/26S proteasome system (UPS) and autophagy. UPS is principally aimed to the selective removal of small regulatory proteins (Smalle and Vierstra, 2004). In contrast, autophagy is considered as a housekeeping system to rapidly remove and recycle cellular debris including non-proteinaceous material, large particles like organelles, and even entire cytoplasmic regions, as a response to stress conditions (principally starvation) or during developmental transitions (Bassham, 2009; Liu and Bassham, 2012). Considering that stress-induced, embryogenic microspores undergo dramatic developmental changes while simultaneously exposed to severe abiotic stresses, it could be argued that autophagy would be a consequence of stress exposure. However, we showed that pollen-like microspores, exposed to heat stress in the same culture environment, do not present any of the autophagy-related features described hereby. Thus, cytoplasmic cleaning would be a direct and exclusive consequence of the androgenic switch. This is in agreement with older studies

reporting that organelle-free cytoplasmic regions occurred only in heat treated, cultured *B. napus* microspores, but not in microspores of heat-treated whole plants (Telmer *et al.*, 1993). It is also consistent with recent reports describing the upregulation of genes involved in the UPS pathway, exclusively during androgenesis induction (Maraschin *et al.*, 2006). This led the authors to propose an association between the acquisition of androgenic competence and UPS-mediated protein degradation. In line with this, we hereby propose an extension of this association no only to proteolysis, but also to autophagy, which would account for the extensive degradation of cytoplasmic material associated to the androgenic switch.

Excretion of autophagosomal cargo appears essential for proper microspore embryogenesis

Perhaps, the most intriguing observation presented in this work relates to the abundant material present between the plasma membrane and the cell wall of embryogenic microspores, and not of microspores before induction, pollen-like microspores, non-embryogenic microspores, or MDEs. We observed many autophagosomes and small vacuoles fusing with the plasma membrane, and exposing their luminal contents to the cell wall, where massive amounts of fibrillar and membranous material were identified. These evidences are clearly divergent from the normal dynamics of autophagosomes. Usually, the final fate of plant autophagosomes is to fuse with lytic vacuoles, the major site for the degradation and recycling of autophagosomal cargo (Bassham, 2009).

Alternatively, autophagosomes may be functionally self-sufficient to digest their cargo in an autonomous manner, or may also fuse opportunistically to other autophagosomes at different stages of their digestive process (Rose *et al.*, 2006). In this work, we provided ultrastructural evidences of the occurrence of these processes in *B. napus* embryogenic microspores (Supplemental Fig.6, yellow arrows). Moreover, we also showed that at least some of the compartments involved in the lytic pathway (including autophagosomes, small vacuoles and prevacuolar compartments) may be alternatively redirected towards the plasma membrane (Fig. 6, red arrows), thus truncating their conventional dynamics. Upon membrane fusion, autophagosomes would release their partially degraded cargo, accounting for the abundant presence of fibrillar and membranous material in the cell wall. Then, a question arises as to

why autophagosome cargo is not recycled, but directly excreted out of the cytoplasm.

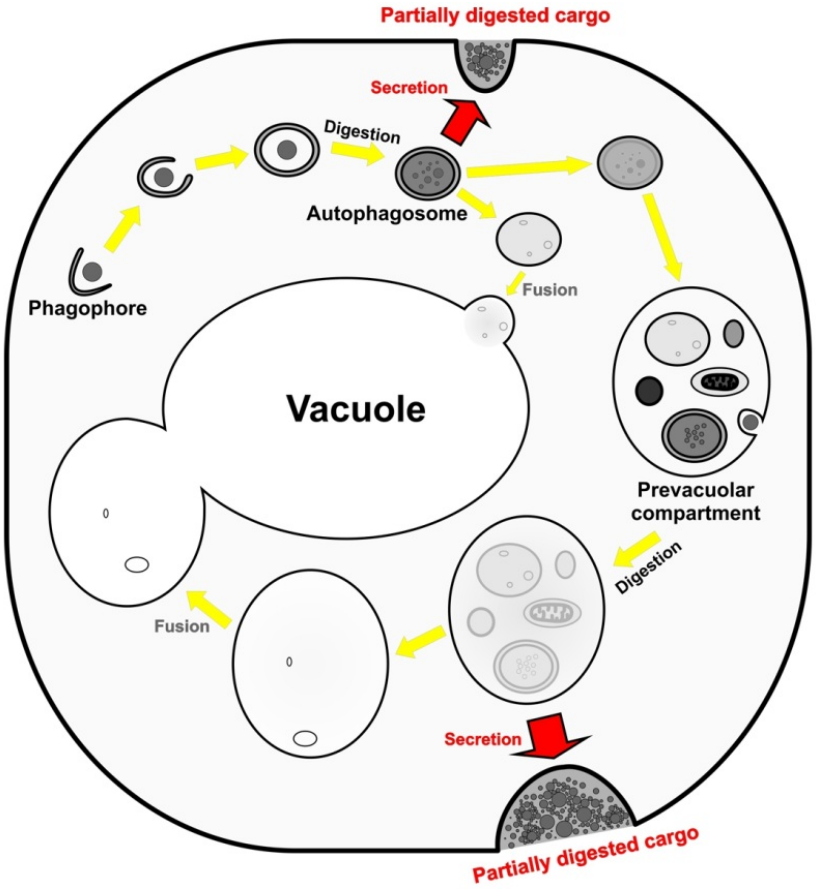

Fig. 6: Proposed model of cytoplasmic cleaning for *B. napus* embryogenic microspores. Cytoplasmic material is engulfed by phagophores and then embedded into autophagosomes. The autophagosomes may follow a conventional lytic pathway (yellow arrows), including digestion of cargo, fusion with lytic vacuoles or with other lytic compartments, and finally recycling of digested contents. In parallel, autophagosomes are deviated from this pathway and directed to the plasma membrane (red arrows), where they fuse and excrete their partially digested cargo to the cell wall.

In our opinion, the most reasonable hypothesis would imply that the cell diverts autophagosomes and prevacuolar compartments to fuse with the

plasma membrane, in order to prevent excessive growth of the vacuolar system and ensure a proper embryogenic development (Fig. 7). Fusion of all autophagosomes/prevacuolar compartments with lytic vacuoles would lead to the formation of several large vacuoles or one giant, central vacuole like those observed in non-embryogenic, few-celled microspores (Fig. 1D). Vacuoles swell by water uptake, mediated by the osmotic pressure exerted by the osmolites accumulated. Conventional fusion of all autophagosomes to vacuoles would contribute significant amounts of membrane, and also of different osmolites, which would cause excessive vacuolar growth. Either way, excessive vacuolation would arrest embryo growth, as we observed in non-embryogenic microspores (Fig. 1D). Such an arrest could be derived from the alteration of essential processes in this zygote-like cell type, such as the establishment of polarity through auxin gradients, the series of programmed divisions necessary to establish the embryo pattern, the formation of the mitotic spindle for caryokinesis or the assembly of the large phragmoplast machinery for cytokinesis.

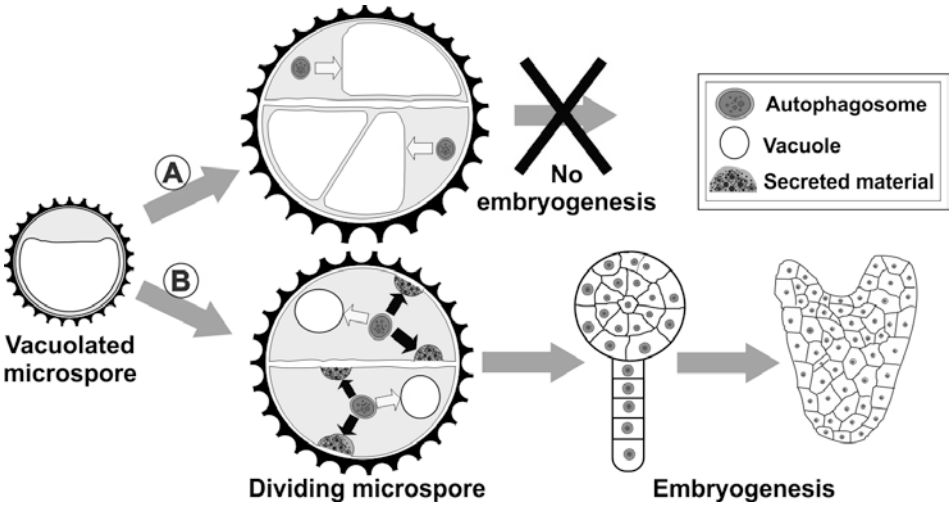

Fig. 7: Relationship of autophagy and excretion with microspore embryogenesis. Vacuolated microspores exposed to the inductive treatment may produce two types of dividing microspores, depending on how they recycle their cytoplasm. Those following the A route produce autophagosomes that follow the conventional lytic pathway (white arrows). This pathway will end up with fusion of vacuoles, digestion, and recycling of the vacuolar content. Such fusion will induce massive vacuolar growth, incompatible with progression of embryogenesis. Those following the B route produce some autophagosomes that follow the conventional lytic pathway (white arrows),

and also others that are diverted towards the plasma membrane (black arrows), in order to fuse with it and excrete their cargo to the cell wall. This way, the cell can retain their proliferative architecture, and embryogenesis progression can continue.

In Arabidopsis meristem cells, the volume of the interphasic vacuolar system is dramatically reduced to nearly 80% of the initial volume to accommodate the phragmoplast microtubule array and associated cell plate-forming structures (Seguí-Simarro and Staehelin, 2006). Conceivably, keeping a reduced vacuolar volume must be essential in embryogenic microspores as well. Vacuole swelling would also displace the division plane, giving rise to asymmetric cells, and inducing an undesired increase in cell size. It is known that cell expansion driven by growth of the central vacuole is a clear sign of cell differentiation. This is consistent with the fact that starch deposits (amiloplasts), known markers of pollen differentiation, were frequently observed in non-embryogenic type of divided microspores (Fig. 1D). Therefore, excretion would be a "safety system" to prevent vacuolar growth and the subsequent transformation of the architecture of early embryogenic microspore cells. Non-excreting cells would recycle their contents and in parallel, would give rise to large, vacuolated and differentiated, non-embryogenic microspores that will eventually stop their growth and die (Fig. 7).

HPF/FS emerges as the best approach to study the subcellular changes associated to the androgenic switch

The present study is not the first focused on the ultrastructural changes associated to induction of microspore embryogenesis. Indeed, different groups followed this approach during the past and the present century (see introduction), providing numerous and valuable results that helped to understand the complexity of this process. However, only few of the results presented in this work have been described before, and in an occasional manner. This is the case of extracytoplasmic vesicles and dense deposits. In *B. napus*, Telmer *et al.* (1993) described that *"cultured embryogenic microspores contained electron-dense deposits at the plasma membrane/cell wall interface, vesicle-like structures in the cell walls and organelle-free regions in the cytoplasm"*. In *Nicotiana tabacum*, Rashid *et al.* (1982) mentioned the presence of *"electron dense inclusions"* in cell walls. Similarly, in *Capsicum annuum* González-Melendi *et al.* (1995) found "*electron opaque deposits in the cytoplasmic vacuoles"* that remained after several cell cycle divisions.

Telmer *et al.* (1995) showed that the presence of such deposits was reversible, since they were absent in *B. napus* proembryos. However, none of these works provided an explanation to their occurrence, possibly due to the lack of reliable evidences pointing to their excretion from the cytoplasm. Aside of these, to our knowledge there were no other previous evidences of the structures and processes described in this work. So the question arises as to why they have not been consistently reported before in literature.

In our opinion, the answer to this relies on the methodology used for sample processing. From the pioneering studies to the most recent reports, all of our current knowledge of the ultrastructural changes undergone by androgenic microspores is derived from studies of chemically fixed cells. Up to now, HPF/FS methods have never been applied to the study of the androgenic switch. As mentioned in the introduction, the most limiting aspect of chemical fixatives is their inability to preserve membranous elements in a reliable manner. An illustrative exercise to realize how HPF/FS may improve the preservation of cell membranes can be done by comparing the appearance of the membranous elements shown in this work (Golgi stacks, tonoplast, ER, nuclear envelope, plasma membrane, etc.) with those from the studies mentioned above. Therefore, it is reasonable to think that in previous studies, many of the new structures hereby described were not observed simply because they were not properly preserved. It is likely that many other membranous structures were previously observed, but due to the effect of chemical fixatives on membranes, they were not considered as real structures, but as artifacts. Conceivably, these "artifacts" and the cells containing them would have been just dismissed. This could well be the case of autophagosomes, never reported in TEM studies of embryogenic microspores despite that TEM is the most reliable method to detect them (Chung, 2011). For example, the works of Dunwell and Sunderland (1975) and Sunderland and Dunwell (1974) in *Nicotiana tabacum* embryogenic microspores described the occasional presence of lysosomes, but their pictures also showed extensive vesiculation of the cytoplasm and profiles suggestive of fusion of large vesicles with the plasma membrane. They could well correspond to the different autophagosomes and vacuolar compartments hereby described. Similar works in *B. napus* by Telmer *et al.* (1993, 1995) showed many vacuole-like structures, filled with substances of variable electron density, and many of them with membranous and multilamellar remnants, but no specific mention was made about them.

Thus, we could conclude that the identification of the structures and processes shown in this work has been possible thanks to the use of cutting-edge technologies for ultrastructural preservation such as HPS/FS. These methodologies have been previously applied to the study of natural processes in a broad spectrum of plant tissues, with remarkable success. However, their use in a complex experimental system such as *in vitro* induction of microspore embryogenesis appears especially useful. The results presented hereby are just an example of their possibilities. Hopefully, they will open the door for further revisions of the ultrastructural changes undergone by the reprogrammed microspore. In parallel, the application of this approach to the study of microspore embryogenesis in recalcitrant species may help to elucidate the cellular basis of such recalcitrance.

Concluding remarks

In this work we have established a clear link between autophagy, excretion, cytoplasmic cleaning, and induction of microspore embryogenesis. It appears that for a successful induction, cells must eliminate all the damaged or useless (gametophytic) machinery, either before triggering of the embryogenic program, or simultaneously as proposed by Malik *et al.* (2007). According to the accepted role of autophagy as a housekeeping process for the breakdown of damaged or unwanted cellular components, the digested and excreted material would include remnants of incomplete or defective cell plates frequently found in embryogenic microspores, and protein aggregates derived from heat shock exposure. Accordingly, large multilamellar bodies would derive from the digestion of organelles damaged by heat shock. Alternatively, the excreted material could also include specific proteins and macromolecules initially destined to pollen differentiation, but no longer needed in the new, embryogenic scenario. This would be consistent with the recent view of autophagy as a highly selective process mediated by recruitment proteins that tether specific cargo to the enveloping autophagosomes (Li and Vierstra, 2012). In summary, autophagy and excretion would be essential processes in the transition towards embryogenesis. Future research should focus on the molecular mechanisms by which embryogenic microspores alter their conventional autophagic pathway. In other words, the analysis of the expression of autophagy-related genes (ATG8, etc.) during these stages would surely help to elucidate the molecular basis of this process.

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Capítulo 5

An efficient method for transformation of pre-androgenic, isolated *Brassica napus* microspores involving microprojectile bombardment and *Agrobacterium*-mediated transformation

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Abstract

The physical barrier imposed by the thick microspore wall constitutes an obstacle for a efficient *Agrobacterium*-mediated transformation of vacuolate microspores prior to androgenic induction and haploid embryogenic commitment. It is thus necessary to implement additional methods to overcome this drawback. In this work, we focused on the optimization of a protocol to allow for the exogenous DNA to enter the microspore in a efficient manner. We tested different options, based on microprojectile bombardment, to be applied prior to agroinfiltration. From them, the best results were obtained through co-transformation by microspore bombardment with DNA-coated microprojectile particles, followed by *Agrobacterium tumefaciens* infection. This method provides an efficient means to integrate extraneous DNA into rapeseed microspores prior to androgenesis induction.

Introduction

In doubled haploid technology, microspores are particularly attractive as targets for genetic transformation, due to their haploid and single-cell nature. Through the application of a wide range of stressing agents, a haploid microspore can be deviated to become a haploid androgenic embryo and, upon chromosome doubling, eventually a doubled haploid plant (Gu et al. 2004; Gu et al. 2003; Seguí-Simarro and Nuez 2008a; b; Zhang et al. 2006a; Zhang et al. 2006b; Zhou et al. 2002a; Zhou et al. 2002b). Transformation of microspores prior to androgenesis induction has the potential to avoid not only hemizygosis but also chimerism, generating embryos and plants where all of their cells express the introduced transgene in homozygosis (Dormann et al. 2001). However, the different attempts to produce transformed microspores have yielded variable results in terms of transformation efficiency. Some authors have reported a relative success for transient and stable integration of introduced marker genes into the microspore/pollen grain genome via different methods such as electroporation (Obert et al. 2004), microinjection (Jones-Villeneuve et al. 1995), microprojectile bombardment (Fukuoka et al. 1998; Jardinaud et al. 1995; Nehlian et al. 2000; Stoger et al. 1995) and infection with *Agrobacterium* (Dormann et al. 1998; Dormann et al. 2001; Pechan 1989), but a reliable, efficient and general protocol is still to be presented.

Among the different procedures used for transformation, the most efficient and widely used is infection with *Agrobacterium* (agroinfiltration). Agroinfiltration has been extensively used to transform a variety of plant tissues and species (Sharma et al. 2005). Additional treatments for physical wounding by sonication, silicon carbide, sand (Singh and Chawla 1999); or microprojectile bombardment (Bidney et al. 1992) prior to agroinfiltration have proven to enhance the efficiency of *Agrobacterium*-mediated transformation. However, agroinfiltration of microspores or pollen grains has been for long reported as extremely difficult if not impossible (Potrykus 1991; Sangwan et al. 1993). The thick cell wall of the microspores has been traditionally considered as the main obstacle for *Agrobacterium* to penetrate the cell, and published alternatives to overcome this physical barrier are limited (Dormann et al. 2001). Therefore, the development of additional or alternative strategies to effectively introduce transgenes still deserves attention.

Direct gene transfer by microprojectile bombardment (biolistics) seems the best method to surpass the microspore wall barrier and reach the cell nucleus (Klein and Fitzpatrick-Mcelligott 1993; Sharma et al. 2005). However, biolistics is known to be less efficient than *Agrobacterium*-mediated gene transfer (Sharma et al. 2005). Thus, it would be desirable to combine the advantages of both techniques into an integrated protocol for microspore transformation. In this work, we focus on the development of an strategy to overcome the difficulties imposed by the microspore wall to the entry of exgenous DNA for microspore transformation. We report on a efficient method to transform *Brassica napus* microspores using an integrated bombardment and agroinfiltration system that combines the advantages of both methods in terms of delivery and expression of exogenous DNA, and generation of microwounds to enhance the efficiency of bacterial infection.

Materials and methods

Plant material

Brassica napus cv. PF_{704} plants were used as donor of microspores, grown in a growth chamber with a 16 h photoperiod (300 µE m^{-2} s^{-1}) and 15/10 ˚C day/night temperature.

Vectors and preparation of bacteria

A pBGWFS7-64 binary vector (kindly provided by Dr. Kim Boutilier, Plant Research International, Wageningen, The Netherlands) carrying the GUS reporter gene under the control of the pollen-specific p28640 promoter (AT2G28640, Exo70 gene family), was used for both microprojectile bombardment and infiltration with *Agrobacterium tumefaciens* (strain LBA4404).

A. tumefaciens strain LBA4404 harboring the binary vector pBGWFS7-64 was taken from a single colony and grown at 28°C for 24 h in LB medium (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, pH 7.5) supplemented with 50 mg/l rifampicin and 100 mg/l streptomycin, under continuous shaking. Finally, bacteria were centrifuged and suspended in liquid NLN-13 medium at different concentrations depending on the experiment (see results).

Preparation of microspores and transformation procedure

Prior to transformation experiments, microspores were isolated, centrifuged and resuspended in NLN-13 medium according to (Seguí-Simarro et al. 2003) For bombardment, 100 µL culture droplets were deposited onto polyester filters over solid NLN medium with different concentrations of sucrose and mannitol. Bombardment was performed either with DNA-free or DNA-coated gold particles of different sizes, using a Bio-Rad PDS-1000/He biolistic particle delivery system. After bombardment, microspores were transferred to liquid NLN medium with the same sugar and mannitol concentration used for bombardment, and 0.5 ml of a suspension of *A. tumefaciens* at different concentrations was added. Then, they were incubated for 24 h at 28 °C in darkness, treated with carbenicilin, washed and analyzed for GUS expression.

GUS histochemical assay

Microspores were analyzed for GUS expression using a procedure modified (Jefferson et al. 1987). The substrate buffer contained 200 mM $NaPO_4$ buffer (pH 7.0), 1 mM $K_3[Fe(CN)_6]$, 1 mM $K_4[Fe(CN)]_6$, 20 mM EDTA, 13% sucrose and 0.1% (w/v) X-GLUC (5-Bromo-4-Chloro-3-Indolyl-D-glucuronic acid). For each sample, 0.5 ml of the substrate buffer was added to 3 ml of solid NLN-13 medium in 10×35 mm Petri dishes. Samples were then placed on top

and incubated for 4 h or 24 h at 37°C. Finally, samples were placed at 4°C until counting and analysis.

Results and discussion

First, we set up the conditions for microprojectile bombardment over rapeseed microspores. We tested three critical parameters previously described as strongly influencing the efficiency of transformation in other systems (Folling and Olesen 2001; Jardinaud et al. 1995; Vain et al. 1993), including the amount of microspores to be bombarded in each shot, the size of the gold microprojectile particles, and the effect of different concentrations of osmoticum in the bombardment medium (Table 1). Our results showed that transformation efficiency is strongly dependent upon microspore density in the bombardment medium, with an optimal density of 10^5 microspores in the 100 µl of the bombarded sample. It is easy to conceive that the density of microspores must have a critical influence on the rate of transformation, since only those microspores of the top layer would be exposed to the gold particles. Thus, it is essential to maximize the number of exposed microspores, but always keeping them distributed as a monolayer (Figures 1B, C). Increasing microspore density beyond 10^5 microspores/100 µl had a negative effect, and similar for the assays with $2x10^5$ microspores/100 µl and those with $3x10^5$ microspores/100 µl. This suggests that higher densities have an additional detrimental influence on microspore viability after bombardment.

Particle size was found to have an effect on the efficiency of transformation. The best results were achieved with a combination of particles of 0.6 and 1.0 µm. This combination clearly overperformed other particle sizes, either combined or alone. The combined use of two gold particle sizes has been proven useful for microspore transformation in wheat (Folling and Olesen 2001). As for wheat, it is likely that in rapeseed highly-sized particles may carry more DNA but could irreversibly damage the microspore, whereas smaller particles are capable of transferring the vector into the nucleus without compromising cell viability.

Table 1. Optimization of parameters for particle bombardment

Parameters	GUS-positive microspores
No. of bombarded microspores	
10^5	530.0 ± 36.56*
$2x10^5$	328.3 ± 34.04
$3x10^5$	325.6 ± 23.81
Particle size (µm)	
0.6	390.3 ± 18.70
1	468.0 ± 42.25
1.6	144.7 ± 31.69
0.6 + 1	576.7 ± 35.18*
0.6 + 1.6	266.3 ± 37.12
1 + 1.6	321.3 ± 25.98
Bombardment medium	
13% Sucrose	514 ± 11.3
6.5% Sucrose + 0.2 M mannitol	672 ± 27.5*
13% Sucrose + 0.2 M mannitol	653 ± 38.6*
13% Sucrose + 0.4 M mannitol	340 ± 42.2

* significance at $p < 0.001$

The presence of additional osmotic agents was also proven positive for transformation efficiency. Whereas different sucrose concentrations did not affect the efficiency, a moderate concentration (0.2 M) of mannitol increased the percentage of GUS-expressing microspores independently of the concentration of sucrose used. Several reports support the notion of a beneficial effect of increased osmolarity before and during bombardment, in the enhancement of transgene expression (Vain et al. 1993). It was previously hypothesized that a mild plasmolysis of the bombarded cells may prevent the cell contents from escaping through the microholes created by bombardment. In our rapeseed microspore system, this effect would be achieved by a 0.2M concentration of mannitol, while higher concentrations would negatively affect cell viability.

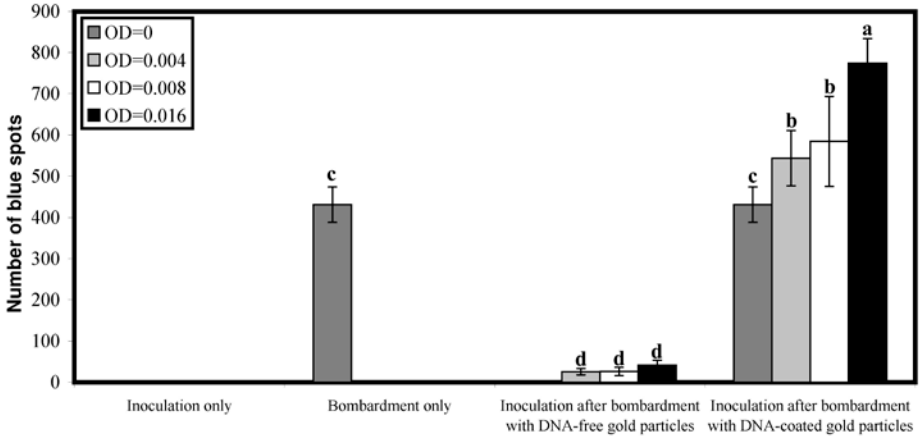

Figure 1. A. Effect on transient GUS expression of the use of agroinfiltration only, bombardment only with DNA-coated particles, integrated bombardment with DNA-free particles and agroinfiltration, and integrated bombardment with DNA-coated particles and agroinfiltration, at different *Agrobacterium* concentrations. Means with the same letter are not significantly different at p = 0.001.

After setting up the best conditions for bombardment, we compared the efficiency, in terms of transient GUS expression, of the optimized bombardment protocol with respect to agroinfiltration alone, agroinfiltration after bombardment with naked (DNA-free) gold particles, and agroinflitration with DNA-coated particles (Table 2). For all of the assays involving *Agrobacterium*–mediated transformation, we tested different bacterial concentrations. Figure 1 and Table 2 show the experiments using concentrations of 0 (control with no bacteria), 4×10^{-3}/100 µl (OD_{600nm}=0.004), 8×10^{-3}/100 µl (OD_{600nm}=0.008), and 16×10^{-3} /100 µl (OD_{600nm}=0.016). For concentrations higher than 16×10^{-3} /100 µl (OD_{600nm}=0.016; data not shown), the liquid nature of the medium for microspore and bacterial coculture, and the presence of high sucrose levels gave rise to excessive *Agrobacterium* proliferation and contamination of cultures.

Table 2. Assessment of different strategies for microspore transformation.

Transformation method	Density of Agrobacterium (OD_{600nm})	GUS-positive microspores

Agrobacterium only	0	0
	$4.x\ 10^{-3}$	0
	8×10^{-3}	0
	1.6×10^{-2}	0
Bombardment only, with DNA-coated particles.	0	431.0 ± 43.03
Bombardment (DNA-free particles) + *Agrobacterium*	0	0
	4×10^{-3}	25.7 ± 7.77
	8×10^{-3}	26.3 ± 10.21
	1.6×10^{-2}	41.7 ± 12.05
Integrated system: *Bombardment (DNA-coated particles)* + Agrobacterium	0	431.0 ± 43.03
	4×10^{-3}	543.7 ± 67.00
	8×10^{-3}	584.7 ± 109.26
	1.6×10^{-2}	774.3 ± 59.70*

*, significance at $p < 0.001$

At these levels of bacterial concentration, contamination was impossible to control even with high concentrations of carbenicilin. Therefore, we decided to focus on a range up to OD_{600nm}=0.016. In terms of GUS expression, bombardment with DNA-coated particles was clearly more effective than single agroinfiltration or agroinfiltration after bombardment with DNA-free particles, for all of the *Agrobacterium* densities tested (Table 2). Bombardment with DNA-bearing microprojectiles seems to be critical for a high rate of GUS expression. This indicates that just perforation of the microspore wall to allow for bacteria to easily access the microspore may not be enough for efficient transgene expression. The ability of gold particles to directly carry DNA fragments into the cell nucleus would allow for the observed high level of GUS espression. This notion can be confirmed by the comparison of the results of transformation with agroinfiltration after bombardment (Figure 1). When DNA-coated particles were used (Figure 2A), GUS-expressing microspores were more abundant than with DNA-free particles (Figure 2B). Although microperforation of the microspore coat is equivalent in both methods, particle coating provided a GUS expression level ~10-fold higher.

Despite of the fact that *Agrobacterium*-mediated transformation is a widely used method to transform a variety of plant tissues (Sharma et al. 2005), including *Brassica* tissues (Cardoza and Stewart 2004), its efficiency for microspore transformation is limited (this study; (Stoger et al. 1995). In parallel, it is known that mechanical wounding may increase the efficiency of *Agrobacterium*-mediated transformation (Hoshi et al. 2004; Singh and Chawla 1999). In this study we demonstrate that the generation of microholes with DNA-free particles is sufficient to allow for *Agrobacterium* penetration and to achieve an effective transformation of rapeseed microspores, otherwise very difficult if not impossible. Similar results were observed in sunflower meristems and tobacco leaves (Bidney et al. 1992), and in soybean(Droste et al. 2000). However, it must be noted that in our case, the difficulties imposed by the exine coat are higher than in somatic tissues. In this scenario, the generation of physical channels through the exine seems critical. Nevertheless, among all of our experiments, the best results were obtained with the combination of bombardment with DNA-coated particles, followed by infiltration with *Agrobacterium* (Figures 1, 2A), in a manner dependent on the bacterial density used (Table 2). This method has the potential to combine the advantages of the *Agrobacterium* transformation system with the high efficiency of biolistic DNA delivery. Besides, the addition of a controlled bacterial coculture step after bombardment does not seem to contribute any additional constraint to the viability of microspores. Although the combined transformation method reported hereby only shows transient transgene expression, we show that this approach is a promising method to incorporate to the experimental protocols for eventually obtaining transgenic, non-chimeric androgenic embryos and double haploid plants. In summary, this work sets up the conditions for an efficient transformation protocol. Based on this protocol, further improvements in both the viability, survival and development of microspores/embryos and the stability of transformation, can now be addressed.

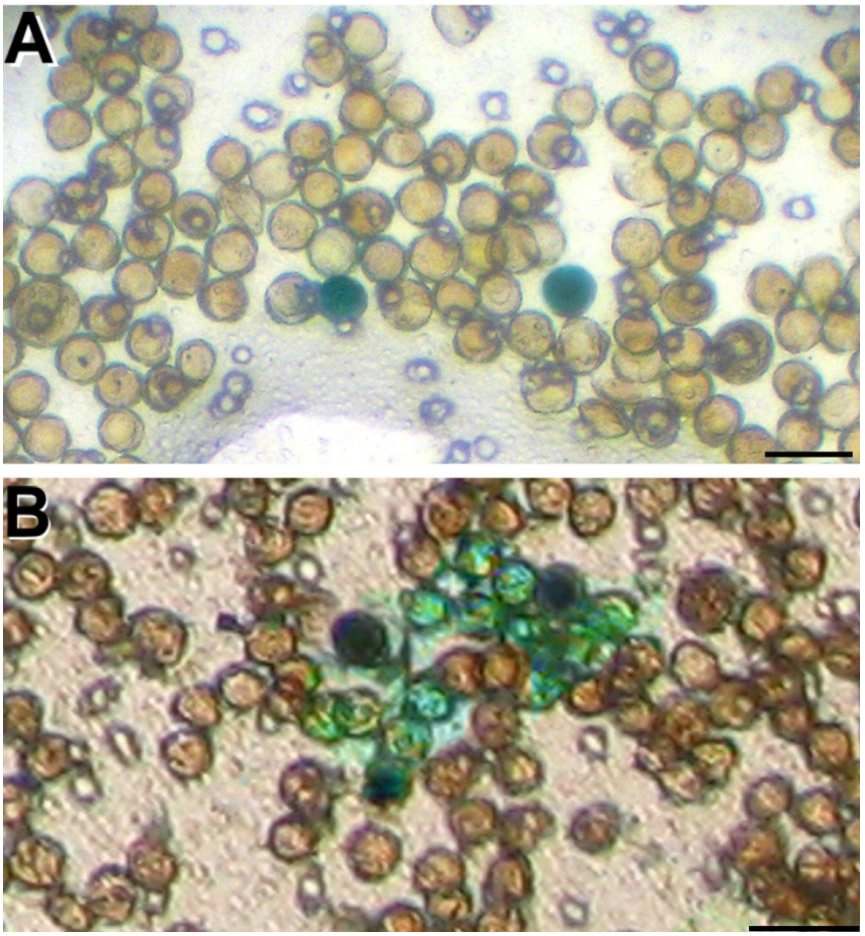

Figure 2: A. GUS-expressing microspores transformed by DNA-free bombardment + agroinfiltration. **B.** Microspores transformed by DNA-coated bombardment + agroinfiltration. Bars: 40 µm.

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Capítulo 6

Enhancing secondary embryogenesis in *Brassica napus* by selecting hypocotyl-derived embryos and using plant-derived smoke extract in culture medium

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Abstract

Induction of secondary embryogenesis on transformed androgenic microspore-derived embryos (MDEs) is a convenient approach to avoid chimerism and hemizygosis for the introduced transgene. In this work, we improved two aspects related to secondary embryogenesis in rapeseed (*Brassica napus* L. cv. Topas) MDEs: the identification of the best source of secondary embryos in the germinated MDE and the increase in the production of secondary embryos (SE). We performed a ploidy analysis of the different organs of MDE-derived plantlets by flow cytometry. Our results showed that 60% of the MDEs-derived plantlets were mixoploid, with 60% of them having different ploidies for different organs. We concluded that hypocotyl-derived SEs present in general higher levels of genome duplication, which makes them a source of SEs better than cotyledons in terms of genetic stability and avoidance of hemizygosis. In order to increase production of SEs, we used plant-derived aqueous smoke extracts. The aim was to verify whether these extracts have a positive effect on secondary embryogenesis and if so, to identify the most efficient conditions of use. We tested smoke extracts at different incubation times, concentrations and methods of application to MDEs. The use of smoke extract, either prior to or during germination of MDEs, markedly enhances secondary embryogenesis. The best results were obtained with the use of smoke extract as a pretreatment, incubating MDEs for no longer than 15 minutes with a 1:250 extract concentration.

Introduction

Secondary embryogenesis, also known as repetitive embryogenesis, usually occurs when single epidermal cells of primary embryos, most commonly from the cotyledons or hypocotyl, enter embryogenesis and give rise to successive cycles of embryos, known as secondary embryos (SE) (Merkle et al. 1990). In the last two decades, this process has been described in a number of angiosperm and gymnosperm species (Raemakers et al. 1995) including cereals (Eudes et al. 2003), carnation (Karami et al. 2008), *Alyssum borzaeanum* (Paunescu 2008) or *Brassica napus* (Abdollahi et al. 2011a; Abdollahi et al. 2009a; Macdonald et al. 1988; Nehlin et al. 1995; Yadollahi et al. 2011). As an experimental, inducible system, secondary embryogenesis has several ad-

vantages over primary somatic embryogenesis, including a higher multiplication rate, less dependence from explants sources and higher repeatability (Raemakers et al. 1995). Furthermore, it has been shown in many species that the efficiency of explants for secondary embryogenesis is higher than for primary embryogenesis (Raemakers et al. 1995). Combined with other inducible embryogenic processes, such as somatic embryogenesis or androgenesis, cyclic secondary embryogenesis has the potential to maintain embryogenic competence for a prolonged period. Haploid lines can be multiplied and maintained by secondary embryogenesis, which constitutes an additional advantage of this process.

However, one of the main advantages of secondary embryogenesis relates to its use in combination with genetic transformation and androgenesis. Androgenesis is a powerful experimental tool to obtain 100% homozygous, doubled haploid lines through microspore culture (Seguí-Simarro 2010). Once isolated and induced, microspores develop into haploid microspore-derived embryos (MDEs), which will finally give rise to doubled haploid MDEs upon chromosome doubling (Seguí-Simarro and Nuez 2008). Transformation of haploid MDEs is a convenient approach to avoid hemizygosis, since the necessary step of genome doubling will fix transgenes, together with the rest of genes, into a homozygous status. However, protocols for transformation of haploid MDEs have as a side consequence the occurrence of chimerism, where transformed and non transformed cells coexist in the same MDE (Abdollahi et al. 2009a). In this context, secondary embryogenesis may be induced to obtain SEs from the MDE. The epidermal and single-cell origin of SEs makes them ideal tools to obtain fully transformed, 100% homozygous individuals through induction of several cycles of secondary embryogenesis under selective conditions over the surface of transformed chimeric embryos (Raemakers et al. 1995).

This combined approach has the potential to improve the efficiency of plant transformation in agronomically interesting crops such as rapeseed (*Brassica napus*). Rapeseed is one of the most important oilseed crops worldwide and a classic model system for the study of androgenesis. Indeed, large amounts of haploid MDEs and plantlets can be obtained through different protocols for microspore culture (Ferrie and Möllers 2011). Rapeseed MDE cells have the capacity to develop directly into SEs in response to induction signals (Abdollahi et al. 2011a; Abdollahi et al. 2009a; Macdonald et al. 1988; Nehlin et al. 1995; Yadollahi et al. 2011). Thus, it is not surprising that combined

induction of androgenesis, MDE transformation through microprojectile bombardment and secondary embryogenesis has been successfully used to obtain transformed rapeseed MDEs and plants (Abdollahi et al. 2011a; Abdollahi et al. 2009b; Chen and Beversdorf 1994; Raemakers et al. 1995). However, two of the current bottlenecks of this approach are (1) the genetic stability of MDE cells induced to develop SEs and (2) the efficiency of the treatments to induce secondary embryogenesis.

With respect to the first of these bottlenecks, it is necessary to know the ploidy level of the MDEs used as primary embryos. Since mixoploidy is a phenomenon whose occurrence has been widely described during androgenesis (Corral-Martínez et al. 2011; Dunwell 2010; Salas et al. 2011; Seguí-Simarro and Nuez 2008), it is also important to know to what extent mixoploidy may constitute a drawback in this system. As occurs with SEs originated from non-transformed cells of chimeric embryos, it would not be desirable to obtain SEs originated from haploid, triploid or tetraploid cells of mixoploid embryos. Therefore, the first aim of this study was to analyze the ploidy of germinated MDEs used as sources of SEs, both at the level of the whole embryo and specifically in the different organs where secondary embryogenesis could be induced. With respect to the second bottleneck, we have focused on the improvement of the protocol to induce the production of SEs through the use of plant smoke extracts. Plant-derived smoke (or aqueous extracts from smoke) is able to stimulate different growth and developmental processes such as flowering in *Cyrtanthus ventricosus* (Keeley 1993), *in vitro* root growth in *Solanum lycopersicum* (Taylor and Van Staden 1998) root initiation and development in the hypocotyls of *Vigna radiata* (Taylor and Van Staden 1996), or seed germination, vigor and survival in many different plant species, including not only those from fire-prone environments but also those with fire-insensitive seeds (Light et al. 2009) and references therein). In addition, plant-derived smoke has been involved with somatic embryogenesis in different species, including *Pelargonium hortorum* (Senaratna et al. 1999), *Pinus wallichiana* (Malabadi and Nataraja 2007), and *Baloskion tetraphyllum,* where these stimulatory effects have been also observed on zygotic embryos (Ma et al. 2006). In view of all these previous indications of a important role of smoke extracts in growth processes, including somatic embryogenesis, the second goal of this work was to evaluate the effect of plant-derived smoke extracts on the promotion of secondary embryogenesis from *Brassica napus* MDE-derived plantlets.

Materials and methods

Plant material

Seeds of *B. napus* L. cv. Topas were sown in 25 cm pots filled with greenhouse soil mixture. Pots were placed in a growth chamber under a 16/8 h photoperiod, a light intensity of 300 µmol m^{-2} s^{-1}, and a day/night temperature of 15/10°C.

Microspore culture and MDE germination

Flower buds of 2.5-3.5 mm (mostly containing vacuolate microspores) were selected, surface sterilized with 5.25% sodium hypochlorite for 5 min, washed three times in sterile distilled water and transferred to a blender and macerated to release the microspores in 30 ml of cold microspore isolation medium (distilled water with 13% sucrose, pH 6.0). Suspended microspores were successively filtered through 106 µm and 53 µm metal sieves, transferred to 50 ml conical tubes and centrifuged at 1270 rpm for 3 min. After discarding the supernatant, the pellet of microspores was resuspended in 5 ml of cold microspore isolation medium. This procedure was repeated twice for a total of three centrifugations and resuspensions, using for the last resuspension the final culture liquid medium, NLN-13, prepared according to (Lichter 1982). Before the last centrifugation step, microspore concentration was calculated using a hemacytometer, and then the required volume of NLN-13 medium was added to adjust suspension to a concentration of 4×10^4 microspores·ml^{-1}. Adjusted microspore suspension was distributed in sterile culture dishes, and induced to androgenesis by incubating at 30°C for 14 days in darkness. Then, cultures were transferred to 25°C, still in darkness and under a 40 rpm agitation regime in a orbital shaker. After 35 days, MDEs at the cotyledonary stage were collected and transferred for germination to 100×15 mm culture dishes containing 20 ml of germination medium, which consisted on B5 medium (Gamborg et al. 1968) supplemented with 0.1 mg·l^{-1} gibberellic acid, 20 g·l^{-1} sucrose and 8 g·l^{-1} agar, pH 5.7.

Flow cytometry

A total of 68 freshly germinated MDE-derived plantlets were used for ploidy analysis through flow cytometry. For each of the 68 plantlets, small pieces of radicle, hypocotyl, cotyledon and leaf tissue were chopped at 4ºC with a razor blade in 400 µl of nuclear extraction buffer (NEB) from the CyStain UV precise P kit from Partec GmbH (Münster, Germany). After incubating chopped tissue for one minute in NEB, 1.6 ml of DAPI-based staining buffer from the same kit was added and incubated for 2 more minutes. The extracted nuclear preparation was filtered through 30 µm CellTricks filters (Partec GmbH) and immediately analyzed in a Partec PA-I Ploidy Analyzer.

Preparation of smoke extract

An aqueous smoke extract was prepared from approximately 5 kg of burning leaf material from adult individuals of the medicinal plant *Tanacetum parthenium* L. (feverfew). Smoke extracts of this species were previously shown to act as strong promoters of germination and seedling growth in milk thistle *Silybum marianum L.* (Abdollahi et al. 2011b). Smoke was bubbled through a column of distilled water (500 ml) for 45 minutes (Baxter et al. 1994). A neutral fraction of smoke extract excluding weak and strong acids, as well as phenols, was prepared (Flematti et al. 2007). One ml of this neutral smoke extract was added to 9, 99, 249, 499, 999 and 1999 ml of distilled water to prepare, respectively, the following concentrations (v/v) of smoke extract: 1:10, 1:100, 1:250, 1:500, 1:1000 and 1:2000. These concentrations were used in the secondary embryogenesis experiments.

Secondary embryogenesis experiments

Different experiments were performed to determine the most convenient incubation time, concentration and method of application of smoke extracts, as described in Results. For each of the treatments analyzed in each experiment, a minimum of five replicates were included. Each replicate consisted of a dish with 10 randomly picked MDEs which upon germination gave rise to MD-derived plantlets, where SE took place. Each experiment was repeated three times. Induced SEs on each MDE-derived plantlet were scored and photographed under a binocular microscope (Nikon, Tokyo, Japan). The effect of smoke extracts on secondary embryogenesis was measured by means

of the following three parameters: (1) the percentage of responding MDEs, defined as the percentage from the total of MDE-derived plantlets that develop SEs; (2) the mean number of SE/MDE, defined as the mean number of SEs formed in those MDE-derived plantlets effectively induced to form SEs; and (3) the EFC index, a combination of the two previous parameters defined as follows: EFC = (mean number of SE/MDE) × (% of responding MDEs)/100. Data were subjected to standard analysis of variance using the Sigmastat software (Systat Software Inc.) and means were separated using a Tukey's test with $p \leq 0.05$.

Results

Induction of microspore embryogenesis and secondary embryogenesis

Upon establishment of microspore cultures, dividing (induced) microspores were first observed within the first week of culture, and suspensor-bearing MDEs appeared by the second week of culture (Figure 1A). As seen in Figure 1B, the MDE population present in a single culture dish after one month of culture was remarkably heterogeneous, covering the different stages of embryogenesis from globular to cotyledonary. However, only mature, cotyledonary MDEs (inset in Figure 1B) were ready to be transferred to germination medium. Transferred MDEs rapidly turned green and germinated into plantlets (Figure 1C), producing SEs at their surface after few days. As seen in Figures 1D-E, developing SEs were morphologically comparable to MDE and zygotic embryos, covering the different stages of embryogenesis including globular, heart-shaped, torpedo (Figure 1D) and cotyledonary (Figure 1E). Once developed, SEs germinated with no dormancy stage (Figure 1F), elongating and developing radicles similarly to MDEs (compare Figures 1F and 1C). SEs originated from the cotyledons and hypocotyl of germinated plantlets. However, the frequency of secondary embryogenesis was different for each organ. From 593 SEs studied, 419 (71% of the total) arose out of the hypocotyl, whereas 174 (29% of the total) arose out of the cotyledons. Thus, hypocotyl cells appeared considerably more prone to undergo secondary embryogenesis than any other part of the freshly germinated plantlet.

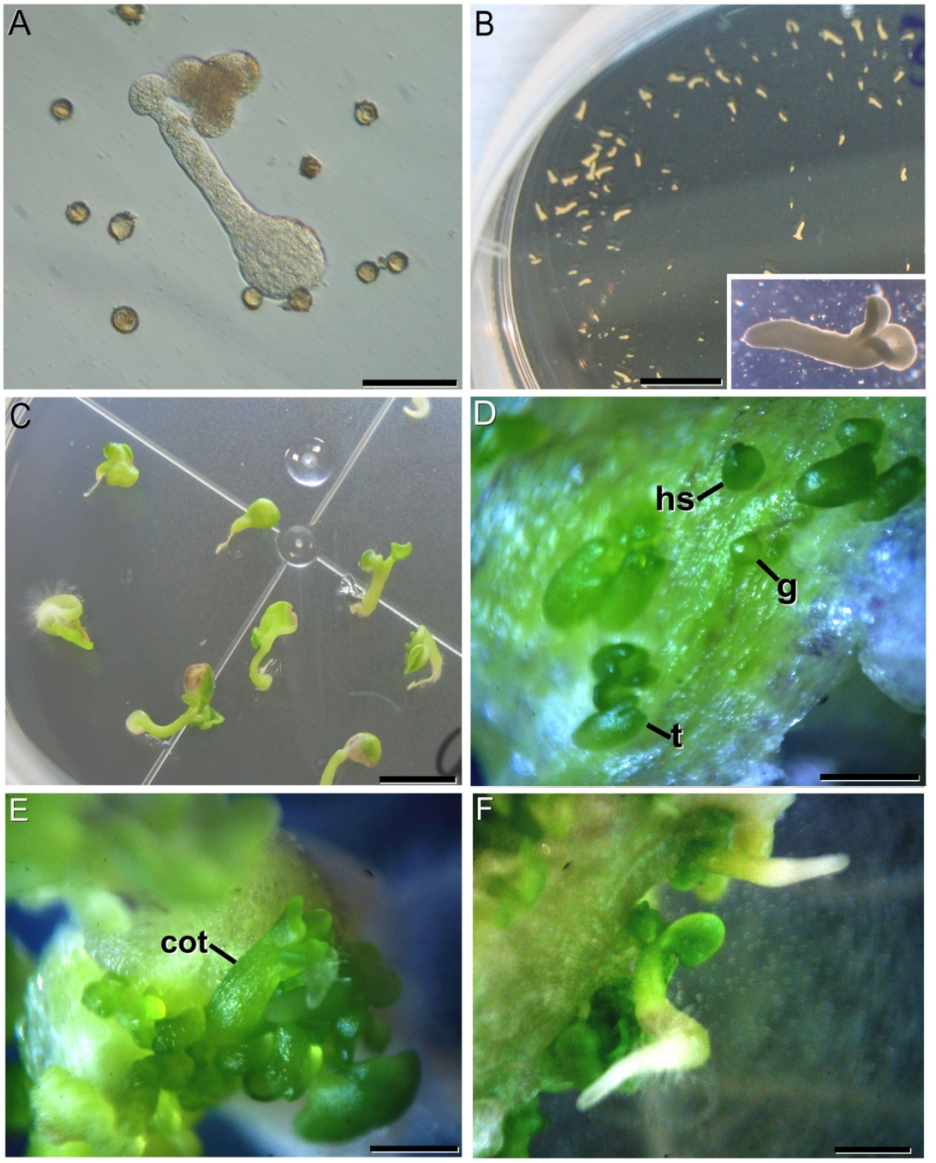

Figure 1: Microspore embryogenesis, germination of MDEs and secondary embryogenesis. A shows a young, globular MDE surrounded by several non-induced microspores, B: Partial view of a culture dish with MDEs at different developmental stages, including cotyledonary (inset), ready for germination. C: Germinating MDEs. D-F show different examples of SEs arisen over the hypocotyl and cotyledons of MDE-derived plantlets. These embryos passed through the same stages than

zygotic embryos, including globular (*g*), heart-shaped (*hs*), torpedo (*t*) and cotyledonary (*cot*). Note the similarity between the cotyledonary MDEs shown in inset of Figure B and the cotyledonary SE in Figure E. F: Germinating SE, still attached to the hypocotyl. Bars: A: 100 µm; B-C: 1 cm; D-F: 1 mm.

Analysis of ploidy levels of MDE-derived plantlets

We studied through flow cytometry the ploidy of 68 randomly chosen germinated plantlets derived from MDEs from five different microspore cultures. As seen in Table 1, nearly 40% of the total were euploids, with equal amounts of haploid and doubled haploid individuals. 60% of the germinated plants were mixoploids. Within mixoploid plants, 40% presented the same ploidies in all plant organs studied (cotyledons, hypocotyl, radicles and leaves). The most frequent mixoploidy observed was C+2C, present in ~93% of this type of mixoploids. The other type of mixoploids, constituting 60% of total mixoploids and 40% of all the plantlets studied, presented ploidy chimerism, with different ploidies in the different organs analyzed. As mentioned, we measured ploidies in cotyledons, hypocotyl, radicles and leaves, but for clarity purposes and considering that SEs only appeared in hypocotyls and cotyledons, we will only refer to these two organs, with their corresponding data.

Table 1 shows that from all the possible combinations of ploidies in cotyledons and hypocotyls, we only observed five, each of them with a different frequency. In this type of mixoploids, it was generally observed that cotyledons had cells with ploidies lower than in hypocotyls. For example, the most abundant combination (33.3% of these mixoploids) consisted of plantlets having 2C (doubled haploid) cotyledons, but a 2C+4C hypocotyl. Another interesting feature was that 70% of the mixoploid hypocotyls were associated to euploid cotyledons (37% C and 33.3% 2C). The remaining 30% of mixoploid cotyledons included only C and 2C cells, whereas mixoploid hypocotyls included cells of up to three different ploidies, from C to 4C. Indeed, tetraploid (4C) cells were found in ~80% of the mixoploid hypocotyls, whereas they were absent from cotyledons. In summary, 40% of the total of plantlets analyzed presented mixoploid hypocotyls, with 11.8% additionally presenting mixoploid cotyledons. All these results strongly suggested that hypocotyl cells are more prone to genome duplication than those of cotyledons. Conversely, cotyledonary cells would be more prone to remain in a haploid status.

Table 1: Flow cytometry analysis of ploidy levels of germinated MDEs. Results are expressed as partial percentages within each category and as percentages from total.

		Partial %	% from total
Euploids			**39.7%**
C (haploids)		48.1%	19.1%
2C (doubled haploids)		48.1%	19.1%
4C (tetraploids)		3.7%	1.5%
Mixoploids			**60.3%**
For all organs		**40%**	**20.6%**
	C+2C	92.9%	19.1%
	C+2C+4C	7.1%	1.5%
For some organs		**60%**	**39.7%**
Cotyledon	*Hypocotyl*		
C	C+2C	22.2%	8.8%
C	C+2C+4C	14.8%	5.9%
2C	2C+4C	33.3%	13.2%
C+2C	C+2C+4C	25.9%	10.3%
C+2C	2C+4C	3.7%	1.5%

Effect of pretreating MDEs with smoke extract

We focused on the improvement of SE production by using plant-derived smoke extracts. First, we used smoke extract diluted in distilled water as a pretreatment for MDEs before transference to germination medium. In order to find out the most adequate incubation time, we performed a preliminary experiment whereby MDEs were pretreated for 5, 10, 15 and 20 min with different smoke extract concentrations. A pretreatment for 20 min markedly affected MDEs, which reduced the production of SEs and lost their green color, adopting a whitish appearance clearly indicative of a negative effect of the exposure to smoke extract for 20 min (data not shown). Exposures of 15 min or less did not show these deleterious effects. Figure 2 shows

that for each concentration, results obtained with incubation times of 5, 10 and 15 min were similar for both the percentage of responding MDEs (Figure 2A) and the mean number of SE/MDE (Figure 2B). Tukey's test ($p \leq 0.05$) confirmed that for each concentration, results obtained with the three incubation times were not significantly different. As a result of this, we decided to fix incubation time to 15 min for the rest of experiments presented in this study.

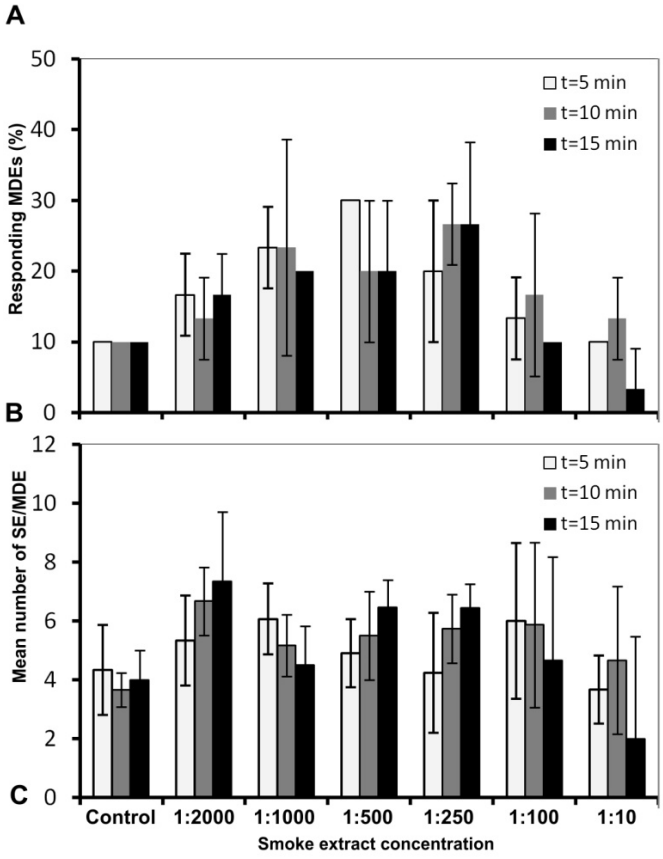

Figure 2: Effect of pretreating MDEs, prior to germination, with aqueous smoke extract at different combinations of time and concentrations. A shows the effect on the percentage of germinated MDE-derived plantlets undergoing secondary embryogenesis (*Responding MDEs %*). B shows the effect on the mean number of SEs produced per MDE-derived plantlet (*mean number of SE/MDE*). For each concentration, no significant differences were found among time 5, 10 and 15 min.

We next focused on the evaluation of the effect of pretreating MDEs with different concentrations of smoke extract (Figure 3). Pretreating embryos with smoke extract concentrations ranging between 1:2000 and 1:250 had a statistically significant positive effect ($p<0.05$) in the induction of secondary embryogenesis (Figure 3A). In other words, there were more MDEs producing SEs. This positive effect was highest with 1:500 and 1:250 extracts, which more than doubled the percentage obtained in control experiments. Concentrations higher than 1:250 were not significantly different than controls with no smoke extract. With respect to the mean number of SE produced in a single MDE, results were not as conclusive as for the previous parameter, with only one concentration (1:2000) showing significant positive differences (Figure 3B). By combining both parameters in the EFC index, a clearer outline of the effect of smoke pretreatments arose (Figure 3C). All concentrations between 1:2000 and 1:250 outperformed controls. Among them, 1:250 was the best with a EFC value 3-fold higher than controls. Thus, it could be concluded that secondary embryogenesis could be notably improved by pretreating MDEs with a smoke extract concentration of 1:250 for 15 min.

Effect of culturing MDEs with smoke extract

Next, we checked the effect of adding smoke extract to B5 germination medium, thus keeping MDEs continuously exposed to smoke extract. From the concentrations used in the previous experiment, we used the intermediate range where maximal response was observed, from 1:1000 to 1:100 (Figure 4). With respect to the percentage of responding MDE-derived plantlets (Figure 4A), the best concentration to apply to culture medium was found to be 1:100, which doubled the percentage observed in control experiments. Besides, a clear positive correlation was observed between extract concentration and percentage of MDEs forming SEs. A mean number of SE per MDE higher than in controls was observed for all the concentrations used (Figure 4B), with no significant differences among them. Finally, the combination of the two previous parameters into the EFC index evidenced a clearly positive and concentration-dependent effect of all concentrations, with a peak at 1:100, which was nearly 4-fold higher than controls (Figure 4C). Thus, it could be concluded that when applied to MDE germination medium, smoke extract is also beneficial, being 1:100 the best concentration to enhance secondary embryogenesis.

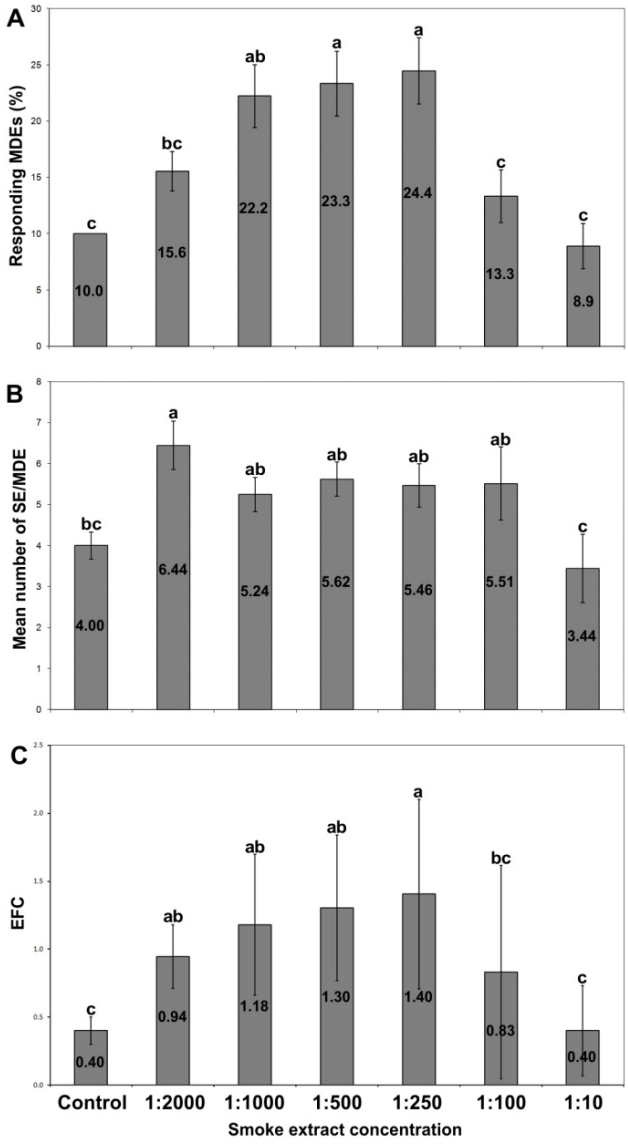

Figure 3: Effect of pretreating MDEs, prior to germination, with aqueous smoke extract at different concentrations. A shows the effect on the percentage of germinated MDE-derived plantlets undergoing secondary embryogenesis (*Responding MDEs %*). B shows the effect on the mean number of SEs produced per MDE-derived plantlet (*mean number of SE/MDE*). C shows the effect on the embryo-forming capacity index (*EFC*). Bars with same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

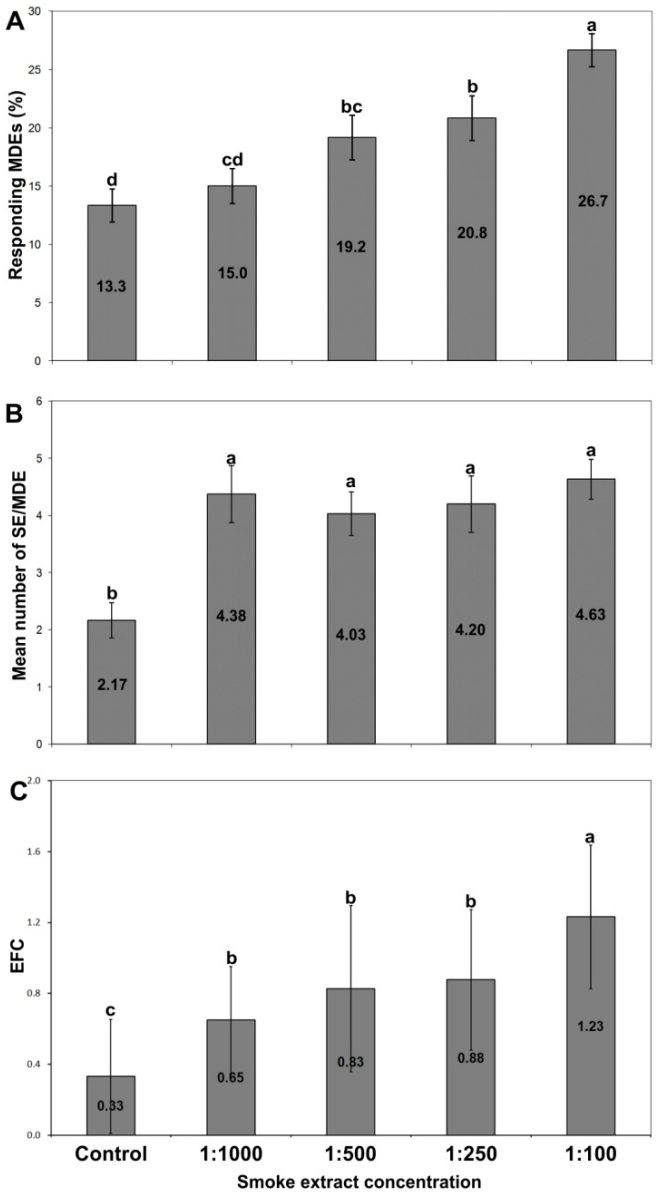

Figure 4: Effect of supplementing the germination medium with different concentrations of smoke extract. A shows the effect on the percentage of germinated MDE-derived plantlets undergoing secondary embryogenesis (*Responding MDEs %*). B shows the effect on the mean number of SEs produced per MDE-derived plantlet (*mean number of SE/MDE*). C shows the effect on the

embryo-forming capacity index (*EFC*). Bars with same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Comparison of different methods of application of smoke extract to MDEs

We compared the efficiency of the two methods of application of smoke extract (as a pretreatment and as a component of the germination medium), together with a third method of application, consisting on the apposition of a filter paper saturated with aqueous smoke extract (1:250) on top of culture dishes containing germination medium (Figure 5). As controls, we performed in parallel experiments completely excluding the use of smoke extracts. Our results showed that among the three ways to apply smoke extracts, the most efficient is the pretreatment of MDEs for 15 min. with water-based extract (1:250) prior to culture, as evidenced for the percentage of responding MDEs (data not shown), for the mean number of SE/MDE (data not shown), and for the EFC index (Figure 5). In terms of efficiency, pretreatment was followed by the addition of smoke extract to the germination medium, and finally by the apposition of a filter paper saturated with smoke extract, which showed levels of responding MDEs, mean number of SE/MDE (data not shown) and of EFC (Figure 5) not significantly different from controls. In order to identify any possible effect of using filter paper as a carrier of the smoke extract, we performed two parallel controls excluding and including filter paper on top of the medium. No differences ($p<0.05$) were found between them, which indicated that the absence of effect observed with the use of filter paper saturated with smoke extract was not due to the use of paper itself, but to the method used.

The positive effect of using smoke extracts was not only reflected in the efficiency of induction of secondary embryogenesis. In addition to this, the use of smoke extract, either as a pretreatment or a part of the germination medium, apparently had a positive effect in the development and maturation of induced SEs. In all experiments, SEs from MDEs exposed to smoke extract developed faster than those of controls, many of them reaching the cotyledonary stage in shorter time (data not shown).

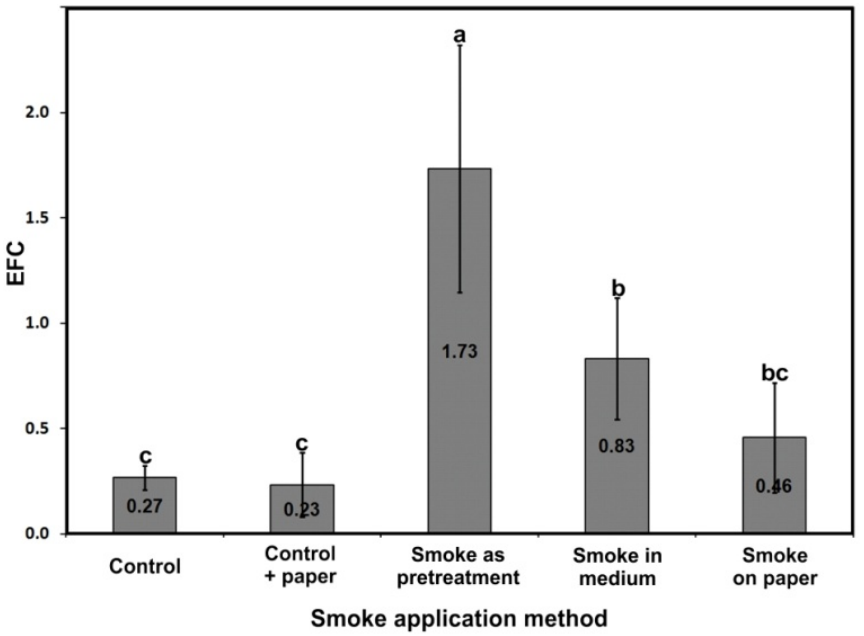

Figure 5: Effect of different methods for application of smoke extract on the embryo-forming capacity index (EFC). Methods consisted on: no application of smoke extract, either without (*control*) or with a filter paper on top of the germination medium (*control + paper*), application of smoke extract to MDEs as a pretreatment prior to germination (*smoke as pretreatment*), supplementation of the germination medium with smoke extract (*smoke in medium*) and addition of a filter paper saturated with smoke extract on top of the germination medium (*smoke on paper*). Bars with same letters are not significantly different ($p \leq 0.05$) according to Tukey's test.

Discussion

In rapeseed MDEs, hypocotyl is the best source of secondary embryos

In this work, we found that from rapeseed MDE-derived plantlets, nearly 70% of SEs arise from hypocotyl cells and 30% from cotyledons. Considering this, it is clear that the hypocotyl is the main source of SEs. But is hypocotyl the best source? It is widely known that in addition to doubled haploids,

haploid, triploid, tetraploid and even mixoploid individuals are frequent in *in vitro* androgenic systems, occurring in both microspore-derived calli and MDEs with variable frequencies (Corral-Martínez et al. 2011; Dunwell 2010; Salas et al. 2011; Seguí-Simarro and Nuez 2008). Since only doubled haploid or mixoploid MDEs (including doubled haploid cells) would be useful for our purposes, it is important to know to what extent mixoploidy and ploidy chimerism are present in rapeseed MDEs. Thus, we analyzed the ploidy of MDE-derived plantlets and of different SE-producing organs. We found that mixoploidy is frequent in MDE-derived plantlets, occurring in ~60% of them. Moreover, 20% of the total were mixoploid for all their organs, and 40% only for some organs (ploidy chimeras). Observing the ploidies of this latter chimeric 40%, we deduced that hypocotyl cells undergo genome duplication more likely than those of cotyledons. Thus, in order to avoid hemizygosis when transforming MDEs, it would be desirable to select hypocotyl-derived SEs, since the chances of obtaining a doubled haploid individual with fixed, homozygous transgenes will be higher. In other words, hypocotyl is the best source of SEs due to its higher rate of secondary embryogenesis and of chromosome doubling.

Pretreatment of MDEs with plant-derived smoke markedly enhances the secondary embryogenic response

In this work we showed that the use of plant-derived smoke extracts have a clear positive effect on the response of MDE-derived plantlets in terms of secondary embryogenesis, expressed as both number of MDE-derived plantlets producing SEs and number of SEs per MDE-derived plantlet. The stimulatory effect of plant-derived smoke on secondary embryogenesis, together with its well documented and similar effect on somatic embryogenesis (Malabadi and Nataraja 2007; Senaratna et al. 1999) points to the presence in plant-derived smoke of one or more bioactive compounds with a clear promoting role in embryogenic processes, among many others. One of these compounds has been previously isolated and identified in plant-derived smoke (Van Staden et al. 2004) and in cellulose (paper)-derived smoke as the butenolide 3-methyl-2H-furo [2,3-c] pyran-2-one (Flematti et al. 2004). Application of this butenolide to somatic and zygotic embryos within seeds confirmed its embryogenesis-stimulatory role (Ma et al. 2006). According to the role proposed by (Ma et al. 2006), bioactive compounds of smoke extracts would not be involved on embryogenesis induction, but rather on embryo growth, allowing for a faster maturation. In the particular caser of secondary embryogene-

sis, we also observed a faster maturation of SEs. However, while the percentage of SE-forming MDEs was highly dependent of concentration (Figures 3A, 4A), the mean number of SE/MDE did not show such a dependence, neither when applied as a treatment (Figure 3B) nor in the germination medium (Figure 4B). Thus, it was difficult to conclude whether the observed positive results are due, in addition to a faster growth and maturation, to a higher induction rate. Notwithstanding this, our results clearly indicate that, at least in rapeseed MDEs, smoke extracts promote embryogenesis, since more plantlets produced SEs and more SEs were produced per plantlet.

Among the different possible applications of smoke extract to germinating MDEs, the most efficient was a 15 min-long pretreatment of MDEs prior to germination with a 1:250 dilution. However, when applied as a part of the germination medium, the best results were observed with higher concentrations. In other words, when added to germination medium, a given concentration of smoke extract is less effective than when used as a pretreatment for MDEs. It appeared that the promoting effect of water-based smoke extract was stronger when used alone than when added to germination medium. Besides, when included in the germination medium, EFC and smoke extract concentration clearly showed a positive linear correlation (Figure 4C), whereas in the case of smoke extract applied as a pretreatment, correlation was not linear, with higher concentrations decreasing the EFC index to levels comparable to controls (Figure 3C). These facts are suggesting that smoke extracts may also include toxic substances with detrimental effects on embryogenesis, as also proposed by several authors (Light et al. 2009; Light et al. 2002; Senaratna et al. 1999). When smoke extracts are applied as a pretreatment, the stimulating effects would be more evident, but so would be the negative, deleterious effects. When added to the culture medium, the rest of compounds would somehow avoid such effects, possibly inactivating or buffering potentially toxic substances. Thus, germination medium would allow for the use of higher extract concentrations with no detrimental effects, but also with less stimulatory effects.

Concluding remarks

The results presented hereby shed light on the frequency of occurrence of genome doubling events in different parts of the rapeseed MDE, which have important implications on the SEs derived from them. In parallel,

our work demonstrated that smoke extracts have a clear and positive effect of the occurrence of secondary embryogenesis in *Brassica napus* germinated MDEs, and that the most efficient way to apply them is to pretreat MDEs with a 1:250 aqueous solution during a maximum time of 15 min, prior to transference of MDEs to germination medium. It is evident that the ideal scenario for *in vitro* applications like secondary embryogenesis would be to directly apply 3-methyl-2H-furo [2,3-c] pyran-2-one, responsible of embryogenesis promotion, instead of using smoke extract. However, obtaining this butenolide, either through chemical synthesis (Flematti et al. 2005) or isolating it from smoke (Flematti et al. 2004), may not be available for all laboratories or companies working on *in vitro* embryogenesis. Thus, the use of smoke extracts may be a quick, easy and inexpensive way to foster secondary embryogenesis in this and other *in vitro* systems. Together, our results have the potential to improve the production of doubled haploid SEs from rapeseed MDE-derived plantlets.

Acknowledgements

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Discusión general

Utilización de la tecnología de los doble haploides para la obtención de líneas puras

La aplicación principal de los doble haploides consiste en su utilización como líneas puras para la obtención de híbridos. Hay especies como maíz, colza, tabaco, arroz, trigo y cebada en las que pueden obtenerse fácilmente un número suficiente de embriones mediante cultivo de anteras y/o microsporas, permitiendo su utilización de forma rutinaria en programas de mejora. Por otro lado, también hay especies muy recalcitrantes para este proceso, que pueden ser muy interesantes desde un punto de vista científico o económico pero en las que no se ha conseguido obtener todavía una respuesta aceptable para una o ambas metodologías, o la eficiencia obtenida es muy baja. Este es el caso de algunas especies de la familia de las Solanáceas como la berenjena y el tomate (Corral-Martínez et al. 2010). Debido a la elevada importancia económica de ambas especies, sería muy interesante disponer de protocolos que permitieran la obtención de doble haploides de forma eficiente. En la presente Tesis Doctoral hemos abordado este objetivo, consiguiendo avances significativos en el conocimiento de las particularidades de este proceso en estas especies, y en la obtención de dichos protocolos, como veremos a continuación.

Obtención de doble haploides a partir del cultivo de anteras de tomate

En el caso del tomate, la eficiencia obtenida hasta el momento mediante cultivo de anteras ha sido extremadamente baja (Seguí-Simarro and Nuez 2007). Debido a la gran importancia económica de este cultivo, quisimos estudiar las posibles causas de la dificultad de obtener individuos doble haploides en esta especie, tan próxima a otras especies en las que se obtienen doble haploides a partir del cultivo de anteras de forma rutinaria (capítulo 1 de la presente Tesis Doctoral). Como material se utilizó una línea androestéril con la mutación *ms10*^{35}, que produce una mayor cantidad de callos comparada con las líneas fértiles (Seguí-Simarro and Nuez 2007). A partir de esta línea, se analizó el proceso de formación de callos en anteras cultivadas *in vitro* en estadio de meiocito. Los regenerantes obtenidos tras el cultivo fueron analizados mediante citometría de flujo, marcadores morfológicos y marcadores microsatélites para determinar su ploidía y su origen androgénico o somático. Tras analizar los resultados se observó un alto porcentaje de callos mixoploides y de origen somático. El estudio de las primeras etapas del cultivo *in vitro*

de las anteras demostró que los meiocitos contenidos en ellas sufren diversos procesos de fusión nuclear que son los responsables de la presencia de individuos mixoploides descrita no solo en esta Tesis, sino también en otros trabajos previos (Seguí-Simarro and Nuez 2007; Shtereva et al. 1998; Zagorska et al. 2004). Sin embargo, esta no parece ser la principal causa de que hasta ahora no se haya obtenido un protocolo eficiente de obtención de doble haploides mediante cultivo de anteras en tomate. En esta tesis hemos demostrado también que el tejido de las paredes de la antera es la principal fuente de aparición de regenerantes. Este hecho no es algo infrecuente, pues ha sido descrito en otras especies, entre ellas en otras solanáceas recalcitrantes como la berenjena y el pimiento (Parra-Vega et al. 2012; Salas et al. 2011). Sin embargo, en el caso del tomate este fenómeno adquiere un peso mucho más importante (83% de las plantas regeneradas). El análisis microscópico de las anteras reveló que en la línea androstéril la proliferación del tejido conectivo presente en el septo intralocular de la antera es la causa de la gran cantidad de regenerantes de origen somático observada. Esto reduce considerablemente la eficiencia del proceso, ya que esta elevada presencia de callos de origen somático junto con los provenientes de fenómenos de fusión, hacen que pueda darse una competencia por los recursos para el crecimiento de los callos. Esto queda reflejado en el hecho de que sólo el 7% de los regenerantes provengan de un único núcleo meiótico, y por lo tanto sean o acaben siendo doble haploides que puedan ser utilizados como líneas puras.

En definitiva, la fusión entre núcleos haploides y la proliferación del tejido de los septos interloculares de las anteras, estarían detrás de la gran cantidad (casi un 94%) de callos mixoploides y de origen somático obtenidos, dificultando así la inducción de androgénesis a partir de los meiocitos. Esto, junto con la dificultad de cultivar meiocitos *in vitro* explicaría la baja eficiencia en la obtención de doble haploides en tomate, descrita a lo largo de más de 40 años de estudio de este problema biotecnológico. También sería la causa de que hasta ahora no se haya podido trasladar esta metodología experimental a la producción a nivel industrial, ya que requiere la evaluación genética de cada regenerante individual para determinar su origen y ploidía, lo que hace muy costoso el proceso y ralentiza en gran medida el resultado final.

Podríamos concluir, por tanto, que el cultivo de anteras de tomate no es una vía eficiente de obtención de doble haploides y que presenta un margen de mejora muy limitado. Así pues, futuras investigaciones deberían apostar por otras vías, como el desarrollo de protocolos a partir de microsporas

aisladas, que eliminaría esta serie de limitaciones observadas. Hasta el momento solamente se ha conseguido inducir las primeras divisiones en estas microsporas pero no se ha podido promover su crecimiento más allá (Bal and Abak 2005; Seguí-Simarro and Nuez 2007), lo que da una idea de la recalcitrancia de la especie. Así pues, habría que continuar con este estudio para conseguir una mínima eficiencia en este proceso que nos permita obtener doble haploides en esta especie tan recalcitrante.

Otra vía, totalmente alejada del abordaje experimental utilizado en esta Tesis, y en todos los demás trabajos realizados hasta la fecha en tomate, sería la de tratar de aplicar la metodología diseñada por Ravi y Chan para la obtención de haploides y doble haploides en *Arabidopsis* (Ravi and Chan 2010), una especie tanto o más recalcitrante que el tomate. Hace tres años, estos investigadores desarrollaron un método por el cual es posible eliminar selectivamente todo el genoma haploide de uno de los parentales tras ser hibridados sexualmente. De este modo, se producen directamente individuos haploides uniparentales mediante semilla. En este procedimiento, un parental es manipulado genéticamente para alterar la histona centromérica CENH3, con el fin de reducir su capacidad de interactuar con la maquinaria mitótica de segregación cromosómica. Cuando los mutantes *cenh3* son hibridados con individuos silvestres, no mutantes (CENH3), el zigoto híbrido contendrá cromosomas con ambos tipos de histonas centroméricas (CENH3 y *cenh3*), pero aquellos cromosomas que contengan las silvestres (CENH3) se verán favorecidos durante la segregación cromosómica de la anafase mitótica. Dicho de otro modo, los cromosomas que contengan *cenh3,* proveniente de un parental, serán finalmente eliminados, y se producirá un individuo haploide, sin ningún tipo de modificación genética, y que podrá fácilmente convertirse en doble haploide mediante técnicas convencionales de duplicación cromosómica. Este innovador abordaje tiene la gran ventaja de que, al menos en teoría, podría ser aplicado virtualmente a cualquier especie, puesto que CENH3 se expresa de forma universal en todas las especies eucariotas. Se trata pues, de una tecnología claramente candidata a ser aplicada en genotipos recalcitrantes como el tomate. Tan solo quedaría el paso quizá más difícil: la traslación práctica de esta tecnología, desarrollada en *Arabidopsis*, al tomate. Es decir, la identificación de homólogos de CENH3 en tomate, su análisis funcional, la obtención de mutantes, y su uso para obtener los haploides.

Obtención de doble haploides a partir del cultivo de microsporas aisladas de berenjena

En berenjena es posible obtener desde hace ya décadas doble haploides mediante cultivo de anteras (Corral-Martínez et al. 2010; Kashyap et al. 2003; Rotino 1996; Rotino et al. 1987; Rotino et al. 2005; Seguí-Simarro et al. 2011), aunque como hemos descrito en la introducción, no sin una serie de limitaciones. Una de ellas es que el proceso para la obtención de doble haploides se ralentiza y encarece mucho al tener que analizar con marcadores moleculares cada regenerante obtenido para descartar un posible origen somático. Pese a las ventajas de la inducción de androgénesis mediante el cultivo de microsporas aisladas, la rentabilidad de esta técnica depende fundamentalmente de que se disponga de un protocolo de inducción de androgénesis y obtención de doble haploides optimizado. Por ello, el objetivo principal en los capítulos 2 y 3 de esta Tesis Doctoral fue poner a punto un protocolo a partir de microsporas aisladas que permitiera obtener líneas puras de forma eficiente. Debido a que uno de los factores que afectan en mayor medida a la inducción de androgénesis es el genotipo de la planta donante (Rotino 1996), en primer lugar se procedió a evaluar diferentes genotipos de interés para diversos programas de Mejora Genética en berenjena y se identificó el de mayor respuesta androgénica. Como punto de partida se ensayó el único protocolo completo publicado previamente (Miyoshi 1996), en el que se demuestra claramente el origen haploide de las plantas obtenidas. Partiendo de este protocolo, se incluyeron modificaciones en la metodología de aislamiento de las microsporas. A continuación se realizó un análisis del cultivo de microsporas y del proceso de regeneración a planta completa. El análisis de los primeros estadios se llevo a cabo mediante microscopía óptica, y se observó que en un primer momento sí se obtienen embriones a partir de las microsporas aisladas, lo cual no había sido descrito previamente. Sin embargo, posteriormente estos embriones sufren una desdiferenciación a callo. A partir de estos callos se consiguen regenerar plantas completas por organogénesis. Posteriormente, los regenerantes obtenidos se analizaron mediante citometría de flujo para determinar su ploidía, y mediante marcadores moleculares microsatélites para caracterizar el origen (somático o androgénico) de los regenerantes. Se confirmó que todos los individuos diploides eran en efecto doble haploides. Continuando con el diseño de un protocolo eficiente para la obtención de doble haploides a partir del cultivo de microsporas aisladas en berenjena, como paso previo al estudio de la regeneración, también se ensayaron diferentes métodos para disgregar los callos obtenidos. El objetivo era

multiplicar el número de callos obtenidos en el cultivo, y así disponer de más material para poder desarrollar protocolos de regeneración eficaces e independientes del genotipo. Al disponer de un material genéticamente idéntico, cualquier respuesta observada en cuanto al crecimiento de los callos y a la formación de brotes y/o raíces a partir de ellos, podremos atribuirla a las diferencias en la composición de los medios ensayados.

Una vez seleccionado el mejor método para la clonación del material, se procedió por último a evaluar diferentes medios de regeneración de plantas completas, ya que uno de los pasos limitantes en la obtención de doble haploides a partir del cultivo de microsporas aisladas de berenjena, es justamente el bajo porcentaje de plantas regeneradas por cada callo.

Como resultado de estos estudios hemos conseguido conocer, con un nivel de detalle no publicado hasta ahora, las particularidades de este proceso experimental en berenjena. De entre ellos destaca el hecho de que, en contra del trabajo previo publicado por Miyoshi (1996), las microsporas aisladas sí son reprogramadas hacia embriogénesis. Es decir, las microsporas de berenjena no se comportarían al menos en un primer momento, de forma distinta a como lo hacen las del resto de especies sensibles a la inducción de embriogénesis de microsporas. No obstante, hay un punto en su desarrollo en el que estos embriones se detienen y sus células entran en una fase de proliferación no regulada por los patrones de desarrollo típicos de la embriogénesis, con lo que los embriones acaban transformándose en callos. Aunque hemos demostrado que es posible obtener plantas haploides y doble haploides a partir de estos callos, es evidente que el primer objetivo de una mejora en el protocolo debería centrarse en evitar el bloqueo de la embriogénesis. Este fue el objetivo principal que se abordó en el capítulo 2, aunque no el único, puesto que si no fuera posible la continuación de la embriogénesis hasta la germinación del embrión, sería al menos deseable aumentar la frecuencia de obtención de callos y de regeneración de plantas haploides y doble haploides a partir de dichos callos. Por todo esto, en los capítulos 2 y 3 se evaluó el papel de una serie de sustancias implicadas, de forma directa o indirecta, en estos procesos: la inducción y promoción de la embriogénesis (cigótica, androgénica o somática), y la regeneración a partir de callos indiferenciados. Tal como se explica en el capitulo 3, de todas las sustancias evaluadas sólo una dio muestras de ser capaz de sortear el bloqueo de la embriogénesis entre las etapas de globular y corazón. Esta sustancia fue la goma arábiga, una mezcla compleja de arabinogalactanos (AGs) y proteínas de arabinogalactanos (AGPs)

(Phillips 2009). De acuerdo con nuestros resultados, los promotores de este efecto serían las AGPs más que los AGs.

Las AGPs son glicoproteínas de la matriz extracelular con un elevado grado de glicosilación que se encuentran en diferentes tejidos reproductivos (Cheung and Wu 1999). Estas proteínas además de estar implicadas en el crecimiento y el desarrollo de la planta, juegan un papel muy importante en la reproducción sexual (Cheung and Wu 1999). Además las AGPs aunque todavía no se sabe claramente cómo, influyen de modo decisivo en la inducción a androgénesis y promueven el desarrollo del embrión androgénico. La aplicación al medio de AGPs y AGs consigue incrementar la tasa de inducción de androgénesis y la supervivencia de las microsporas en cultivo en trigo (Letarte et al. 2006). En maíz, las AGPs presentes en los medios de cultivo de microsporas androgénicas, son capaces de promover el desarrollo no sólo de embriones androgénicos sino también zigóticos (Paire et al. 2003) y permitir que tenga lugar la embriogénesis de microsporas en genotipos de maíz que eran recalcitrantes para este proceso (Borderies et al. 2004).

Nuestros resultados confirman estas propiedades de las AGPs en la embriogénesis vegetal, en el sistema, todavía no explorado de la berenjena. Sin embargo, nuestros resultados con AGPs no fueron todo lo positivos que hubiera sido deseable, pues no se consiguieron embriones completos, con un meristemo apical del tallo bien formado. De esto se infiere que será necesario seguir trabajando en sortear el cuello de botella del bloqueo de la embriogénesis en embriones androgénicos de berenjena, quizá mediante el uso de otros factores junto con las AGPs.

El resto de sustancias evaluadas en el capítulo 2 y 3: polietilenglicol (PEG), manitol, ácido abcísico (ABA), epibrasinólido, distintas concentraciones y diferentes ratios de auxinas y citoquininas no mostraron un efecto significativo en la solución del mencionado bloqueo. Sin embargo, algunos de ellos sí evidenciaron un claro potencial de mejorar la eficiencia de la inducción de embriogénesis, como fue la aplicación del PEG, del epibrasinólido y la reducción de la concentración de los factores de crecimiento añadidos al medio de cultivo. Este incremento en la inducción a embriogénesis fue superior en algunos casos al combinar varios de estos factores entre ellos, consiguiéndose también en algunas combinaciones aumentar el porcentaje de regeneración de plantas haploides o doble haploides a partir de los callos, una vez estos se han formado.

Por ello podemos concluir que la inclusión de AGPs, PEG y epibrasinólido en el protocolo descrito en el capítulo 2, podría aumentar aún más la eficiencia de obtención de plantas doble haploides a partir del cultivo *in vitro* de microsporas aisladas de berenjena. De este modo, y aunque el protocolo muestre aún claros márgenes de mejora en cuanto al sostenimiento del desarrollo embriogénico, podríamos decir que está en condiciones de proporcionar a los mejoradores un protocolo capaz de producir doble haploides con una eficiencia superior a la obtenida mediante cultivo de anteras. La principal ventaja de este método en cuanto a su utilización a escala comercial sería la eliminación de los distintos inconvenientes inherentes al cultivo de anteras mencionados en la introducción, lo cual redundaría en una mayor rapidez, sencillez, seguridad y economía en la obtención de líneas puras de berenjena.

Utilización de la especie modelo *Brassica napus* para el estudio a nivel ultraestructural de la inducción a embriogénesis de microsporas

Como ya hemos dicho, la principal utilidad de los doble haploides es su uso como líneas puras para la obtención de híbridos en Mejora Genética Vegetal. Pero para favorecer este proceso en especies recalcitrantes donde la eficiencia no es suficiente para utilizar este sistema experimental para la obtención de doble haploides, puede resultar muy útil tener conocimientos a un nivel más básico de lo que ocurre cuando las microsporas son inducidas a un cambio en el programa en el desarrollo. Saber qué es lo que pasa en la microspora y qué procesos se desencadenan en ella tras la inducción a ese cambio en el desarrollo nos puede dar información para conocer las causas de la dificultad de conseguir doble haploides en aquellas especies recalcitrantes y permitir mejorar su eficiencia.

Este tipo de estudios a nivel básico en este sistema experimental, se han llevado a cabo en la especie modelo *Brassica napus* y comenzaron poco después de que se descubriera esta ruta en 1964 (Guha and Maheshwari 1964). Pero gracias al desarrollo tecnológico que ha tenido lugar en las últimas décadas se han podido retomar este tipo de estudios y obtener resultados que antes eran impensables. Han tenido lugar grandes avances en muchos campos, por ejemplo, en el campo de la genómica, proteómica y metabolómica, que han permitido la identificación de genes y metabolitos que se expresan cuando tiene lugar este cambio en el programa del desarrollo (Joosen et al. 2007).

En el campo de la microscopía electrónica también ha habido avances, entre ellos la fijación por alta presión (HPS), que en combinación con la criosustitución (FS), ha hecho posible una notable mejora en la preservación de la ultraestructura del tejido (Wilson and Bacic 2012). En la HPF, la combinación de bajas temperaturas con una presión elevada permite preservar la ultraestructura de forma mucho más cercana a la realidad y por lo tanto la información que obtenemos es más precisa, además permite evitar la presencia de los artefactos producidos por el uso de los fijadores químicos. Esta técnica de fijación no había sido utilizada anteriormente para el estudio de las microsporas tras la inducción de embriogénesis. Su aplicación a este sistema experimental nos ha permitido, volver a abordar el estudio a nivel ultraestructural de los cambios que tienen lugar en la microspora durante el cambio en el programa de desarrollo, tal como se ha descrito en el capítulo 4. Tras este estudio se ha observado que tienen lugar procesos de autofagia, y que éstos se dan de forma exclusiva en aquellas microsporas que han sido inducidas a embriogénesis, por lo que son consecuencia directa del cambio en el programa de desarrollo. Anteriormente, se habían realizado estudios a nivel ultraestructural en colza y también en otras especies. En estos estudios se describieron fenómenos de remodelado del citoplasma (Hause et al. 1992; Simmonds and Keller 1999), cambios en la expresión de proteínas de choque térmico (Seguí-Simarro et al. 2003), entre otros, pero en ningún caso fenómenos de autofagia como los descritos en el capítulo 4 de esta tesis doctoral.

La autofagia es un mecanismo habitual para la degradación del contenido celular en plantas (Bassham 2007). Los procesos de autofagia pueden desencadenarse por distintas causas, entre las que destaca el estrés abiótico, principalmente el ayuno (Aubert et al. 1996). Por otro lado, también se da en condiciones normales del desarrollo para eliminar orgánulos y proteínas anormales o dañadas (Inoue et al. 2006), o en procesos de senescencia (Bassham 2007; Gaffal et al. 2007; Inoue et al. 2006; Liu and Bassham 2012). En todos estos casos, los procesos de autofagia están caracterizados por la incorporación de orgánulos y macromoléculas citosólicas a vacuolas líticas para su digestión y reciclado (Bassham 2009). Sin embargo, esto no es lo que hemos observado en el caso de las microsporas inducidas a embriogénesis, ya que aquí junto con el fenómeno de autofagia tiene lugar una excreción masiva. En este caso, material de distintos tipos y en distintos grados del proceso de digestión, es excretado al espacio entre la membrana plasmática y la pared celular modificando así la dinámica de un proceso de autofagia habitual.

En estudios previos en otras especies, se han descrito evidencias indirectas que apuntaban a que al inicio de la embriogénesis de microsporas tiene lugar una limpieza del citoplasma (Hosp et al. 2007; Maraschin et al. 2005). En microsporas inducidas se observaron regiones libres de orgánulos y con menor número de ribosomas (Maraschin et al. 2005). Sin embargo, hasta la fecha, no había observaciones directas de que esta limpieza del citoplasma se estuviera llevando a cabo mediante procesos de autofagia. Esta limpieza consiste en la eliminación de todo lo que no se necesita del antiguo programa de desarrollo gametofítico (proteínas, mRNAs, enzimas…(Hosp et al. 2007)) consistente en el desarrollo del grano de polen y su preparación para la polinización y la fecundación. Todo este material que ya no se necesita se elimina mediante autofagia. Pero, además, como hemos observado en este estudio, esta limpieza del citoplasma está mediada también por procesos de excreción masiva al espacio extracelular, ayudando así a eliminar la gran cantidad de material del antiguo programa de desarrollo.

Si solamente tuvieran lugar fenómenos de autofagia masiva, todos los autofagosomas y compartimentos prevacuolares acabarían fusionándose y formando una o varias vacuolas líticas de gran tamaño. Esta vacuola además, continuaría aumentando por acumulación de agua para compensar la presión osmótica producida por los osmolitos acumulados en su interior. La formación de una vacuola de estas dimensiones, por lo que hemos observado en nuestro estudio, haría que se detuviera el desarrollo embriogénico de las microsporas inducidas, probablemente porque se alteran procesos clave para este tipo de desarrollo. Así pues, pensamos que la fusión de gran parte de estos autofagosomas y compartimentos prevacuolares con la membrana para su excreción, es un mecanismo de defensa para poder asegurar la continuación del desarrollo embriogénico. En este trabajo hemos determinado, que la autofagia y la excreción parecen ser procesos esenciales en la transición al desarrollo embriogénico. Además de esto, pensamos que estos dos procesos podrían estar relacionados con la mayor recalcitrancia de las distintas especies para entrar en embriogénesis. La facilidad que una microspora inducida tenga para activar estos procesos que hacen posible la continuación del desarrollo embriogénico podría ser la clave. De esta forma, en especies modelo como la colza, la elevada sensibilidad a la inducción a embriogénesis podría deberse a que un elevado porcentaje de microsporas inducidas activan procesos de autofagia y excreción favoreciendo su supervivencia y su continuación en el desarrollo embriogénico, esto ocurriría en mucha menor medida en las microsporas inducidas de especies recalcitrantes. Para verificar esta hipótesis, sería intere-

sante en un futuro, ver si en especies recalcitrantes y también en otras con un grado intermedio de recalcitrancia se dan estos fenómenos y en qué medida, puesto que no hay nada publicado al respecto hasta la fecha. Para ello, el mejor abordaje experimental sería la aplicación de HPS, FS y MET tal como hemos hecho en la presente Tesis.

Con estos estudios hemos conseguido avanzar en el conocimiento de lo que está sucediendo en la microspora en el momento del cambio en el programa del desarrollo a un nivel ultraestructural. Continuar con el estudio a un nivel básico para conocer lo que ocurre en esta ruta experimental y lo que implica este cambio de desarrollo a nivel celular puede ser esencial para la comprensión de este proceso y para conseguir favorecerlo.

Utilización de la tecnología de los doble haploides para aumentar la eficiencia de la transformación genética

La tecnología de los doble haploides puede ser útil también en otros campos diferentes a la obtención de híbridos, como es el caso de la transgénesis. Una de las aproximaciones posibles, consiste en transformar las microsporas con el transgén de interés y a continuación inducir estas microsporas transformadas a embriogénesis para obtener individuos doble haploides, que tendrán por lo tanto dos copias del transgén. La utilidad de este procedimiento se debe a que se evita la aparición de individuos hemicigotos, al transformarse células haploides que posteriormente son sometidas a inducción de embriogénesis (Dormann et al. 2001). Esto posibilita la obtención de individuos homocigotos para el transgén en una sola generación en lugar de en dos como es habitual en un proceso de transformación genética. Además, se obtienen en una proporción mayor, un 50% de los regenerantes en lugar del 25%. De esta forma estamos acortando el tiempo de obtención de plantas transgénicas y aumentando su eficiencia. También, debido a que se transforma una única célula se evita la formación de quimeras, que si aparecen con la transformación de otro tipo de tejidos vegetales (Dormann 2001).

Los estudios realizados en esta Tesis Doctoral donde se combina la tecnología de los doble haploides y la transformación genética los hemos llevado a cabo también en colza. Hasta el momento no existía un protocolo eficiente para la transformación de microsporas, ya que el método de transformación más utilizado en plantas, la agroinfiltración (Sharma et al. 2005), es poco eficaz en este caso ((Sangwan et al. 1993). En la transformación de mi-

crosporas lo que ocurre es que la gruesa pared de la microspora actúa como una barrera física a la transformación (Dormann et al. 2001), dificultando así la entrada de *Agrobacterium* al interior. Para transformar microsporas el método más eficaz en cebada y tabaco es el bombardeo de microproyectiles ya que permite salvar esa barrera física y se consiguen plantas transformadas (Jahne and Becker 1994; Stoger et al. 1995). La limitación en el caso de la transformación mediante bombardeo de micropartículas es que no se controla la inserción, por lo que se insertan múltiples copias y fragmentos incompletos del gen (Dormann et al. 2001). Para solventar las limitaciones existentes, en este trabajo se combinó la técnica de bombardeo de partículas con la agroinfiltración, debido a que tras el bombardeo se producen microheridas que facilitan la entrada de ADN exógeno al interior, facilitando así la entrada de *Agrobacterium* en la microspora.

Para desarrollar un protocolo eficiente para la transformación, se buscó optimizar tres parámetros que en trabajos previos se ha visto que son críticos para la eficiencia en la transformación: la densidad de las microsporas sometidas al proceso de transformación, intentando maximizar el número de microsporas expuestas, el tamaño de las partículas de oro de los microproyectiles de forma que no se comprometa la viabilidad de las microsporas (Folling and Olesen 2001) y la presencia y concentración óptima de un agente osmótico adicional (Vain et al. 1993), para incrementar la osmolaridad y evitar que el contenido celular salga al exterior. Tras definir estos parámetros, se realizaron varios ensayos comparando ambos métodos de transformación, solos y en combinación. La eficiencia de la transformación se midió en términos de expresión transitoria del gen reportero GUS, obteniendo mayor respuesta con la combinación de ambas técnicas de transformación y en concreto cuando las partículas de oro están recubiertas con la construcción con la que se pretende transformar, consiguiendo aumentar en este caso hasta en 10 veces la expresión del gen reportero GUS. Por lo tanto, hemos conseguido desarrollar los primeros pasos de un protocolo útil para la integración transitoria de ADN externo dentro de las microsporas de colza antes de la inducción a androgénesis. Partiendo de este protocolo se puede continuar el estudio para conseguir mejorar la supervivencia de las microsporas, la continuación de su desarrollo, y conseguir también una expresión estable del transgén.

Por otra parte, a pesar de la facilidad del método descrito anteriormente y de todas las ventajas que presenta, la frecuencia de producción de haploides transgénicos a partir de la transformación de microsporas es muy

baja (Abdollahi et al. 2009). Esto es debido, a que en un proceso de transformación genética además del método de transformación, otro de los cuellos de botella es la regeneración de las plantas desde las células transformadas (Sharma et al. 2005). En este caso, el proceso de transformar las microsporas e inducirlas después a androgénesis disminuye mucho la supervivencia de este tipo celular, reduciendo el número de microsporas que continúan con el desarrollo para formar embriones completos. Por este motivo, se han estudiado otras alternativas, como es la transformación de embriones derivados de microsporas (MDEs). La transformación de MDEs tiene el inconveniente de la posible formación de quimeras, debido a que algunas de las células del embrión serán transformadas pero otras no (Abdollahi et al. 2009). Para solventar este problema, una solución es la obtención de embriones secundarios (SEs) obtenidos a partir de los MDEs previamente transformados, de forma que al provenir de una única célula, si ésta ha sido transformada, el embrión secundario obtenido tendrá el transgén en todas sus células (Raemakers et al. 1995), evitando así el quimerismo y la hemicigosis. La embriogénesis secundaria se ha descrito en al menos 80 gimnospermas y angiospermas, demostrando ser muy repetible y manteniéndose a lo largo del tiempo la capacidad embriogénica (Raemakers et al. 1995). Este hecho, junto con la facilidad con la que es posible inducir SEs a partir de los MDEs en colza (Abdollahi et al. 2009; Nehlin et al. 1995), hace que se pueda llegar a obtener un sistema de transformación que permita mejorar la eficiencia de la transformación de plantas en cultivos de interés.

Sin embargo, esta aproximación presentaba dos limitaciones. La primera de ellas, la estabilidad genética de las células de los MDEs a partir de las cuales tendrá lugar la embriogénesis secundaria. Por ello, en el capítulo 6 estudiamos mediante citometría de flujo, las distintas partes de los MDEs, para determinar su ploidía y estimar la estabilidad genética del tejido para seleccionar la mejor fuente de embriogénesis secundaria. En trabajos previos realizados en colza, se vio que la capacidad embriogénica del hipocotilo como explante era muy superior (Nehlin et al. 1995). En nuestro trabajo, además de confirmar estos resultados ya que el 71% de los SEs provenían del hipocotilo, gracias al análisis de la ploidía, podemos confirmar que es la mejor fuente de SEs ya que las células del hipocotilo son más propensas a la duplicación genética, y por lo tanto la posibilidad de obtener doble haploides con transgénes en homocigosis será mayor.

El otro cuello de botella era la eficiencia de inducción de embriogénesis secundaria. Por lo tanto en el capitulo 6, se trató de incrementar el número de SEs mediante la aplicación de extracto de humo a los MDEs obtenidos. El extracto de humo se ha utilizado en varias especies para favorecer distintos procesos del crecimiento y desarrollo, incluyendo la embriogénesis somática, donde se ha conseguido incrementar el potencial embriogénico (Bhatia et al. 2004; Senaratna et al. 1999). En el capitulo 6 se probaron distintos modos de aplicación del compuesto, distintos tiempos de exposición y un rango de concentraciones del mismo, seleccionando para el protocolo final los más eficientes en cada caso: pretratamiento antes de la inducción de SE durante 15 minutos con una concentración de 1:250. La aplicación en estas condiciones resultó beneficiosa para promover la embriogénesis secundaria en MDEs de colza, aunque por los resultados obtenidos, no podemos concluir si el efecto positivo observado se debe a una mayor inducción de SEs o a que favorece la maduración de los embriones como se ha observado en otras especies (Ma et al. 2006). Así pues aplicando a los MDEs transformados un pretratamiento con extracto de humo podemos aumentar la eficiencia de obtención de embriones secundarios de forma rápida, sencilla y económica, lo que nos permite disponer de un protocolo eficiente para obtener individuos doble haploides transgénicos.

Por lo tanto, hemos conseguido desarrollar dos protocolos de partida, a partir de los cuales se puede seguir trabajando para poder utilizar la tecnología de los doble haploides de forma eficiente para trabajos de transgénesis. La principal utilidad de disponer de estos protocolos, consiste en la posibilidad de transferir un gen con función conocida a un cultivo de interés, añadiendo además las ventajas antes mencionadas del uso de la tecnología de los doble haploides.

Conclusiones

1.- En esta Tesis Doctoral, se han identificado nuevos procesos asociados y factores que influyen positivamente en el cambio de programa de desarrollo que sufren las microsporas o sus precursores (en el caso del tomate), con un impacto potencial muy notable en el resultado final de este proceso experimental.

Los procesos incluyen:

- la limpieza de citoplasma mediada por procesos masivos de autofagia y excreción del material parcialmente digerido en microsporas aisladas de colza.
- la elevada tasa de regeneración a partir de tejido somático y la fusión de núcleos meióticos haploides, que tienen un papel destacado en la baja eficiencia del cultivo de anteras de tomate.

Los factores incluyen:

- un pretratamiento con extracto de humo vegetal en cultivos de microsporas de colza
- la aplicación al medio de cultivo de AGPs, epibrasinólido, ácido abscísico y polietilenglicol, solos o combinados, en cultivos de microsporas de berenjena.

2.- Gracias a la aplicación al cultivo de los factores mencionados, se ha conseguido mejorar la eficiencia de la obtención de doble haploides en cultivos de microsporas de berenjena.

3.- Se ha avanzado en la posibilidad de combinar inducción de embriogénesis y transformación genética en un sistema modelo como colza, gracias a:

- la combinación de incubación con *Agrobacterium* y bombardeo de micropartículas, tanto en microsporas como en los embriones obtenidos de ellas.
- la utilización de hipocotilos de embriones transformados como fuente de embriones secundarios.

4.- Finalmente, se ha demostrado que la mejor metodología actual para la preservación de la ultraestructura de las microsporas inducidas a embriogénesis es la combinación de fijación por alta presión y criosustitución.

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