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"El *cross-talk* etileno/giberelinas y la rotura de la dormición de semillas de *Sisymbrium officinale* L. provocada por el after-ripening"

Tesis Doctoral

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INFORMA:

Que el trabajo titulado "El *cross-talk* etileno/giberelinas y la rotura de la dormición de semillas de *Sisymbrium officinale* L. provocada por el after-ripening", presentado por Dña. Raquel Iglesias Fernández para optar al Título de Doctora en Biología, ha sido realizado bajo mi dirección. Considerándolo finalizado, autorizo su presentación y defensa.

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I. INTRODUCCIÓN GENERAL

I.1. – Concepto y Morfología de la Semilla

Las semillas representan el propágulo elegido por las plantas Espermatófitas (Gimnospermas y Angiospermas) tanto para su dispersión como para la propagación de su estirpe (Lohe y Chaudhury, 2002). La independencia del agua durante la fecundación o el control de la germinación a través de la dormición son importantes estrategias adaptativas que han hecho de las Espermatófitas los seres más evolucionados del Reino Vegetal (Baskin y Baskin, 1998, 2004; Probert, 2000; Sacandé *et al.*, 2004). Desde una perspectiva socio-económica, las semillas son una sustancial fuente de recursos alimentarios (Bewley, 1997b); esto se hace patente en la producción de cereales, arroces, pastas, harinas y aceites entre otros productos derivados de las mismas. Todas estas razones hacen necesario e interesante el estudio fisiológico, bioquímico y molecular de la semilla con el fin de optimizar y garantizar la sostenibilidad de su explotación.

Una vez ocurre la doble fertilización, comienza el desarrollo de la semilla en Angiospermas. La célula huevo (dotación haploide) y la célula central diploide del gametófito femenino son fecundadas por los núcleos espermáticos procedentes del grano de polen; originando el zigoto y el *endosperma*, respectivamente. Posteriormente, el zigoto es sometido a una serie de procesos de diferenciación que darán lugar al embrión maduro y al *suspensor*; estructura efímera que sostiene al embrión, física y fisiológicamente, durante las fases tempranas del desarrollo del mismo. El embrión consta principalmente de dos regiones: el eje, a partir del cual se formará el cuerpo vegetativo de la planta y el(s) cotiledón(es) que a menudo funciona como órgano de reserva en las Dicotiledóneas (West y Harada, 1993; Goldberg *et al.*, 1994). La célula central fertilizada sufre una serie de divisiones celulares carentes de citocinesis, provocando la formación de un *sincitio*, que posteriormente se celularizará (Olsen, 2004), originando al endosperma. El endosperma puede ser una estructura transitoria o permanente; todo depende de la especie en cuestión. En las semillas no-endospérmica*s*, como la soja (*Glycine max*) y el cacahuete (*Arachis hypogea*), el endosperma es absorbido completamente por el embrión en desarrollo. En las semillas endospérmica*s* existe gran variación en el grado de presencia del mismo. Así por ejemplo, las semillas de *Arabidopsis thaliana* presentan una única capa celular endospérmica, mientras que en las semillas de cereales el endosperma constituye la mayor parte del cuerpo de la semilla. Otras especies también presentan *perisperma*, que deriva de las células *nucelares* del óvulo. En algunas especies, este *perisperma* persiste a lo largo del desarrollo de la semilla y es utilizado como órgano de reserva (Bewley y Black, 1994).

La cubierta seminal o testa es otra parte importante de la semilla. Está constituida por diferentes capas celulares de origen materno (Bewley y Black, 1994; Boesewinkel y Bouman, 1995) las cuales proporcionan una importante interfaz entre el embrión y el ambiente externo durante procesos tan importantes como la embriogénesis, la dormición y la germinación (Bewley y Black, 1994; Debeaujon y Koornneef, 2000; Finch-Savage y Leubner-Metzger, 2006; Müller *et al.*, 2006; Bethke *et al.*, 2007). Además, la testa también funciona como órgano protector (Mohamend-Yasseen *et al.*, 1994; Weber *et al.*, 1996) y participa en la dispersión (Huang *et al.*, 2000, 2008).

I.2. – El Desarrollo de la Semilla

Los procesos de desarrollo de la semilla están divididos en dos fases: la morfogénesis y la maduración. Atendiendo al momento de la abscisión del fruto, la maduración se divide fisiológicamente en dos etapas: la maduración temprana, que engloba todos aquellos procesos que se desarrollan antes de la abscisión, y la maduración tardía que abarca aquellos que tienen lugar después de la misma.

Durante la maduración temprana se produce la síntesis de reservas en la semilla, por lo que parece lógico que sean los genes relacionados con la síntesis de proteínas de reserva los que se expresen en este estadío (Fig. 1).

La adquisición de tolerancia a la desecación es una cualidad que se adquiere durante la embriogénesis tardía (Fig. 1). Es en esta fase donde se expresan genes de proteínas LEA (Late Embryogenesis Abundant). Las proteínas LEA son proteínas hidrofílicas que están estructuralmente divididas en al menos seis grupos (Wise y Tunnacliffe, 2004). Se las ha relacionado con la adquisición de la tolerancia a la desecación, aunque en realidad su función todavía es un enigma.

Tanto el ácido abscísico (ABA) como las giberelinas (GAs) son hormonas necesarias para el crecimiento del embrión a lo largo del desarrollo del mismo. Así, la sobreexpresión de *GA2ox2* de guisante (la GA2-oxidasa es un enzima implicada en la desactivación de las GAs bioactivas) en *Arabidopsis* aumenta la proporción de abortos en la semilla (Singh *et al.*, 2002). Mutantes deficientes en ABA, como *aba2*, muestran un importante incremento en la proporción de abortos en semillas comparados con el fenotipo silvestre (Cheng *et al.*, 2002).

La germinación precoz de los mutantes *lec* (*leafy cotyledon*) y *fus* (*fusca*) de *Arabidopsis* se suprime con una mutación deficiente en GAs, indicando que la biosíntesis de GAs activas está negativamente regulada durante el desarrollo de la semilla, evitando así el viviparismo (Raz *et al.*, 2001).

Por el contrario, la acumulación de ABA a lo largo del desarrollo de la semilla juega un papel importante en la inducción de los procesos de maduración y en la imposición de la dormición. Hay que subrayar la existencia de una acumulación bifásica de ABA a lo largo del desarrollo de la semilla (Karssen *et al.*, 1983). El primer pico de ABA aparece tanto en el zigoto como en

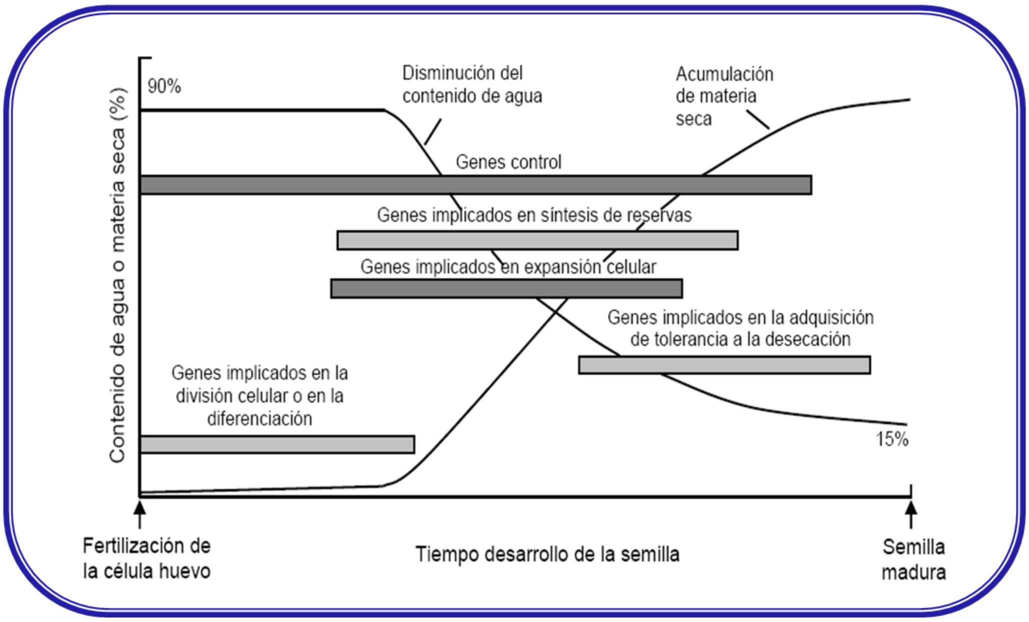

Figura 1: Esquema general de los principales eventos que ocurren durante el desarrollo embrionario de la colza. (Adaptado de Bradford y Bewley, 2003).

los tejidos maternos, mientras que el segundo pico sólo se produce en el embrión. El ABA de origen zigótico es esencial para inducir y mantener la dormición de la semilla. Sin embargo, el ABA de origen materno parece estar implicado en la inhibición del viviparismo y en los procesos de maduración de la semilla (Koornneef *et al.*, 1989; Raz *et al.*, 2001).

I.3. – La Dormición de la Semilla

Durante la maduración de la semilla, el ciclo celular cesa, la dependencia molecular a la planta madre desaparece, el contenido en agua disminuye, se sintetizan los productos de almacenamiento, se acumula ABA y finalmente la dormición primaria se establece (Hilhorst y Toorop, 1997; Raz *et al.*, 2001; Finkelstein *et al.*, 2002; Kermode, 2005; Weber *et al.*, 2005; Finch-Savage y Leubner-Metzger, 2006; Holdsworth *et al.*, 2008a).

La dormición, que está definida como un impedimento para germinar de la semilla madura, seca y viable bajo condiciones favorables, es considerada como un rasgo adaptativo dirigido a optimizar la germinación en el momento más adecuado para que la semilla complete su ciclo vital (Finch-Savage y Leubner-Metzger, 2006; Bentsink *et al.*, 2007). Por lo tanto, la dormición debe desaparecer para que la germinación tenga lugar (Finkelstein *et al.*, 2008). Otros autores consideran a la dormición como una característica de la semilla, ya que la intensidad de la dormición define las condiciones que deberían de existir para permitir la germinación (Vleeshouwers *et al.*, 1995).

Uno de los sistemas de clasificación más reciente está basado en el hecho de que la dormición está determinada tanto por las propiedades morfológicas como fisiológicas de la semilla (Nikolaeva, 1977). Basándose en este esquema Baskin y Baskin (2004) propusieron un sistema de clasificación, el cual incluye cinco clases de dormición:

- Fisiológica (DF)
- Morfológica (DM)
- Morfofisiológica (DMF)
- Física (DFs)
- Combinada (DF+DFs)

i. Dormición Fisiológica (DF)

La DF es el tipo de dormición más abundante en el banco de semillas del suelo y entre las semillas de Gimnoespermas y en la gran mayoría de las Angiospermas. Se puede dividir en tres niveles: profunda, intermedia y no-profunda (Baskin y Baskin, 2004).

DF Profunda (DFP): Se dice que una semilla está en DFP cuando una vez separado el embrión es incapaz de germinar; ni siquiera en presencia de GAs se rompe esta dormición. Solamente largos períodos de frío (subtipo a) o de calor (subtipo b) son capaces de provocar la germinación. La **DF intermedia (DFI)** necesita períodos más cortos de frío o calor para romper la dormición.

DF no-Profunda (DFnP) o dormición primaria: La gran mayoría de las especies tienen dormición no profunda (Baskin y Baskin, 2004). Los embriones escindidos de estas semillas desarrollan plántulas normalmente. El tratamiento con GAs puede romper la dormición, así como la escarificación, el after-ripening (AR), y la estratificación (tanto a bajas como a altas temperaturas).

ii. Dormición Morfológica (DM)

Este tipo de dormición está presente en todas aquellas semillas cuyos embriones están fisiológicamente subdesarrollados; pero completamente diferenciados en cotiledones e hipocótilo-radícula. Estos embriones no son fisiológicamente durmientes, sino que necesitan más tiempo para desarrollarse y

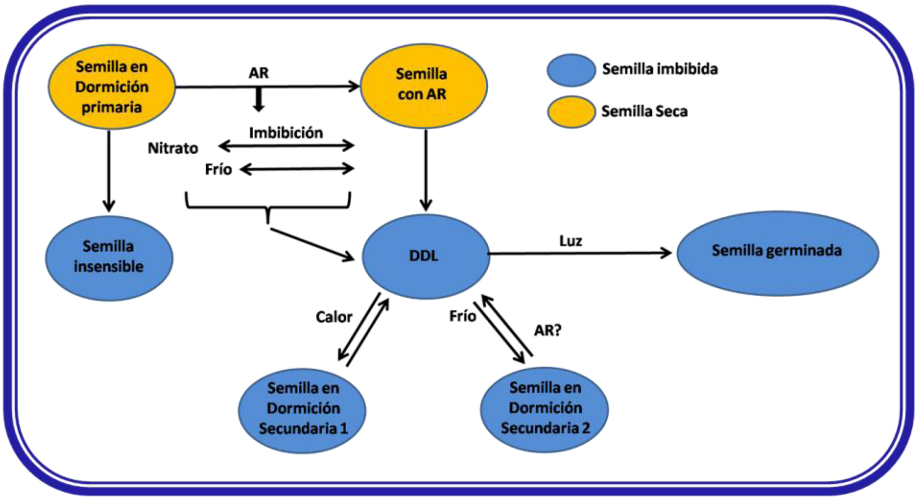

Figura 2: Ciclo de dormición y estatus fisiológico en el transcurso de la germinación de semillas de *Arabidopsis thaliana* ecotipo Cvi. Inicialmente las semillas están en estado de dormición primaria y necesitan de un período de AR para adquirir sensibilidad a los factores ambientales. Una vez imbibidas entran en un estado de dormición dependiente de la luz (DDL); sin ella entran en dormición secundaria. (Adapatado de Holdsworth *et al.*, 2008b)

germinar. Dentro de este tipo de dormición se encuentran semillas de *Apium graveolens* (apio).

iii. Dormición Morfofisiológica (DMF)

Las semillas con DMF también contienen embriones subdesarrollados, pero además existe un componente fisiológico añadido a su dormición (Baskin y Baskin, 2004). Estas semillas, por lo tanto, necesitan un tratamiento para romper su dormición, como la estratificación o la aplicación de GAs.

iv. Dormición Física (DFs)

Este tipo de dormición está causada por las envueltas impermeables de células en empalizada de las semillas o de los frutos que controlan los movimientos del agua. Tanto la escarificación mecánica como química pueden romper este tipo de dormición (Finch-Savage y Leubner-Metzger, 2006).

v. Dormición Combinada (DF+DFs)

Ocurre en aquellas semillas que tienen cubiertas impermeables (DFs) y DF en el embrión (Baskin y Baskin, 2004).

I.4. – El Estudio de la Dormición

A pesar de la existencia de la clasificación anteriormente citada, y en aras de una mayor simplificación, los autores prefieren utilizar una clasificación basada en la temporalidad en la que ocurre el proceso de dormición. La **dormición primaria** designa al tipo de dormición que ocurre antes de la dispersión de la semilla y que forma parte del proceso de desarrollo de la misma (Hilhorst y Toorop, 1997). Una semilla madura entra en **dormición secundaria** después de haber sido imbibida en condiciones desfavorables para poder germinar (Amen, 1968).

Dado que el único modo de estudiar el fenómeno de la dormición es observar cuales son los mecanismos necesarios para romperla, los medios a través de los cuales se puede abordar su estudio son limitados. Además, debido a que una semilla se considera no dormida cuando puede germinar, y el proceso de germinación nada tiene que ver con el de dormición, ambos procesos suelen solaparse y llevar al investigador a errores interpretativos. Consecuentemente, existe gran dificultad a la hora de abordar cuales son aquellas condiciones necesarias para romper la dormición y cuales las implicadas en

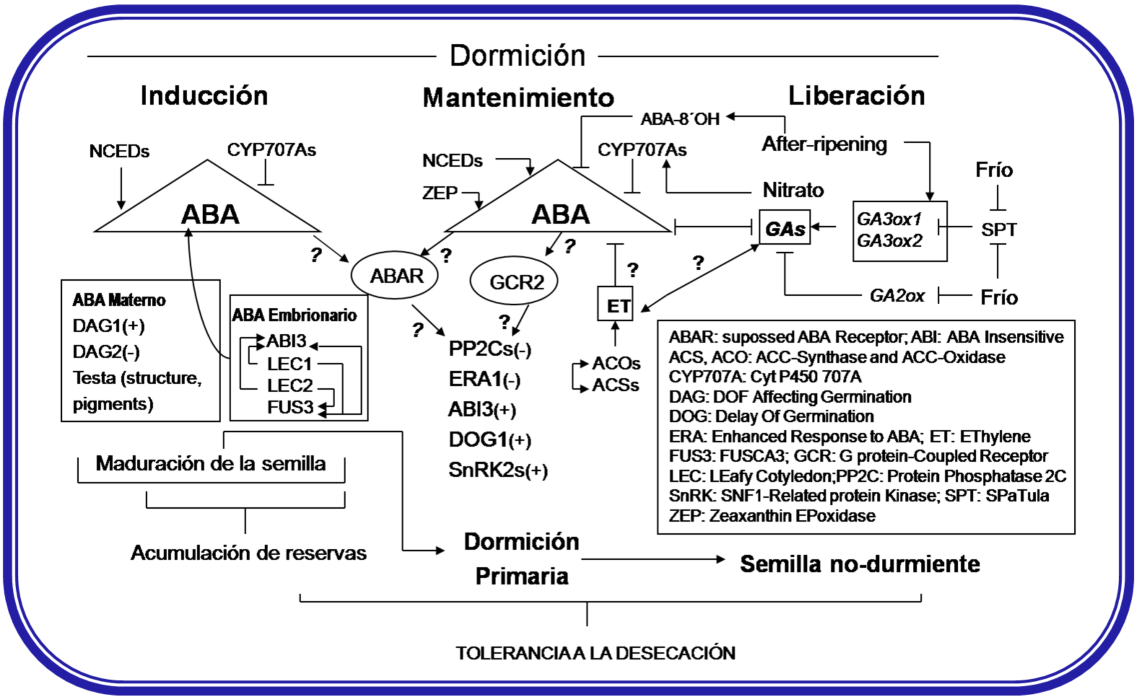

Figura 3: Posible regulación de la dormición a través de ABA y su interacción con GAs (*cross-talk*) y factores ambientales (principalmente frío y after-ripening). Las abreviaturas ABA, ET y GAs significan sus niveles relativos debido a la acción de sus enzimas anabólicos y catabólicos. Las flechas y barras señalan efectos promotores o represores, respectivamente. Los símbolos de interrogación muestran la ausencia de datos para confirmar dicho efecto. (Adaptado de Filkenstein *et al.*, 2008; Holdsworth *et al.*, 2008a; Iglesias-Fernández y Matilla, 2009; Matakiadis *et al.*, 2009).

promover la germinación. Los estudios genéticos combinados con los ensayos de germinación en condiciones óptimas, son una buena estrategia tecnológica para abordar el estudio de la dormición, uno de los procesos menos entendidos de la biología (Finch-Savage y Leubner-Metzger, 2006).

Tal y como se ha citado en el párrafo anterior, la dormición es un proceso muy poco conocido. Sin embargo, existen algunas especies donde se ha realizado un estudio más pormenorizado. Es el caso de *A. thaliana* (L.) ecotipo Cvi (Cabo Verde Island). Sus semillas presentan DFnP (Baskin y Baskin, 1998), existe un gran abanico de factores ambientales que pueden liberar de este tipo de dormición (Cadman *et al.*, 2006; Finch-Savage *et al.*, 2007) tales como el almacenamiento en seco (AR), la baja temperatura, la luz y el nitrato. Sin embargo, para que la rotura de la dormición tenga lugar, estas señales deben seguir un orden determinado. La semilla madura en dormición primaria es insensible a los factores ambientales y requiere AR para desarrollar dicha sensibilidad. La sensibilidad a la luz se adquiere después de un largo proceso de AR, lo mismo ocurre con el frío y el nitrato; entra en estado durmiente dependiente de luz (DDL), factor indispensable para romper la dormición. Si esta semilla DDL es imbibida y privada de luz entrará en dormición secundaria. La dormición secundaria se puede subsanar con frío o calor; pero si las semillas no se exponen a la luz volverán de nuevo a entrar en dormición secundaria (este proceso es conocido como **ciclo de la dormición**). La naturaleza reversible de la dormición fisiológica permite la existencia de ciclos de dormición en los bancos de semillas del suelo, provocados por cambios estacionales de temperaturas (Hilhorst *et al.*, 1996). El ciclo de la dormición es crucial para el establecimiento y la supervivencia de numerosas comunidades vegetales (Fig. 2).

I.5. – Factores Implicados en la Dormición

Existen varios reguladores de la dormición que han sido identificados. La acumulación de estos reguladores está correlacionada con el estatus de dormición y con la preparación para la germinación (visión general en la Fig. 3).

- **Ácido Abscisico (ABA)**

El estudio de la dormición utilizando mutantes ha evidenciado la necesidad de ABA en el establecimiento de la dormición durante la maduración del embrión (Finkelstein *et al.*, 2002; Nambara y Marion-Poll, 2003; Kermode, 2005; Finch-Savage y Leubner-Metzger, 2006; Feurtado y Kermode, 2007). El ABA puede inhibir la germinación y su acumulación está relacionada con el establecimiento de la dormición. Aquellos mutantes deficientes en ABA presentan dormición reducida (Nambara y Marion-Poll, 2003; Lin *et al.*, 2007). El ABA de origen materno o el administrado exógenamente no es suficiente para inducir la dormición (Finkelstein *et al.*, 2002; Finch-Savage y Leubner-Metzger, 2006). Este hecho parece suponer la existencia de una ruta de biosíntesis de ABA dependiente del embrión o del endosperma en el establecimiento de la dormición (Finkelstein, *et al.*, 2008).

Los niveles de ABA disminuyen en semillas secas durmientes debido a su catabolismo mediante la ABA 8′-hidroxilasa codificada por un gen del la familia del citocromo P450 *CYP707A* (Okamoto *et al.*, 2006). Mediante estudios genéticos y de expresión génica se han hallado genes de la familia *AtNCED* (dioxigenasa 9-cis-epoxicarotenoide), tales como *NCED6* y *NCED9*, que son esenciales para la síntesis de ABA, tanto en el embrión como en el endosperma, durante la inducción de la dormición (Lefebvre *et al.*, 2006). Así mismo, se han correlacionado los niveles de expresión de algunos genes como *NCED6*, *NCED9* y *ZEP* (zeoxantina epoxidasa) con el mantenimiento de la dormición y liberación de la misma, vía catabolismo de ABA (CYP707A2) (Cadman *et al.*, 2006; Millar *et al.*, 2006).

- **Especies Reactivas de Oxígeno (ERO)**

La pérdida de agua es un proceso altamente estresante para la semilla. No sorprende que otros mecanismos implicados en el proceso de deshidratación y adquisición de la tolerancia a la desecación sean los mecanismos de defensa antioxidantes. Existe una notable correlación entre la generación de especies reactivas de oxígeno (ERO) y semillas intolerantes a la desecación. Gran cantidad de semillas durante la maduración aumentan su actividad catalasa (Bailly, 2004; Berjak, 2006), acumulan glutation y peroxiredoxinas (Stacy, 1996; Haslekås *et al.*, 1998).

- **Componentes de la cubierta seminal**

La variación genética de las estructuras y/o pigmentos de la cubierta seminal (testa) o de las estructuras envolventes de la semilla, tales como el pericarpo en los granos, avocan a muchas semillas a distintos estados de dormición como también a la variación en la longevidad de las mismas (Groos *et al.*, 2002; Sweeney *et al.*, 2006; Debeaujon *et al.*, 2007). Los pigmentos son generalmente componentes fenólicos (ej. flavonoides). Las semillas de *Arabidopsis* presentan principalmente proantocianidinas y flavonoles glicósidos. Las proantocianidinas (PA) son polímeros que establecen enlaces con los componentes de la pared celular durante la maduración de la semilla; lo que provoca un engrosamiento de las capas celulares que proporciona una mayor resistencia mecánica y reduce la permeabilidad al agua, a los gases y a las hormonas. Además, la naturaleza antioxidante de los flavonoides les permite inhibir la degradación oxidativa del ABA, impidiendo de esta forma el AR y la germinación. Sin embargo, aunque las semillas con mayor pigmentación presentan elevados grados de dormición, otros parámetros, tales como los niveles hormonales y la sensibilidad a los mismos, pueden aumentar la dormición de las semillas con menos pigmentación.

- **Giberelinas (GAs)**

Las GAs estimulan la germinación mediante la inducción de enzimas hidrolíticas que debilitan los tejidos que se oponen a la emergencia radicular (ej. endosperma y la cubierta seminal), movilizan las sustancias de reserva de la semilla, y estimulan la expansión del cotiledón (Bewley y Black, 1994). Las GAs también parecen estimular la germinación favoreciendo la vía de transición del desarrollo embriogénico al vegetativo. La estimulación podría estar mediada, entre otros, por el factor de remodelación de la cromatina PICKLE (PKL) (Henderson *et al.*, 2004). Además, las GAs intervienen en la desaparición de la proteína de identidad embrionaria FUSCA3 (FUS3), la cual estimula positivamente la síntesis de ABA y regula negativamente la síntesis de GAs (Gazzarini *et al.*, 2004).

Figura 4: Representación esquemática de la ruta de biosíntesis de las GAs, se muestran las reacciones finales catalizadas por las GA dioxigenasas (Adaptado de Fagoaga *et al.*, 2007).

El papel de las GAs en la liberación de la dormición y en el proceso de germinación es controvertido, ya que los tratamientos con GAs no estimulan la germinación en todas las especies o en las semillas completamente durmientes de *Arabidopsis* (Derkx *et al.*, 1994; Bewley, 1997b; Ali-Rachedi *et al*, 2004). Antes de que los niveles de GAs y se estimule su sensibilidad puede que se requiera una disminución de los niveles de ABA (Jacobsen *et al.*, 2002; Ali-Rachedi *et al*, 2004). Los niveles de GAs y la sesibilidad a la luz aumentan con el AR en *Arabidopsis* (Derkx y Karssen, 1993b). Así mismo, los niveles de GAs incrementan en *Arabidopsis* con la estratificación (Yamauchi *et al.*, 2004). La acumulación del transcripto *GA3ox2* (GA3-oxidasa), uno de los principales genes implicados en la producción de GAs bioactivas, aumenta 40v en semillas con AR; mientras que la expresión *GA2ox1*, enzima implicada en la desactivación de GAs, es más alta en semillas durmientes de *Arabidopsis* ecotipo Cvi (Finch-Savage *et al.*, 2007). La estratificación aumenta la expresión de genes implicados en la síntesis de GAs, tales como *GA20ox1* (GA20-oxidasa), *GA20ox2* y *GA3ox1* (GA3-oxidasa) y disminuye la expresión de los genes implicados en el catabolismo (*GA2ox2*) (Yamauchi *et al.*, 2004) (Fig. 4).

Las GAs son críticas para la germinación en *Arabidopsis* y en tomate ya que los mutantes defectuosos en genes que codifican enzimas implicadas en la síntesis de GAs, son incapaces de germinar (Mitchum *et al.*, 2006; Steber, 2007). El tratamiento con GAs de estos mutantes es capaz de permitir la germinación.

- **Brasinoesteroides (BRs)**

Los BRs son hormonas vegetales involucradas en la elongación del tallo, el desplegado de la hoja (Clouse, 2001) y la promoción de la germinación de las semillas. Los mutantes en la biosíntesis y señalización de estas hormonas germinan bien. Sin embargo, son más sensibles a los tratamientos con ABA que las semillas del fenotipo silvestre, sugiriendo que estas hormonas disminuyen el potencial germinativo (Clouse y Sasse, 1998; Steber y McCourt, 2001). En *Arabidopsis*, la aplicación de epibrasinólido (EBR) y de brasinólido (BL) a mutantes de síntesis de GAs y al mutante *sleepy 1* (*sly1*) (mutante de señalización de GAs) (Steber y McCourt, 2001) es capaz de restaurar su germinación.

Tanto los BRs como las GAs son capaces de estimular la germinación promoviendo la ruptura del endosperma en semillas de tabaco durmientes; sin embargo, los mecanismos que utilizan son distintos. Solamente las GAs son capaces de estimular la actividad βGLU1 (β-1,3-glucanasa I) que es esencial para la rotura del endosperma en semillas de tabaco (Leubner-Metzger, 2001). Por lo tanto, los BRs pueden estimular la germinación, aunque los mecanismos que emplean son independientes de GAs.

- **Etileno (ET)**

El ET podría promover la rotura de la dormición a través de la interacción con la señalización de ABA. Los mutantes *etr1* (*ethylene receptor 1*) y *ein2/era3* (*ethylene insensitive2/enhanced response to aba 3*) presentan un incremento en su dormición correlacionado con el aumento de la sensibilidad y síntesis de ABA (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Chiwocha *et al.*, 2005). Sin embargo, el mutante *ctr1* (*constitutive triple response 1*) y los tratamientos de semillas del fenotipo silvestre con ACC (aminociclopropano carboxílico; precursor inmediato del ET) provocan una disminución de la sensibilidad al ABA (Ghassemian *et al.*, 2000). El mutante *etr1-2* también acumula GAs, sugiriendo que el incremento de la dormición provocado por la acumulación de ABA es parcialmente subsanado por este incremento en los niveles de GAs, y que el ET podría ser una molécula mediadora entre ambas hormonas a lo largo de la dormición y germinación de las semillas. Este tema se abordará más adelante.

- **Compuestos nitrogenados**

El NO, el nitrito (NO_2^-) y el nitrato (NO_3^-) pueden promover la germinación, afectando al metabolismo, al estado de oxidación, o a la señalización de la semilla (Bethke *et al.*, 2007). Tanto el NO_3^- como las condiciones que disminuyen la dormición inducen la expresión de genes implicados en la síntesis de enzimas de la ruta de las pentosas fosfato, la cual proporciona nutrientes esenciales para la germinación (Finch-Savage *et al.*, 2007). El NO, NO_2^- y el NO_3^- podrían estimular la ruta de las pentosas incrementando la oxidación de NADPH a $NADP^+$, aceptor de electrones que es factor limitante. EL NO inhibe la actividad catalasa, provocando la acumulación de H_2O_2, producido por la β-oxidación de los ácidos grasos almacenados en la semilla. El H_2O_2 podría estimular la germinación actuando como sustrato para las peroxidasas, permitiendo así la oxidación de NADPH a $NADP^+$. El H_2O_2 también podría estimular el catabolismo de ABA (Bailly, 2004). El NO_3^- modifica los niveles hormonales mediante la inducción de la expresión de enzimas que catalizan la desactivación del ABA (ej. CYP707A2) y de enzimas implicadas en la síntesis de GAs (GA3ox1) (Finch-Savage *et al.*, 2007). En *Arabidopsis*, el NO puede estimular la germinación provocando la vacuolización y el debilitamiento de las paredes celulares de la capa de aleurona (Bethke *et al.*, 2007).

I.6. – Análisis Post-Genómico de la Dormición

- **Inducción y mantenimiento**

Reguladores transcripcionales y post-transcripcionales

Existe una gran variedad de mutantes que presentan defectos en la maduración de sus semillas. En el caso de los mutantes de en *Arabidopsis leafy cotyleon class* (*lec1*, *lec2*, y *fus3*) y los mutantes *ABA-insensitive 3* (*abi3*), cuyas semillas son no durmientes (Finkelstein *et al.*, 2002; Finch-Savage y Leubner-Metzger, 2006). Sin embargo, estos mutantes presentan efectos pleiotrópicos, además de no producir semillas viables tolerantes a la desecación; por tanto, estas características dificultan su estudio. Uno de los mutantes más interesantes es *abi3*, ya que produce semillas secas viables que son no durmientes. Este mutante proporciona un buen material de estudio para poder abordar la dormición. Los factores de transcripción ABI4 y ABI5 parecen estar implicados en el control de la germinación en respuesta al ABA o al estrés, pero no parecen afectar a la dormición (Finkelstein *et al.*, 2008).

Opuestamente a los loci *ABI*, los loci *DAG1* y *DAG2* [*DOF* (*DNA binding with one zinc finger*) *AFFECTING GERMINATION*] codifican factores de transcripción altamente similares que se expresan en el tejido materno y que tienen un efecto opuesto sobre el potencial germinativo (Gualberti *et al.*, 2002; Papi *et al.*, 2002). *DAG1* parece tener dominancia epistática sobre *DAG2*. Esto parece sugerir que DAG1 activa los genes maternos que estimulan la dormición y que DAG2 reprime esta acción, mediante la unión al mismo *elemento en cis* o uniéndose directamente al factor DAG1.

Estudios recientes han proporcionado evidencias genéticas sobre el control transcripcional a través de la remodelación de la cromatina. El locus *RDO4* (*REDUCED DORMANCY*) (Peeters *et al.*, 2002) codifica una *zinc finger protein* con homología a enzimas modificadoras de histonas de otras especies (Liu Y *et al.*, 2007). Las mutaciones en este gen provocan fallos en la mono-ubiquitinación de la histona H2B, por lo que el locus fue renombrado a *HISTONE MONOUBIQUITINATION* (*HUB1*). Defectos en su homólogo *HUB2* provocan una disminución en el estatus de la dormición. Ensayos con dobles mutantes sugieren que estas proteínas forman parte de un heterotetrámero necesario para la ubiquitinación.

Proteínas fosfatasas y quinasas

Existen mutantes *abi* de *Arabidopsis* que son no durmientes y que incluyen dos mutaciones dominantes en los loci *ABI1* y *ABI2* (Koornneef *et al.*, 1984), los cuales codifican miembros relacionados con la familia de proteín fosfatasas 2C (PP2C) del grupo-A. Curiosamente, los mutantes *knockout* en estos loci, que tienen su actividad fosfatasa reducida, presentan hipersensibilidad al ABA e hiperdormición (Merlot *et al.*, 2001). Mutantes *knockout*/*knockdown* en algunos genes de esta familia muestran mayor dormición que el fenotipo silvestre, resultando ser genes con elevada expresión durante la maduración; es el caso de *ahg1* y *ahg3* (Yoshida *et al.*, 2006; Nishimura *et al.*, 2007). Las PP2Cs probablemente actúen sobre numerosos sustratos; pero se han identificado pocos (Christmann *et al.*, 2006).

La actividad de algunos factores de transcripción tipo bZIP está controlada por sus estados de fosforilación. La cascada de señalización de las MAP (Mitogen Activated Protein) quinasas activa a ABI5 (Lu *et al.*, 2002), probablemente de forma indirecta. Los miembros de las familias de proteín-quinasas dependientes de calcio (CDPK) y las proteín-quinasas relacionadas con SNF1 (SnRK) interactúan *in vitro* con múltiples miembros de los factores de transcripción bZIP de la clase AREB/ABF/ABI5 (Choi *et al.*, 2005; Kobayashi *et al.*, 2005; Furihata *et al.*, 2006; Chae *et al.*, 2007; Fujii *et al.*, 2007). La fosforilación de los factores de transcripción bZIP está relacionada con su activación. Consistentemente con esto, las familias redundantes de las kinasas SnRK2.2 y SnRK2.3 son reguladores positivos de la dormición y de la sensibilidad a ABA durante la germinación (Fujii *et al.*, 2007). Una de las interacciones mejor conocidas que afectan al potencial germinativo es la fosforilación del factor bZIP en trigo, TaABF, por la SnRK

PKABA1 (Jonhson *et al.*, 2002), provocando la represión de los genes inducidos por GAs, incluyendo los factores de transcripción GA-Myb, y la activación de los genes inducidos por ABA (Gómez-Cadenas *et al.*, 1999; 2001).

Regulación de la estabilidad y la localización proteica

La regulación de la estabilidad y la localización proteica también controla la función de ciertos reguladores ABI. Así, ABI3 parece ser diana de AIP2 (ABI3 ITERACTION PROTEIN), la cual se expresa a lo largo del desarrollo (Zhang *et al.*, 2005). Sin embargo, la sobre-expresión de esta proteína tiene efectos suaves en la sensibilidad al ABA, produciendo un fenotipo similar al del alelo *abi3-1*.

Las modificaciones lipídicas producidas por la farnesilación pueden promover la unión a la membrana plasmática, la estabilización proteica, y las interacciones proteína-proteína en plantas (Galichet y Gruissem, 2003). *ERA1*, el cual codifica para la β-subunidad de la proteína farnesilasa, estimula la respuesta a ABA durante la germinación (Cutler *et al.*, 1996). Los mutantes *era1* son altamente pleiotrópicos, y muestran hipersensibilidad a ABA en múltiples respuestas, incluyendo la dormición.

Receptores de ABA

Actualmente, se han descrito tres posibles receptores de ABA: una proteína de unión a ARN que regula la floración, FLOWERING TIME CONTROL (FCA) (Razem *et al.*, 2006); una quelatasa de Mg^{2+} implicada en la síntesis de clorofila y en la comunicación entre el núcleo y el plastidio, ABAR/CHLH/GENOMICS UNCOUPLED 5 (GUN5) (Shen *et al.*, 2006); y finalmente un receptor asociado a proteína G (GCR2) (Liu X *et al.*, 2007). Sin embargo, publicaciones recientes han negado la unión de FCA a ABA (Risk *et al.*, 2009) y el control de la acción del ABA por GCR2 (Guo *et al.*, 2008). Hasta ese momento sólo los dos últimos se habían relacionado con la respuesta de la semilla al ABA. Las líneas *knockout* de ABAR son plantas letales, que carecen de proteínas de reserva y lípidos. Las líneas *knockdown* ABAR muestran germinación resistente al ABA, expresión reducida de los genes *LEA* y de los factores de transcripción *ABI* y *MYB*, que regulan positivamente la respuesta al ABA. A este respecto, el receptor ABAR/CHLH/GUN5 parece jugar un papel central en la señalización que media la respuesta a ABA en las semillas.

- **Pérdida de la dormición**

La síntesis de GAs está regulada a lo largo del desarrollo en respuesta a señales ambientales. Un factor de transcripción *zinc finger* GATA, BME3 (BLUE MYCROPYLAR END), promueve la expresión de genes relacionados con la síntesis de GAs, *GA3ox1* y *GA20ox3*, en la región *micropilar* de la semilla. Estos enzimas estimulan la producción de GAs y la relajación de los tejidos circundantes al ápice radicular antes de la emergencia. La importancia de de la biosíntesis de GAs se hace patente en el retraso de la germinación en el mutante *bme3* (Liu PP *et al.*, 2005).

Los cambios en la expresión de *GA3ox1* y *GA20ox3*, provocados por la imbibición en frío y luz, están regulados por dos factores de transcripción del tipo *helix-loop-helix* (bHLH): SPATULA (SPT) y PHYTOCHROME-INTERACTING FACTOR 3-LIKE 5 (PIL5) (Oh *et al.*, 2004; 2006, 2007; Pendfield *et al.*, 2005). SPT inhibe la expresión de *GA3ox1* y *GA3ox2* durante la imbibición de la semilla en frío. Los mutantes *spt* presentan dormición más pronunciada cuando son expuestos a la luz, sugiriendo que SPT media el *cross-talk* entre el frío y la luz en la regulación de la dormición. PIL5 previene la germinación de la semilla en la oscuridad a bajas temperaturas mediante la represión de la expresión de *GA3ox1* y *GA3ox2*, y la inducción de la expresión de *GA2ox2* (Pendfield *et al.*, 2005; Oh *et al.*, 2006). La proteína PIL5 desaparece con la luz, aparentemente provocada por la degradación estimulada por el fitocromo y mediada por el proteosoma 26s (Oh *et al.*, 2006). Esto sugiere que la estratificación estimula la germinación incrementando el potencial de la semilla para acumular GAs activas. PIL5 parece reprimir la acumulación de GAs estimulando la síntesis de los represores de la respuesta a GA, DELLA, tales como *RGA* (*REPRESSOR OF GA1-3*), *GAI* (*GA-INSENSITIVE*) (Oh *et al.*, 2007). Estos resultados sugieren la implicación de las proteínas DELLA en el control ambiental de la dormición.

I.7. – El After-Ripening (AR)

El estatus de la dormición de la semilla aumenta durante la maduración de la misma, alcanzando su máximo en las semillas completamente maduras (Karssen *et al.*, 1983; Ooms *et al.*, 1993). A lo largo del almacenamiento posterior en seco de la semilla (AR), el estatus de la dormición disminuye progresivamente. La duración del AR, así como del estatus de la dormición, puede variar (Donohue *et al.*, 2005) en función de la maduración, tipo de semilla, almacenamiento y condiciones de germinación (Donohue *et al.*, 2005). Los mecanismos moleculares que disminuyen el estatus de la dormición durante el AR no están bien definidos, pero algunos estudios recientes proporcionan evidencias sobre su actuación a distintos niveles, desde la transcripción hasta las reacciones enzimáticas y no enzimáticas.

Las semillas maduras y secas contienen un gran número de diversas especies de ARN. El almacenamiento de ARNm en semillas fue por primera vez observado en algodón (Dure y Waters, 1965) y parece ser universal para todas las especies de plantas con flor. (Ishibashi *et al.*, 1990; Kuligowski *et al.*, 1991; Almoguera y Jordano, 1992). La función de este ARNm “almacenado” permanece desconocida; sin embargo, se cree que estos transcritos no son solamente productos remanentes de la embriogénesis o de la maduración de la semilla, sino que alguno de ellos proporcionan especies de ARNm para la síntesis de proteínas durante la fase temprana de la germinación (imbibición). Estudios post-genómicos han revelado que más de la mitad de los genes están representados por sus ARNm$_s$ en semilla seca madura (Nakabayashi *et al.*, 2005). Transcriptos de todas las categorías ontológicas fueron observados. Sin embargo, el 2-3 % de los transcriptos que presentan los valores más altos de expresión pertenecen a genes que codifican funciones asociadas con el metabolismo, así como con la degradación y síntesis proteica. El dominio ABRE (*ABA binding response element*) está presente en los promotores de los genes con mayor expresión en el transcriptoma de la semilla seca (Nakabayashi *et al.*, 2005). Estos autores también muestran que los ARNm presentes en la semilla seca están agrupados en los cromosomas y situados no aleatoriamente.

Recientemente, se ha propuesto que la semilla seca tiene competencia transcripcional. Sin embargo hay discusión sobre el tema debido al bajo contenido de humedad de la misma (Comai y Harada, 1990). Se han observado bajos niveles de transcripción y traducción del gen de la β-1,3-glucanasa durante el AR de la semilla de tabaco, estos niveles provocan la pérdida de la dormición (Leubner-Metzger, 2005). Esta idea ha sido apoyada por estudios posteriores de transcriptómica y proteómica que comparan las semillas recién recolectadas (durmientes) y las semillas con AR. El análisis de los cDNA-AFLP en *Nicotiana plumbaginifolia* (Bove *et al.*, 2005) y en cebada (Leymarie *et al.*, 2007) permitió identificar algunos transcriptos que aumentaban su expresión durante el AR de la semilla seca. Estos estudios también revelaron que una gran cantidad de transcriptos disminuían su abundancia durante el AR en comparación con aquellos que aumentaban (Bove *et al.*, 2005; Leymarie *et al.*, 2007). El análisis transcriptómico global en *Arabidopsis* permitió mostrar la existencia de 30 genes que disminuyen durante el AR, incluyendo *DOG1* (*DELAY OF GERMIANTION*) (Finch-Savage *et al.*, 2007).

El análisis proteómico global en *Arabidopsis* permitió identificar algunas proteínas que se acumulan en la semilla seca durante el AR (Chibani *et al.*, 2006). Muchas de ellas corresponden a proteínas de almacenamiento o a proteínas con funciones metabólicas, las cuales podrían preparar a la semilla para la germinación, ya que no presentan una función clara en la pérdida de la dormición. En contraste con los estudios transcriptómicos, los análisis proteómicos mostraron pocas proteínas que disminuyen sus niveles durante el AR, en comparación con aquellas que incrementan (Chibani *et al.*, 2006).

Los cambios transcriptómicos y proteómicos mostrados en estos estudios parecen revelar la existencia en la semilla seca de un proceso específico durante el AR, ya que la mayoría de los transcriptos y proteínas no muestran cambios en sus niveles de expresión durante el AR. Los mecanismos responsables de estos cambios en la expresión génica son hasta la fecha desconocidos, pero podrían jugar un papel bastante importante en la pérdida de la dormición. Estos resultados sugieren que la transcripción y la traducción son posibles bajo condiciones de baja hidratación en semillas. De acuerdo con esto, se ha demostrado en *Bromus tectorum* (Bair *et al.*, 2006) y en *Lolium rigidum* (Steadman *et al.*, 2003) que la pérdida de la dormición a través del AR disminuye o está ausente en condiciones de muy baja hidratación. Bajo condiciones normales de hidratación, la transcripción y la traducción no tienen lugar en el conjunto de toda la semilla, sino en áreas específicas con un mayor contenido de humedad. Es probable que haya zonas en la semilla seca donde el contenido de humedad es relativamente alto (por encima de un umbral) para permitir estas alteraciones (Manz *et al.*, 2005). Por lo tanto, el proceso de AR en semilla seca viable podría alterar positiva o negativamente los niveles de algunos transcriptos (Bove *et al.*, 2005; Finch-Savage *et al.*, 2007; Leymarie *et al.*, 2007) y proteínas (Chibani *et al.*, 2006) en estas regiones más hidratadas.

Mediante resonancia magnética nuclear se obtuvieron micro-imágenes en semillas secas que demuestran la existencia de regiones con mayor contenido en agua (Leubner-Metzger, 2005). Uno de los principales propósitos para investigaciones futuras será conocer si en dichas regiones altamente hidratadas de la semilla seca ocurre la transcripción y la traducción. De hecho, el término semilla seca es problemático en estudios de fisiología, ya que una semilla puede presentar diversos estados de hidratación, y por lo tanto, "seca" debería de ser reemplazada por el término "baja-hidratación" (Holdsworth *et al.*, 2008a).

Recientemente, se han relacionado redes génicas muy complejas con el AR (Finch-Savage y Leubner-Metzger, 2006; Holdsworth *et al.*, 2008b). Sin embargo, aunque la necesidad de AR parece evidente en algunas especies, el abordaje molecular de este proceso con respecto a los cambios inducidos durante el AR en la semilla seca y viable, y su impacto del mismo durante la imbibición, es complejo (Kucera *et al.*, 2005; Finch-Savage y Leubner-Metzger, 2006; Holdsworth *et al.*, 2008b).

Las reacciones no enzimáticas parecen jugar un papel bastante importante en la pérdida de la dormición. Por ejemplo, las ERO se producen durante el AR (Oracz *et al.*, 2007). La incubación en presencia de metilviológeno (compuesto generador de ERO) predispone a las semillas durmientes a la pérdida de la dormición.

Un concepto reciente de AR sugiere que este proceso actúa incrementando la

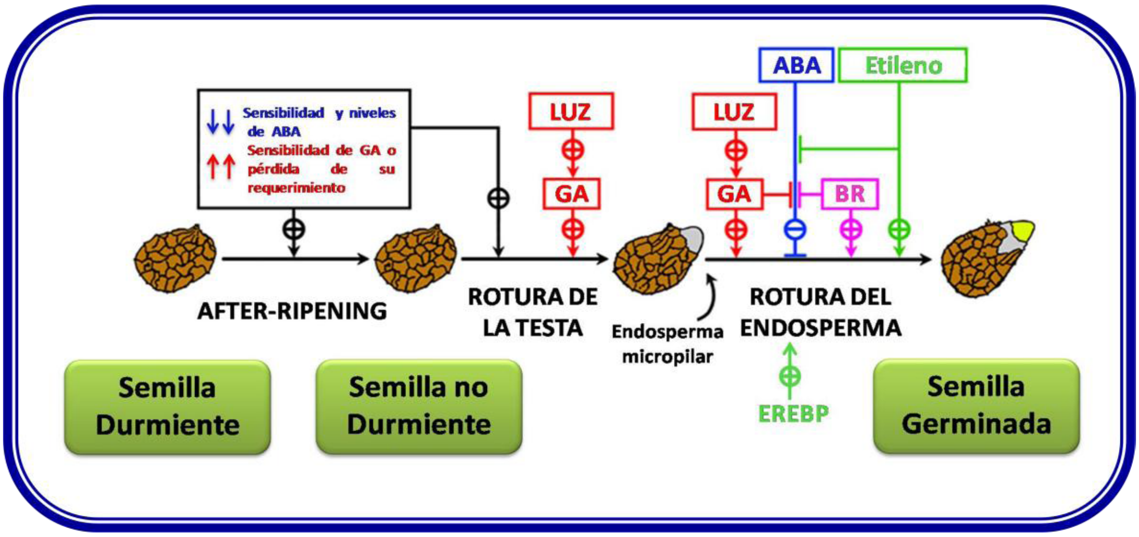

Figura 5: Interacciones hormonales a lo largo de la dormición, AR y germinación, así como de sus efectos en la ruptura de la testa y del endosperma, de semillas de tabaco. ABA: Ácido abscísico; BR: Brasinoesteroides. (Adaptado de Kucera y *et al.*, 2005).

sensibilidad de la semilla a las condiciones ambientales, promoviendo de esta forma la germinación; al mismo tiempo que disminuye la sensibilidad a las condiciones que reprimen la germinación (Finch-Savage y Leubner-Metzger, 2006). El AR en la semilla está determinado por el grado de hidratación, el contenido de aceites, la cubierta seminal y la temperatura. Además, y como anteriormente se ha citado, el AR requiere que el contenido de humedad se encuentre por encima de un valor umbral (Manz *et al.*, 2005).

Los principales efectos del AR en la semilla:

- Mayor rango de temperatura de germinación (Oracz *et al.*, 2007).
- Disminución en los niveles de ABA y sensibilidad al mismo, y aumento de la sensibilidad a GAs o pérdida de su requerimiento (Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004; Cadman *et al.*, 2006) (Fig. 5).
- Pérdida del requerimiento de la luz para aquellas semillas que no germinan en oscuridad (Derkx y Karssen, 1993a) y un incremento en la sensibilidad a la luz en semillas que no germinan incluso con luz (Derkx y Karssen, 1993a; Batlla y Benech-Arnold, 2005).
- Pérdida de los requerimientos de NO_3^- (Derkx y Karssen, 1993a; Alboresi *et al.*, 2005).
- Aumento de la velocidad de germinación (Finch-Savage y Leubner-Metzger, 2006; Holdsworth *et al.*, 2008a).

1.8. – El Control Hormonal de la Germinación

Muchas hormonas vegetales han sido relacionadas con la germinación (Kucera *et al.*, 2005). Entre ellas, las más ampliamente conocidas son las GAs, relacionadas con la estimulación de la germinación, y el ABA con su inhibición (Finkelstein *et al.*, 2002, 2008; Yamaguchi y Kamiya, 2002; Yamaguchi y Nambara, 2006). Sin embargo, el papel del ET parece menos obvio que el del ABA y las GAs, ya que su intervención durante el mantenimiento de la dormición y durante la transición dormición-germinación está relacionada con una red compleja de señalizaciones cuyos pasos están por descubrir (Vandenbussche y van der Straeten, 2007) (Fig. 5). Por lo tanto, existen diversas opiniones concernientes al momento del desarrollo durante el cual el ET regula la dormición. Algunos autores sugieren que el

ET actúa mínimamente durante el establecimiento de la dormición y que su principal acción es durante la imbibición con el fin de finalizar la dormición y/o iniciar la germinación (Matilla y Matilla-Vázquez, 2008). En estudios donde fueron usados mutantes de *Arabidopsis* que tenían afectada la respuesta a ET, el ET endógeno provocó la germinación mediante la disminución de la sensibilidad al ABA endógeno (Beaudoin *et al.*, 2000). El ET parece por tanto ser un regulador negativo del ABA durante la germinación (Ghassemian *et al.*, 2000). En definitiva, el ET puede que actúe como un antagonista del ABA durante la terminación de la dormición y, en colaboración con las GAs, promueva la transición hacia la germinación. Aunque el ET y las GAs están implicados en este proceso, la participación de la GAs en la emergencia radicular parece ser tanto cuantitativa como cualitativamente más importante. Hasta la fecha, los datos publicados indican que el ET no es la hormona que dispara la transición dormición-germinación, pero sí forma parte de una compleja red de señalización involucrada en la dormición. No obstante, los detalles de dicha señalización son actualmente difíciles de descifrar. La mutación *etr1-2* producen poblaciones de semillas maduras que exhiben dormición primaria más pronunciada (Chiwocha *et al.*, 2005). Además, los mutantes *etr1-2* presentan desequilibrio homeostático de las rutas de ABA, auxina, citoquinina y GAs (Chiwocha *et al.*, 2005).

Aunque las señales de la germinación en las semillas comienzan a ser visibles con la emergencia radicular, es incuestionable que durante el período de maduración (Nakabayashi *et al.*, 2005; Holdsworth *et al.*, 2008a), imbibición (Yamauchi *et al.*, 2004; Finch-Savage y Leubner-Metzger, 2006) y almacenamiento en seco (Grappin *et al.*, 2000; Holdsworth *et al.*, 2008a) ocurren una serie de procesos preparatorios destinados a la rotura de las cubiertas seminales. Sin embargo, la identidad de estos procesos y su regulación hormonal está lejos de ser conocida en detalle a nivel molecular (Kucera *et al.*, 2005). Mucha de la información conocida hasta ahora sobre el AR ha sido proporcionada por estudios hechos en tabaco y *Arabidopsis*. Sin embargo, *Arabidopsis thaliana* ecotipo Ler (Lansberg erecta) y Col (Columbia) presentan un tipo de dormición débil que es eliminada por cortos períodos de AR (van de Schaar *et al.*, 1997), haciendo estas especies menos adecuadas para los estudios de dormición. En contraste, el ecotipo Cvi, el cual presenta dormición profunda, ya que requiere algunos meses de AR, es usado actualmente en estudios genéticos y moleculares de la dormición y AR (Alonso-Blanco *et al.*, 2003; Ali-Rachedi *et al.*, 2004; Bentsink *et al.*, 2006; Carrera *et al.*, 2008; Holdsworth *et al.*, 2008b).

1.9. – La Germinación

La germinación representa un rasgo clave tanto ecológico como agronómico, ya que determina cuando las especies vegetales entran en los ecosistemas (Bewley y Black, 1994; Wilkinson *et al.*, 2002). Por lo tanto, es obvio que este proceso está altamente regulado tanto interna como externamente por factores que determinan el estatus de la dormición y el potencial germinativo. La germinación está definida como la emergencia radicular a través de cubiertas envolventes de la semilla (Bewley, 1997b; Baskin y Baskin, 2004; Finch-Savage y Leubner-Metzer, 2006). En *Arabidopsis thaliana*, la dormición de su semilla está clasificada como no profunda (DFnP), y se pierde mediante la estratificación o AR (Baskin y Baskin, 2004). En *Arabidopsis,* el embrión está rodeado por una sola capa de células endospérmica*s* (también llamada *capa de aleurona*), la cual procede de la doble fecundación (Finch-Savage y Leubner-Metzger, 2006), y la testa (Debeaujon *et al.*, 2000).

Investigaciones a nivel genético, fisiológico y molecular en *Arabidopsis*

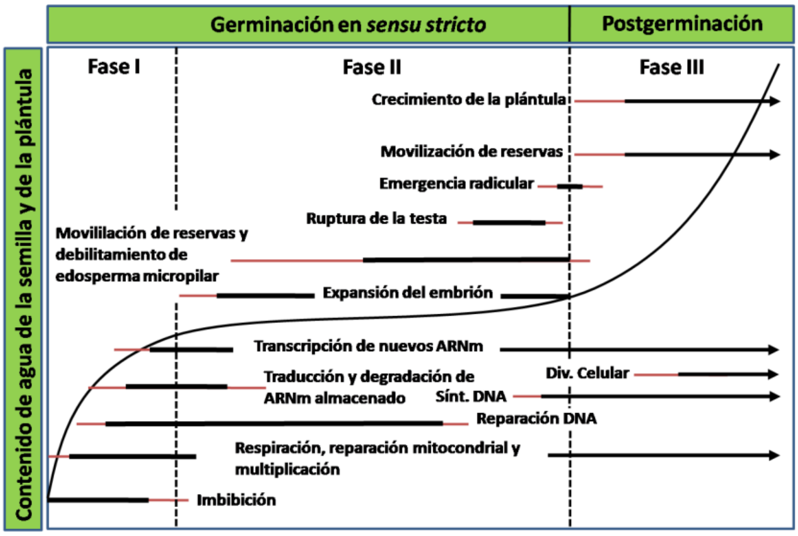

Figura 6: Transcurso de la imbibición y momentos más importantes asociados con la germinación y el crecimiento de la plántula. (Adaptado de Bewley *et al*., 2000)

revelaron la existencia de muchos componentes relacionados con la inducción y mantenimiento de la dormición, y la germinación (léase luz, temperatura y hormonas). La principal conclusión es que el ABA es un regulador positivo de la dormición, mientras que las GAs liberan a la semilla de la dormición y promueven la germinación, contrarrestando los efectos del ABA (Bewley y Black, 1994). Por esa razón, los primeros loci de germinación identificados en *Arabidopsis* mediante mutaciones incluyen aquellos que actúan en la biosíntesis y percepción de GAs y ABA (Koornneef, 1978; Koornneef y van der Veen, 1980; Koornneef *et al*., 1982; 1984).

Los primeros pasos de la germinación están relacionados con la toma de agua con el fin de activar los procesos metabólicos. La entrada inicial de agua es un proceso físico y está presente en todas las semillas, tanto vivas como muertas. En muchos casos, las semillas viables muestran un perfil de toma de agua dividido en tres fases: entrada de agua rápida (fase I), fase de meseta (fase II) de duración variable, y nueva fase de toma rápida (Fase III) asociada con el crecimiento del embrión (Bewley *et al*., 2000) (visión general en Fig.5).

La velocidad de imbibición está inicialmente controlada por la permeabilidad de la testa, la cual contiene células lignificadas. La entrada de agua suele producirse por aquellos puntos menos impermeables, que en el caso de muchas especies vegetales es la región micropilar (McDonald *et al*., 1988, 1994). La toma de agua podría estar afectada por la presencia de canales de agua (acuaporinas) (Maurel *et al*., 1997).

- **La testa o cubierta seminal y su implicación en la germinación**

Los mutantes en alguna característica de la cubierta seminal exhiben dormición reducida o aumentada, demostrando que la presencia y/o propiedades de los tejidos externos al embrión pueden afectar al tiempo y a la consecución de la germinación (Debeaujon *et al*., 2000; Downey *et al*., 2004). En algunas semillas (ej. *Brassicaceae*), la testa y las cubiertas envolventes son delgadas y relativamente débiles; y por consiguiente, se ropen relativamente fácil durante el proceso de imbibición. Son barreras poco resistentes al paso del embrión (Schopfer *et al*, 1979). En otros casos, tales como el tabaco, la testa se rompe durante la imbibición, pero la germinación es controlada por el tejido endospérmico que rodea al embrión (Manz *et al*., 2005). Son los casos del tomate (*Licopersicum esculentum*), pimiento (*Capsicum annuum*), estramonio (*Datura*), o lechuga (*Latuca sativa*) (Watkins y Cantliffe, 1983;

Groot y Karssen, 1987; Sánchez *et al.*, 1990; Sung *et al.*, 1998); la testa podría contribuir pero con una incidencia mucho menor (Hilhosrt y Downie, 1996). Aún en ausencia de resistencias provocadas por las envueltas circundantes al embrión, los tejidos embrionarios deben expandirse; por lo que los procesos relacionados con el crecimiento celular también regulan la germinación (de Miguel y Sánchez, 1992; Toorop *et al.*, 1998). En definitiva, el balance entre la resistencia ejercida por los tejidos envolventes y el potencial de crecimiento provocado por el embrión, determinará si la emergencia radicular ocurre o no.

- **Proteínas DELLA y germinación**

Las proteínas DELLA son reguladores negativos de las diversas respuestas a GAs (Sun y Gubler, 2004). Las GAs estimulan la germinación mediante la desaparición de estas proteínas (Ariizumi y Steber, 2007; Tyler *et al.*, 2004). Las proteínas de la familia DELLA presentan un dominio DELLA en el extremo N-terminal que es necesario para la unión a GAs, y un dominio GRAS en el extremo C-terminal, que es importante para su correcto funcionamiento (Peng *et al.*, 1999; Pysh *et al.*, 1999). Dentro de esta familia de proteínas nos encontramos GAI (GA-INSENSITIVE), RGA (REPRESSOR OF GA1-3) y SCARECROW. Mutaciones en el dominio DELLA provocan una estimulación de la señalización de las GAs produciendo fenotipos similares a mutantes deficientes en GAs; mientras que la pérdida de función en el dominio GRAS resulta en un aumento o una respuesta constitutiva a GAs (Thomas *et al.*, 2005).

RGL2 (*RGA-LIKE*) parece ser una de las principales proteínas DELLA implicadas en la germinación, ya que la pérdida de la función *RGL2* parcialmente restaura la germinación en semillas deficientes en GAs (Lee *et al.*, 2002; Tyler *et al.*, 2004). Sin embargo, mutaciones únicas en cualquiera de los otros miembros no son suficientes, aunque combinaciones de RGA, GAI y RGL1 pueden incrementar la restauración de la germinación (Cao *et al.*, 2005). Evidencias recientes sugieren que las proteínas DELLA promueven la dormición de las semillas a través de la inhibición de la expansión del cotiledón (Pendfield *et al.*, 2006), probablemente reprimiendo la expresión de enzimas hidrolíticas y modificadoras de la pared celular.

Las GAs son capaces de provocar la inhibición de las proteínas DELLA en la germinación mediante la activación del proteosoma 26S, cuya actividad se inicia mediante poli-ubiquitinación producida por la E3 ubiquitina ligasa SCF$^{SLY1/GID2}$ (proteína Skp1-Cdc53/CUL-1/F-box, en este caso SLY1/GID2) (Ariizumi y Steber, 2007). Además de los mecanismos de regulación de la concentración de las proteínas DELLA a través de la destrucción de las mismas, parecen existir mecanismos de regulación post-traduccional, como la fosforilación o las mutaciones fosfomímicas que parecen estabilizar al RGL2 (Hussain *et al.*, 2005; 2007). El mutante *sly1* es capaz de soportar la dormición mediante la mutación de *RGL2*, sin embargo, estos mutantes presentan elevada acumulación de RGL2, sugiriendo mecanismos de regulación post-traduccionales (Ariizumi y Steber, 2007). También las mutaciones en *Arabidopsis* del gen *SPY* [O-linked β-N-acetilglucosamina (O-GlcNAc) transferasa SPINDLY] estimulan la germinación independiente de GAs e incrementan la estabilidad de las proteínas DELLA, sugiriendo que la modificación O-GlcNAc puede activarlas o estimular su *turnover* (Jacobsen *et al.*, 2002; Shimada *et al.*, 2006; Silverstone *et al.*, 2007).

- **Otros genes relacionados con la germinación**

Algunos estudios genéticos han resaltado la idea de la importancia de la interrupción del catabolismo de triacilgliceroles (TAG) en la liberación de la dormición y/o germinación. Los mutantes defectuosos en las rutas catabólicas lipídicas y en la β-oxidación [*kat2* (*ketacil-CoA thiolase-2*) y *acx1acx2* (*acylcoA oxidase*) o el mutante doble *csy2csy3* (*citrato synthase*)] (Footitt *et al.*, 2006; Pindfield-Wells *et al.*, 2005; Pracharoenwattana *et al.*, 2005) o en el transporte de ácidos grasos de cadena larga al peroxixoma (*cts*, *COMATOSE*) (Russell *et al.*, 2000; Footitt *et al.*, 2002; Theodoulou *et al.*, 2005) son profundamente durmientes.

Solamente se ha identificado y clonado un QTL (Quantitative Trait Locus) relacionado con la germinación en *Arabidopsis*, DOG1. Este gen se expresa solo en semillas, y parece ser esencial y específico de la dormición. Codifica para cuatro transcriptos alternativamente escindidos que codifican para proteínas de función desconocida. Existe correlación entre el grado de dormición y los niveles de expresión de *DOG1* (Bentsink *et al.*, 2006).

- **Enzimas implicadas en el debilitamiento de las cubiertas seminales y los tejidos envolventes**

Los mecanismos que provocan el debilitamiento del endosperma tienen gran interés desde los años 60, cuando Ikuma y Thimann (1963) sugirieron, basándose en sus estudios de germinación en semillas de lechuga, que el debilitamiento del endosperma era producto de la degradación provocada por enzimas. Debido a que las paredes celulares son las responsables de la forma y resistencia de las células vegetales, el estudio de las hidrolasas de las mismas se ha convertido en el principal foco de estudio en relación a la emergencia radicular. La emergencia radicular requiere, por una parte, la disminución de la resistencia mecánica de los tejidos circundantes al *micrópilo* (*loosening*); y por otra, el incremento de la fuerza interna producida por la expansión de los cotiledones y el eje embrionario (Fig. 6) (Nonogaki *et al.*, 2007).

Expansinas

Las expansinas son proteínas de la pared celular que tienen la capacidad de producir la extensión de paredes celulares aisladas (Cosgrove *et al.*, 2002). Existen tres genes de expansinas que se expresan a lo largo de la germinación en semillas de tomate. Una de ellas, *LeEXPA4* (Kende *et al.*, 2004), se expresa específicamente en el endosperma micropilar a las 12 h de imbibición, coincidente con el comienzo del debilitamiento del endosperma (Chen y Bradford, 2000). Las GAs estimulan la expresión de *LeEXPA4* en las semillas de tomate de mutantes deficientes en GAs (*gib-1*). Sin embargo, la expresión de *LeEXPA4* no es inhibida por ABA, apoyando la idea de que el ABA no está implicado en el debilitamiento del *endosperma micropilar*.

Xiloglucan-endotransglicosilasa/hidrolasas (XTHs)

Las XTHs modifican el xiloglucano, el cual es uno de los componentes principales de las paredes celulares en Dicotiledóneas (Carpita y Gibeaut, 1993). El gen *LeXET4* se expresa en semillas de tomate entre las 12-24 h de imbibición. Además, está localizado exclusivamente en la región del endosperma micropilar. Al igual que *LeEXPA4*, la expresión de *LeXET4* también es dependiente de GAs, pero independiente de ABA (Nonogaki *et al.*, 2007) (Fig. 7).

Endo-β-mananasa, α-galactosidasa y β–manosidasa

Los mananos son polisacáridos hemicelulósicos presentes en el endosperma. La degradación de los mismos involucra a las endo-β-mananasas, α-galactosidasas y β -manosidasas, las cuales han sido identificadas a lo largo de la germinación de numerosas semillas.

Las **endo-β-mananasas** hidrolizan los enlaces internos de los β-1,4-D-manopiranosil presentes en las cadenas de mananos. En las semillas de numerosas especies se produce un importante incremento de la actividad endo-β-mananasa durante la germinación. A lo largo de la germinación de las semillas de tomate se hallaron dos genes de endo-β-mananasas, *LeMAN1* y *LeMAN2.* La expresión de *LeMAN2* es detectable a las 12-18 h de imbibición, mientras que *LeMAN1* no se expresa hasta que la germinación se ha completado, a las 48 h (Bewley *et al.*, 1997a; Nonogaki *et al.*, 2000). Ambos genes se expresan en tejidos diferentes, *LeMAN2* se expresa solamente en la región del endosperma micropilar, mientras que *LeMAN1* está localizada en el *endosperma lateral* (Fig. 7). LeMAN1 probablemente es la responsable de la movilización de las reservas en el endosperma lateral que proporciona nutrientes para el crecimiento de la plántula. LeMAN2 parece implicada en la degradación del galactomanano del endosperma micropilar. La expresión de *LeMAN2* es inducida por GAs en el mutante *gib-1*, pero no es inhibida por ABA en las semillas del fenotipo silvestre (Nonogaki *et al.*, 2000).

En *Datura ferox*, la luz roja estimula la expresión de una endo-β-mananasa (*DfMan*) en el endosperma micropilar, así como estimula la actividad enzimática (Arana *et al.*, 2006). La actividad endo-β-mananasa de lechuga es estimulada por GAs e inhibida por ABA (Halmer *et al.*, 1976; Dulson *et al.*, 1988). Además, se han encontrado tres isoformas de endo-β-mananasa durante la germinación de (Nonogaki y Morohashi, 1999; Wang *et al.*, 2004). Por otra parte, el tratamiento con ET incrementa significativamente la actividad endo-β-mananasa en estas semillas (Nascimento *et al.*, 2000), lo que hace aventurar que la promoción de la germinación después del tratamiento con ET es debido al debilitamiento del endosperma micropilar provocado por un aumento de la actividad endo-β-mananasa (Nascimento *et al.*, 2004). Sin embargo, el gen *LsMan1* se expresa específicamente en el endosperma de lechuga, pero cuando la germinación ha finalizado (Nonogaki y Morohashi, 1999). Teniendo en cuenta estos resultados, es probable que la actividad endo-β-mananasa en lechuga esté relacionada con la movilización de reservas y no con el debilitamiento del endosperma micropilar.

En semillas de café fueron clonadas dos endo-β-mananasas, *manA* y *manB* (Marraccini *et al.*, 2001). Ambos genes sólo se expresan en semillas germinadas, y no se han observado en otros tejidos. Su expresión es coincidente con la iniciación de la emergencia radicular y decrece una vez ésta ha finalizado (Marraccini *et al.*, 2001). La actividad endo-β-mananasa se encuentra en el endosperma micropilar, pero a diferencia del tomate, es inhibida por ABA (da Silva *et al.*, 2004).

Las **α-galactosidasas** hidrolizan los residuos de galactosa unidos al esqueleto de mananos de los galactomananos. La actividad α-galactosidasa ha sido detectada en la región micropilar y lateral del endosperma de semillas de tomate. *LeαGal*, está presente en la región micropilar y lateral del tejido endospérmico, aunque también en el embrión (Feurtado *et al.*, 2001) (Fig. 7).

Las **β-manosidasas** hidrolizan los residuos termianles no reductores de β-D-manosas de los oligomananos liberados por la acción de las endo-β-mananasas y las α-galactosidasas. La actividad β-manosidasa ha sido detectada en el endosperma micropilar de las semillas de varias especies, incluyendo las de tomate (Mo y Bewley, 2002), *Datura* (de Miguel *et al.*, 2000) y café (da Silva *et al.*,

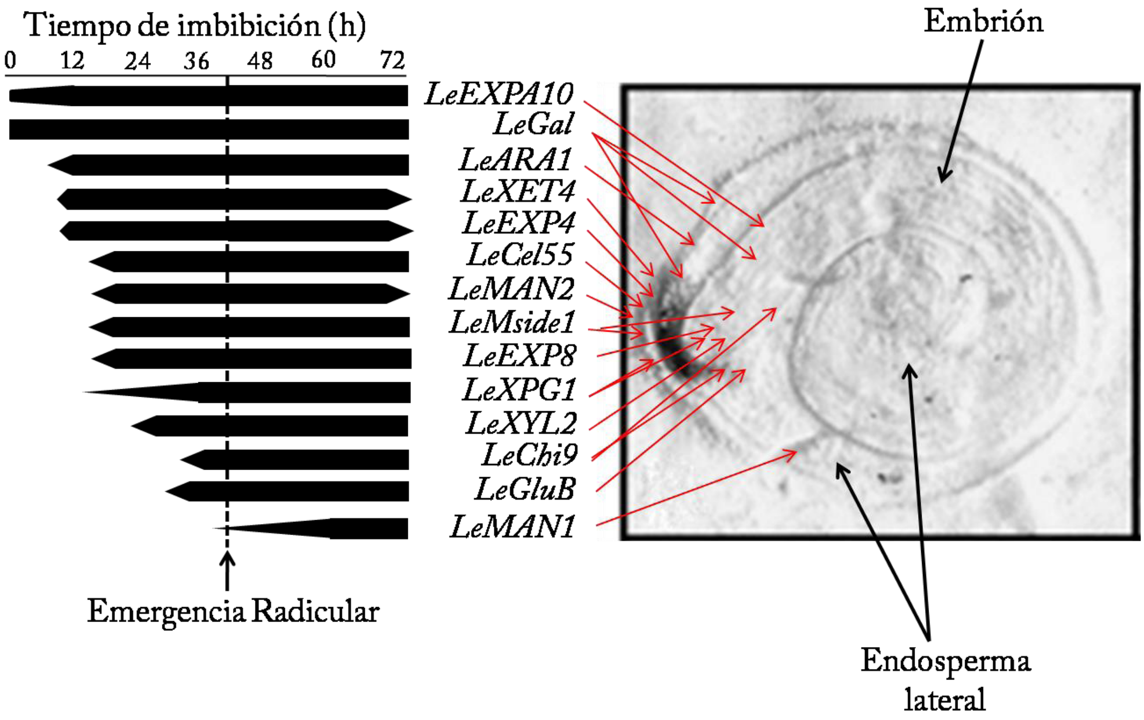

Figura 7: Localización espacial y temporal de la expresión de genes de enzimas hidrolíticas en semillas de tomate. La región teñida muestra el endosperma micropilar (Adaptado de Nonogaki *et al.*, 2007)

2005). Tanto en semillas de café como tomate, la actividad β-manosidasa incrementa con un perfil temporal similar al de la actividad endo-β-mananasa (Mo y Bewley, 2002; da Silva *et al.*, 2005). El transcripto de una β-manosidasa en tomate, *LeMside1*, fue detectado en la región del endosperma micropilar y lateral así como en el embrión de esta semilla (Mo y Bewley, 2002) (Fig. 7).

Celulasa

La enzima clave para degradar la celulosa es la endo-β-1,4-glucanasa o celulasa. La actividad celulasa ha sido detectada a lo largo de la germinación de semillas de numerosas especies vegetales. En semillas de café hay un incremento significativo de la actividad celulasa durante la fase inicial del ablandamiento del endosperma micropilar (da Silva *et al.*, 2004). El transcripto del gen *LeCel55* incrementa su expresión en la radícula y en el endosperma micropilar de las semillas de tomate antes de que la germinación finalice (Bradford *et al.*, 2000) (Fig. 7).

Otros enzimas hidrolíticos

También se encontró el gen (*LeARA1*) de una α-L-arabinofurosidasa que se expresa en el endosperma lateral durante la germinación de las semillas de tomate (Bradford *et al.*, 2000) (Fig. 7). Este se expresa antes de la emergencia radicular, su localización sugiere que está principalmente implicado en procesos post-germinativos.

Un gen relacionado con la hidrólisis de las pectinas (exopoligalacturonasa, *LeXPG1*) se expresa durante la germinación de las semillas de tomate, encontrándose principalmente expresado en el endosperma micropilar y en el ápice radicular (Sitrit *et al.*, 1999) (Fig. 7). El grado de esterificación de las pectinas también parece estar relacionado con el debilitamiento del endosperma radicular, ya que la actividad pectin-metilestearasa (PME) se ha encontrado en el endosperma micropilar de las semillas de tomate durante la germinación, aunque la mayor actividad se halló en el embrión (Downie *et al.*, 1998).

El papel de la β-1,3-glucanasa (βGLU) se ha estudiado ampliamente a lo largo de la germinación de las semillas de tabaco. El análisis de la expresión del gen *βGLU* así como su actividad enzimática incrementa justo antes de la ruptura del endosperma (Leubner-Metzger *et al.*, 1995). El análisis de βGLU por inmunolocalización en semillas transgénicas de tabaco reveló que se encuentra localizada principalmente en el endosperma micropilar y en el tegumento interno de la testa (Leubner-Metgzer, 2002). Además la expresión sentido de *βGLU* es capaz de reemplazar parcialmente el requerimiento de AR de las semillas de tabaco (Leubner-Metzger y Meins, 2000) y la expresión antisentido aumenta el período de dormición de las mismas (Leubner-Metzger y Meins, 2001).

BIBLIOGRAFÍA

Alboresi A, Gestin C, Leydecker M-T, Bedu M, Meyer C, Truong HN (2005) Nitrate, a signal relieving seed dormancy in *Arabidopsis*. *Plant, Cell Environ* **28**: 500-12

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, *et al.* (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219**: 479-88

Almoguera C, Jordano J (1992) Developmental and environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and LEA messenger-RNAs. *Plant Mol Biol* **19**: 781-92

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn-de Vries H, Koornneef M (2003) Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genet* **164**: 711-29

Amen RD (1968) A model of seed dormancy. *Bot Rev* **34**: 1-31

Arana MV, de Miguel LC, Sánchez RA (2006) A phytocrome-dependent embryonic factor modulates gibberellin responses in the embryo and mycropylar endosperm of *Datura ferox* seeds. *Planta* **223**: 847-57

Ariizumi T, Steber CM (2007) Seed germination of GA-insensitive *sleepy1* mutants does not require RGL2 protein disappearance in *Arabidopsis*. *Plant Cell* **19**: 791-804

Bailly C (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci Res* **14**: 93-107

Bair NB, Meyer SE, Allen PS (2006) A hydrothermal after-ripening time model for seed dormancy loss in *Bromus tectorum* L. *Seed Sci Res* **16**: 17-28

Baskin, CC & Baskin, JM (1998) *Seeds: ecology, biogeography, and evolution of dormancy and germination*. Academic Press, San Diego

Baskin, CC & Baskin, JM (2004) A classification system for seed dormancy. *Seed Sci Res* **14**: 1-16

Batlla D, Benech-Arnold RL (2005) Changes in the light sensitivity of buried *Polygonum aviculare* seeds in relation to cold-induced dormancy loss: development of a predictive model. *New Phytol* **165**: 445-52

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signaling cascades. *Plant Cell* **12**: 1103-15

Bentsink L, Jowett J, Hanhart CJ, Koornneef M (2006) Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. *Proc Natl Acad Sci* USA **103**: 17042-47

Bentsink L, Soppe W, Koornneef (2007) Genetic aspects of seed dormancy. *Annu Plant Rev* **27**: 176-223

Berjak P (2006) Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Sci Res* **16**: 1-15

Bethke PC, Libourel IG, Aoyama N, Chung YY, Still DW, Jones RL (2007)The *Arabidopsis* aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. *Plant Physiol* **143**: 1173-88

Bewley JD (1997a) Breaking down the walls – a role for endo-betamannanase in release from seed dormancy? *Plant Sci* **2**: 464-69

Bewley JD (1997b) Seed germination and dormancy. *Plant Cell* **9**(7): 1055-66

Bewley JD, Black M (1994) *Seeds: physiology of development and germination*. Pp. 367. Plenum, New York

Bewley JD, Hempel FD, McCormick, Zambryski (2000) Reproductive development. En: *Biochemistry & molecular biology of plants* (eds. Buchanan B, Gruissem W, Jones R) Pp. 988-1043. American Society of Plant Biologist, Rockville, MD

Boesewinkel FD, Bouman F (1995) The seed: structure and function. En: *Seed development and germination* (eds. Kigel J, Galili G) Pp. 1-24. Marcel Dekker, New york

Bove J, Lucas P, Godin B, Oge L, Jullien M, Grappin P (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Mol Biol* **57**: 593-612

Bradford KJ, Bewley JD (2003) Seeds: Biology, Technology and Role in Agriculture. En: *Plants, genes and crop biotechnology* (eds. Chrispeels MJ, Sadava DE) Pp. 212-39. Jones and Bartlett, Massachusetts, USA

Bradford KJ, Chen F, Cooley MB, *et al.* (2000) Gene expression prior to radicle emergence in imbibed tomato seeds. En: *Seed Biology: Advances and Applications* (eds. Black M, Bradford KJ, Vázquez-Ramos J) Pp. 231-51. CABI, Wallingford, UK

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE (2006) Gene expression profiles of *Arabidopsis* Cvi seed during cycling through dormant and non-dormant states indicate a common underlying dormancy control mechanism. *Plant J* **46**: 805-22

Cao DN, Hussain A, Cheng H, Peng JR (2005) Loss of function of four DELLA genes leads to light- and gibberellin-independent seed germination in *Arabidopsis*. *Planta* **223**: 105-13

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J* **3**: 1-30

Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL, Holdsworth MJ (2008) Seed after-ripening is a discrete developmental pathway associated with specific gene networks in *Arabidopsis*. *Plant J* **53**: 214-24

Chae MJ, Lee JS, Nam MH, Cho K, Hong JY, *et al.* (2007) A rice dehydration-inducible SNF1-related protein kinase 2 phosphorylates an abscisic acid responsive element binding factor and associates with ABA signaling. *Plant Mol Biol* **63**: 151-69

Chen F, Bradford KJ (2000) Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiol* **124**: 1265-74

Cheng WH, Endo A, Zhou L, Penney J, Chen HC, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* **14**: 2723-43

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P (2006) Proteomic analysis of seed dormancy in *Arabidopsis*. *Plant Physiol* **142**: 1493-1510

Chiwocha SD, Cutler AJ, Abrams SR, Ambrose SJ,Yang J, *et al.* (2005) The *etr1-2* mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. *Plant J* **42**: 35-48

Choi HI, Park HJ, Park JH, Kim S, Im MY, *et al.* (2005) *Arabidopsis* calcium-dependent protein kinase AtCPK32 interacts with ABF4, a transcriptional regulator of abscisic acid-responsive gene expression, and modulates its activity. *Plant Physiol* **139**: 1750-61

Christmann A, Moes D, Himmelbach A, Yang Y, Tang Y, Grill E (2006) Integration of abscisic acid signalling into plant responses. *Plant Biol* **8**: 314-25

Clouse SD (2001) Brassinosteroids. *Curr Biol* **11**: R904

Clouse SD, Sasse JM (1998) Brassinosteroids: essential regulators of plant growth and development. *Annu Rev Plant Physiol Plant Mol Biol* **49**: 427-51

Comai L, Harada JJ (1990) Transcriptional activities in dry seed nuclei indicate the timing of the transition from embryogeny to germination. *Proc Natl Acad Sci, USA* **87**: 2671-74

Cosgrove DJ, Li LC, Cho HT, Hoffmann-Benning S, Moore RC, Blecker D (2002) The growing world of expansions. *Plant Cell Physiol* **43**: 1436-44

Cutler S, Ghassemian M, Bonetta D, Cooney S, McCourt P (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in *Arabidopsis*. *Science* **273**: 1239-41

da Silva EAA, Toorop PE, Nijsse J, Bewley JD, Hilhorst HWM (2005) Exogenous gibberellins inhibit coffee (*Coffea arabica* cv. Rubi) seed germination and cause cell death in the embryo. *J Exp Bot* **56**: 1029-38

da Silva EAA, Toorop PE, van Aelst AC, Hilhorst HWM (2004) Abscisic acid controls embryo growth potential and endosperm cap weakening during coffe (*Coffea arabica* cv. Rubi) seed germination. *Planta* **220**: 251-61

Debeaujon I, Koornneef M (2000) Gibberellin requirement for *Arabidopsis* seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiol* **122**: 415-24

Debeaujon I, Lepiniec L, Pourcel L, Routaboul JM (2007) Seed coat development and dormancy. En: *Seed development, dormancy and germination* (eds. Bradford K y

Nonogaki H) Pp. 25-49. Blackwell Publishing, Oxford

de Miguel L, Burgin MJ, Casal JJ, Sánchez RA (2000) Antagonistic action of low-fluence amd high irradiance modes of response of phytocrome on germination and β-mannanase activity in *Datura ferox* seeds. *J Exp Bot* **51**: 1127-33

de Miguel L, Sánchez RA (1992) Phytochrome-induced germination, endosperm softening and embryo growth-potential in *Datura ferox* seeds. Sensitivity to low water potential and time to escape to Fr reversal. *J Exp Bot* **43**: 969-74

Derkx MPM, Karssen CM (1993a) Changing sensitivity to light and nitrate but not to gibberellins regulates seasonal dormancy patterns in *Sisymbrium officinale* seeds. *Plant, Cell Environ* **16**: 469-79

Derkx MPM, Karssen CM (1993b) Effects of light and temperature on seed dormancy and gibberellins stimulated germination in *Arabidopsis thaliana*: studies with gibberellins deficient and -insensitive mutants. *Physiol Plant* **89**: 360-68

Derkx MPM, Vermeer E, Karssen CM (1994) Gibberellins in seeds of *Arabidopsis thaliana*: biological activities, identification, and effect of light and chilling on endogenous levels. *Plant Growth Regul* **15**: 223-34

Donohue K, Dorn L, Griffith C, Kim E, Aguilera A, Polisetty CR, Schmitt J (2005) The evolutionary ecology of seed germination of *Arabidopsis thaliana*: variable natural selection on germination timing. *Evol* **59**: 758-70

Downey AB, Dirk LMA, Hadfield KA, Wilkins TA, Bennett AB, Bradford KJ (1998) A gel diffusion assay for quantification of pectin methylestearase activity. *Anal Biochem* **264**: 149-57

Downey AB, Dirk LMA, Xu QL (2004) A physical, enzymatic and genetic characterization of perturbations in the seeds of the brown seed tomato mutants. *J Exp Bot* **55**: 961-73

Dulson J, Bewley JD, Johnston RN (1988) Abcisic acid is an endogenous inhibitor in the regulation of mannanase production by isolated lettuce (*Latuca sativa* cv. Grand Rapids) endosperms. *Plant Physiol* **87**: 660-65

Dure L, Waters L (1965) Long-lived messenger RNA: evidence from cotton seed germination. *Science* **147**: 410-12

Fagoaga C, Tadeo FR, Iglesias DJ, Huerta L, Lliso I, Vidal AM, Talón M, Navarro L, García-Martínez JL, Peña L (2007) Engineering of gibberellin levels in citrus by sense and antisense overexpression of a *GA20-oxidase* gene modifies plant architecture. *J Exp Bot* **58**: 1407-20

Feurtado AJ, Banik M, Bewley JD (2001) the cloning and characterization of α-galactosidase present during and following germination of tomato (*Lycopersicon esculentum* Mill.) seed. *J Exp Bot* **52**: 1239-49

Feurtado AJ, Kermode AR (2007) A merging of paths: abscisic acid and hormonal cross-talk in the control of seed dormancy maintenance and alleviation. En: *Seed development, dormancy and germination* (eds. Bradford K y Nonogaki H) Pp. 176-223. Blackwell Publishing, Oxford

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW (2007) Seed dormancy release in *Arabidopsis* Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directly by environment specific sensing. *Plant J* **51**: 60-78

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* **171**: 501-23

Finkelstein R, Campala SSL, Rock CD (2002) Abcisic acid signaling in seeds and seedlings. *Plant Cell* **14**: S15-S45

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* **59**: 387-415

Footitt S, Slocombe SP, Larner V, Kurup S,Wu Y, *et al.* (2002) Control of germination and lipid mobilization by COMATOSE, the *Arabidopsis* homologue of human ALDP. *EMBO J* **21**: 2912-22

Footitt S, Marquez J, Schmuths H, Baker A, Theodoulou FL, Holdsworth M (2006) Analysis of the role of *COMATOSE* and peroxisomal beta-oxidation in the determination of germination potential in *Arabidopsis*. *J Exp Bot* **57**: 2805-14

Fujii H, Verslues PE, Zhu JK (2007) Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in *Arabidopsis*. *Plant Cell* **19**: 485-94

Furihata T, Maruyama K, Fujita Y, Umezawa T, Yoshida R, *et al.* (2006) Abscisic acid dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc Natl Acad Sci, USA* **103**: 1988-93

Galichet A, Gruissem W (2003) Protein farnesylation in plants—conserved mechanisms but different targets. *Curr Opin Plant Biol* **6**: 530-35

Gazzarrini S, Tsuchiya Y, Lumba S, Okamoto M, McCourt P (2004) The Transcription Factor FUSCA3 controls developmental timing in *Arabidopsis* through the hormones gibberellin and abscisic acid. *Develop Cell* **7**: 373-38

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of abscisic acid signaling by the ethylene response pathway in *Arabidopsis*. *Plant Cell* **12**: 1117-26

Goldberg RB, De Paiva G, Yadegari R (1994) Plant embryogenesis: zygote to seed. *Science* **266**: 605-14

Gómez-Cadenas A, Verhey SD, Holappa LD, Shen Q, Ho T-HD, Walker-Simmons MK (1999) An abscisic acid-induced protein kinase, PKABA1, mediates abscisic acid suppressed gene expression in barley aleurone layers. *Proc Natl Acad Sci, USA* **96**: 1767-72

Gómez-Cadenas A, Zentella R, Walker-Simmons MK, Ho T-HD (2001) Gibberellin/ abscisic acid antagonism in barley aleurone cells: Site of action of the protein kinase PKABA1 in relation to gibberellin signaling molecules. *Plant Cell* **13**: 667-79

Grappin P, Bouinot D, Sotta B, Miginiac E, Julien M (2000) Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta* **210**: 279-85

Groos C, Gay G, Perretant MR, Gervais L, Bernard M, *et al.* (2002) Study of the relationship between preharvest sprouting and grain color by quantitative trait loci analysis in a white × red grain bread-wheat cross. *Theor Appl Genet* **104**: 39-47

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening-a study with gibberellins-deficient mutants. *Planta* **171**: 525-31

Gualberti G, Papi M, Bellucci L, Ricci I, Bouchez D, *et al.* (2002) Mutations in the Dof zinc finger genes *DAG2* and *DAG1* influence with opposite effects the germination of *Arabidopsis* seeds. *Plant Cell* **14**: 1253-63

Guo J, Zeng Q, Emami M, Ellis BE, Chen JG (2008) The GCR2 gene family is not required for ABA control of seed germination and early seedling development in *Arabidopsis*. *PLoS ONE* **3**: e2982

Halmer P, Bewley JD, Thorpe TA (1976) Enzyme to degrade lettuce endosperm cell walls-appearance of a mannanase following phytochrome-induced and gibberellins-induces germination. *Planta* **130**: 189-96

Haslekås C, Stacy RA, Nygaard V, Culiánez-Macià FA, Aalen RB (1998) The expression of a peroxiredoxin antioxidant gene, *AtPer1*, in *Arabidopsis thaliana* is seed-specific and related to dormancy. *Plant Mol Biol* **36**: 833-45

Henderson JT, Li HC, Rider SD, Mordhorst AP, Romero-Severson J, *et al.* (2004) PICKLE acts throughout the plant to repress expression of embryonic traits and may play a role in gibberellin-dependent responses. *Plant Physiol* **134**: 995-1005

Hilhorst HWM, Derkx MPM, Karssen CM (1996) An integrating model for seed dormancy cycling: characterization of reversible sensitivity. En: *Plant dormancy: physiology, biochemistry and molecular biology* (ed. Lang G) Pp. 342-60. CAB International, Wallingford, UK

Hilhorst HWM, Downie B (1996) Primary dormancy in tomato (*Lycopersicon esculentum* cv. Moneymaker): studies with the *sitiens* mutant. *J Exp Bot* **47**: 89-97

Hilhorst HWM, Toorop, PE (1997) A review on dormancy, germinability and germination in crop and weed seeds. *Adv Agron* **61**: 111-65

Holdsworth MJ, Bentsink L, Soppe WJJ (2008a) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytol* **179**: 33-54

Holdsworth MJ, Finch-Savage WE, Grappin P, Job D (2008b) Post-Genomics dissection of seed dormancy and germination. *Trends Plant Sci* **13**: 7-13

Huang Z, Huc Z, Gutterman Y (2000) Structure and function of mucilaginous achenes of *Artemisa monosperma* inhabiting the Negev desert of Israel. *Israel J Plant Sci* **48**: 255-66

Huang Z, Boubriak I, Osbosne DJ, Dong M, Gutterman Y (2008) Possible role of pectin-containing mucilage and dew in repairing embryo DNA of seeds adapted to desert conditions. *Ann Bot* **101**(2): 277-83

Hussain A, Cao D, Cheng H, Wen Z, Peng J (2005) Identification of the conserved serine/ threonine residues important for gibberellin-sensitivity of *Arabidopsis* RGL2 protein. *Plant J* **44**: 88-99

Hussain A, Cao D, Peng J (2007) Identification of conserved tyrosine residues important for gibberellin sensitivity of *Arabidopsis* RGL2 protein. *Planta* **226**: 475-83

Iglesias-Fernández R, Matilla AJ (2009) After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during early imbibition of *Sisymbrium officinale* L. seeds. *J Exp Bot* **60**: 1645-61

Ikuma H, Thimann KV (1963) The role of the seed-coats in germination of photosensitive lettuce seeds. *Plant Cell Physiol* **4**: 169-85

Ishibashi N, Yamauchi D, Minamikawa T (1990) Stored mRNA in cotyledons of *Vigna unguiculata* seeds: nucleotide sequence of cloned cDNA for a stored mRNA and induction of its synthesis by precocious germination. *Plant Mol Biol* **15**: 59-64

Jacobsen JV, Pearce DW, Poole AT, Pharis RP, Mander LN (2002) Abscisic acid, phaseic acid and gibberellin contents associated with dormancy and germination in barley. *Physiol Plant* **115**: 428-41

Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK (2002) The abscisic acid responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant Physiol* **130**: 837-46

Karssen CM, Brinkhorst-van der Swan DLC, Breekland AE, Koornneef M (1983) Induction of dormancy during seed development by endogenous abscisic acid: Studies on abscisic acid deficient genotypes of *Arabidopsis thaliana* (L.) Heynh. *Planta* **157**: 158-65

Kende H, Bradford KJ, Brummell, *et al.* (2004) Nomenclature for members of the expansin superfamily of genes and proteins. *Plant Mol Biol* **55**: 311-14

Kermode AR (2005) Role of Abscisic Acid in Seed Dormancy. *J Plant Growth Regul* **24**: 329-44

Kobayashi Y, Murata M, Minami H, Yamamoto S, Kagaya Y, *et al.* (2005) Abscisic acid activated SNRK2 protein kinases function in the gene-regulation pathway of ABA signal transduction by phosphorylating ABA response element-binding factors. *Plant J* **44**: 939-49

Koornneef M (1978) Gibberellin sensitive mutants in *Arabidopsis thaliana. Arabidopsis Inform Serv* **15**: 17-20

Koornneef M, Jorna ML, Brinkhorst van der Swan DLC, Karssen CM (1982) The isolation of abscisic acid (ABA) deficient mutants by selection of induced revertants in non-germinating gibberellin sensitive lines of *Arabidopsis thaliana* (L.) Heynh. *Theoret Appl Genet* **61**: 385-93

Koornneef M, Hanhart CJ, Hilhorst HWM, Karssen CM (1989) *In vivo* imbibition of seed development and reserve protein accumulation in recombinants of abscisic acid biosynthesis and responsiveness mutants in *Arabidopsis thaliana. Plant Physiol* **90**: 439-69

Koornneef M, Reuling G, Karssen C (1984) The isolation and characterization of abscisic acid-insensitive mutants of *Arabidopsis thaliana. Physiol Plant* **61**: 377-83

Koornneef M, van der Veen JH (1980) Induction and analysis of gibberellin-sensitive mutants in *Arabidopsis thaliana* (L.) Heynh. *Theoret Appl Genet* **58**: 257-63

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Sci Res* **15**: 281-307

Kuligowski J, Ferrand M, Chenou E (1991) Stored mRNA in early embryos of a fern *Marsilea vestita*: a paternal and maternal origin. *Mol Rep Develop* **30**: 27-33

Lee S, Cheng H, King KE,Wang W, He Y, *et al.* (2002) Gibberellin regulates *Arabidopsis* seed germination via *RGL2*, a *GAI/RGA*-like gene whose expression is up-regulated following imbibition. *Genes Dev* **16**: 646-58

Lefebvre V, North H, Frey A, Sotta B, Seo M, *et al.* (2006) Functional analyses of *Arabidopsis NCED6* and *NCED9* genes indicates that ABA synthesized in the endosperm is involved in the induction of seed dormancy. *Plant J* **45**: 309-19

Leubner-Metzger G, Frundt C, Vogeli-Lange R, Meins F (1995) Class-I β-1,3-glucanases in the endosperm of tobacco during germination. *Plant Physiol* **109**: 751-59

Leubner-Metzger G, Meins F (2000) Sense transformation reveals a novel role for class-I β-1,3-glucanase in tobacco seed germination. *Plant J* **23**: 215-21

Leubner-Metzger G (2001) Brassinosteroids and gibberellins promote tobacco seed germination by distinct pathways. *Planta* **213**: 758-63

Leubner-Metzger G, Meins F (2001) Antisense-transformation reveals a novel roles for class-I β-1,3-glucanase in tomato seed after-ripening and photodormancy. *J Exp Bot* **52**: 1753-59

Leubner-Metzger G (2002) Seed after-ripening and over-expression of Class-I β-1,3-glucanase confer maternal effects on tobacco testa rupture and dormancy release. *Planta* 215: 959-68

Leubner-Metzger G (2005) Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant J* **41**: 133-45

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F (2007) Identification of transcripts potentially involved in barley seed germination and dormancy using cDNA-AFLP. *J Exp Bot* **58**: 425-37

Lin PC, Hwang SG, Endo A, Okamoto M, Koshiba T, Cheng WH (2007) Ectopic expression of *ABSCISIC ACID 2/GLUCOSE INSENSITIVE 1* in *Arabidopsis* promotes seed dormancy and stress tolerance. *Plant Physiol* **143**: 745-58

Liu PP, Koizuka N, Martin RC, Nonogaki H (2005) The BME3 (Blue Micropylar End 3) GATA zinc finger transcription factor is a positive regulator of *Arabidopsis* seed germination *Plant J* **44**: 960-71

Liu X, Yue Y, Li B, Nie Y, Li W, *et al.* (2007) A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science* **315**: 1712-16

Liu Y, Koornneef M, Soppe WJ (2007) The absence of histone H2B monoubiquitination in the *Arabidopsis hub1* (*rdo4*) mutant reveals a role for chromatin remodeling in seed dormancy. *Plant Cell* **19**: 433-44

Lohe AR, Chaudhury A (2002) Genetic and epigenetic processes in seed development. *Curr Opin Plant Biol* 5(1): 19-25

Lu C, Han M-H, Guevara-Garcia A, Fedoroff N (2002) Mitogen-activated protein kinase signaling in postgermination arrest of development by abscisic acid. *Proc Nat Acad Sci, USA* **99**: 15812-17

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G (2005) Water uptake and distribution in germinating tobacco seeds investigated *in vivo* by nuclear magnetic resonance imaging. *Plant Physiol* **138**: 1538-51

Marraccini P, Rogers WJ, Allard C, *et al.* (2001) Molecular and biochemical characterization of endo- β-mannanase from germinating coffe (*Coffea Arabica*) grains. *Planta* **213**: 296-308

Matakiadis T, Alboresi A, Jikumaru Y, Tatematsu K, Pichon O, Renou JP, Kamiya Y, Nambara E, Truong HN (2009) The *Arabidopsis* abscisic acid catabolic gene CYP707A2 plays a key role in nitrate control of seed dormancy. *Plant Physiol* **149**: 949-60

Matilla AJ, Matilla-Vázquez MA (2008) Involvement of ethylene in seed dormancy. *Plant Sci* **175**: 87-97

Maurel C, Chrispeels M, Lurin C, *et al.* (1994) Function and regulation of seeds aquaporins. *J Exp Bot* **48**: 421-30

McDonald MB, Sullivan J, Lauer MJ (1994) The pathway of water-uptake in maize seeds. *Seed Sci Tech* **22**: 79-90

McDonald MB, Vertucci CW, Roos EE (1988) Soybeen seed imbibition-water absorption by seed parts. *Crop Sci* **28**: 993-97

Merlot S, Gosti F, Guerrier D, Vavasseur A, Giraudat J (2001) The ABI1 and ABI2 protein phosphatases 2C act in a negative feedback regulatory loop of the abscisic acid signaling pathway. *Plant J* **25**: 295-303

Millar AA, Jacobsen JV, Ross JJ, Helliwell CA, Poole AT, *et al.* (2006) Seed dormancy and ABA metabolism in *Arabidopsis* and barley: the role of ABA 8-hydroxylase. *Plant J* **45**: 942-54

Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, *et al.* (2006) Distinct and overlapping roles of two gibberellin 3-oxidases in *Arabidopsis* development. *Plant J* **45**: 804-18

Mo BX, Bewley JD (2002) β-mannosidase (EC 3.2.1.25) activity during and following germination of tomato (*Lycopersicon esculentum* Mill.) seeds. Purification, cloning and characterization. *Planta* **215**: 141-52

Mohamend-Yasseen Y, Barringer SA, Splittstoesser WE, Costanza S (1994) The role of seed coats in seed viability. *Bot Rev* **60**: 426-39

Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited *Brassicaceae* seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiol* **47**: 864-77

Nakabayashi K, Okamoto M, Koshiba T, Kamiya Y, Nambara E (2005) Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *Plant J* **41**: 697-709

Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. *Trends Plant Sci* **8**: 213-17

Nascimento WM, Cantliffe DJ, Huber DJ (2000) Thermotolerance in lettuce seeds: association with ethylene and endo-β-mannanase. *J Am Soc Hort Sci* **125**: 518-24

Nascimento WM, Cantliffe DJ, Huber DJ (2004) Ethylene evolution and endo- β-mannanase activity during lettuce seed germination at high temperature. *Sci Agric* **61**: 156-63

Nikolaeba MG (1977) Factors controlling the seed dormancy pattern. En: *The physiology and biochemistry of seed dormancy and germination* (ed. Khan AA) Pp. 51-74. Nort-Holland, Amsterdam.

Nishimura N, Yoshida T, Kitahata N, Asami T, Shinozaki K, Hirayama T (2007) *ABA Hypersensitive Germination1* encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in *Arabidopsis* seed. *Plant J* **50**: 935-49

Nonogaki H, Morohashi Y (1999) Temporal and spatial pattern of the development of endo- β-mannanase activity in germinating and germinated lettuce seeds. *J Exp Bot* **50**: 1307-13

Nonogaki H, Gee OH, Bradford KJ (2000) A germination-specific endo-β-mannanase gene is expressed in the mycropilar endosperm cap of tomato seeds. *Plant Physiol* **123**: 1235-45

Nonogaki H, Chen F, Bradford KJ (2007) Mechanisms and genes involved in germination in *sensu stricto*. En: *Seed development, dormancy and germination* (eds. Bradford K y Nonogaki H) Pp. 264-304. Blackwell Publishing, Oxford

Oh E, Kim J, Park E, Kim JI, Kang C, Choi G (2004) PIL5, a phytochrome-interacting basic helix-loop-helix protein, is a key negative regulator of seed germination in *Arabidopsis thaliana*. *Plant Cell* **16**: 3045-58

Oh E, Yamaguchi S, Kamiya Y, Bae G, Chung WI, Choi G (2006) Light activates the degradation of PIL5 protein to promote seed germination through gibberellin in *Arabidopsis*. *Plant J* **47**: 124-39

Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, *et al.* (2007) PIL5, a phytochrome-interacting bHLH protein, regulates gibberellin responsiveness by binding directly to the *GAI* and *RGA* promoters in *Arabidopsis* seeds. *Plant Cell* **19**: 1192-208

Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, *et al.* (2006) *CYP707A1* y *CYP707A2*, which encode abcisic acid 8´-hydroxylases, are indispensable for proper control of seed dormancy and germination in *Arabidopsis*. *Plant Physiol* **142**: 97-107

Olsen AD (2004) Nuclear Endosperm Development in Cereals and *Arabidopsis thaliana*. *Plant Cell* **16**: S214-S27

Ooms JJJ, Léon-Kloosterziel KM, Bartels D, Koornneef M, Karssen CM (1993) Acquisition of desiccation tolerance and longevity in seeds of *Arabidopsis thaliana* (a comparative-study using abscisic acid-insensitive *abi3* mutants). *Plant Physiol* **102**: 1185-91

Oracz K, Bouteau HEM, Farrant JM, Cooper K, Belghazi M, Job C, Job D, Corbineau F, Bailly C (2007) ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *Plant J* **50**: 452-65

Papi M, Sabatini S, Altamura MM, Hennig L, Schafer E, *et al.* (2002) Inactivation of the phloem-specific Dof zinc finger gene

DAG1 affects response to light and integrity of the testa of *Arabidopsis* seeds. *Plant Physiol* **128**: 411-17

Peeters AJ, Blankestijn-de Vries H, Hanhart CJ, Leon-Kloosterziel KM, Zeevaart JA, Koornneef M (2002) Characterization of mutants with reduced seed dormancy at two novel *rdo* loci and a further characterization of *rdo1* and *rdo2* in *Arabidopsis*. *Physiol Plant* **115**: 604-12

Penfield S, Josse EM, Kannangara R, Gilday AD, Halliday KJ, Graham IA (2005) Cold and light control seed germination through the bHLH transcription factor SPATULA. *Curr Biol* **15**: 1998-2006

Penfield S, Gilday AD, Halliday KJ, Graham IA (2006) DELLA-mediated cotyledon expansion breaks coat imposed seed dormancy. *Curr Biol* **16**: 2366-70

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, *et al.* (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**: 256-61

Pinfield-Wells H, Rylott EL, Gilday AD, Graham S, Job K, *et al.* (2005) Sucrose rescues seedling establishment but not germination of *Arabidopsis* mutants disrupted in peroxisomal fatty acid catabolism. *Plant J* **43**: 861-72

Pracharoenwattana I, Cornah JE, Smith SM (2005) *Arabidopsis* peroxisomal citrate synthase is required for fatty acid respiration and seed germination. *Plant Cell* **17**: 2037-48

Probert RJ (2000) The role of temperature in the regulation of seed dormancy and germination. En: *Seeds: the ecology of regeneration in plant communities.* (ed. Fenner M) Pp. 261-92. CAB International, Wallingford

Pysh LD, Wysocka-Diller JW, Camilleri C, Bouchez D, Benfey PN (1999) The *GRAS* gene family in *Arabidopsis*: sequence characterization and basic expression analysis of the *SCARECROW-LIKE* genes. *Plant J* **18**: 111-19

Raz V, Bergervoet JH, Koornneef M (2001) Sequential steps for developmental arrest in *Arabidopsis* seeds. *Development* **128**: 243-52

Razem FA, El-Kereamy A, Abrams SR, Hill RD (2006) The RNA-binding protein FCA is an abscisic acid receptor. *Nature* **439**: 290-94

Risk JM, Day CL, Macknight RC (2009) Reevaluation of abscisic acid-binding assays shows that G-protein-coupled receptor2 does not bind abscisic acid. *Plant Physiol***150**: 6-11

Russell L, Larner V, Kurup S, Bougourd S, Holdsworth M (2000) The *Arabidopsis COMATOSE* locus regulates germination potential. *Development* **127**: 3759-67

Sacandé M, Pritchard HW, Dudley A (2004) Germination characteristics of *Prunus africana* seeds. *New Forest* **27**: 239-50

Sánchez RA, Sunell L, Labavitch JM, Bonner BA (1990) Changes in the endosperm cell walls of two *Datura* speciesbefore radical protrusion. *Plant Physiol* **64**: 822-27

Schopfer P, Bajracharya D, Plachy (1979) Control of seed germination by abcisic acid. I. Time course of action in *Sinapis alba* L. *Plant Physiol* **64**: 822-27

Shen YY,Wang XF,Wu FQ, Du SY, Cao Z, *et al.* (2006) The Mg-chelatase H subunit is an abscisic acid receptor. *Nature* **443**: 823-26

Shimada A, Ueguchi-Tanaka M, Sakamoto T, Fujioka S,Takatsuto S, *et al.* (2006) The rice *SPINDLY* gene functions as a negative regulator of gibberellin signaling by controlling the suppressive function of the DELLA protein, SLR1, and modulating brassinosteroid synthesis. *Plant J* **48**: 390-402

Silverstone AL, Tseng TS, Swain SM, Dill A, Jeong SY, *et al.* (2007) Functional analysis of SPINDLY in gibberellin signaling in *Arabidopsis*. *Plant Physiol* **143**: 987-1000

Singh DP, Jermakow AM, Swain SM (2002) Gibberellins are required for seed development and pollen tube growth in *Arabidopsis*. *Plant Cell* **14**: 3133-47

Sitrit Y, Hadfield KA, Bennett AB, Bradford KJ, Downie AB (1999) Expression of a polygalacturonase associated with tomato seed germination. *Plant Physiol* **121**: 419-28

Stacy RA, Munthe E, Steinum T, Sharma B, Aalen RB (1996) A peroxiredoxin antioxidant is encoded by a dormancy-related gene, *Per1*, expressed during late development in the aleurone and embryo of barley grains. *Plant Mol Biol* **31**: 1205-16

Steadman KJ, Crawford AD, Gallagher RS (2003) Dormancy release in *Lolium* *rigidum* seeds is a function of thermal after-ripening time and seed water content. *Funct Plant Biol* **30**: 345-52

Steber C (2007) De-repression of seed germination by GA signaling. En: *Seed development, dormancy and germination* (eds. Bradford K y Nonogaki H) Pp. 248-64. Blackwell Publishing, Oxford

Steber C, McCourt P (2001) A role for brassinosteroids in germination in *Arabidopsis*. *Plant Physiol* **125**: 763-69

Sun TP, Gubler F (2004) Molecular mechanism of gibberellin signaling in plants. *Annu Rev Plant Biol* **55**: 197-223

Sung Y, Cantliffe DJ, Nagata R (1998) Using a puncture test to identify the role of seed coverings on thermotolerant lettuce seed germination. *J Am Soc Hort Sci* **123**: 1102-6

Sweeney MT, Thomson MJ, Pfeil BE, McCouch S (2006) Caught red-handed: *Rc* encodes a basic helix-loop-helix protein conditioning red pericarp in rice. *Plant Cell* **18**: 283-94

Theodoulou FL, Job K, Slocombe SP, Footitt S, Holdsworth M, *et al.* (2005) Jasmonic acid levels are reduced in COMATOSE ATP-binding cassette transporter mutants. Implications for transport of jasmonate precursors into peroxisomes. *Plant Physiol* **137**: 835-40

Thomas SG, Rieu I, Steber CM (2005) Gibberellin metabolism and signaling. *Vitam Horm* **72**: 289-338

Toorop PE, van Aelst AC, Hilhorst HWM (1998) Endosperm cap weakening and endo-β-mannanase activity during priming of tomato (*Lycopersicon esculentum* cv. Moneymaker) seeds are initiated upon crossing a threshold water potential. *Seed Sci Res* **8**: 483-91

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, *et al.* (2004) DELLA proteins and gibberellin-regulated seed germination and floral development in *Arabidopsis*. *Plant Physiol* **135**: 1008-19

Vandenbussche F, van Der Straeten D (2007) One for all and all for one: cross-talk of multiple signals controlling the plant phenotype. *J Plant Growth Regul* **26**: 78-187

van der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Cansen RC, van Ooijen JW, Koornneef M (1997) QTL analysis of seed dormancy in *Arabidopsis* using recombinant inbred lines and MQM mapping. *Heredity* **79**: 190-200

Vleeshouwers LM, Bouwmeester HJ, Karssen CM (1995) Redefining seed dormancy: an attempt to integrate physiology and ecology. *J Ecol* **83**: 1031-37

Wang AX, Li JR, Bewley JD (2004) Molecular cloning and characterization of an endo-β-mannanase gene expressed in the lettuce endosperm following radicle emergence. *Seed Sci Res* **14**: 267-76

Watkins JY, Cantliffe DJ (1983) Mechanical resistance of the seed coat and endosperm during germination of *Capsicum annuum* at low temperature. *Plant Physiol* **72**: 146-50

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. *Annu Rev Plant Biol* **56**: 253-79

Weber H, Buchner P, Borisjuk L, Wobus U (1996) Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. *Plant J* **9**: 841-50

West MA, Harada JJ (1993) Embryogenesis in higher plants: an overview. *Plant Cell* **5**: 1361-69

Wilkinson MD, McKibbin RS, Bailey PC, Flintham JE, Gale MD, Lenton JR, Holdsworth MJ (2002) Use of comparative molecular genetics to study pre-harvest sprouting in wheat. *Euphytica* **126**: 27-33.

Wise MJ,Tunnacliffe A (2004) POPP the question: what do LEA proteins do? *Trends Plant Sci* **9**(1): 13-17

Yamaguchi S, Kamiya Y (2002) Gibberellins and light-stimulated seed germination. *J Plant Growth Regul* **20**: 369-76

Yamaguchi S, Nambara N (2006) Seed development and germination. En: *Plant hormone signaling* (eds. Hedden P, Thomas SG) Pp. 311-38. Blackwell Publishing, Oxford

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S (2004) Activation of gibberellins biosynthesis and response pathways by low temperature during imbibitions of *Arabidopsis thaliana* seeds. *Plant Cell* **16**: 367-78

Yoshida T, Nishimura N, Kitahata N, Kuromori T, Ito T, *et al.* (2006) *ABA-hypersensitive germination3* encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among *Arabidopsis* protein phosphatase 2Cs. *Plant Physiol* **140**: 115-26

Zhang X, Garreton V, Chua N-H (2005) The AIP2 E3 ligase acts as a novel negative regulator of ABA signaling by promoting ABI3 degradation. *Genes Dev* **19**: 1532-43

ANEXO:

"Three notable hormones involved in the control of seed germination" (2007) *Current Topics in Phytoch* **8**: 59-71

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Review

Three notable hormones involved in the control of seed germination

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ABSTRACT

Seed dormancy is a primary component of the different life-history strategies of annual plants. The evolution of the seed habit has permitted higher plants to colonize a large number of ecological niches due to the excellence of orthodox seeds as dispersal units relatively impervious to harsh environmental conditions. Seed dormancy, defined as the inability of a viable seed to germinate under conditions that allow germination, is assumed to be an important adaptive trait in nature. The overall fitness of maternal plants can favour production of cohorts of seeds having dramatically different germination criteria so that germination is distributed over time. This review provides an overview of current knowledge of three hormonal factors (i.e. gibberellins, abscisic acid and ethylene) and genes controlling seed dormancy.

KEYWORDS: abscisic acid, after-ripening, β-1,3-glucanase, cell-wall loosening, endo-β-mannanase, endospermic and non-endospermic seeds, ethylene, gibberellins, mucilage, radicle, seed, seed-coat, testa

INTRODUCTION

The appearance of the seed, the organ by which angiosperms disperse and propagate, carries far-reaching evolutionary implications, assuring the survival and perpetuation of the mother plant [1]. Seeds are heterotrophic organs, totally dependent on nutrients imported (nutrient loading) from the parent plant for their growth and development [2, 3]. A typical angiosperm seed contains an embryo (diploid) surrounded by an endosperm (triploid; with two-thirds of its genome of maternal origin) and a seed coat (diploid) [4, 5]. The endosperm is formed by alive cells with high metabolic activity, while the seed coat is composed of a tissue of maternal origin (i.e. ovular tissue) in which the cells would have died during the late seed maturation. Depending on the species, the endosperm is visible in the dry seed (i.e. endospermic seeds) or else disappear completely or partially during zygotic embryogenesis (i.e. non-endospermic seeds), the cotyledons providing the embryo with nutrients [6, 7]. Under an appropriate temperature, the germination of a mature, viable, and non-dormant seed begins with gradual water uptake, which triggers a burst of respiration. Water uptake, the fundamental requirement for the initiation and completion of dry seed germination, is triphasic: rapid initial uptake (phase I, i.e. imbibition) followed by a plateau in water uptake (phase II), and a further increase after germination is completed, as the embryo axes elongate (phase III, i.e. post-germination phase of water uptake) [8, 9]. The abscisic acid (ABA), synthesised with plastidial aid

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(Fig. 1), inhibits phase III water uptake and the transition from germination to post-germination growth [10]. The dormant seeds does not complete germination as it lacks phase III. The germination process ends when the radicle tip has protruded through the covering structures (i.e. endosperm and seed coat in endospermic seeds) [11, 12]. Seed coat and endosperm always rupture at the micropylar level. Completion of radicle emergence during seed germination depends essentially on embryo extension, which is a turgor-driven growth process. Thus, cell elongation is necessary and probably sufficient for radicle protrusion to start; however, cell division is not required [13]. Radicle protrusion depends on embryo expansion, which is a process driven by water imbibition and cell-wall loosening [14]. In most endospermic seeds (e.g. coffee, lettuce, tobacco or tomato) the tissue that makes up the endosperm is a germination-limiting barrier, and so that germination is completed, the growth potential of the radicle should be high enough to overcome the resistance of the endospermic tissue [8, 11, 14, 15]. The role of the seed coat as a germination constraint has been studied by using testa mutants of Arabidopsis [14, 16]. The seed coat and endosperm rupture are temporally separated in model plants as tobacco and Arabidopsis [15, 17, 18].

Dormancy as a survival mechanism

The seed constitutes a key life-cycle stage in terms the mother plant survival as a species. It is the dispersal unit and is able to survive the period between seed maturation and the establishment of the next generation as a seedling after germination [19]. To survive in a particular location, plants have developed mechanisms that regulate seed germination to coincide with the most appropriate season of the year. One such mechanism for proper

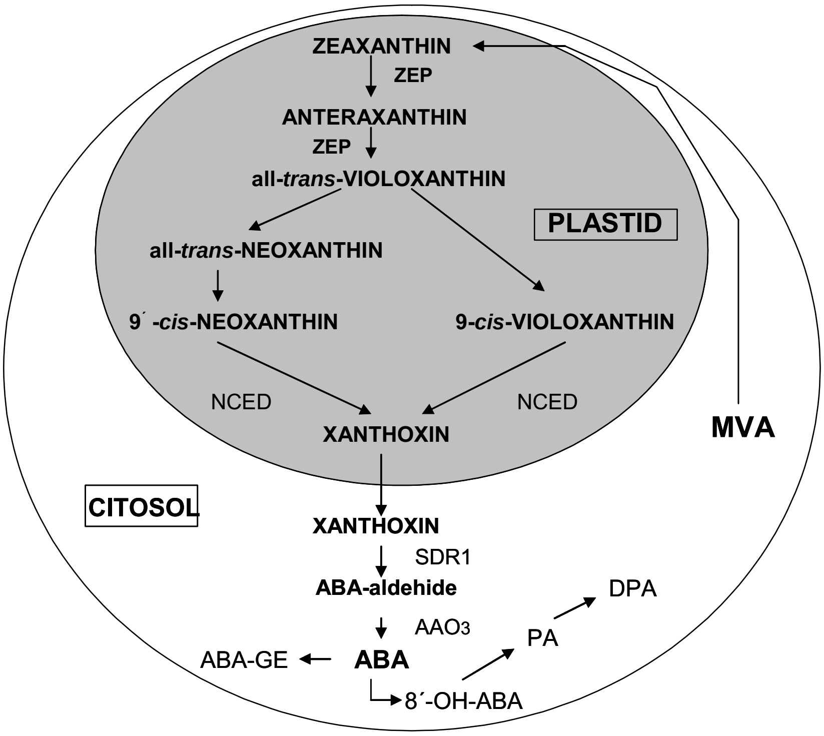

Fig. 1. ABA biosynthesis pathway. AAO3: abscisic aldehide oxidase; DPA: dihydrophaseic acid; MVA: mevalonic acid; NCED: nine-*cis*-epoxicarotenoid-dioxigenase; PA: phaseic acid; SDR1: short-chain dehydrogenase/reductase; ZEP: zeaxanthin epoxidase.

timing of seed germination is seed dormancy, which can be defined as the temporary failure of an intact viable seed to complete germination under favourable conditions [20]. However, still very little is known about the process by which the embryo emerges from the seed to complete germination and how embryo emergence is blocked in dormant seeds [8, 11]. During seed maturation, water content decreases, ABA accumulate, and dormancy is established [21, 22]. Dormancy of many species can be altered by after-ripening (i.e. storage of mature seeds for several weeks under dry, warm conditions [22, 23]. This pause in the plant life cycle called dormancy also allows growth of the seedling to occur under conditions favourable and in a season that provides sufficient time for completion of the next generation. Therefore, seed dormancy is an adaptive mechanism to ensure plant survival. Dormancy can be overcome in many seeds by exposure to chilling, light, plant hormones, temperature, or osmotic shock [8, 24, 25]. Although several hypotheses have been proposed to explain the action of these factors, dormancy is still one of the least understood phenomena in the field of seed biology. Genetic evidence strongly indicates that ABA is central to the establishment and maintenance of seed dormancy [12, 14, 21, 25] and that gibberellins (GAs) are important for germination and for counteracting ABA effects in seed dormancy [12, 26, 27]. In general, ABA delays or prevents seed germination and determines the depth of dormancy during development, whereas GAs break dormancy and promote germination upon imbibition by some mature seeds [28]. However, the ABA signalling in the life cycle of seeds is still little known. The seeds of most angiosperms are dormant at maturity, and dormancy must be lost before germination can occur [8, 11]. Dormancy is a property of an intact seed, but several parts within the seed can contribute to seed dormancy [11, 12, 29]. Dormancy is genetically determined, and seeds with some genotypes are dormant after months or years of dry storage, whereas seeds with other genotypes lose dormancy within weeks [30]. This process of dormancy loss can be hastened or slowed by environmental conditions [19]. In seeds, ABA is also involved in the acquisition of nutritional reserves, development, and maturation and desiccation tolerance [14]. Besides GAs, seed dormancy can be unblocked by ethylene (ET), which promotes seed germination in some seeds and also counteracts ABA effects in dormant seeds [12, 31].

Germinating seeds of many endospermic species exhibit “coat-enhanced” dormancy in which radicle emergence is physically restrained by the endosperm and in some cases by additional covering layers [8, 11, 23]. In Arabidopsis and in non-endospermic seeds, which contain only one endospermic cell layer, the testa properties are responsible mainly for the imposed coat-dormancy [16, 26]. Nevertheless, the Arabidopsis seed [32, 33], is like many seeds in that both the embryo and seed coats have been related to the control of dormancy and germination [18, 19, 26, 29]. This seed-coat-imposed dormancy is widespread and more common than true embryo dormancy, where the embryo fails to initiate growth even when removed from the constraints imposed by the seed coverings [19, 27]. However, this imposition is reportedly absent in dicotyledons, such as *Brassica napus* [34] and *Phaseolus vulgaris* seeds [35]. By contrast, in endospermic seeds, both the testa and endosperm layers can be involved in coat-enhanced dormancy [12, 22].

Seed dormancy is a sufficiently complex process to be controlled by a single endogenous or exogenous factor. Since dormancy is regulated at different developmental phases, in interaction with environmental factors, it is difficult to detect when the genetic and physiological differences are established. This difficulty arises because all assays are based on seed germination, which results from the balance between the degree of dormancy and the capacity of the embryo to overcome dormancy. Thus, mutants that germinate better or faster can represent genes that promote dormancy or those that repress germination. The interaction between hormonal factors and the strong effect of the environment, both during seed development and during imbibition, make seed dormancy very complex. The challenge in dormancy research is to identity the nature of the crucial regulator(s) responsible from preventing the onset of germination (dormancy) and for triggering the germination process and their mutual interaction. According to current knowledge, at least three hormones (i.e. ABA, GAs and ET) (Fig. 2) are involved in the signalling that maintains and/or eliminates seed dormancy [36].

The mutants, key tools to advance knowledge of seed dormancy

ABA-mutants

It is well known that *de novo* synthesis of ABA during the imbibition of dormant mature seeds contributes to dormancy maintenance [37]. Several mutants that produce seeds with reduced dormancy have been raised from Arabidopsis. An important group of mutants is constituted by those specifically involved in seed dormancy (i.e. mutants with altered ABA biosynthesis or signalling). Seeds which lack ABA do not undergo ABA-induced dormancy and germinate without GAs [38]. Genetic studies show that ABA produced by the embryo itself, and not maternal ABA, is necessary to impose dormancy [39]. Reciprocal crosses between wild type (WT) and the ABA-deficient *aba1* mutants also showed that dormancy is regulated by the ABA genotype of the embryo and not by that of the mother plant. Likewise, inhibitors of ABA biosynthesis promote germination and indicate that the maintenance of dormancy in imbibed seeds is an active process involving *de novo* ABA synthesis as was also found for *N. plumbaginifolia* [40]. On the other hand, ABA-insensitive (*abi*) mutants *abi1*, *abi2, abi3*, *abi4,* and *abi5* are able to germinate in the presence of ABA levels that are inhibitory to the WT. The Arabidopsis *aba1*, *aba2,* and *aba3* mutants are defective in zeaxanthin epoxidase [41], short-chain dehydrogenase/ reductase [42] and a molybdenum co-factor sulfurase genes [43], respectively. More recently, *aba4* was identified in a screen for paclobutrazol-resistant germination (i.e. paclobutrazol, inhibitor of GAs biosynthesis), and the *ABA4* gene appears to be involved in violaxanthin transformation [44]. All of the *aba4* mutants showed similar paclobutrazol-resistance phenotypes compared with WT and reduced endogenous ABA levels in seeds, and the germination showed lower dormancy than did the WT [44]. No dormancy was observed in *abi5* mutant [45], except reduction of some seed-maturation-specific mRNAs [46]. However, germination of the *era1*, *era2,* and *era3* (*ERA*, enhanced response to ABA) mutant seed is prevented by low concentrations of exogenous ABA that normally permit germination of the WT [47]. Moreover, mutations at any of *ABI3* (*ABA INSENSITIVE3*), *FUS3* (*FUSCA3*) or

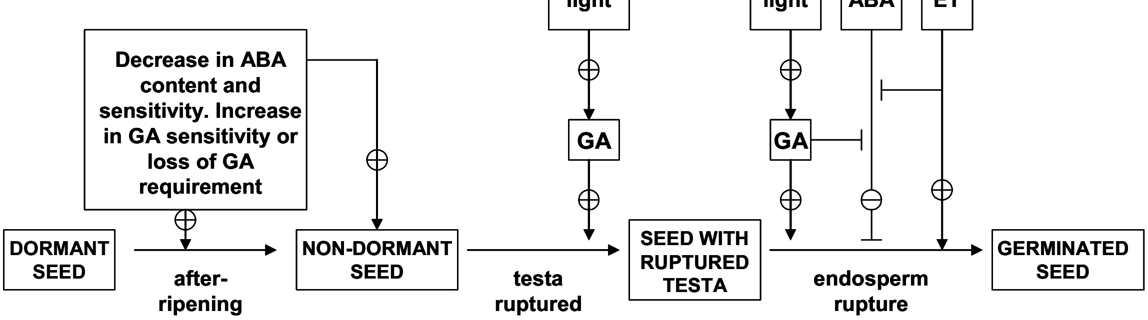

Fig. 2. Possible hormonal interactions during tobacco seed after-ripening, dormancy release and germination and their effects on testa and endosperm rupture. Adapted from Leubner-Metzger, G. (2003b).

LEC1 and *LEC2* (*LEAFY COTYLEDON*) loci affect acquisition of dormancy and desiccation tolerance, and seed maturation [48]. It is possible that the non-dormant phenotype of *lec1*, *fus3,* and *abi3* mutants is due to defective seed maturation and that mutant seeds germinate because this is the default state [49]. Most of the Arabidopsis ABA-deficient mutants identified to date have been demonstrated to present germination that is resistant to paclobutrazol or equivalent GA-biosynthesis inhibitors [50, 51, 52]. In an ABA-deficient mutant the GA requirement for germination is thus less than in WT seed [53, 54], which is related to the antagonistic effect of ABA and GA on germination.

GAs-mutants

GAs are habitually required to overcome the ABA-induced dormant state. Many mutants that increase seed dormancy or reduce the germination capacity have mutations in the biosynthesis of signalling pathways of plant hormones that stimulate seed germination such as GAs and ET. GAs can promote germination by their ability to overcome germination constraints that exist in seeds requiring after-ripening, light, and cold. Light may induce GAs biosynthesis during the early phases of germination (Fig. 3). One of two 3-β hydroxylase enzymes, encoded by the *GA4H* gene, is induced in germinating seeds of Arabidopsis by phytochrome [55]. Cold treatments do not stimulate GAs biosynthesis in some seeds, but rather increase their sensitivity to GAs [26]. Two different mechanisms of action have been proposed to explain the role of endogenous GAs in the control of germination: (i) induction of genes encoding enzymes that reduce the mechanical resistance to radicle protrusion; and (ii) direct effect on the growth potential of the embryo. A single dominant mutant (*gai1*) with decreased GA-signal transduction has been described [14, 30]. *Gai1* has reduced sensitivity to GAs but does not exhibit strongly reduced germination. However, loss-of-function (LOF) alleles of this locus require slightly less GAs for growth than WT [56]. Mutations in the *COMATOSE* (*CTS*) gene also results in a marked reduction in germination potential. Thus, mature *cts* seeds do not respond to GAs, and prolonged chilling of imbibed seeds only partially restores the germination capacity.

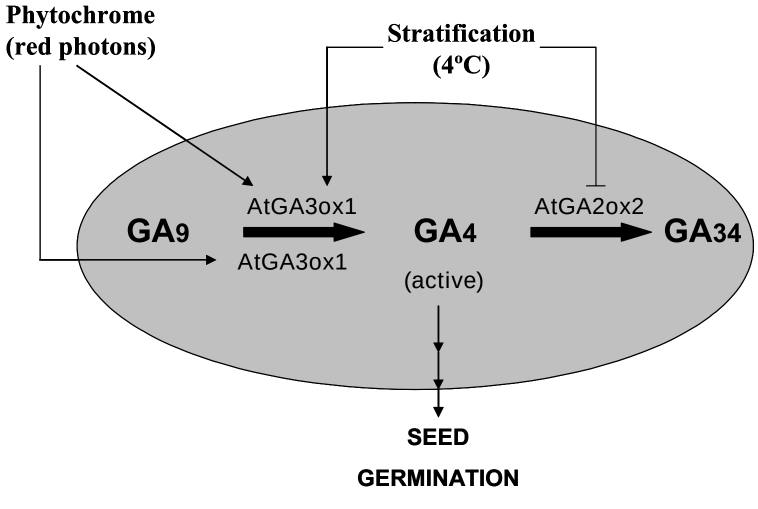

Fig. 3. Light and temperature regulation of GAs synthesis in Arabidopsis seeds. *AtGA3ox* and *AtGA2ox* encode GA3-oxidases which transcripts are localized during imbibition period in the cortical and endodermal cells of embryonic axis. Adapted from Yamaguchi and Nambara (2006). In: P. Hedden and S.G. Thomas (Eds.) Plant Hormone Signaling. Blackwell Publishing Ltd, Oxford OX4 2DQ, UK. pp. 311-338.

It is suggested that *CTS* represses embryo dormancy and might be involved in GAs signalling specific for seeds [57].

Ethylene-mutants

Opinions vary about the developmental stage during which ET regulates dormancy, and some suggest that the role of ET during dormancy inception is minimal and that its major action is during imbibition to terminate dormancy and/or initiate germination [58]. Seeds that respond to ET are often light sensitive for germination [31, 59]. Stratification in water at 4ºC or moist pre-chilling at restricted water content is able to break dormancy in seed-bearing woody plants [60, 61], and ET accelerates the breaking of dormancy from the first week of treatment [62, 63]. Although ET can fully rescue the germination defect of some mutants (e.g. *ga-1*, [64]) and some mutants in ET signalling are affected in their germination response [65], little is known about the molecular mechanism of ET action during the breaking of dormancy. However, has been suggested that some component(s) of ET signalling may be involved in this process. In beechnut seeds, FsERF1-mRNA is absent in dormant seeds and increases during pre-chilling, which induces a progressive release from seed dormancy [60]. The ET treatment provokes a drastic increase in transcript levels of FsERF1 (a functional ET-responsive DNA-binding protein) [66]. The mutation *etr1-1* insensitizes Arabidopsis seeds to ET and intensifies sensitivity to ABA; while seeds produced by *ctr1* are less sensitive to ABA than are those of the WT [67]. There are antagonistic effects of ET and ABA on dormancy and germination. Thus, ET may promote germination by interfering with the inception and/or maintenance of seed dormancy by ABA [58]. For example, the greater proportion of dormant seeds in a population of mature seeds of ET-insensitive mutants (e.g. *etr1*), as compared with those of the WT, may be a consequence of their increased sensitivity to ABA [67]. Likewise, increased ABA biosynthesis in the *ein2* mutant indicates that ET sensitivity and ABA biosynthesis are coupled in Arabidopsis [58]. By other hand, *etr1-2* mutation confers dominant ET insensitivity and results in a larger proportion of mature seeds that exhibit primary dormancy. Likewise, a role for ET perception in determining the length of time Arabidopsis seeds remain in the lag phase prior to radicle protrusion was concluded by using ET-insensitive gain-of-function (GOF) receptor mutants in tomato and Arabidopsis, as well as single and double LOF receptor mutants in Arabidopsis [68]. It appears that EIN2, a membrane associated and positive regulator of ET responses [65], is a negative regulator of seed sensitivity with respect to ABA. Thus, the mutation *ein2* in Arabidopsis produces seeds with great sensitivity to ABA and very little to ET. Consequently, ABA and ET signalling pathways appear to be closely related [67]. These facts indicate that seed dormancy in Arabidopsis is negatively regulated by endogenous ET, a fact that has been suspected [69] but not demonstrated until now [58, 67]. In short, ET alone is incapable of positively regulating seed germination, but an interaction between the signalling of ABA and ET (Fig. 1) appears to be necessary at some point of the germinative process [70, 71]. Probably, ET counteracts the effect of ABA by diminishing sensitivity of the seed to endogenous ABA. More recently, it was reported that the hormone profiles during germination of seeds obtained from an *etr1-2* mutant (dominant ET insensitivity and higher level of seed dormancy) also suggest a requirement of GA levels higher than in the WT in order to promote germination in absence of a functional ET signalling pathway [72]. These results also suggest that ET signalling modulates the metabolism of all the other plant-hormone pathways in seeds. The ET mimics GAs action as applied ET allows germination of seeds of the GAs-deficient mutants [73]. ET insensitive mutants such as *etr* and *ein2* germinate less well or after a longer period of after-ripening than WT [67]. In short, ET negatively regulates seed dormancy by inhibiting ABA action [67].

Seed envelop mutants

Mutants of Arabidopsis with an altered seed coat or testa also show reduced seed dormancy [16]. The development of Arabidopsis seed coat, which is formed from two integuments of epidermal origin that surround the mature ovule, has been described [74]. The morphological differentiation of the outer integument, which excretes mucilage upon imbibition of some seeds [75], has been also studied [32, 76]. Within the Cruciferae, several aspects of seed coat development and maturation have been described, including cytology of mucilage production [77]. In *Arabidopsis*, mucilage, a soluble hydrophilic polysaccharide, is produced by the developing seed and deposited in the outer cell layer of the seed coat, a property known as myxospermy. Upon imbibition or initial water uptake, the dehydrated mucilage is rapidly released from the seed coat providing a gelatin-like coating surrounding the mature seed. Although the role of mucilage is unknown, it is thought to aid in the dispersal and/or protection of the emerging seedling during imbibition and germination. Such seed-coat mucilage cells are necessary for neither viability nor germination under normal laboratory conditions. Seed-coat mutants consist of two major groups: (i) affected in flavonoid pigmentation that is represented by the *transparent testa* (*tt*) [16] and *transparent testa glabra* (*ttg*); the *ttg1*-mutants lacks mucilage and trichomes and is affected in the morphology of the outer layer of the seed coat as well as in pigment production; and (ii) affected in testa structure (e.g. *ats*, *aberrant testa shape*) [78].

Hormonal control of enzymatic proteins related to dormancy signalling

Cell-wall-dismantling enzymes and seed dormancy

It has been suggested that the weakening of the endosperm cell walls is required to allow for protrusion of the radicle from the seed [8]. However, in some seeds, such as lettuce, there is neither transcription nor an increased activity of the cell-wall-degrading enzyme (e.g. endo-β-mannanase; EC 3.2.1.78) prior to radicle emergence or any obvious degradation of the micropylar endosperm cell walls [79, 80]. The means by which emergence occurs in lettuce is at present unknown. In tomato seeds, the walls of the endosperm adjacent to the cotyledons are thicker than those adjacent to the radicle, and in both regions the cells exhibit thickened walls [81]. Changes in the cell walls occur prior to germination, coinciding with an increase in endo-β-mannanase activity; the degradation of the micropylar endosperm cell walls to permit germination is likely to require the activities of several hydrolases [8, 11, 82]. In some hard seeds, the cells of the micropylar endosperm adjacent to and overlying the radicle tip have relatively thin walls [83, 84]. This localized modification of endosperm cellular structure (i.e. micropylar endosperm) suggests that it offers little resistance to radicle emergence. In contrast to the above, in date seeds (e.g. lettuce, tomato, and tobacco) the majority of the cells in the micropylar endosperm that surround the radicle are thick-walled. There has been controversy about whether the increase in endo-β-mannanase in the micropylar endosperm during germination is sufficient to permit the radicle to emerge and the consensus appears to be that, whilst this enzyme is required for endosperm weakening, it is not, by itself, sufficient to allow germination to be completed [8, 11, 85]. Two cDNA clones for tomato endo-β-mannanase (i.e. *LeMan1* and *LeMan2*) have been reported [86], *LeMan1* being expressed following germination in the lateral endosperm region and *LeMan2* in the micropylar endosperm prior to the completion of germination [86]. Besides endo-β-mannanase other enzymes associated with the hydrolysis of galactomannans have been studied in endospermic seeds. Recently, β-mannosidase has been purified from tomato, and a cDNA (*LeMside1*) clone obtained [87]. β-mannosidase and endo-β-mannanase increase in activity prior to the completion of germination, particularly in the micropylar endosperm, and both increase in the lateral endosperm following germination [87]. This change in activity parallels a greater expression of transcripts for both enzymes in the micropilar endosperm, and the increase in both β-mannosidase activity and transcripts in the lateral endosperm precedes those of endo-β-mannanase by about 12 h [87].

In the case of endosperm-limited germination, it is believed that hydrolytic enzymes facilitate weakening of the endosperm surrounding the radicle tip by hydrolysing cell-wall materials [8, 11, 88]. The first morphological event following imbibition of tobacco seeds is the rupture of the seed coat (testa). This is followed by rupture of the endosperm, which is the limiting step of tobacco-seed germination [12, 15, 29]. Also, β-1,3-glucanase (βGLU) activity is induced after testa rupture and just prior to the onset of endosperm rupture. This activity, localized in the micropylar endosperm at the site where the radicle will emerge, results from transcriptional induction of class I β-1,3-glucanase (βGLU I) [10]. The Leubner-Metzger's group provided direct evidence that βGLU I has a role in rupture of endosperm, which is the limiting step in tobacco-seed germination [89]. Moreover, other results from this group support the hypothesis that a threshold βGLU content is required for endosperm rupture, and suggests that accumulation of βGLU is necessary but not sufficient for endosperm weakening. The induction of βGLU I and endosperm rupture are tightly linked in response to physiological factors known to affect the incidence and timing of germination (e.g. ABA treatment specifically delays endosperm rupture and inhibits the induction of βGLU I) [90, 91, 92]. Taken together, these findings support the view that germination control by coat-covering layers is achieved by the collaborative or successive action of several cell-wall-modifying enzymatic proteins and various molecular mechanisms. On the other hand, little is known about the molecular basis for the modulation of dormancy during after-ripening [8, 23]. Over-expression of βGLU I during germination replaced the after-ripening effect on endosperm rupture, but did not influence photodormancy. Physiological studies and recent results obtained with sense transformants strongly suggest that βGLU I has a causal role in endosperm rupture [89].

Hormonal control of dismantling enzymes

The ruptures of micropylar-endosperm and testa in Arabidopsis seeds are temporally separated, the former being ABA inhibited but not the latter rupture [17]. Data are scarce regarding the molecular mechanisms of both endosperm and testa weakening. The participation of a single enzyme in the process appears to be ruled out at present. Conversely, evidence exists for the contribution of several cell-wall-dismantling enzymes (e.g. endo-β-1,4-mannanases and endo-β-1,3-glucanases) [8, 11, 12, 14, 80, 85]. Expression of endo-β-1,3-glucanase in the micropylar endosperm, its inhibition by ABA and the inhibition of endosperm rupture by ABA is widespread among the Solanaceae [12, 15, 92]. The ABA inhibition of endosperm weakening is partially reversed in transgenic tobacco seeds that overexpress endo-β-1,3-glucanase in the seed-covering layers under the control of an ABA-inducible transgene promoter [93]. In the Discussion of this last work, it is stated that endo-β-1,3-glucanase is involved in cell-wall weakening; but it is not the only enzyme. During after-ripening of tobacco seeds, falling ABA levels and possibly decreasing sensitivity to ABA eventually permits βGLU I expression needed for endosperm rupture during seed imbibition [89], but could also permit βGLU I expression in the dry state, which results in the release of dormancy. Decline in ABA content, lower sensitivity to ABA and higher sensitivity to GAs correlates with after-ripening of many species [8, 22, 23]. In general, modulation of dormancy during after-ripening results in a broadening of the germination responses to environmental conditions [8, 11, 23]. The fact that βGLU I expression is regulated by hormones and environmental factors [28, 91] suggests that βGLU I could be a key factor in modulating dormancy and germination in response to the environment. Leubner-Mezger and Meins proposed that βGLU I expression in tobacco dry seeds contributes to the after-ripening-mediated release of seed dormancy [94]. The after-ripening-mediated promotion of tobacco-seed germination is due mainly to: (i) βGLU I contribution to the after-ripening-mediated release of dormancy in the dry seed state, which is manifested in the promotion and ABA-insensitive testa rupture during imbibition; and (ii) ABA-sensitive expression of βGLU I in the micropylar endosperm, which contributes to endosperm rupture [91]. Likewise, the after-ripening-mediated release of tobacco dormancy is also correlated to a decrease in GAs requirement for testa rupture during dark imbibition. Thus, as in Arabidopsis and tomato, testa-imposed dormancy of tobacco appears to be regulated by an indirect ABA-GAs interaction. The importance of testa characteristics appears to be a common feature during the after-ripening-mediated release of coat-enhanced dormancy in endospermic and non-endospermic seeds. It is possible, but not proven, that tomato endosperm weakening is a biphasic process and only the second phase is inhibited by ABA [90]. βGLU I, which contributes to endosperm rupture of tobacco seeds, is induced by GAs just prior to tomato endosperm rupture and is inhibited by ABA [90]. ABA inhibits the induction of βGLU I genes, specifically delays endosperm rupture. The results of Leubner-Mezger and Meins support the view that a threshold βGLU I content is required, but not sufficient, for endosperm rupture [89, 94]. Tobacco germination is accompanied by ET evolution, and endogenous ET is required for the promotion of endosperm rupture and high-level βGLU I expression of light-imbibed seeds and non-photodormant dark-imbibed seeds [95]. ET does not affect the spatial and temporal pattern of βGLU I expression and does not break photodormancy or affect the kinetics of testa rupture. All the above taken together, ET promotes endosperm rupture by enhancing βGLU I expression while the GAs/light pathway and ET regulate βGLU I induction in the micropylar endosperm and seem to control endosperm weakening [12].

Recently, the β-mannosidase (EC 3.2.1.25) was purified and molecular studies were carried out from intact tomato seeds [98]. The authors concluded that β-mannosidase and β-mannanase work in collaboration in the mobilization of the galactomannan-containing cell walls of tomato seeds. Whether they control processes or elements in common remains to be determined. Germination of intact tomato seeds was completely inhibited by ABA. The production of endo-β-mannanase in the micropylar endosperm is not initially sensitive to ABA during germination, leading to the view that the presence of this enzyme alone is not in itself sufficient to allow germination occur [96]. The decline in endo-β-mannanase activity after longer times of imbibition has also been noted previously and has been related to the failure of a second phase of endosperm weakening necessary for germination to be completed [81]. All this considered, the initial presence of both mannan-degrading enzymes, endo-β-mannanase and β-mannosidase, is insufficient to permit germination to occur. An increase in activity of β-mannosidase occurred in the lateral endosperm in seeds imbibed in water whereas ABA weakly reduced this activity. Conversely, the effect of ABA on β-mannosidase activity in the micropylar endosperm was relatively weak but there was more sensitivity to ABA [98]. On the other hand, isolated endosperms of the *gib-1* mutant of tomato seeds, a line that does not synthesise GAs, respond to applied GAs (i.e. GA_4) by synthesising endo-β-mannanase [97]. In the absence of GAs there was a small amount of β-mannosidase, although no seeds imbibed on water completed germination, and there was negligible endo-β-mannanase activity. In the presence of GAs, activities of both enzymes were induced in all regions, particularly in the micropylar and lateral endosperm regions following the completion of germination. Thus, in the intact *gib-1* seed, the activities of both enzymes responded similarly, being low in the absence of a germination stimulus, but both increasing in a similar temporal manner in the presence of GAs. The behaviour of the enzymes in the GA-induced *gib-1* mutant seeds is also similar to that of the WT seeds [98]. In conclusion, the most up-to-date information indicates that the increase or decrease in both endo-β-mannanase and β-mannosidase activity in tomato seeds are regulated by ABA and GAs in a similar way, particularly in the micropylar endosperm.

Future prospects

For the data published to date, there are several types of dormancy, which can be broken in the laboratory by altering different processes and behavioural traits of the seed [20, 29]. Genetic and biochemical analyses of plant hormone-response mutants in Arabidopsis are providing invaluable information on the different components involved in seed-dormancy signalling [14, 30, 39, 44, 52, 57, 65]. However, although there are a number of genes suspected of reducing dormancy, none has yet been isolated and characterized as being directly involved in the complete breaking of dormancy. That is, no mutation is known to inhibit the action of the phytochrome or of the low temperatures, light, or stratification needed by seeds to break dormancy. Nor is any mutation known that impedes germination in viable seeds that require storage in a dry environment in order to germinate (after-ripening). In other words, no researcher has yet been able to unblock the impediment to germination in viable, mature seeds. A more than probable explanation may be that dormancy is the result of the intervention of a good number of endogenous and genetically regulated environmental factors. These factors are clearly not under the control of a single gene. However, it also appears clear that dormancy, to occur effectively, needs the integrated and strongly regulated action of a good number of genes, some of which may have been isolated already from wood plants seeds in different laboratories [61, 62, 63, 66]. The lack of test specifics to evaluate dormancy without having to resort to germination as an indicator of the presence or absence of this state clearly complicates the understanding of this critical phase in the perpetuation and survival of plants.

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REFERENCES

1. Boesewinkel, F. D., and Bouman, F. 1995, In: Seed Development and Germination. (J. Kigel and G. Galili, eds.). Marcel Deker, New York, pp. 1-24.
2. Patrick, J. W., and Offler, C. E. 2001, J. Exp. Bot., 52, 551.
3. Zhang, W-H., Zhou, Y., Dibley, K. E., Tyerman, S. D., Furbank, R. T., and Patrick, J. W. 2007, Funct. Plant Biol., 34, 314.
4. Raghavan, V. 1986, In: Embryogenesis in Angiosperms. A Developmental and Experimental Study (P. W. Barlow, P. B. Green, C. C. Wylie, eds.). Cambridge University Press, Cambridge CB2 1RP, UK, pp. 13-45.
5. Torres-Ruiz, R. A., 1998, In: Arabidopsis. Ann. Plant Rev. vol. 1 (M. Anderson, M., J. A. Roberts, eds.). Boca Raton, Florida, CRC Press. USA, pp. 223-261.
6. Friedman, W. E. 1998, Sex. Plant Reprod., 11, 6.
7. Forbis, T. A., Floyd, S. K., and Queiroz, A. 2002, Evol., 56, 2112.
8. Bewley, J. D. 1997a, Plant Cell, 9, 1055.
9. Manz, B., Müller, K., Kucera, B., Volke, F., and Leubner-Metzger, G. 2005, Plant Physiol., 138, 1538.
10. Leubner-Metger, G., Frundt, C., Vogeli-Lange, R., and Meins, Jr. F. 1995, Plant Physiol., 109, 751.
11. Bewley, J. D. 1997b, Trends in Plant Sci., 2, 464.
12. Kucera, B., Cohn, M. A., and Leubner-Metzger, G. 2005, Seed Sci. Res., 15, 281.

13. Barroco, R. M., Van Poucke, K., Bergervoet, J. H. W., De Veylder, L., Groot, S. P. C., Inze, D., and Engler, G. 2005, Plant Physiol., 137, 127.
14. Koornneef, M., Bentsink, L., and Hilhorst, H. 2002, Curr. Opin. Plant Biol., 5, 33.
15. Leubner-Metzger, G., Kucera, B., and Müller, K. 2006, In: Seeds: Biology, Development and Ecology (S. Navie, S. Adkins, S. Ashmore, eds.). CAB International, Wallingford, Oxon, UK.
16. Debeaujon, I., Léon-Kloosterziel, K. M., and Koornneef, M. 2000, Plant Physiol., 122, 403.
17. Liu, P. P., Koizuka, N., Homrichhausen, T. M., Hewitt, J. R., Martin, R. C., and Nonogaku, H. 2005, Plant J., 41, 936.
18. Müller, K., Tintelnot, S., and Leubner-Metzger, G. 2006, Plant Cell Physiol., 47, 864.
19. Bewley, J. D., and Black, M. 1994, Seeds: Physiology of Development and Germination. Plenum Press, NY.
20. Baskin, J. M., and Baskin, C. C. 2004, Seed Sc. Res., 14, 1.
21. Hilhorst, H. W. M., and Toorop, P. E. 1997, Adv. Agron., 61, 111.
22. Hilhorst, H. W. M. 1995, Seed Sci. Res., 5, 61.
23. Li, B. L., and Foley, M. E. 1997, Trends Plant Sci., 2, 384.
24. Schneider, W. L., and Gifford, D. J. 1994, Physiol. Plant., 90, 246.
25. Jullien, M., Bouinot, D., Ali-Rachedi, S., Sotta, B., and Grappin, P. 2000. In: Dormancy in Plants: From Whole Plant Behaviour to Cellular Control (J-D. Viemont, J. Crabbe, eds.). CAB International, Wallingford, Oxon, UK.
26. Debeaujon, I., and Koornneef, M. 2000, Plant Physiol., 122, 415.
27. Ogawa, M., Hanada, A., Yamauchi, Y., Kuwahara, A. Kamiya, Y., and Yamaguchi, S. 2003, Plant Cell, 15, 1591.
28. Leubner-Metzger, G. 2003a, Seed Sci. Res., 13, 17.
29. Finch-Savage, W. E., and Leubner-Metzger, G. 2006, New Phytol., 171, 501.
30. Koornneef, M., Alonso-Blanco, C., Bentsink, L., Blankenstein-de Vries, H., Debeaujon, I., Hanhart, C. J., Leon-Kloosterciel, K. M., Peeters, A. J. M., Raz, V. 2000, The Genetics of Seed Dormancy. CAB International, Wallingford, Oxon, UK.
31. Matilla, A. J. 2000, Seed Sci. Res., 10, 111.
32. Windsor, J. B., Symonds, V. V., Mendenhall, J., and Lloyd, A. M. 2000, Plant J., 22, 483.
33. Debeaujon, I., Nesi, N., Perez, P., Devic, M., Grandjean, O., Caboche, M., and Lepiniec, L. 2003, Plant Cell, 15, 2514.
34. Schopfer, P., and Plachy, C. 1984, Plant Physiol., 76, 155.
35. Petruzzelli, L., Coraggio, I., and Leubner-Metzger, G. 2000, Planta, 211, 144.
36. Finkelstein, R. R., Gampala, S. S. L., and Rock, C. D. 2002, Plant Cell., 14, S15.
37. Kermode, A. R. 2004, In: Handbook of Plant Biotechnology (H. Klee, P. Christou, eds). JohnWiley & Sons, Ltd, UK.
38. Léon-Klosterziel, K. M., van de Bunt, G. A., Zeevaart, J. A. D., and Koornneef, M. 1996, Plant Physiol., 110, 233.
39. Frey, A., Godin, B., Bonnet, M., Sotta, and Marion-Poll, A. 2004, Planta, 218, 958.
40. Grappin, P., Bouinot, D., Sotta, B., Miginiac, E., and Jullien, M. 2000, Planta, 210, 279.
41. Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A., and Marion-Poll, A. 1996, EMBO J., 15, 2331.
42. Cheng, W. H., Endo, A., Zhou, L. Penney, J., Chen, H-Ch., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., and Sheen, J. 2002, Plant Cell, 14, 2723.
43. Bittner, F., Oreb, M., and Mendel, R. R. 2001, J. Biol. Chem., 276, 40381.
44. North, H. M., de Almeida, A., Boutin, J. P., Frey, A., To, A., Botran, L., Sotta, B., and Marion-Poll, A. 2007, Plant J., 50, 810.

45. Finkelstein, R. R. 1994, Plant J., 5, 765.
46. Finkelstein, R. R., and Lynch, T. J. 2000, Plant Cell, 12, 599.
47. Cutler, S., Ghassemian, M., Bonetta, D., Cooney, S., and McCourt, P. 1996, Sci., 273, 1239.
48. Parcy, F., Valon, C., Kohara, A., Miséra, S., and Giraudat, J. 1997, Plant Cell, 9, 1265.
49. Nambara, E., and Marion-Poll, A. 2005, Ann. Rev. Plant Biol., 56, 165.
50. González-Guzmán, M., Abia, D., Salinas, J., Serrano, R., and Rodríguez, P. L. 2004, Plant Physiol., 135, 325.
51. Seo, M., Aoki, H., Koiwai, H., Kamiya, Y., Nambara, E., and Koshiba, T. 2004, Plant Cell Physiol., 45, 1694.
52. Lefebvre, V., North, H., Frey, A., Sotta, B., Seo, M., Okamoto, M., Nambara, E., and Marion-Poll, A. 2006, Plant J., 45, 309.
53. Nambara, E., Akazawa, T., and McCourt, P. 1991, Plant Physiol., 97, 736.
54. Nambara, E., Kawaide, H., Kamiya, Y., and Naito, S. 1998, Plant Cell Physiol., 39, 853.
55. Yamaguchi, S., Smith, M. W., Brown, R. G., Kamiya, Y., and Sun, T. 1998, Plant Cell, 10, 2115.
56. Peng, J., Carol, P., Richards, D. E., King, K. E., Cowling, R. J., Murphy, G. P., and Harberd, N. P. 1997, Genes Dev., 11, 3194.
57. Russell, L., Larner, V., Kurup, S., Bougourd, S., and Holdsworth, M. J. 2000, Devel., 127, 3759.
58. Ghassemian, M., Nambara, E., Cutler, S., Kawaide, H., Kamiya, Y., and Mc Court, P. 2000, Plant Cell, 12, 1117.
59. Kepczynski, J., and Kepczynska, E. 1997, Physiol. Plant., 101, 720.
60. Mortensen, L-Ch., Rodríguez, D., Nicolás, G., Ericksen, E. N., and Nicolás, C. 2004, Seed Sci. Res., 14, 27.
61. Nicolás, C., Nicolás, G., and Rodríguez, D. 1996, Physiol. Plant, 96, 244.
62. Calvo, A. P., Jiménez, J. A., Nicolás, C., Nicolás, G., and Rodríguez, D. 2003, In: The Biology of Seeds: Recent Research Advances (G. Nicolás, K. J. Bradford, D. Côme, H. Pritchard, eds.). CAB International, Wallingford, UK, pp. 141.
63. Calvo, A. P., Nicolás, C., Nicolás, G., and Rodríguez, D. 2004, Physiol. Plant, 120, 623.
64. Koornneef, M., and Karssen, C. M. 1994, In: Arabidopsis (E. M. Meyerowitz, C. R. Somerville, eds.). Cold Spring Harbor Laboratory Press, N.Y. pp. 313.
65. De la Torre, F., Rodríguez-Gacio, M., and Matilla, A. J. 2006, Plant Signaling Behavior, 1, 231.
66. Jiménez, J. A., Rodríguez, D., Calvo, A. P., Mortensen, L-Ch., Nicolás, G., and Nicolás, C. 2005, Physiol. Plant, 125, 373.
67. Beaudoin, N., Serizer, C., Gosti, F., and Giraudat, J. 2000, Plant Cell, 12, 1103.
68. Siriwitayawan, G., Geneve, R. L., and Downie, A. B. 2003, Seed Science Research, 13, 303.
69. Abeles, F. B., Morgan, P. W., and Saltveit, Jr. M. E. 1992, Ethylene in Plant Biol, 2nd edn. San Diego, USA.
70. Gazzarrini, S., and McCourt, P. 2001, Curr. Op. Plant Biol., 4, 387.
71. Brady, S. M., and McCourt, P. 2003, Journal of Plant Growth Regulation, 22, 25.
72. Chiwocha, S. D. S., Cutler, A. J., Abrams, S. R., Ambrose, S. J., Yang, J., Ross, A. R. S., and Kermode, A., Plant J. 2005, 42, 35.
73. Karssen, C. M., Zagórski, S., Kepzcyski, J., and Groot, S. P. C. 1989, Ann. Bot., 63, 71.
74. Beeckman, T., De Rycke, R., Viane, R., and Inze, D. 2000, J. Plant Res., 113, 139.
75. Willats, W. G. T., McCartney, L., and Knox, J. P. 2001, Planta, 213, 37.
76. Western, T. L., Skinner, D. J., and Haughn, G. W. 2000, Plant Physiol., 122, 345.
77. Van Caeseele, L., Mills, J. T., Sumner, M., Gillespie, R. 1981, Canadian Journal of Botany, 59, 292.
78. Léon-Kloosterziel, K. M., Keijzer, C. J., and Koornneef, M. 1994, Plant Cell, 6, 385.

79. Dirk, L. M. A., Griffen, A., Downie, B., and Bewley, J. D. 1995, Phytochem., 40, 1045.
80. Wang, A., Li, J., and Bewley, J. D. 2004, Seed Sci. Res. 14, 267.
81. Toorop, P. E., van Aelst, A. C., and Hilhorst, H. W. M. 2000, J. Exp. Bot., 51, 1371.
82. Bradford, K. J., Chen, F., Cooley, M. B., Dahal, P., Downie, B., Fukunaga, K. K., Gee, O. H., Gurusingbe, S., Mella, R. A., Nonogaki, H., Wu, C-T., Yang, H., and Yim K-O. 2000, In: Seed biology: advances and applications (M. Black, K. J. Bradford and J. Vázquez-Ramos, eds.). Wallingford, UK: CABI Publishing, pp. 231.
83. Williams, H. A., Bewley, J. D., Greewood, J. S., Bourgault, R., and Mo, B. 2001, Seed Sci. Res., 11, 305.
84. da Silva, E. A. A., Toorop, P. E., van Aelst, A. C., and Hilhorst, H. W. M. 2005, Planta, 220, 251.
85. Hilhorst, H. W. M., Groot, S. P. C., and Bino, R. J. 1998, Acta Bot. Neerl., 47, 169.
86. Nonogaki, H., Gee, O. H., and Bradford, K. J. 2000, Plant Physiol., 123, 1235.
87. Mo, B., and Bewley, J. D. 2002, Planta 215, 141.
88. Sitrit, Y., Hadfield, K. A., Bennet, A. B., Bradford, K., and Bruce Downie, A. 1999, Plant Physiol., 121, 419.
89. Leubner-Metzger, G., and Meins, F. Jr. 2000, Plant J., 23, 215.
90. Wu, C., Leubner-Metzger, G., Meins, F., and Bradford, K. J. 2000, Plant Physiol., 126, 1299.
91. Leubner-Metzger, G. 2003b, In: The Biology of Seeds. Recent Research Advances (G. Nicolás, K. J. Bradford, D. Côme, H. W. Pritchard, eds.). CAB International, Wallingford, Oxon, UK. pp. 101.
92. Petruzzelli, L.,Müller, K.,Hermann, K., and Leubner-Mettzer, G. 2003, Seed Sci. Res., 13, 139.
93. Leubner-Metzger, G. 2002, Planta, 215, 959.
94. Leubner-Metzger, G., and Meins, F. Jr. 2001, J. Exp. Bot., 52, 1553.
95. Leubner-Metzger, G., Petruzzelli, L., Waldvogel, R., Vögeli-Lange, R., and Meins, F. Jr. 1998, Plant Mol. Biol., 38, 785.
96. Toorop, P. E., Bewley, J. D., and Hilhorst, H. W. M. 1996, Planta, 200, 153.
97. Groot, S., Kieliszewska-Rokicka, B., Vermeer, E., and Karssen, C. M. 1988, Planta, 174, 500.
98. Mo, B., and Bewley, J. D. 2003, J. Exp. Bot., 54, 2503.

II. OBJETIVOS

II. – Objetivos

Las plantas superiores han elegido a la semilla como órgano reproductivo y altamente evolucionado para facilitarles la adaptación adecuada a las condiciones medioambientales y a la vez para que la planta madre se perpetúe a la generación siguiente. Sin embargo, la evolución ha determinado que la planta madre tendría más posibilidades para mantenerse y afianzarse al nicho ecológico si produjese semillas con diferentes características físicas y fisiológicas. Aparecieron las plantas productoras de semillas heterogéneas, dentro de las que las *Brasicaceae* engloban un buen número de especies. Por otra parte, y siempre en aras a la adaptación óptima al ambiente adecuado, las semillas han adquirido una serie de estrategias para inhibir la germinación cuando todavía permanecen integradas en la planta madre dentro del fruto o bien cuando las condiciones ambientales no son las adecuadas. Este estatus, denominado dormición, es altamente complejo y está regulado por el programa de desarrollo y una compleja red de señalizaciones en la que las fitohormonas y los factores ambientales, entre otros, tienen especial relevancia (revisado por R. Iglesias-Fernández en el 2007, y añadida como un anexo de la Introducción General).

El Proyecto de Investigación (CGL2004-01996/BOS) que sufraga esta Memoria incluye una serie de protocolos tendentes al estudio del porqué el fruto de Crucíferas es tan diferente en lo tocante a los habitáculos seminales que poseen. Es decir, partiendo de la base de la existencia de frutos compartimentalizados en valvas y rostros (ambos con semillas), y frutos que solamente poseen valvas o rostros (con semillas cada uno), el objetivo de nuestro Grupo de Investigación es el estudio de las semillas de cada uno de eso frutos para conocer si eran heterogéneas en una serie de parámetros morfológicos y fisiológicos (caracteres físicos, síntesis de ET y GAs, y presencia o no de dormición, entre otros).

Dada la complejidad del estudio y la dificultad de obtener abundante material de algunas especies, nos hemos centrado para esta Memoria de Doctorado en las semillas pertenecientes a la especie *Sisymbrium officinale* L. Fueron varias las razones que nos llevaron hacia esta decisión. Quizás las más sobresalientes hayan sido: (i) el nicho ecológico de esta especie es rico en nitrógeno (planta nitrófila) y, por tanto, era factible que la dependencia de NO_3^- para germinar fuese más que probable; el NO_3^- es muy interesante porque posee señalización; (ii) existencia de datos en la bibliografía sobre algunos caracteres de esta semilla que han sido de gran apoyo para el inicio de nuestro estudio; (iii) rapidez en la germinación, lo cual siempre es un factor muy aprovechable en el laboratorio; (iv) el fruto de esta especie solamente posee valvas con multitud de semillas, las cuales son heterogéneas en color, tamaño y masa; y (v) es una semilla *endospérmica* parecida a *Arabidopsis*.

Nos hemos centrado en los siguientes aspectos de la fisiología de las semillas de *S. officinale* L.:

1.- Estudio pormenorizado de las condiciones de germinación de los lotes oscuro y claro en que hemos dividido las semillas. Estos resultados han sido publicados como en: "*Ripe fruits of Sisymbrium officinale L. contain heterogeneous endospermic seeds with different germination rates*" (2007) *Seed Sci. Biotech.* **1**: 18-24, y figuran como anexo del cap. 1.

2.- Análisis histológico de la cubierta seminal (incluyendo el estudio de la producción de *mucílago*), capa *endospérmica* (monoestratificada) y embrión y cotiledones. Este estudio se llevó a cabo con semillas secas de ambos lotes, y durante la imbibición y germinación.

3.- Aislamiento de material vegetal de diferentes etapas de la embriogénesis, y de semillas imbibidas y germinadas con el fin de estudiar: (i) la expresión de *SoACS*, *SoACO*, *SoGA3ox*, *SoGA20ox* y *SoGA2ox* durante el desarrollo de la semilla, en semilla seca y durante el proceso germinativo; (ii) los niveles de ACC y MACC; y (iii) las alteraciones en la actividad endo-β-mananasa (MAN) durante la germinación. Estos resultados han sido enviados a publicar en marzo del 2009 a la revista *Annals of Botany* (Londres).

4.- A lo largo de este estudio con semillas de *S. officinale* L. hemos demostrado que el *after-ripening* (AR) provoca un fuerte incremento en la velocidad de germinación con respecto a las semillas oscuras no tratadas. Dado que se desconoce el mecanismo de acción del AR, se estudió en profundidad el efecto que provoca el AR sobre los genes estudiados en el cap.3, así como sobre la actividad MAN. Los resultados de este estudio aparecen publicados como: "*After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during early imbibition of Sisymbrium officinale L. seeds*" (2009) *J Exp Bot* **60**: 1645-61, y se adjuntan como anexo en el cap. 3.

III. CAPÍTULO 1

La heterogeneidad en semillas de *S. officinale* L.

III.1. – Introducción

III.1.1. La Evolución en las Semillas

Las semillas representan el propágulo elegido por las plantas Espermatófitas para su dispersión y propagación (Lohe y Chaudhury, 2002; Yamaguchi y Nambara, 2006). La independencia del agua durante la fecundación y el control de la germinación mediante el proceso de dormición son importantes estrategias adaptativas que han hecho de las Espermatófitas los seres más evolucionados del Reino Vegetal (Probert, 2000; Baskin y Baskin, 2004; Sacandé *et al.* 2004). Desde una perspectiva socio-económica las semillas son una fuente sustancial de recursos alimentarios como la producción de cereales, pastas, harinas y aceites, entre otros (Bewley, 1997b). Actualmente, las semillas son también utilizadas en otros sectores como el industrial. Es el caso del arroz, que se emplea como sustrato en la producción de biocombustibles, encareciendo así su precio en el mercado internacional. Todas estas y otras razones hacen necesario el estudio fisiológico, bioquímico y molecular de la semilla con el fin de optimizar y garantizar la sostenibilidad de su explotación. Uno de los aspectos de interés es el estudio de la transición entre la dormición y germinación, lo cual representa una fase crítica en el ciclo de vida de plantas superiores y a su vez constituye un aspecto ecológico y comercial de enorme trascendencia. Si se consigue un balance apropiado entre la dormición y la germinación habremos logrado que las semillas tengan una germinación homogénea y reducir la germinación temprana (*pre-harvest sprouting*) que provoca una fuerte reducción de la calidad del grano y viabilidad de la semilla. El *pre-harvest sprouting* constituye una de las mayores pérdidas para la industria (Gubler *et al.*, 2005).

La semilla está constituida por tejidos de origen materno y embriogénico. El primero procede de las paredes del primordio seminal u óvulo y da origen a la cubierta seminal o testa (Haugh y Chaudhury, 2005), la cual suele presentar un orificio denominado *micrópilo* por el cual se produce habitualmente la emergencia radicular (para actualizar ver la Web: "The seed biology place", http://www.seedbiology.de). El tejido embriogénico es el resultado de una doble fecundación. Como consecuencia de ello se origina el *endopserma* (3n), tejido de reserva que nutrirá al esporófito (embrión) durante la maduración y germinación de Monocotiledóneas hasta que la semilla se convierta en un organismo fotosintéticamente competente (Müntz *et al.*, 2001; Drews y Yadegari, 2002; Dolfini *et al.*, 2007). En algunas familias, es el perisperma el que cumple la función de tejido nutricio (Karssen, 1976; Hermann *et al.*, 2007). Otra consecuencia de la doble fecundación afecta a la formación del embrión propiamente dicho (Nawaschin, 1898; Guignard, 1899; Dresselhaus, 2006). Este órgano está constituido por el/los cotiledón(es), hojas primordiales que funcionan como tejido de reserva, la radícula y la yema caulinar (plúmula) (Borisjuk *et al.*, 2004; Weber *et al.*, 2005; Müller *et al.*, 2006). La parte comprendida entre la *radícula* y la *plúmula* se denomina *hipocótilo* (Jürgens *et al.*, 1995; Lohe y Chaudhury, 2002) (Fig. 1).

III.1.2. Organización del Primordio Seminal y Gametofito Femenino

Los primordios seminales están constituidos en las Espermatófitas por el saco embrionario (8n) y los tejidos de origen materno (2n) (Paniagua *et al.*, 1997; Drews y Yadegari, 2002; Yadegari y Drews, 2004). Un rudimento seminal *átropo* (léase, posición erecta) presenta uno (Gimnospermas) o dos (Angiospermas) tegumentos (Frohlich y Chase, 2007) que envuelven un tejido nutricio (*nucela*) que engloba al saco embrionario. La zona de contacto entre los tegumentos y la nucela se denomina *chalaza*, habitualmente dispuesta en la región diametralmente opuesta a la *zona micropilar.*

Figura 1: Estructura de la semilla madura de *Lepidium sativum* (*Brassicaceae*). Sección longitudinal de semilla imbibida 2-3 h y teñida con azul de toluidina, analizada en microscopio de campo claro. Se observa un embrión completamente desarrollado, el endosperma y la testa o cubierta seminal. (Adaptado de Müller *et al.*, 2006).

La estructura que mantiene al primordio seminal unido a la planta madre se llama *funículo* (Strasburger *et al.*, 1994). Cuando el rudimento seminal está curvado, es decir, el micrópilo y la chalaza se disponen al mismo nivel, se denomina *campilótropo* (ej. *Arabidopsis thaliana*; Christensen *et al.*, 1997) (Fig. 2). El gametófito femenino es de tipo "*Poligonum*" en el 70% de las Angiospermas. Es el caso de las familias *Brassicaceae* (ej. *Arabidopsis*, *Capsella*, *Brassica*), *Gramineae* (ej. maíz, arroz y trigo), *Malvaceae* (ej. algodón), *Leguminoseae* (ej. judía y soja) y *Solanaceae* (ej. pimiento, tabaco, tomate, patata y petunia) (Maheshwari, 1950; Willemse y van Went, 1984; Haig, 1990; Huang B-Q y Russell, 1992). La denominación "*Poligonum*" hace referencia a la primera descripción hecha en *Poligonum divaricatum* (Strasburger, 1879; Maheshawari, 1950; Yadegari y Drews, 2004). El gametófito femenino o saco embrionario de las Angiospermas está constituido por ocho núcleos. La distribución inicial de este saco octonucleado es la siguiente (Christensen *et al.*, 1997; Paniagua *et al.*, 1997):

- Cuatro núcleos dispuestos en la zona micropilar (*oogangio*).
- Cuatro núcleos situados en la zona chalazal (aparato *antipodal*).

Posteriormente, se produce la migración de uno de los núcleos de cada aparato al centro del saco embrionario, dando lugar a los *núcleos polares*, seguida de la formación de membranas celulares divisorias y paredes celulares primarias que determinan la configuración del saco embrionario en siete células (y no en ocho, ya que los núcleos polares no están rodeados de membrana) (Paniagua *et al.*, 1997; Drews *et al.*, 1998; Drews y Yadegari, 2002) (Fig. 2) Por lo tanto, la configuración final del saco embrionario se dispone del siguiente modo:

- Dos células *sinérgidas*, situadas en el extremo micropilar del saco embrionario.
- La *oosfera*, que se sitúa adosada a las sinérgidas e inmediatamente por debajo de ellas, y que constituye la *ovocélula*.

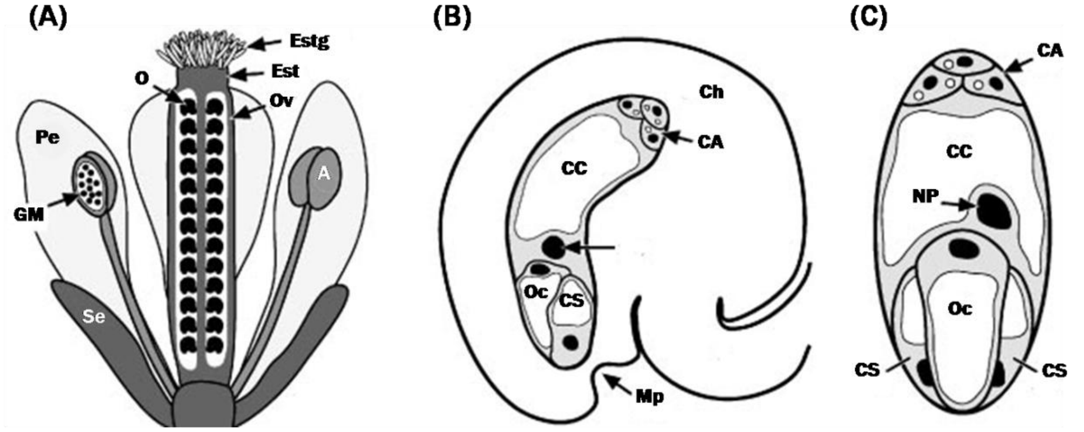


Figura 2: Descripción de las estructuras reproductoras de *Arabidopsis thaliana*. (A) flor, (B) óvulo y (C) gametófito femenino. La vista en (C) es perpendicular a (B). En (B) y (C), las áreas grises representan el citoplasma, las blancas las vacuolas y las negras los núcleos. A: antera; CA: células antípodas; CC: célula central; CS: células sinérgidas; Ch: chalaza; Estg: estigma; Est: estilo; GM: gametófito femenino; Mp: micrópilo; NP: núcleos polares; O: óvulo; Oc: ovocélula; Ov: ovario; Pe: pétalo; Se: sépalo. (Adaptado de Drews y Yadegari, 2002).

- Tres células *antípodas*, situadas en la *zona chalazal*.
- Los *núcleos polares* (superior e inferior) que se sitúan en el centro del saco embrionario. Como no están rodeados de membrana plasmática se puede considerar al saco embrionario como su citoplasma.

III.1.3. El Grano de Polen y el Tubo Polínico

La germinación del grano de polen, si las condiciones son adecuadas, tiene lugar en el estigma y se caracteriza por la emisión de una proyección citoplasmática denominada tubo polínico (Hiscock y Allen, 2008), que por su crecimiento, recuerda a un pelo radical (Samaj *et al.*, 2006). Poco antes de la germinación se produce un aumento en el número de polisomas y de la síntesis de ARN y proteínas. Esta y otras actividades se mantienen durante el crecimiento del tubo polínico (Boavida *et al.*, 2005; Marton *et al.*, 2005; Han *et al.*, 2006; Higashiyama y Hamamura 2008; Pradoa *et al.* 2008).

El citoplasma del tubo polínico, como el de los pelos radicales, se acumula en el extremo en crecimiento (Samaj *et al.*, 2006). Es en este extremo donde se encuentra el *núcleo vegetativo* seguido de dos *núcleos generativos*, así como orgánulos citoplasmáticos, principalmente dictiosomas y retículo endoplasmático liso. Los únicos orgánulos que se observan en el extremo apical son vesículas procedentes de los dictiosomas. En el interior de estas vesículas se acumulan polisacáridos, pectinas y proteínas. Las vesículas se desplazan hacia la membrana plasmática fusionándose con ella. El contenido de las vesículas es liberado al exterior originando la formación de la pared celular de esta región apical en crecimiento (Paniagua *et al.*, 1997; Higashiyama *et al.*, 2003; Bosch *et al.* 2005; Higashiyama y Hamamura, 2008). Parecen existir señales moleculares producidas por el gametófito femenino para guiar al tubo polínico (Higashiyama, 2002; Johnson MA y Preuss, 2002; Higashiyama *et al.*, 2003). El NO puede representar una molécula clave en la direccionalidad del tubo polínico (Pradoa *et al.*, 2008). En el caso de *Arabidopsis* existen dos rutas de movimiento del tubo polínico: *funicular* y micropilar. La *vía funicular* podría ser un mecanismo de control para evitar que un mismo ovario sea fecundado por dos tubos polínicos diferentes (Higashiyama *et al.*, 2003, Higashiyama y Hamamura, 2008) (Fig. 3).

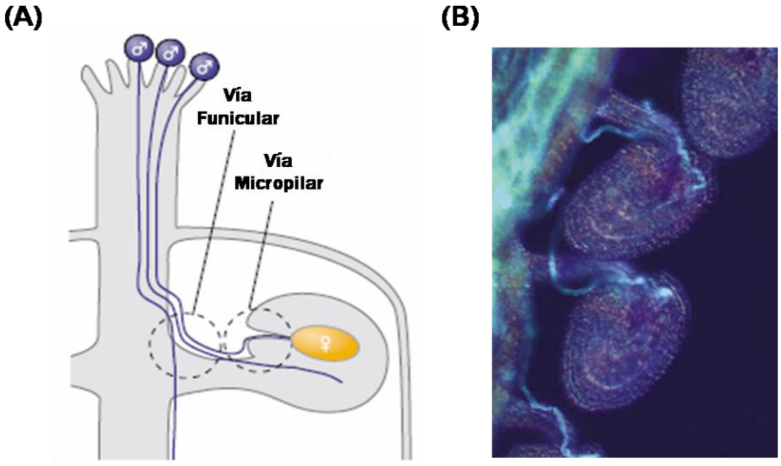


Figura 3: Guía del tubo polínico en *Arabidopsis*. (A)Representación esquemática: las líneas azules representan el crecimiento del tubo polínico hacia el gametófito femenino. Algunos tubos polínicos se pierden (regiones rodeadas por círculos) cuando el gametófito femenino es defectuoso. (B) Imagen fluorescente de óvulos teñidos con azul de anilina. (Adaptado de Higashiyama *et al.*, 2003).

III.1.4. La Formación de la Semilla: Doble Fecundación

La formación del tubo polínico ocurre una vez que el grano de polen alcanzó el estilo. El tubo polínico va descendiendo por el tejido de transmisión hasta contactar con el saco embrionario. La vía de entrada al gametófito femenino suele ser el micrópilo (*fecundación porogámica*); aunque también puede ocurrir en algunas especies a través de la chalaza (*fecundación calazogámica*) (Paniagua *et al.*, 1997).

Los tres núcleos (uno *vegetativo* y dos *generativos*) se encuentran en el extremo apical del tubo polínico (Bosch *et al.*, 2005). El *vegetativo* tiene funciones tróficas (Honys y Twell, 2003; Sano y Tanaka, 2007) y los *generativos* realizan la *doble fecundación* (Russell, 1992; Raghavan, 2003; Dresselhaus, 2006; Berger *et al.*, 2008). Uno de los *núcleos generativos* se une a la oosfera dando lugar al *zigoto* (2n) y el otro lo hace a los *núcleos polares* (3n). La primera unión es la auténticamente sexual. En la otra se constituye una célula triploide, la cual, por sucesivas divisiones dará origen al órgano nutricio en Monocotiledóneas, denominado *albúmen* o endosperma (Dresselhaus, 2006; Dumas y Rogowsky, 2008). Las células sinérgidas de *Arabidopsis* podrían estar implicadas en la formación del *aparato filiforme*, sistema membranoso que guiaría al tubo polínico desde la entrada del micrópilo hasta el gametófito femenino (Kasahara *et al.*, 2005; Berger *et al.*, 2008). El *núcleo vegetativo*, las sinérgidas y las antípodas están destinados a desaparecer (Berger *et al.*, 2008). Sin embargo, en algunas especies las *antípodas* logran dividirse y formar agrupaciones celulares de hasta 60 células (Yadegary y Drews, 2004). La nucela es habitualmente digerida durante el desarrollo embrionario; sin embargo, en algunas especies como *Beta vulgaris* se utiliza de tejido de reserva (*perisperma*) (Hermann *et al.*, 2007).

Finalmente, mediante complejas redes de señalización, las paredes del ovario originan el fruto, y el rudimento seminal (óvulo) origina la semilla (Raven *et al.*, 1999; Patrick y Offler, 2001; Haughn y Chaudhury, 2005).

III.1.5.a. La Embriogénesis Zigótica

La embriogénesis zigótica es un proceso que engloba los cambios morfológicos, estructurales y de expresión génica que tienen lugar desde que se forma el zigoto hasta que se produce un embrión maduro dispuesto a germinar (revisado en Matilla, 2008). Las primeras divisiones del zigoto ocurren con una orientación periclinal (paralela al eje principal), la cual es asimétrica y de ella resulta una pequeña célula apical y

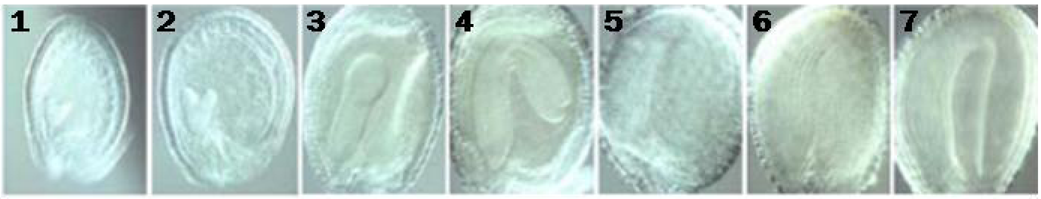


Figura 4: Embriogénesis zigótica en *Arabidopsis thaliana*. Estadío de corazón (1-2); estadío de torpedo (3); estadío de bastón (4); estadíos maduros del embrión (5-7). Imágenes obtenidas con microscopía diferencial de contraste de interferencia (DIC). (Tomado de Hewitt, 2007).

una célula basal mayor (*polaridad del embrión*). La célula apical forma el *proembrión* y la célula basal el *suspensor* (zona chalazal), que mantiene la unión al tejido materno. Al *suspensor* se le atribuye la función de facilitar la absorción de nutrientes desde el endosperma (Yeung y Meinke, 1993; Schwartz *et al.*, 1994). Posteriormente, se van sucediendo divisiones mitóticas periclinales y anticlinales que darán origen a los distintos estadíos de desarrollo del embrión y finalmente al embrión maduro (Harada, 1999; Berleth y Chatfield, 2002) (Fig. 4).

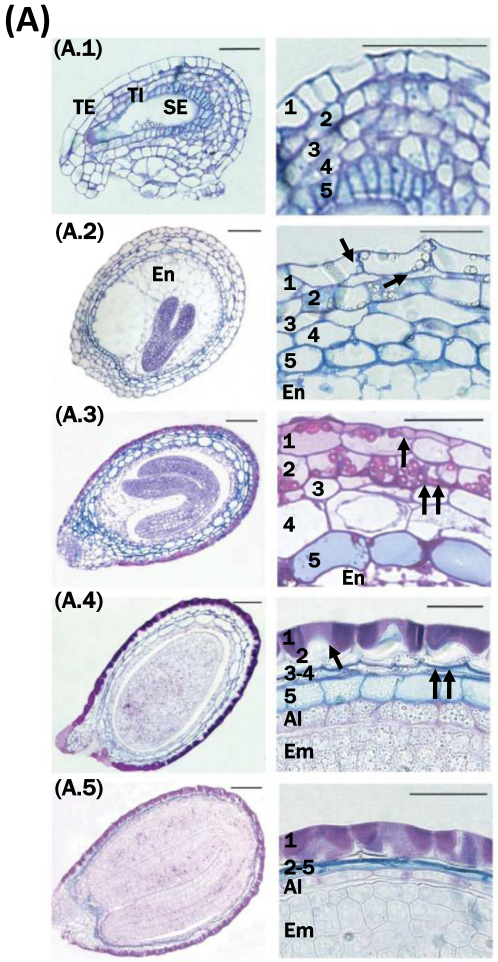
El ovario, una vez producida su fecundación, se transforma en fruto; este proceso está regulado por el programa de desarrollo y se caracteriza por una estricta regulación hormonal, provocando numerosas modificaciones estructurales, bioquímicas y moleculares (Lelièvre *et al.*, 1997; Agustí, 2000; White, 2002; Giovannoni, 2004). El fruto es el órgano de dispersión de la(s) semilla(s) en Angiospermas (Srivastava, 2002; Bewley, 2003). Primitivamente, las Espermatófitas se reproducían mediante la producción de semillas que carecían de estructuras protectoras (Gimnospermas). Sin embargo, a lo largo de la evolución ha habido una tendencia a la fusión y comunicación de los órganos florales y la semilla con el fin de protegerla, alimentarla y facilitar su dispersión (Patrick y Offler, 2001). De esta forma, la formación de las semillas a partir de los primordios seminales (*megaesporangios*) va acompañada de un recubrimiento por tejidos de origen carpelar. Por lo tanto, el desarrollo de la semilla y del fruto es paralelo (Agustí, 2000; Ferrándiz, 2002). Así, se ha observado en muchos frutos que el crecimiento y desarrollo normal del *pericarpo* requiere la presencia de semillas en desarrollo, las cuales podrían ser las responsables del correcto crecimiento del fruto mediante el mantenimiento de los niveles hormonales en los tejido adyacentes (Ozga *et al.*, 2002; Ozga y Reinecke, 2003).

Los principales tipos de frutos pueden distinguirse en base a como se desarrolló la pared del ovario. De esta forma podemos distinguir, frutos secos, subdivididos en dehiscentes e indehiscentes y frutos carnosos. El fruto de la familia *Brassicaceae*, donde está incluida *Sisymbrium officinale*, es un fruto seco de tipo capsular y dehiscente tipo silicua. Es decir, formado por dos carpelos que constituyen las valvas donde se almacenan las semillas. La valva están separadas por un *pseudoseptum* en la región central (revisado en Matilla, 2007). La arquitectura de la silicua de crucíferas, sus modificaciones evolutivas e importancia en la dispersión de la semilla ha sido descrita en detalle (Morgan *et al.*, 1998; Matilla, 2007).

III.1.5.b. Desarrollo de la Cubierta Seminal

-Generalidades

La fertilización en Angiospermas involucra la activación y coordinación de distintas rutas del desarrollo cuyo fin es la formación del embrión, del endosperma y de la cubierta seminal o testa (Western *et al.*, 2001; Lohe y Chaudhury, 2002; Haughn y Chaudhury, 2005). La cubierta seminal está constituida por diferentes capas celulares de origen materno que proporcionan una



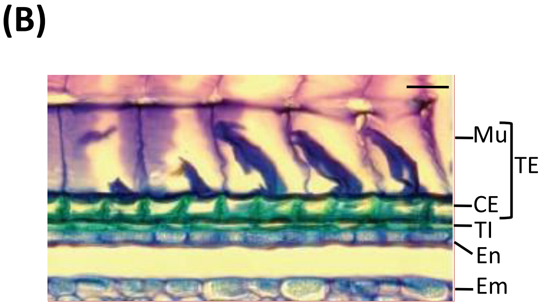


Figura 5: (A) Diferenciación de los tegumentos del óvulo en la cubierta seminal de *Arabidopsis thaliana*. Se muestran en detalle los distintos estadíos de desarrollo de la semilla (izda.) y de los tegumentos (dcha.). (A.1) Dos capas celulares en el tegumento externo (1 y 2) y tres capas celulares en el tegumento interno (3-5), que corresponden a un periodo de crecimiento durante los 5 primeros días después de la fertilización (DDF). (A.2-3) Comienza la diferenciación celular, endotelio (5), capa en empalizada (2) y epidermis (1); este proceso de diferenciación se completa aproximadamente a los 10 DDF. (A. 4-5) Se inicia el proceso de maduración de la semilla (15 DDF), las células de todas las capas celulares se mueren y son aplastadas conjuntamente, excepto la epidermis (1); la cual mantiene la estructura y la forma de la semilla mediante las paredes celulares secundarias de la columella. Se observan amiloplastos (flecha, A.2), mucílago (flecha, A.3), formación de pared celular secundaria en la epidermis (flecha, A.4) y formación de pared secundaria en la capa en empalizada (dobles flechas, A.3-4). Escala de la barra 80 μm (izda.) y 40 μm (dcha.). Adaptado de Haughn y Chaudhury (2005). (B) Estructura de la cubierta seminal de *Lepidium sativum* teñida con azul de toluidina. Escala de la barra 50 μm. Adaptado de Müller *et al*., (2006). Al, capa de aleurona; CE; capa en empalizada; Em, embrión; En, endosperma; Mu, mucílago; SE, saco embrionario; TE, tegumento externo; TI, tegumento interno.

barrera importante entre el embrión y el ambiente externo a lo largo de la embriogénesis, dormición e imbibición de la semilla (Bewley y Black *et al.*, 1994; Debeaujon y Koornneef, 2000; Finch-Savage y Leubner-Metzger, 2006; Müller *et al.*, 2006; Bethke *et al.*, 2007). Sin olvidar el papel de las cubiertas como órgano protector (Mohamend-Yasseen *et al.*, 1994; Weber *et al.*, 1996) y participante en la dispersión (Huang *et al.*, 2000, 2008).

La diferenciación de la cubierta seminal, a partir de los tegumentos internos del óvulo, incluye cambios celulares profundos que pueden observarse durante el desarrollo de la semilla y que culminan con la muerte de las células que constituyen la cubierta. Estos tipos especializados de células, aunque muertas, confieren protección y están implicadas en la dormición y germinación (Haughn y Chaudhury, 2005). La cubierta seminal de *Arabidopsis*, que se diferencia en 2-3 semanas (Debeaujon *et al.*, 2001), se origina a partir de 5 capas celulares (dos procedentes del tegumento externo del óvulo y tres del tegumento interno) (Windsor *et al.*, 2000; Haugh y Chaudhury, 2005; Lepiniec *et al.*, 2006) (Fig. 5A).

La capa celular más interna, el *endotelio* (forma parte del tegumento interno), sintetiza un tipo de pigmentos denominados protoantocianidinas (PAs), componentes flavonoides, también conocidos como taninos condensados (Dixon *et al.*, 2005; Lepiniec *et al.*, 2006). Las PAs se acumulan en la vacuola central durante la primera semana después de la fertilización, para oxidarse más tarde y conferir el color marrón característico de la cubierta seminal de *Arabidopsis* (Debeaujon *et al.*, 2000; Abrahams *et al.*, 2002; Lepiniec *et al.*, 2006). Por el contrario, las otras dos capas celulares del tegumento interno no sufren ningún tipo de diferenciación y son aplastadas progresivamente conforme avanza el desarrollo seminal (Debeaujon *et al.*, 2000; Haughn y Chaudhury, 2005). Este proceso también se describió en *Brassica napus* (Wan *et al.*, 2002) (Fig. 5A).

Previo a la especialización de las células que constituyen las dos capas del tegumento externo de *Arabidopsis* tiene lugar durante las fases de crecimiento la acumulación de almidón en los *amiloplastos*. La capa sub-epidérmica (capa 2) produce una pared celular muy gruesa en las paredes tangenciales dando lugar a la denominada *capa en empalizada* (Haughn y Chaudhury, 2005). Las células de la capa epidérmica sintetizan y secretan al apoplasto una gran cantidad de *mucílago* (polímero polisacarídico rico en pectinas); esta secreción se produce específicamente en los puntos de unión entre las paredes radiales y tangenciales. A medida que ocurre la deposición del mucílago, la vacuola se contrae y se origina una columna citoplasmática en el centro de la célula rodeada por espacio apoplástico repleto de mucílago. Una vez finalizada la síntesis y acumulación de mucílago se produce la deposición de la pared celular secundaria, dando lugar a la *columella* (Western *et al.*, 2000; Pendfield *et al.*, 2001; Western *et al.*, 2001; Dean *et al.*, 2007) (Fig. 5A). Recientemente, se ha demostrado que la salida del mucílago al exterior de la semilla requiere la participación de una serín-proteasa (subtilasa) (Rautengarten *et al.*, 2008).

Durante los estadíos tardíos de la embriogénesis zigótica las células de todas las capas de la cubierta seminal se mueren. La estructura de las células epidérmicas es preservada por el mucílago y la *columella*; y el resto de capas son aplastadas en el proceso final de la maduración. Las PAs son aparentemente liberadas por las células endoteliales, impregnando las tres capas celulares del tegumento interno (Debeaujon *et al.*, 2000; Haughn y Chaudhury, 2005; Nakaune *et al.*, 2005; Lepiniec *et al.*, 2006) (Fig. 5A).

Los mecanismos por los que se controla el desarrollo del embrión y endosperma son muy complejos (García *et al.*, 2003, 2005). Mutaciones en el gen *HAIKU* provocan un crecimiento limitado del endosperma sincitial (García *et al.*, 2003). Esta reducción en el crecimiento tiene efectos sobre el desarrollo de la testa (p. ej. restricciones en el crecimiento por expansión y no por división). Estos resultados sugieren que el crecimiento del endosperma regula la extensión de los tegumentos, procesos relacionados con el desarrollo de la cubierta seminal. Por el contrario, las mutaciones con pérdida de función (LOF) en el gen *TRANSPARENT TESTA GLABRA2 (TTG2)* (Johnson *et al.*, 2002) restringen la elongación celular de la cubierta seminal, lo cual limita el crecimiento del endosperma. Este "*cross-talk*" entre el endosperma en desarrollo y la cubierta seminal parece coordinar el crecimiento entre ambos órganos (Haughn y Chaudhury, 2005).

Las semillas maduras de la mayor parte de las Angiospermas son *endospérmicas*; aunque a lo largo de la evolución se ha tendido a la pérdida del endosperma y a la utilización de los cotiledones como órganos de reserva. Esta tendencia se hace evidente en la familia *Brassicaceae*, ya que a lo largo de la evolución se ha limitado la presencia del endosperma a una sola capa celular (*capa de aleurona*) (Ruiz y Escale, 1995; Nguyen *et al.*, 2000; Müller *et al.*, 2006) (Fig. 5B), o a ninguna, en cuyo caso la capa de aleurona se origina a partir de tegumento interno de la cubierta seminal. Esto último ocurre en ciertos géneros como *Sinapis*, *Raphanus* y *Brassica* (Schopfer y Plachy, 1984; Bergfeld y Schopfer, 1986; Schopfer, 2001).

-La Cubierta Seminal de *Arabidospis*

A pesar de la existencia de diferencias estructurales en las cubiertas seminales de semillas maduras de la familia *Brassicaceae*, éstas presentan un patrón general que bien puede estar representado en las semillas maduras de la planta modelo *Arabidopsis thaliana*. La estructura de su cubierta seminal está dispuesta del siguiente modo (Fig 5A):

- Tegumento externo

❖ **Epidermis**, constituida por *células mucilaginosas* (***mixosperma***) ricas en mucílago y columellas (Boesewinkel y Bouman, 1995; Western *et al.*, 2000; Pendfield *et al.*, 2001; Western *et al.*, 2001, 2004; Dean *et al.*, 2007).

❖ **Capa en empalizada**, constituida por células con paredes tangenciales engrosadas. Se ha relacionado con el intercambio de gases y fluidos. Finalmente, en el proceso final de la maduración esta capa sufre un proceso de aplastamiento, junto con las capas del tegumento interno (Windsor *et al.*, 2000; Haughn y Chaudhury, 2005).

- Tegumento interno

❖ **Tres capas comprimidas**, producto del aplastamiento de las capas del tegumento interno (Debeaujon *et al.*, 2000; Haughn y Chaudhury, 2005; Nakaune *et al.*, 2005; Lepiniec *et al.*, 2006).

- Restos del Endosperma

❖ **Capa de aleurona** monoestratificada derivada del endosperma (O´Brien y McCully, 1969; Vaughan *et al.*, 1971; Corner, 1976; Ruiz y Escale, 1995; Nguyen *et al.*, 2000).

-Funciones de la Cubierta Seminal

Los papeles atribuidos a la cubierta seminal son: promover/mantener la dormición física y su relación con el proceso germinativo, y la protección y dispersión de las semillas (Bewley y Black, 1994; Mohamend-Yasseen *et al.*, 1994; Weber *et al.*, 1996; Debeaujon y Koornneef, 2000; Huang *et al.*, 2000; Finch-Savage y Leubner-Metzger, 2006; Müller *et al.*, 2006; Bethke *et al.*, 2007; Huang *et al.*, 2008).

❖ Función de la Epidermis

Tejido constituído por células mucilaginosas (mixosperma). Aunque la función del mucílago no está clara, existen algunas teorías: (i) implicación en procesos fisiológicos tan importantes como la imbibición y la germinación, controlando la entrada de agua y la disponibilidad de oxígeno; (ii) relación con la dispersión, permitiendo la adhesión de la semilla a vehículos de dispersión (ej. extremidades de los animales); (iii) función protectora frente a agentes químicos tóxicos; y (iv) vinculación a los procesos de reparación del ADN genómico del embrión (Huang y Gutterman, 1998; Huang *et al.*, 2000; Western *et al.*, 2000, 2004; Huang *et al.*, 2008). Los mutantes con poca cantidad de mucílago presentan reducida la capacidad germinativa respecto al fenotipo silvestre bajo condiciones limitantes de agua (Pendfield *et al.*, 2001). Este hecho sugiriere que el mucílago proporciona un ambiente hidratado que colabora a la germinación.

❖ Función de la Capa en Empalizada y las Tres Capas Comprimidas

La existencia de paredes celulares secundarias engrosadas proporciona apoyo, protección e impermeabilidad al agua y al oxígeno. La presencia de PAs ha sido relacionada con la resistencia a patógenos (Debeaujon *et al.*, 2000; Debeaujon y Koornneef, 2000).

Muchos mutantes relacionados con la cubierta seminal, incluyendo aquellos específicamente defectuosos en la síntesis de PAs (ej. *ban*) y paredes secundarias de las capas más externas (ej. *ap2*), pero no aquellos mutantes específicamente defectuosos en la síntesis del mucílago (ej. *gl2*), tienen cubiertas seminales que fueron significativamente más permeables que las del fenotipo salvaje (Abrahams *et al.*, 2002). Algunos mutantes (ej. *tt4*, *tt12* y *ttg1*) germinan más fácilmente en respuesta a tratamientos con GA_3 (Debeaujon *et al.*, 2000; Debeaujon y Koornneef, 2000; Clerkx *et al.*, 2004).

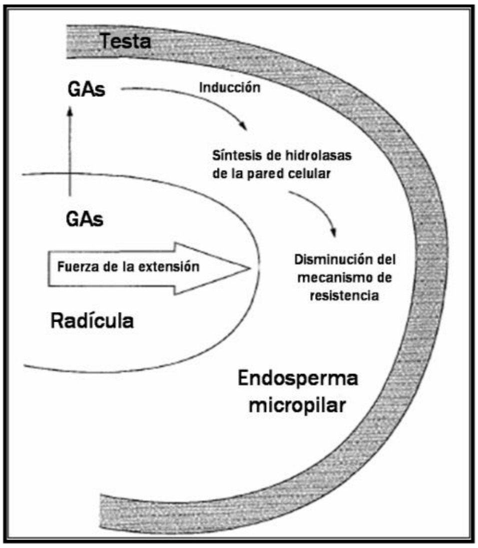


Figura 6: Región micropilar de una semilla madura de tomate. Las GAs, transportadas desde el embrión al endosperma micropilar inducen la síntesis de enzimas hidrolíticas de la pared celular. La acción de éstas disminuye la resistencia en esta parte del endosperma facilitando la emergencia radicular. (Adaptado de Bewley, 1997a).

Estos datos apoyan la hipótesis de que los PAs, las paredes celulares secundarias engrosadas y el mucílago están relacionados con el intercambio de gases y fluidos entre el ambiente y la semilla, prolongando la vida del embrión durmiente previniendo así la germinación hasta que las condiciones ambientales sean las adecuadas (Haughn y Chaudhury, 2005).

❖ Función de la Capa de Aleurona

Es el único tejido vivo de la cubierta seminal (Debeaujon *et al.*, 2000). Suele almacenar proteínas de reserva y lípidos durante la maduración de la semilla (Declercq *et al.*, 1990; Penfield *et al.*, 2004). En semillas de *Arabidopsis* se le ha atribuido la función de aportar azúcares a la plántula en crecimiento (Pendfield *et al.*, 2004). Mutantes que presentan alteraciones en el metabolismo de lípidos en la capa de aleurona reflejan defectos en la germinación y crecimiento temprano de la plántula (Eastmond *et al.*, 2000; Footitt *et al.*, 2002). La movilización de los lípidos almacenados en la capa de aleurona requiere GAs y no está bloqueada por ABA (Pendfield *et al.*, 2004). Por otra parte, se ha relacionado la emergencia radicular con el debilitamiento del *endosperma micropilar* (Fig. 1). Es el caso de semillas de tomate, tabaco, pimiento, lechuga, *Datura* y *Annona crassiflora* (Leubner-Mezger, 2003; Müller *et al.*, 2006; da Silva *et al.*, 2007; Finkelstein *et al.*, 2008). En estas especies la rotura de la testa se produce antes de que tenga lugar la del endosperma micropilar; sin ambas roturas. La emergencia radicular no se completa. Se han propuesto algunas enzimas hidrolíticas relacionadas con la degradación de las paredes celulares (*loosening*) de las células del endosperma micropilar y del adyacente a éste (ej. endo-β-mananasa, β-manosidasa, α-galactosidasa, xiloglucan-endo-transglicosidasa, β-1,3-glucanasa, quitinasa, peroxidasa y expansinas; Bewley , 1997a; Welbaum *et al.*, 1998; Chen y Bradford, 2000; Leubner-Mezger y Meins, 2000; Ren y Kermode, 2000; Mo y Bewley, 2002; Ren *et al.*, 2008). A pesar de las diversas enzimas que se han relacionado con este proceso, no parece que exista una imprescindible. Por lo tanto, se cree que el control del debilitamiento del endosperma micropilar y de la cubierta seminal podría llevarse a cabo por la acción conjunta de varias enzimas hidrolíticas (da Silva *et al.*, 2007). Las GAs parecen regular positivamente la producción de estas enzimas hidrolíticas en el endosperma micropilar (revisado en Holdsworth *et al.*, 2008) (Fig. 6). En esta regulación no podemos descartar al etileno y ABA (revisado por Matilla *et al.*, 2007). Finalmente, también se ha barajado como una posible función del endosperma micropilar la secreción de enzimas hidrolíticas de las paredes celulares de la testa (Dubreucq *et al.*, 2000; Leubner-Metzger, 2002).

III.1.6. La Heterogeneidad en las Semillas

Las plantas superiores tienen diferentes estrategias para auto-perpetuarse bajo condiciones medioambientales adecuadas (Imbert, 2002). La producción de semillas heterogéneas es una de esas estrategias. Es decir, para asegurar la supervivencia de la siguiente generación, un individuo puede producir semillas heterogéneas con respecto al grado de dormición, dispersión y persistencia en el banco de semillas del suelo. La heterogeneidad puede afectar no solamente a ciertas propiedades fisiológicas y moleculares relacionadas con el proceso germinativo sino también a características como el color (Khan *et al.*, 1996), el tamaño o la forma (Thompson *et al.*, 1993). En semillas heterogéneas estas propiedades determinan, entre otras propiedades, el comportamiento germinativo (revisado en Matilla *et al.*, 2005).

Aunque la heterogeneidad parece estar relacionada con la supervivencia, se conoce muy poco en la actualidad sobre su control hormonal. En algunas *Brassicaceae*, la heterogeneidad se adquiere porque la maduración no está sincronizada en la planta madre e incluso dentro del propio fruto (silicua). Así, el *shattering* (rozamiento) produce enormes pérdidas en especies de alto valor socio-económico o la caída de semillas en especies consideradas como "malas hierbas" que afectan a determinados cultivos (revisado en Matilla, 2007). Recientemente, se ha demostrado en semillas de *Brassica rapa* que uno de los factores que desencadenan las diferencias en la germinación de semillas heterogéneas puede ser la señalización del etileno y ABA (Puga-Hermida *et al.*, 2003; Rodríguez-Gacio *et al.*, 2004; revisado en Matilla *et al.*, 2005; revisado en de la Torre *et al.*, 2006).

Las semillas de la crucífera *S. officinale*, el material biológico de esta Memoria, están incluidas en una silicua que posee numerosas semillas heterogéneas en masa y color. Para el trabajo presentado en este capítulo hemos elegido lotes de semillas de color oscuro y claro. En la fase inicial del Proyecto (CGL2004-01996/BOS) que sufraga esta Investigación, se estudió en detalle en ambos lotes de semillas: (i) la estructura microscópica de la semilla seca viable y las alteraciones más notables que acontecen en la misma durante el proceso de imbibición temprano; y (ii) la capacidad de excretar mucílago (mixospermia), velocidad de imbibición y germinación en semillas claras y oscuras en presencia de NO_3^-, ET, GA_{4+7} y compuestos que alteran la producción de ambas fitohormonas. Los resultados del apartado (ii) fueron publicados, y así se presentan en esta Memoria, en la Revista *Seed Science and Biotechnology* (Global Science Books ISSN: 1752-3966) bajo el nombre "*Ripe fruits of Sisymbrium officinale L. contain heterogeneous endospermic seeds with different germination rates*" (2007) **1**: 18-24. Una parte de los resultados obtenidos en esta publicación nos ha dado información para, mediante aproximaciones de Biología Molecular, llevar a cabo estudios exhaustivos sobre el control hormonal de la germinación e imbibición de semillas de *S. officinale* pertenecientes al lote oscuro, que se presentan en los Cap. 2 y 3, respectivamente.

III.2. – Materiales y Métodos

III.2.1. Material Biológico

Las plantas secas de la especie *Sisymbrium officinale* (L.) Scopoli fueron recogidas en 2006-2007 en ambientes nitrófilos de la provincia de Ourense y del Campus Sur de la Universidad de Santiago de Compostela, respectivamente. Las semillas (Fig. 7) se obtuvieron a partir de los frutos una vez eliminados de los mismos los pedicelos, el replum y las valvas (para visualizar estas partes en frutos de Crucíferas ver Matilla, 2007). Todas las semillas *S. officinale* están en el compartimento valvar ya que el rostro es rudimentario en esta especie. Para este estudio se dividieron las semillas maduras en dos lotes atendiendo al color de las cubiertas seminales (**oscuras** y **claras**). La relación cuantitativa entre ambas semillas fue de 2 ± 0.1. Se almacenaron en una atmósfera con baja HR a 21 ± 0.2ºC.

III.2.2. Microscopía de Campo Claro de las Semillas

Las semillas objeto de estudio fueron fijadas en tampón fosfato sódico (50 mM, pH 6.8), que contenía *p*-formaldehido al 2% (p/v) y glutaraldehido al 2% (v/v), 6 d a 4ºC. La fijación fue seguida por un lavado en tampón fosfato (50 mM, pH 6.8), deshidratación con etanol diluido e infiltración e inclusión con resina LR-White (Cuadro I).

Se realizaron secciones longitudinales semifinas (2 µm) en un ultra-microtomo de rotación "Reichert-Jung Ultracut 701701" (Leyca, Alemania). Posteriormente se

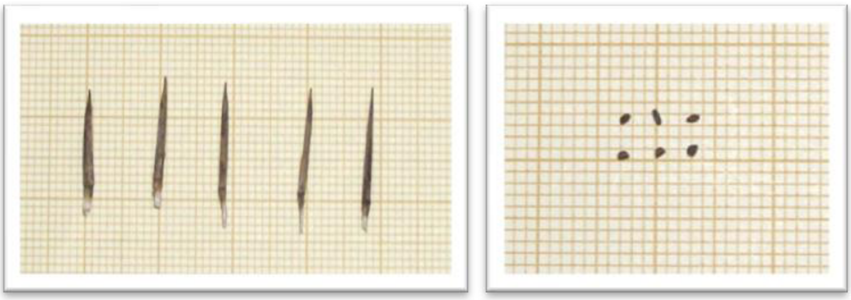


Figura 7: Silicuas secas (izda.) y semillas maduras y secas oscuras (dcha.) de *S. officinale*

Cuadro I: Condiciones preparatorias para la microscopía

TRATAMIENTO	REACTIVO	TIEMPO	Tª
FIJACIÓN	*p*-formaldehído 2% (p/v) Glutaraldehído 2% (v/v)	6 d	4°C
LAVADOS	Tampón fosfato sódico 50 mM pH 6.8 (p/v)	3 x 8 h	4°C
DESHIDRATACIÓN	Alcohol 50° Alcohol 70° Alcohol 96° Alcohol absoluto	6 d 6 d 6 d 3 x 6 d	4°C
INFILTRACIÓN	LR-White Resin: Alcohol absoluto (1:3) LR-White Resin: Alcohol absoluto (3:1) LR-White Resin	7 d 7 d 7 d	4°C
INCLUSIÓN	LR-White Resin	24 h	60°C

procedió a la tinción de las muestras. El método usado fue PAS-Naphthol Blue Black, adaptado a resinas acrílicas (LR- White). Es una tinción específica para carbohidratos (color rosa) y proteínas (color azul). Los tiempos de tinción se muestran en el cuadro II.

Las secciones fueron analizadas en un microscopio Optiphot-2 (Nikon, Japón). Finalmente, se tomaron fotografías con una cámara Coolpix 8400 (Nikon, Japón).

Cuadro II: Condiciones de tinción

REACTIVO	TIEMPO	Tª
Ác. Periódico 0.5 % (p/v)	30′	30°
Agua destilada	5′	TA
Reactivo de *Schiff* (oscuridad)	30′	TA
Agua destilada	Segundos	TA
Metabisulfito sódico 0.5 % (p/v)	6′	TA
Agua destilada	5′	TA
Naphthol Blue Black 1% (p/v)	45′	TA
Ác. Acético glacial 0.5 % (v/v)	5′	TA
Agua	Segundos	TA

Cuadro descriptivo del origen de los reactivos utilizados

REACTIVO	FABRICANTE
Ác. Acético glacial	Panreac (España)
Ác. Periódico	Merck (Alemania)
Alcohol absoluto	Panreac (España)
Bifosfato ácido de sodio	Sigma-Aldrich (España)
Bifosfato sódico	Panreac (España)
Glutaraldehído 25%	Merck (Alemania)
LR-White Resin	Sigma-Aldrich (España)
Metabisulfito sódico	Merck (Alemania)
Naphthol Blue Black	Sigma-Aldrich (España)
Nitrato potásico (KNO_3)	Duchefa Biochemia (Holanda)
p-formaldehído	Panreac (España)
Reactivo de *Schiff*	Merck (Alemania)
Rojo de Rutenio	Fluka (Alemania)

III.2.3. Detección de Mucílago (Rojo Rutenio)

La presencia de mucílago fue determinada como está descrito en Western *et al.* (2000), con ligeras modificaciones. Las semillas fueron imbibidas en NO_3K (20 mM) durante 30′ a 24°C y posteriormente teñidas durante 10 min con rojo de rutenio 0.2 % (p/v). Finalmente, se analizaron y fotografiaron en un estereomicroscopio B061 (Olympus, Japón).

III.2.4. Ensayos de Germinación

Se colocaron tres réplicas de 50 semillas de *S. officinale* en placas de Petri (90 mm de diámetro) que contenían dos círculos de papel de filtro (Whatman No. 1). Se añadieron 3 ml de NO_3K (20 mM) que contenían los compuestos adecuados para cada experimento: etileno (10 µM etrel, Sigma-Aldrich, España).

Para los experimentos de germinación las semillas no se esterilizaron a fin de no influir en el estatus de dormición. No se detectaron contaminaciones por hongos. La germinación se llevó a cabo e una cámara a 24ºC y un fotoperíodo de 16h/8h. Las semillas se consideraron germinadas cuando fue visible la emergencia radicular.

III.3. – Resultados y Discusión

III.3.1. Histología General de la Semilla

Las semillas de *S. officinale* miden aprox. 1 mm de largo y presentan una forma irregular no esférica. Las semillas maduras contienen un embrión completamente diferenciado rodeado por el endosperma ó capa de aleurona (monoestratificada) y por la cubierta seminal (testa) (Fig. 8). La orientación del embrión está condicionada por la anatomía de su óvulo, que es *campilótropo*. La zona micropilar y la chalazal se encuentran al mismo nivel, lo que está reflejado en la organización de la semilla madura. El embrión consta de dos *cotiledones* (Dicotiledónea), el hipocótilo (radícula) y la plúmula (meristema apical del tallo).

La capa de aleurona rodea toda la periferia de la semilla excepto en la *zona* chalazal. En este punto se observa la presencia de restos nucelares, y en determinadas secciones haces vasculares (Fig. 10B). Éstos últimos, pertenecientes a las conexiones vasculares existentes entre el embrión y la planta madre, vía funicular, están implicados en el aporte de nutrientes a la semilla. Precisamente, la existencia de restos nucelares, dispuestos entre la capa de aleurona y la cubierta seminal, es la que nos induce a pensar en el origen embriogénico de esta capa y no en un origen materno. Es decir, que en *S. officinale* el origen de esta capa sea el endosperma, y por lo tanto consideramos a esta semilla como endospérmica. Este modelo embriogénico también ha sido propuesto para *Arabidopsis thaliana* (Haughn y Chaudhury, 2005).

La principal fuente de reservas de estas semillas parecen ser las proteínas, tal y como revela la tinción PAS-naphthol blue-black (Fig. 8). Las células cotiledonares, las de la radícula y las células de la capa de aleurona están repletas de cuerpos proteicos.

No parecen existir diferencias anatómicass entre las semillas oscuras y claras de *S. officinale* (resultados no mostrados).

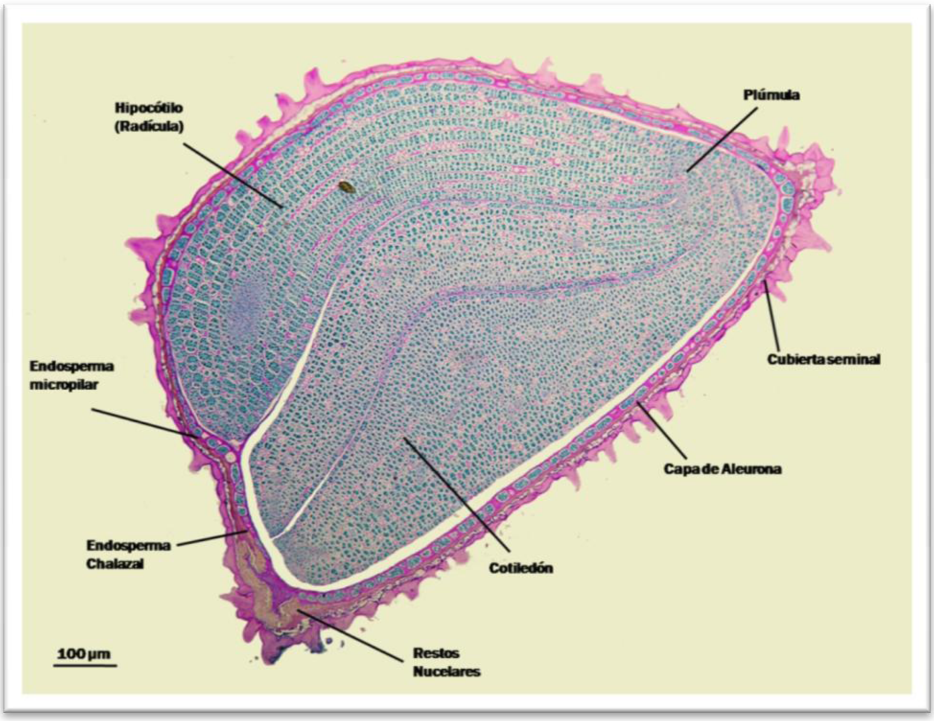


Figura 8: Corte longitudinal de una semilla seca y viable de *S. officinale.*

III.3.2. Histología de la Cubierta Seminal de *S. officinale*

La cubierta seminal de *S. officinale* presenta cierta homología con la de *A. thaliana* (Debeaujon *et al.*, 2007). Las semillas de ambas presentan un *tegumento externo* y otro *interno*. Este último sufre un proceso de aplastamiento a lo largo del desarrollo embriogénico y durante la maduración de la semilla. Al igual que en *Arabidopsis*, las semillas de *S. officinale* son *mixospérmicas*; es decir, exhiben una epidermis hialina, compuesta por *células secretoras de mucílago* (Fig. 9B). El papel del mucílago en las semillas dista mucho de conocerse. Algunos autores apuntaron que era necesario para la hidratación y viabilidad de la semilla (Huang *et al.*, 2008). Sin embargo, esta necesidad no parece haberse demostrado fehacientemente en *Arabidopsis* (Western *et al.*, 2000, 2001, 2004), en cuyas semillas se han caracterizado varios factores de transcripción implicados en la síntesis del mucílago y desarrollo de la cubierta seminal (ej. SBT1, TTG1, TTG2; APETALA2, GLABRA2, EGL3, TT8; Rautengarten *et al.*, 2008 y referencias citadas). Sin embargo, el lote de semillas claras de *S. officinale*, cuya germinación es menos intensa que las del lote oscuro, apenas tienen capacidad de excretar mucílago y poseen una velocidad de imbibición mucho mayor; lo que puede ocasionar el retraso en la emergencia radicular (Iglesias-Fernández *et al.*, 2007). Resultados parecidos se obtuvieron al comparar el mutante *atsbt1.7*, incapaz de excretar mucílago, con el WT (Rautengarten *et al.*, 2008). Mutantes con capacidad reducida de

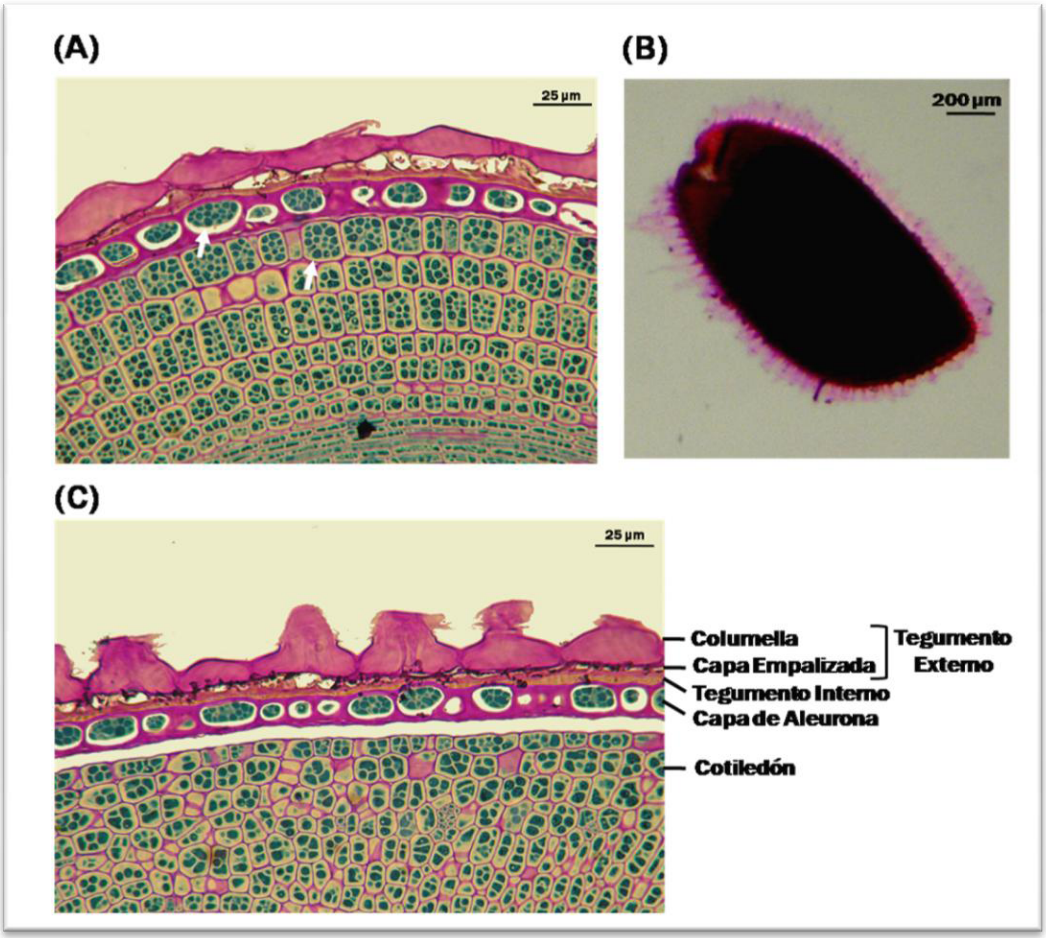


Figura 9: Características notables de la cubierta seminal de semillas secas y maduras de *S. officinale*: (A) visión de la testa a nivel del eje embrionario; (B) visión del mucílago envolvente; y (C) partes constitutivas de la testa a nivel del cotiledón. Las flechas muestran los cuerpos proteicos

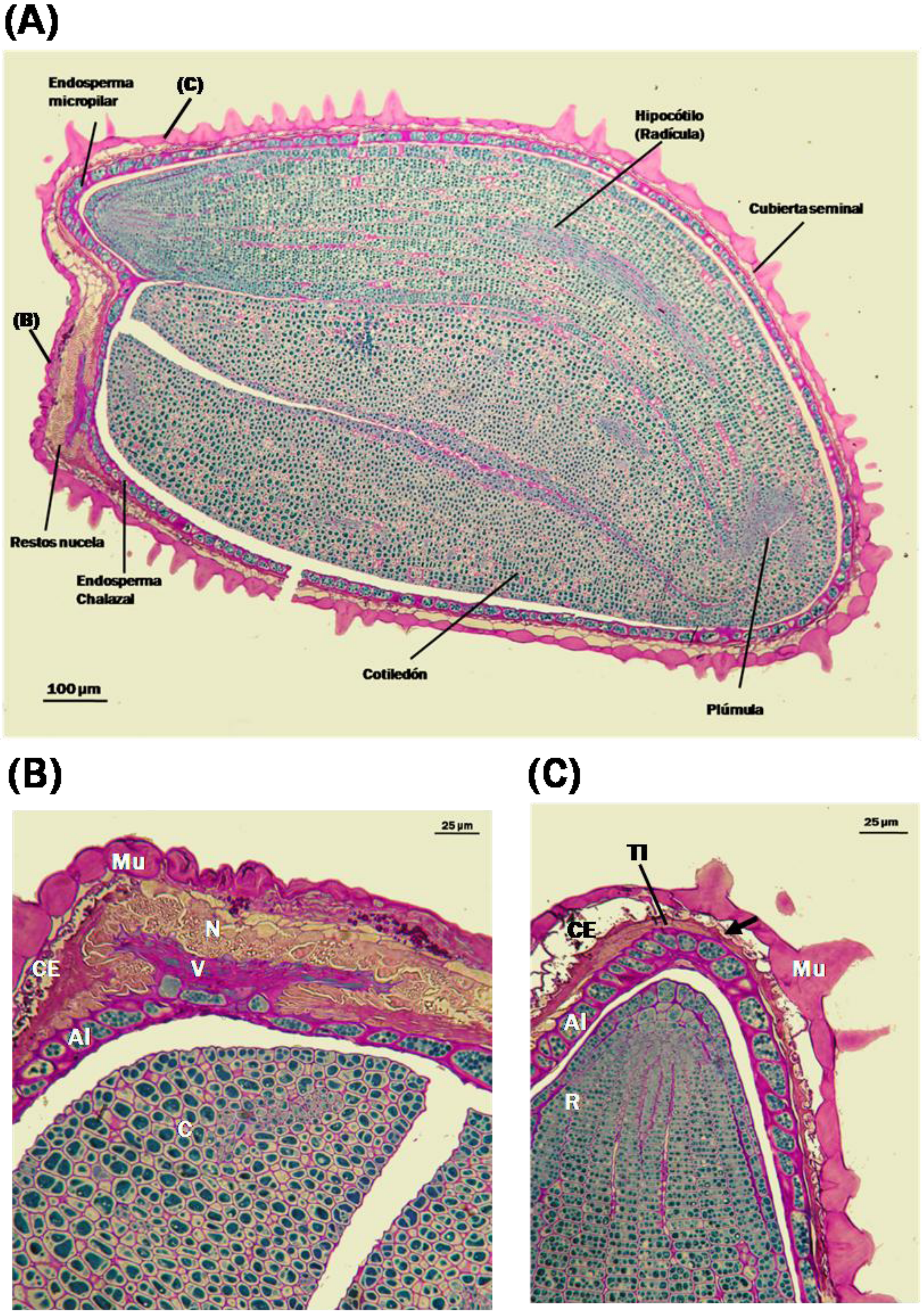


Figura 10: (A) Corte longitudinal de la semilla de *S. officinale* a las 4 h de imbibición. (B) Detalle de la cubierta seminal a nivel del endosperma chalazal. (C) Detalle a nivel de endosperma micropilar. La flecha indica la pared celular secundaria de la capa en empalizada. Al, capa de aleurona; C, cotiledón; CE, capa en empalizada; Mu, mucílago; N, nucela; R, radícula; TI, tegumento interno; V, haces vasculares.

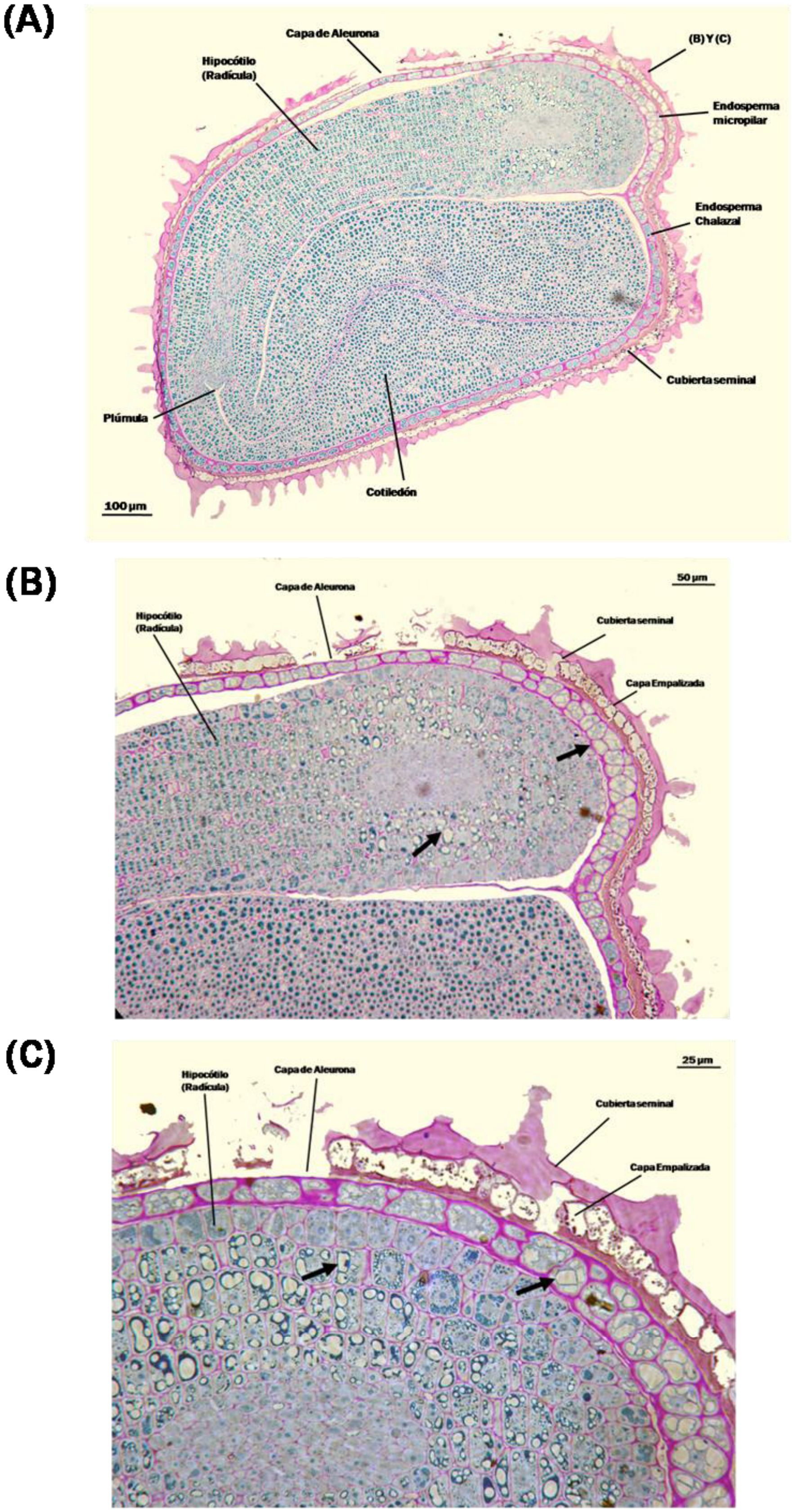


Figura 11: (A) Corte longitudinal de la semilla de *S. officinale* a las 22 h de imbibición. (B y C) Detalle de la cubierta seminal a nivel del endosperma micropilar. Las flechas muestran los cuerpos proteicos vacíos de la capa de aleurona y de las células del ápice radicular.

sintetizar mucílago tienen disminuido el porcentaje de germinación en condiciones de potenciales hídricos muy negativos (Penfield *et al.*, 2001). Nuestros resultados confirman que ambos lotes de semillas de *S. officinale* son fenotípicamente heterogéneos con respecto a la capacidad de excretar mucílago, velocidad de imbibición y capacidad germinativa (Iglesias-Fernández *et al.*, 2007; Rautengarten *et al.*, 2008).

Estructuralmente, la cubierta seminal de *S. officinale* posee las siguientes partes:

- Tegumento externo

❖ **Epidermis**: constituida por células secretoras de mucílago; se puede observar la *columella* (Fig. 9A, C).

❖ **Capa en empalizada** (Fig. 9A, C): esta capa en *S. officinale* parece corresponder al tipo-A de los ocho tipos descritos por Vaughan *et al.* (1971). Es decir, ausencia de engrosamiento en las paredes celulares. Al igual que en *A. thaliana* podría sufrir un proceso de aplastamiento a lo largo de la maduración de la semilla.

- Tegumento interno

❖ **Conjunto de capas celulares comprimidas** (Fig. 9A, C): difícilmente se puede dar un número exacto de estas capas sin un previo estudio del desarrollo y la maduración de la cubierta seminal de esta especie. El número de estas varía según la especie, en *Arabidopsis* procede del aplastamiento de tres capas celulares.

Actualmente existen un buen número de mutaciones en genes que están relacionados con el control de diferentes aspectos de la cubierta seminal. Como se apuntó previamente en esta Memoria, todos los genes que afectan a la estructura y fisiología de la cubierta seminal presentan herencia materna. Así, las semillas del mutante *A. thaliana transparent testa 1* (*tt1*) son amarillas debido a la falta de pigmentos tipo taninos en la cubierta (Sagasser *et al.*, 2002).

- Restos de endosperma

❖ Capa de aleurona, presenta paredes celulares muy engrosadas, y el interior celular está repleto de cuerpos proteicos (Fig. 9C).

III.3.3. Histología de la Cubierta Seminal a lo largo de la Imbibición de *S. officinale*

La cubierta seminal a las 4 h y 22 h de imbibición

A las 4 h de imbibición se observa en la cubierta seminal un importante cambio en los niveles de hidratación de la capa de aleurona. El citoplasma de las células de esta capa se muestra hidratado y distendido. Esta hidratación del citoplasma celular se puede observar a lo largo de todo el perímetro de la semilla (Fig. 10A, B, C).

A las 22 h de imbibición se observa en la región micropilar (apical y lateral) que los cuerpos proteicos presentes en las células de la capa de aleurona están vacios. Sin embargo, este vaciado proteico sólo se observa en esta región y no en el resto de esta capa endospérmica. Además, también se puede observar que en la región apical del eje embrionario los cuerpos proteicos de algunas de las células también están vacíos (Fig. 11A, B, C). Cabe destacar que la germinación en *S. officinale* ocurre en dos pasos, al igual que en tabaco (Leubner.Metzger *et al.*, 1995; Kucera *et al.*, 2005), es decir, primero ocurre la rotura de la testa y finalmente la rotura del endosperma (Fig. 11A, B, C).

Estos resultados indican que los procesos preparatorios necesarios para desencadenar la germinación de estas semillas podrían iniciarse en las primeras horas de imbibición, ya que la capa de aleurona sufre cambios morfológicos relacionados con la hidratación a las 4 h de imbibición. La gran importancia de la capa de aleurona, durante la germinación de diversas especies, ha sido sugerida por numerosos autores (Groot y Karseen, 1989; Bewley 1997a, b; Wang *et al.*, 2004; Kucera *et al.*, 2005;). Además, el vaciado de los cuerpos proteico de las células de la capa de aleurona, presente a las 22 h de imbibición, así como la rotura del endosperma como paso final de la germinación apuntan hacia una función importante de la misma durante la germinación de *S. officinale*. Dicha función podría estar relacionada con la liberación de enzimas hidrolíticas implicadas en la degradación de las paredes celulares de las células de la cubierta seminal, así como de su propio debilitamiento (Dubreucq *et al.*, 2000; Marraccini *et al.*, 2001; Leubner-Metzger, 2002; Kende *et al.*, 2004; Ogawa *et al.*, 2003; Wang *et al.*, 2004; Nonogaki *et al*, 2007).

Una explicación similar podría tener el vaciado de los cuerpos proteicos de las células de la región apical del eje embrionario. Éstos podrían liberar enzimas hidrolíticas implicadas en la degradación de los componentes de las paredes celulares de dichas células, así como también enzimas implicadas en la movilización de las reservas, permitiendo de esta forma el crecimiento en expansión celular, y por lo tanto proporcionando al embrión la fuerza necesaria para permitir la protrusión radicular (Bewley *et al.*, 1997b; Nonogaki *et al.*, 2007).

El balance entre el debilitamiento de las cubiertas envolventes que rodean al embrión y la fuerza ejercida por el mismo parece ser la piedra angular sobre el que se sustenta la germinación de numerosas semillas (Bewley *et al.*, 1997b; Nonogaki *et al.*, 2007).

No se observaron diferencias morfológicas entre las semillas oscuras y claras a lo largo del proceso de imbibición. El tratamiento con ET de las semillas tampoco proporcionó ningún resultado destacable (resultados no mostrados).

Posteriores estudios podrán arrojar luz sobre la importancia de la capa de aleurona a lo largo de la germinación de *S. officinale*, así como de la naturaleza de las proteínas incluidas en los cuerpos proteicos, tanto de las células de la capa de aleurona como de las del eje embrionario.

BIBLIOGRAFÍA

Abrahams S, Tanner GJ, Larkin PJ, Ashton AR (2002) Identification and biochemical characterization of mutants in the proanthocyanidin pathway in *Arabidopsis*. *Plant Physiol* **130**: 561-76

Agustí M (2000) Crecimiento y maduración del fruto. En: *Fundamentos de Fisiología Vegetal* (eds. Azcón-Bieto J, Talón M) Pp. 419-35. McGraw-Hill Interamericana, Madrid España

Baskin, CC & Baskin, JM (2004) A classification system for seed dormancy. *Seed Sci Res* **14**: 1-16

Berger F, Hamamura Y, Ingouff M, Higashiyama T (2008) Double-fertilization-caught in the act. *Trends Plant Sci* **13**: 437-43

Bergfeld R, Schopfer, P (1986) Differentiation of a functional aleurone layer within the seed coat of *Sinapis alba* L. *Ann Bot* **57**: 25-33

Berleth T, Chatfield S (2002) Embryogenesis: pattern formation from a single cell. *The Arabidopsis book* (eds. Somerville CR, Meyerowitz EM) Pp. 1-22. American Society of Plant Biologists, Rockville, MD

Bethke PC, Libourel IG, Aoyama N, Chung YY, Still DW, Jones RL (2007)The *Arabidopsis* aleurone layer responds to nitric oxide, gibberellin, and abscisic acid and is sufficient and necessary for seed dormancy. *Plant Physiol* **143**: 1173-88

Bewley JD (1997a) Breaking down the walls – a role for endo-betamannanase in release from seed dormancy? *Plant Sci* **2**: 464-69

Bewley JD (1997b) Seed germination and dormancy. *Plant Cell* **9**: 1055-66

Bewley JD (2003) Seeds of hope; seeds of conflict. En: *The biology of seeds: recent research advances* (eds. Nicolás G, Bradford KJ, Côme D, Pritchard HW) Pp. 1-10. CABI Publishing, Oxon, UK

Bewley JD, Black M (1994) *Seeds: physiology of development and germination.* Pp. 367. Plenum, New York

Boavida LC, Vieira AM, Becker JD, Feijoó JA (2005) Gametophyte interaction and sexual reproduction: how plants make a zygote. *Int J Dev Biol* **49**: 615-32

Boesewinkel FD, Bouman F (1995) The seed: structure and function. En: *Seed development and germination* (eds. Kigel J, Galili G) Pp. 1-24. Marcel Dekker, New York

Borisjuk L, Rolletschek H, Radchuk R, Weschke E, Wobus U, *et al.* (2004) Seed development and differentiation: a role for metabolic regulation. *Plant Biol* **6**: 375-86

Bosch M, Cheung AY, Hepler PK (2005) Pectin methylesterase, a regulator of pollen tube growth. *Plant Physiol* **138**: 1334-46

Chen F, Bradford KJ (2000) Expression of an expansin is associated with endosperm weakening during tomato seed germination. *Plant Physiol* **124**: 1265-74

Christensen CA, King EJ, Jordan JR, Drews GN (1997) Megagametogenesis in *Arabidopsis* wild type and the *Gf* mutant. *Sex Plant Reprod* **10**: 49-64

Clerkx EJM, Blankestijn-De Vries H, Ruys GJ, Groot SPC, Koornneef M (2004) Genetic differences in seed longevity of various *Arabidopsis* mutants. *Physiol Plant* **121**: 448-61

Corner EJH (1976) *The seeds of dicotyledons. Cambridge University Press, UK*

da Silva EEA, de Melo DLB, Davide AC, de Bode N, Abreu GB, Faria JMR, Hilhorst HWM (2007) Germination ecophysiology of *Annona crassiflora* Mart. seeds. *Ann Bot* **99**: 823-30

Dean GH, Zheng H, Tewari J, Young DS, Hwang YT, Western TL, Carpita NC, McCann MC, Mansfield SD, Haughn GW (2007) The *Arabidopsis* MUM2 gene encodes a β-galactosidase required for the production of seed coat mucilage with correct hydration properties. *Plant Cell* **19**: 4007-21

Debeaujon I, Koornneef M (2000) Gibberellin requirement for *Arabidopsis* seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiol* **122**(2): 415-24

Debeaujon I, Léon-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in *Arabidopsis*. *Plant Physiol* **122**: 403-14

Debeaujon I, Peeters AJM, Léon-Kloosterziel KM, Koornneef M (2001) The *TRANSPARENT TESTA12* gene of *Arabidopsis* encodes a multidrug secondary transporter–like protein required for flavonoid sequestration in vacuoles of the seed coat endothelium. *Plant Cell* **13**: 853-72

Debeaujon I, Lepiniec L, Pource L, Routaboul J-M (2007) Seed coat development and dormancy. En: *Seed development, dormancy and germination* (eds. Bradford K y Nonogaki H) Pp. 25-49. Blackwell Publishing, Oxford

Declercq A, Vandewiele M, Derycke R, Vandamme J, Vanmontagu M, Krebbers E, Vandekerckhove J (1990) Expression and processing of an *Arabidopsis* 2s albumin in transgenic tobacco. *Plant Physiol* **92**: 899-907

de la Torre F, Rodríguez-Gacio MC, Matilla AJ (2006) How ethylene works in the reproductive organs of higher plants. A signaling update from the third millennium. *Plant Sign Behavior* **1**: 231-42

Dixon RA, Xie DY, Sharma SB (2005) Proanthocyanidins—A final frontier in flavonoid research? *New Phytol* **165**: 9-28

Dolfini D, Consonni G, Viotti C, dal Prà M, Saltini G, Giulini A, Pilu R, Malgioglio A, Gavazzi G (2007) A mutational approach to the study of seed development in maize. *J Exp Bot* **58**: 1197-205

Dresselhaus T (2006) Cell-cell communication during double fertilization. *Curr Opin Plant* **9**: 41-7

Drews GN, Lee D, Christensen GA (1998) Genetic analysis of female gametophyte development and function. *Plant Cell* **10**: 5-17

Drews GN, Yadegari R (2002) Development and function of the Angiosperm female gametophyte. *Annu Rev Genet* **26**: 99-124

Dubreucq B, Berger N, Vincent E, Boisson M, Pelletier G, Caboche M, Lepiniec L (2000) The *Arabidopsis AtEPR1* extensin-like gene is specifically expressed in the endosperm during seed germination. *Plant J* **23**: 643-52

Dumas C, Rogowsky P (2008) Fertilization and early seed formation. *C R Biologies* **331**: 715-25

Eastmond PJ, Germain V, Lange PR, Bryce JH, Smith SM, Graham IA (2000) Postgerminative growth and lipid catabolism in oilseeds lacking the glyoxylate cycle. *Proc Natl Acad Sci, USA* **97**: 5669-74

Ferrándiz C (2002) Regulation of fruit dehiscence in *Arabidopsis. J Exp Bot* **53**: 2031-38

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* **171**: 501-23

Finkelstein R, Reeves W, Ariizumi T, Steber C (2008) Molecular aspects of seed dormancy. *Annu Rev Plant Biol* **59**: 387-415

Footitt S, Slocombe SP, Larner V, Kurup S,Wu Y, *et al.* (2002) Control of germination and lipid mobilization by COMATOSE, the *Arabidopsis* homologue of human ALDP. *EMBO J* **21**: 2912-22

Frohlich MW, Chase MW (2007) After a dozen years of progress the origin of Angiosperms is still a great mystery. *Nature* **450**: 1184-89

García D, Saingery V, Chambrier P, Mayer U, Jürgens G, Berger F (2003) *Arabidopsis* haiku mutants reveal new controls of seed size by endosperm. *Plant Physiol* **131**: 1661-70

García D, Fitz Gerald JN, Berger F (2005) Maternal control of integument cell elongation and zygotic control of endosperm growth are coordinated to determine seed size in *Arabidopsis. Plant Cell* **17**: 52-60

Giovannoni JJ (2004) Genetic regulation of fruit development and ripening. *Plant Cell* **16**: 170-80

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening-a study with gibberellins-deficient mutants. *Planta* **171**: 525-31

Gubler F, Millar AA, Jacobsen JV (2005) Dormancy release, ABA and pre-harvest sprouting. *Curr Opin Plant Biol* **8**: 183-87

Guignard ML (1899) Sur les anthérozoïdes et la double copulation sexuelle chez les végétaux angiospermes. *Rev Gén Bot* **11**:129-35

Haig D (1990) New perspectives on the angiosperm female gametophyte. *Bot Rev* **56**: 236-74

Han F, Lamb JC, Birchler JA (2006) High frequency of centromere inactivation resulting in stable dicentric chromosomes of maize. *Proc Natl Acad Sci, USA* **103**: 3238-43

Harada JJ (1999) Signalling in plant embryogenesis. *Curr Opin Plant Biol* **2**: 23-27

Haugh G, Chaudhury A (2005) Genetic analysis of seed coat development in *Arabidopsis. Trends Plant Sci* **10**: 472-77

Hermann K, Meinhard J, Dobrev P, Linkies A, Pesek B, Hess B, Machácková I, Fischer

U, Leubner-Metzger G (2007) 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds. *J Exp Bot* **58**: 3047-60

Hewitt J (2007) Study of *Arabidopsis thaliana* seed development: occurrence of germinability. Thesis of Master of Science. Oregon State University

Higashiyama T (2002) The synergid cell: Attractor and acceptor of the pollen tube for double fertilization. *J Plant Res* **115**: 149-60

Higashiyama T, Kuroiwa H, Kuroiwa T (2003) Pollen-tube guidance: beacons from the female gametophyte. *Curr Opin Plant Biol* **6**: 36-41

Higashiyama T, Hamamura Y (2008) Gametophytic pollen tube guidance. *Sex Plant Reprod* **21**: 17-26

Hiscock SJ, Allen AM (2008) Diverse cell signalling pathways regulate pollen-stigma interactions: the search for consensus. *New Phytol* **179**: 286-317

Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytol* **179**: 33-54

Honys D, Twell D (2003) Comparative Analysis of the *Arabidopsis* Pollen Transcriptome. *Plant Physiol* **132**: 640-52

Huang B-Q, Russell SD (1992) Female germ unit: organization isolation, and function. *Int Rev Cytol* **140**: 233-92

Huang Z, Gutterman Y (1998) *Artemisia monosperma* achene germination in sand: effects of sand depth, sand/water content, cyanobacterial sand crust and temperature. *J Arid Environ* **38**: 27-43

Huang Z, Huc Z, Gutterman Y (2000) Structure and function of mucilaginous achenes of *Artemisa monosperma* inhabiting the Negev desert of Israel. *Israel J Plant Sci* **48**(4): 255-66

Huang Z, Boubriak I, Osbosne DJ, Dong M, Gutterman Y (2008) Possible role of pectin-containing mucilage and dew in repairing embryo DNA of seeds adapted to desert conditions. *Ann Bot* **101**: 277-83

Iglesias-Fernández R, Matilla AJ, Pulgar I, de la Torre F (2007) Ripe fruits of *Sisymbrium officinale* L. contain heterogeneous endospermic seeds with different germination rates. *Seed Sci Biotech* **1**: 18-24

Imbert E (2002) Ecological consequences and ontogeny of seed hetromorphism. *Perspect Plant Ecol, Evol System* **5**: 13-36

Johnson MA, Preuss D (2002) Plotting a course: multiple signals guide pollen tubes to their targets. *Dev Cell* **2**: 273-81

Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK (2002) The abscisic acid responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element binding factor, TaABF, and phosphorylates TaABF peptide sequences. *Plant Physiol* **130**: 837-46

Jürgens G, Mayer U, Busch M, Lukowitz W, Laux T (1995) Pattern formation in the *Arabidopsis* embryo: a genetic perspective. *Philos Trans R Soc London B Biol Sci* **350**: 19-25

Karssen CM (1976) Uptake and effect of abscisic acid during induction and progress of radicle growth in seeds of *Chenopodium album*. *Physiol Plant* **36**: 259-63

Kasahara RD, Portereiko MF, Sandaklie-Nikolova L, Rabiger DS, Drews GN (2005) *MYB98* is required for pollen tube guidance and synergid cell differentiation in *Arabidopsis*. *Plant Cell* **17**: 2981-92

Kende H, Bradford KJ, Brummell, *et al.* (2004) Nomenclature for members of the expansin superfamily of genes and proteins. *Plant Mol Biol* **55**: 311-14

Khan M, Cavers PB, Kane M, Thaompson K (1996) Role of the pigmented seed coat of proso millet (*Panicum miliaceum* L.) in imbibition, germination and seed persistence. *Seed Sci Res* **7**: 21-5

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Sci Res* **15**: 281-307

Lelièvre JM, Latchhé A, Jones B, Bouzayen M, Pech JC (1997) Ethylene and fruit ripening. *Physiol Plant* **101**: 727-39

Lepiniec L, Debeaujon I, Routaboul J-M, *et al.* (2006) Genetics and biochemistry of seed flavonoids. *Annu Rev Plant Biol* **57**: 405-30

Leubner-Metzger G, Frundt C, Vogeli-Lange R, Meins F (1995) Class-I β-1,3-glucanases in the endosperm of tobacco during germination. *Plant Physiol* **109**: 751-59

Leubner-Metzger G, Meins F (2000) Sense transformation reveals a novel role for class-I β-1,3-glucanase in tobacco seed germination. *Plant J* **23**: 215-21

Leubner-Metzger G (2002) Seed after-ripening and over-expression of Class-I β-1,3-glucanase confer maternal effects on tobacco testa rupture and dormancy release. *Planta* **215**: 959-68

Leubner-Metzger G (2003) Functions and regulation of β-1,3-glucanase during seed germination, dormancy release and after-ripening. *Seed Sci Res* **13**: 17-34

Lohe AR, Chaudhury A (2002) Genetic and epigenetic processes in seed development. *Curr Opin Plant Biol* **5**: 19-25

Maheshwari P (1950) *An Introduction to the Embryology of Angiosperms*. McGraw-Hill, New York

Marraccini P, Rogers WJ, Allard C, *et al.* (2001) Molecular and biochemical characterization of endo- β-mannanase from germinating coffe (*Coffea Arabica*) grains. *Planta* **213**: 296-308

Marton ML, Cordts S, Broadhvest J, Dresselhaus T (2005) Micropylar pollen tube guidance by egg apparatus 1 of maize. *Science* **307**: 573-6

Matilla AJ, Gallardo M, Puga-Hermida MI (2005) Structural, physiology and molecular aspects of heterogeneity in seeds: a review. *Seed Sci Res* **15**: 63-76

Matilla AJ (2007) How is the silique fruit dismantled over its maturation? *Funct Plant Sci Biotech* **1**: 85-93

Matilla AJ, de la Torre F, Iglesias-Fernández R (2007) Three notable hormones involved in the control of seed germination. *CurrTop Phytochem* **8**: 59-71

Matilla AJ (2008) Desarrollo y germinación de semillas. En: *Fundamentos de Fisiología Vegetal* (eds. Azcón-Bieto J, Talón M) Pp. 537-58. McGraw-Hill Interamericana, Madrid, España

Mo BX, Bewley JD (2002) β-mannosidase (EC 3.2.1.25) activity during and following germination of tomato (*Lycopersicon esculentum* Mill.) seeds. Purification, cloning and characterization. *Planta* **215**: 141-52

Mohamend-Yasseen Y, Barringer SA, Splittstoesser WE, Costanza S (1994) The role of seed coats in seed viability. *Bot Rev* **60**: 426-39

Morgan CL, Bruce DN, Child R, Ladbrooke ZL, Arthur AE (1998) Genetic variation for pod shatter resistance among lines of oilseed rape developed from synthetic *B. napus. Field Crops Res* **58**: 153-65

Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited *Brassicaceae* seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana. Plant Cell Physiol* **47**: 864-77

Müntz K, Belozersky MA, Dunaevsky YE, Schlereth A, Tiedemann (2001) Stored proteinases and the initiation of storage protein mobilization in seeds during germination and seedling growth. *J Exp Bot* **52**: 1741-52

Nakaune S, Yamada K, Kondo M, *et al.* (2005) A vacuolar processing enzyme, δVPE, is involved in seed coat formation at the early stage of seed development. *Plant Cell* **17**: 876-87

Nawaschin SG (1898) Resultate einer revision der befruchtungsvorgaenge bei Lilium martagon und Fritillaria tenella. *Bull Acad Imp Sci St Petersburg* **9**: 377-382

Nguyen H, Brown RC,Lemmon BE (2000) The specialized chalazal endosperm in *Arabidopsis* thaliana and *Lepidium virginicum* (*Brassicaceae*). *Protoplasma* **212**: 99-110

Nonogaki H, Chen F, Bradford KJ (2007) Mechanisms and genes involved in germination in *sensu stricto*. En: *Seed development, dormancy and germination* (eds. Bradford K y Nonogaki H) Pp. 264-304. Blackwell Publishing, Oxford

O'Brien TP, McCully E (1969) *Plant structure and development: a pictorial and physiological approach*. MacMillan, New York

Ogawa M, Harada A, Yamauchi Y, Kuwalhara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during *Arabidopsis* seed germination. *Plant Cell* **15**: 1591-604

Ozga JA, van Huizen R, Reinecke DM (2002) Hormonal and seed-specific regulation of pea fruit growth. *Plant Physiol* **131**: 1379-89

Ozga JA, Reinecke DM (2003) Hormonal interactions in fruit development. *J Plant Growth Regul* **22**: 73-81

Paniagua R, Nistal M, Sesma P, Álvarez-Uría M, Fraile B, Anádón R, Sáez FJ, de Miguel MP (1997) Parte IV: Histología y organografía vegetal. Órganos reproductores y reproducción sexual. En: *Citología e histología vegetal y animal*. Pp. 891-924. McGraw-Hill·Interamericana, España

Patrick JW, Offler CE (2001) Compartmentation of transport and transfer events in developing seeds. *J Exp Bot* **52**: 551-64

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW (2001) MYB61 is required for mucilage deposition and extrusion in the *Arabidopsis* seed coat. *Plant Cell* **13**: 2777-791

Penfield S, Rylott EL, Gilday AD, Graham S, Larson TR, Graham IA (2004) Reserve mobilization in the *Arabidopsis* endosperm fuels hypocotyl elongation in the dark, is independent of abscisic acid, and requires PHOSPHOENO-LPYRUVATE CARBOXYKINASE1. *Plant Cell* **16**: 2705-18

Pradoa AM, Colacxoa R, Morenoa N, Silvaa AC, Feijo JA (2008) Targeting of pollen tubes to ovules is dependent on nitric oxide (NO) signalling. *Mol Plant* **1**: 703-14

Probert RJ (2000) The role of temperature in the regulation of seed dormancy and germination. In: *Seeds: the ecology of regeneration in plant communities.* (ed. Fenner M) Pp. 261-92. CAB International, Wallingford

Puga-Hermida MI, Gallardo M, Rodríguez-Gacio MC, Matilla, AJ (2003) The heterogeneity of turnip-tops (*Brassica rapa*) seeds inside the silique affects germination, the activity of the final step of the ethylene pathway, and abscisic acid and polyamine content. *Funct Plant Biol* **30**: 767-75

Raghavan V (2003) Some reflections on double fertilization, from its discovery to the present. *New Phytol* **159**: 565-83

Rautengarten C, Usadle B, Neumetzler L, Hartmann J, Büssis D, Altmann T (2008) A subtilisin-like serine protease essential for mucilage release from *A. thaliana* seed coat. *Plant J* **54**: 466-80

Raven PH, Evert RF, Eichhorn SE (1999) *Biology of plants*. Pp. 944. Freeman, New York

Ren C, Kermode AR (2000) An increase in pectin methyl esterase activity accompanies dormancy breakage and germination of Yellow Cedar Seeds. *Plant Physiol* **124**: 231-42

Ren Y, Bewley JD, Wang X (2008) Protein and gene expression patterns of endo-β-mannanase following germination of rice. *Seed Sci Res* **18**: 139-149

Rodríguez-Gacio MC, Nicolás, C, Matilla AJ (2004) The final step of the ethylene biosíntesis pathway in turnip tops (*Brassica rapa*): Molecular characterization of the ACC-oxidase (*BrACO1*) throught zygotic embryogenesis and germination of heterogeneous seeds. *Physiol Plant* **121**: 132-40

Ruiz ZT, Escale M (1995) Ultramicroscopic seed morphology of *Cleome* L. (*Capparidaceae*) in relation to their taxonomy and dispersal syndromes. *Ernstia* **5**: 139-60

Russell SD (1992) Double fertilization. *Int Rev Cytol* **140**: 357-88

Sacandé M, Pritchard HW, Dudley A (2004) Germination characteristics of *Prunus africana* seeds. *New Forest* **27**: 239-50

Sagasser M, Lu G-H, Hahlbrock K, Weisshaar B (2002) *A. thaliana* TRANSPARENT TESTA 1 is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins Genes. *Develop* **16**: 138-49

Šamaj J, Müller J, Beck M, Böhm N, Menzel D (2006) Vesicular trafficking, cytoskeleton and signalling in root hairs and pollen tubes. *Trends Plant Sci* **11**: 594-600

Sano Y, Tanaka I (2007) Detection of differentially expressed variant histone H3.3 in the vegetative nucleus of lily pollen. *Sex Plant Reprod* **20**: 27-33

Schopfer P, Plachy C (1984) Control of seed germination by abscisic acid. II. Effect on embryo water uptake in *Brassica napus* L. *Plant Physiol* **76**: 155-60

Schopfer P (2001) Hydroxyl radical-induced cell wall loosening in vitro and in vivo: implications for the control of elongation growth. *Plant J* **28**: 679-88

Schwartz BW, Yeung EC, Meinke DW (1994) Disruption of morphogenesis and transformation of the *suspensor* in abnormal *suspensor* mutants of *Arabidopsis*. *Development* **120**: 3235-45

Srivastava LM (2002) Seed development and maturation. En: *Plant growth and development: hormones and environment* (ed. Srivastava LM) Pp 431-71. Academic Press, London, UK

Strasburger E (1879) Die *Angiospermen und die Gymnospermen.* Fischer, Jena, Germany

Strasburger E, Noll F, Schenck H, Schimper AFW, *et al.* (1994) *Tratado de Botánica.* Omega, S.A. Barcelona, España

Thompson K, Band SR, Hodgson JG (1993) Seed size and shape predict persistence in soil. *Funct Ecol* 7: 236-41

Vaughan JG, Whitehouse FLS, Whitehouse JM (1971) Seed structure and the taxonomy of the *Cruciferae. Bot J Linn Soc* **64**: 383-409

Wan L, Xia Q, Qiu X, Selvaaraj G (2002) Early stages of seed development in *Brassica napus*: a seed coat-specifc cysteine proteinase associated with programmed cell death of the inner integument. *Plant J* **30**: 1-10

Wang AX, Li JR, Bewley JD (2004) Molecular cloning and characterization of an endo-β-mannanase gene expressed in the lettuce endosperm following radicle emergence. *Seed Sci Res* **14**: 267-76

Weber H, Buchner P, Borisjuk L, Wobus U (1996) Sucrose metabolism during cotyledon development of *Vicia faba* L. is controlled by the concerted action of both sucrose-phosphate synthase and sucrose synthase: expression patterns, metabolic regulation and implications for seed development. *Plant J* **9**: 841-50

Weber H, Borisjuk L, Wobus U (2005) Molecular physiology of legume seed development. *Annu Rev Plant Biol* **56**: 253-79

Welbaum GE, Bradford KJ, Yim K, Booth DT, Oluoch MO (1998) Biophysical, physiological and biochemical processes regulating seed germination. *Seed Sci Res* **8**: 161-72

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. *Plant Physiol* **122**: 345-56

Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis. Plant Physiol* **127**: 998-1011

Western TL, Diana S, Young DS, Dean GH, Tan WL, Samuels AL, Haughn GW (2004) MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the *Arabidopsis* seed coat. *Plant Physiology* **134**: 296-306

White PJ (2002) Recent advances in fruit development and ripening: an overview. *J Exp Bot* **53**: 1995-2000

Willemse MTM, van Went JL (1984) The female gametophyte. En: *Embryology of Angiosperms* (ed Johri BM) Pp. 159-96. Springer-Verlag, Berlín

Windsor JB, Symonds VV, Mendenhall J, Lloyd AM (2000) *Arabidopsis* seed coat development: Morphological differentiation of the outer integument. *Plant J* **22**: 483-93

Yadegari R, Drews GN (2004) Female gametophyte development. *Plant Cell* **16**: S133-S141

Yamaguchi S, Nambara N (2006) Seed development and germination. In: *Plant hormone signaling* (eds. Hedden P, Thomas SG) Pp. 311-38. Blackwell Publishing, Oxford

Yeung EC, Meinke DW (1993) Embryogenesis in angiosperms: development of the suspensor. *Plant Cell* **5**: 1371-81

ANEXO:

"Ripe fruits of *Sisymbrium officinale* L. contain heterogeneous endospermic seeds with different germination rates" (2007) *Seed Sci Biotech* **8**: 18-24

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Ripe Fruits of *Sisymbrium officinale* L. Contain Heterogeneous Endospermic Seeds with Different Germination Rates

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ABSTRACT

The seeds of *Sisymbrium officinale* are largely heterogeneous in mass and colour within the ripe fruit. In order to initiate the characterization of the germinative process from these heterogeneous seeds, we have used two different seed lots (i.e. brown and light-brown), and studied several physical and physiological properties and their possible influence on the germination rate. The most notable features from this work were: (i) the mass of the brown seeds was higher than the light-brown ones, which imbibed more quickly; (ii) light seeds contain much less mucilage (myxospermy) than do the brown; (iii) under optimal germination conditions for the brown seeds, the light-brown ones germinated 6-fold less; and (iv) very similar profiles of germination stimulation was found in the presence of gibberellins (i.e. GA_{4+7}) and ET in brown seeds, but both hormones were not capable of stimulating the germination in light-brown ones. As a general conclusion, we have demonstrated the coexistence in ripe fruits of *S. officinale* of two seed lots with remarkable physiological and physical heterogeneity that could be used as a tool to increase our knowledge about the germination process.

Keywords: ethylene, gibberellins, heterogeneous seeds, imbibition rate, myxospermy, seed density

INTRODUCTION

The appearance of the seed, the propagule chosen by spermatophytic plants for their dispersion and propagation, has great evolutionary implications, since this organ represents the survival and perpetuation of the mother plant (Boesewinkel and Bouman 1995). A typical seed of an angiosperm contains an embryo (diploid) surrounded by an endosperm (triploid; with two-thirds of its genome of maternal origin) and a seed coat (diploid) (Raghavan 1986; Torres-Ruiz 1998). The endosperm is formed by live cells with high metabolic activity, while the seed coat is comprised of a tissue of maternal origin (i.e. ovular tissue), the cells of which die during late seed maturation. Depending on the species, the endosperm is visible in dry seed (i.e. endosperm seeds) or else completely or partially disappears during zygotic embryogenesis (i.e. non-endospermic seeds), the cotyledons exercising the nutritional role of the embryo (Friedman 1998; Forbis *et al*. 2002).

The seeds of most angiosperms are dormant at maturity, and dormancy must be lost before germination can occur (Bewley 1997a, 1997b; Finch-Savage and Leubner-Metzger 2006). The seed transformation from dormant (i.e. failure of an intact, viable seed to complete germination under favourable conditions) to non-dormant stage is associated with changes in gene expression and protein patterns (Cadman *et al*. 2006; Chibani *et al*. 2006; Lee *et al*. 2006). The germination of a mature, viable, and non-dormant seed begins with the gradual uptake of water, which has three phases: rapid initial uptake (phase I, i.e. imbibition) followed by a plateau in water uptake (phase II) and a further increase after germination is completed, as the embryo axes elongate (phase III) (Bewley 1997a; Manz *et al*. 2005). Dormant seeds do not complete germination and therefore lack phase III. The germination process ends when the radicle tip has protruded through its covering structures (i.e. endosperm and seed coat in endospermic seeds) (Bewley 1997b; Kucera *et al*. 2005). Seed coat and endosperm rupture occur at the micropylar level. Cell elongation of the radicle is necessary and possibly sufficient for the completion of protrusion. However, cell division is not essential (Barroco *et al*. 2005). That is, the radicle protrusion depends on embryo expansion, which is a process driven by water uptake and cell-wall loosening (Koornneef *et al*. 2002). In most endospermic seeds (e.g. coffee, lettuce, tobacco, or tomato) the tissue that constitutes the endosperm is a germination-limiting barrier, and so that germination is completed the growth potential of the radicle must be high enough to overcome the resistance of the endospermic tissue (Bewley 1997a; Koornneef *et al*. 2002; Leubner-Metzger *et al*. 2006). The role of the seed coat as a germination constraint has been studied by using testa mutants of Arabidopsis (Debeaujon and Koornneef 2000; Koornneef *et al*. 2002). The seed coat and endosperm rupture are temporally separated in model plants as tobacco and Arabidopsis (Liu *et al*. 2005; Leubner-Metzger *et al*. 2006; Müller *et al*. 2006).

Environmental factors (i.e. light and temperature) and phytohormones (i.e. gibberellins, GAs; abscisic acid, ABA; and ethylene, ET) have been associated with the promotion of germination of endospermic and non-endospermic seeds (Hilhorst and Toorop 1997; Matilla 2000; Toorop *et al*. 2000; Koornneef *et al*. 2002; Kucera *et al*. 2005; Müller *et al*. 2006). Thus, two functions for embryonic GAs have been proposed to be needed during endospermic-seed germination: (i) to increase the embryo growth potential; and (ii) to overcome the mechanical restraint conferred by the seed-covering layers by weakening of the tissues surrounding the radicle (Hilhorst and Downie 1995; Koornneef *et al*. 2002; Leubner-Metzger 2003; Bassel *et al*. 2004; Yamauchi *et al*. 2004; da Silva *et al*. 2005). Nevertheless, GAs can conduct the germination positively by means a yet well known cross-talk with ABA and environmental factors (i.e. light or temperature). Thus, light (i.e. red light) and low temperature (i.e. 4ºC) can modulate the spatial expression pattern of GA biosynthetic genes. Recently, Yamauchi *et al*. (2004) demonstrated that a gene involved in GA biosynthesis in seeds of *A. thaliana* was activated by cold stratification at 4ºC. The increase in tissue sensitivity to GAs during

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cold stratification is another factor that may be involved in controlling seed germination (Derkx and Karssen 1993; Koornneef *et al.* 2002). In some seeds (e.g. *Nicotiana attenuata*), GAs can replace red-light to trigger dark-germination (Schwachtje and Baldwin 2004). From recent evidences in Arabidopsis it can be concluded that the GA requirement for seed germination is determined by seed-coat characteristics, embryonic growth potential and by embryonic ABA (Debeaujon and Koornneef 2000; Debeaujon *et al.* 2000). The above and other recent data indicate that GAs release coat and embryo dormancy, promote germination and counteract inhibitory ABA effects, directly or indirectly.

Also, it has been demonstrated that ABA inhibits: (i) phase-III water uptake but does not inhibit phase I or initial embryo-extension growth (Homrichhausen *et al.* 2003; Frey *et al.* 2004; Manz *et al.* 2005; Müller *et al.* 2006); (ii) the embryo growth potential and endosperm cap weakening during coffee-seed germination (da Silva *et al.* 2004); (iii) the endosperm rupture but not seed coat rupture in tobacco and petunia seeds (Toorop *et al.* 2000; Petruzzeli *et al.* 2003; Kucera *et al.* 2005). In seeds in which the seed-coat and endosperm rupture is not easy to detect visually (e.g. tomato), germination is also inhibited by ABA (Wu *et al.* 2000; Leubner-Metzger 2003); but deficiency in ABA in sit^w mutant is associated with a thinner seed coat (Hilhorst and Downie 1995; Toorop *et al.* 2000). On the other hand, during development and germination of some seeds, ABA and ET action appear to be antagonistic. Thus, it was proposed that ABA increases tissue sensitivity to ET, affecting seed germination, whereas ET may suppress seed dormancy by inhibiting ABA action (Beaudoin *et al.* 2000; Grossmann and Hansen 2001; Koornneef *et al.* 2002).

ET promotes germination and counteracts ABA effects in many seeds (Kepczynski and Kepczynska 1997; Beaudoin *et al.* 2000; Ghassemian *et al.* 2000; Matilla 2000; Koornneef *et al.* 2002) and its production is higher in non-dormant compared to dormant seeds (Matilla 2000). However, even though great progress has been made in elucidating ET signalling, the mechanism(s) of ET action remains to be explained in dormant and germinating seeds (Kucera *et al.* 2005; de la Torre *et al.* 2006). ET biosynthesis and sensitivity are both important for the germination (Beaudoin *et al.* 2000; Siriwitayawan *et al.* 2003; Matilla *et al.* 2005) and transcripts as β-1,3-glucanase or ACC-oxidase, into others, are ET-altered during germination of some seeds (Leubner-Metzger *et al.* 1998; Pretruzzeli *et al.* 2003a, 2003b; Puga-Hermida *et al.* 2003; Rodríguez-Gacio *et al.* 2004). Summarily, although all findings until now published support the view that ET can promote seed germination and counteract ABA inhibitory effects by interfering with ABA signalling, cross-talk between ET and GAs cannot be discarded (Kucera *et al.* 2005).

Fig. 1 Adult plant of *S. officinale*.

In the present study, wild mature dry seeds of *Sisymbrium officinale*, collected in north-western Spain, were used. In this species, seed-coat and endosperm rupture are temporally separated at the beginning of germination and the hormonal control of both process can be studied. This plant is common in fields and path borders and can be identified by its yellow flowers, basal leaves divided with a large and round terminal lobe, and erect fruits held close to the stem. *S. officinale* produces endospermic seeds housed into a fruit which contains numerous heterogeneous in mass and colour seeds (**Fig. 1**). Although the heterogeneity appears to be related in some cases to survival, little is known about how the seeds are affected physiologically (Puga-Hermida *et al.* 2003; Matilla *et al.* 2005). To evaluate the impact of heterogeneity on the germination process of *S. officinale* seeds, in this work we used two lots (brown and light-brown) of the above seeds to investigate some of theirs morphologic characteristics and response to ET and GAs.

MATERIALS AND METHODS

Chemicals

Aminoethoxyvinylglycine (AVG), 1-aminocyclopropane-1-carboxylic acid (ACC), paclobutrazol, silver nitrate, ruthenium red, GA$_{4+7}$, and Co$_2$Cl were purchased from Sigma-Aldrich (Spain), whereas potassium nitrate was from Merck (Germany).

Plant material

Ripe wild seeds of *Sisymbrium officinale* were collected in Galicia

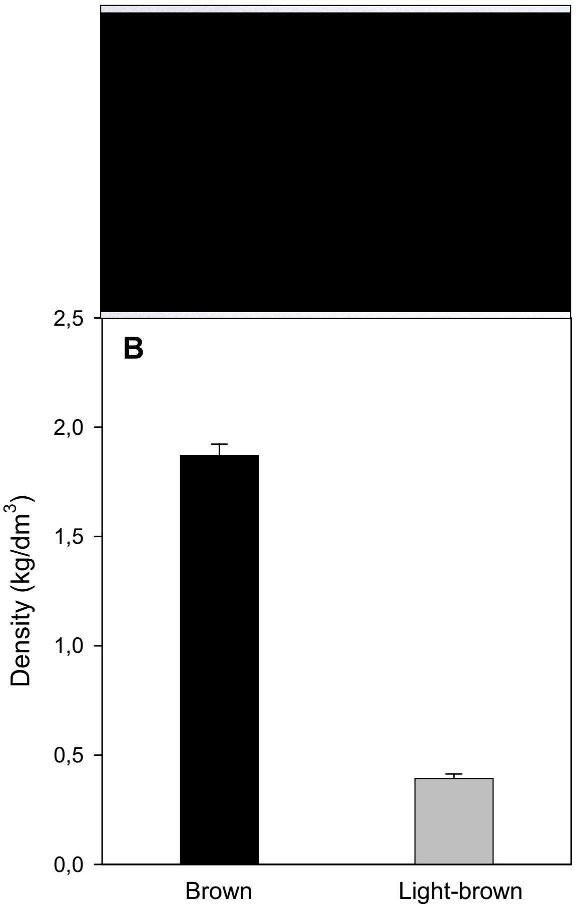

Fig. 2 External aspect (A) and density (B) of brown and light-brown lots of *S. officinale* dry seeds. Data are the means ± standard error (S.E.) of 3-4 independent experiments. Differences in density between lots were highly significant ($P<0.001$) as determined by the LSD test.

(north-western Spain) during July-August, 2006. Seed samples were separated from ripe fruit residues (i.e. pedicel, replum and valve) by means of a suitable sieve, divided into two populations (i.e. brown and light-brown; see **Fig. 2A**) according to the colour of seed coat, weighed in lots of 100 seeds (21.8 ± 0.3 and 13.3 ± 0.6 mg for dark and light, respectively) and stored at 21 ± 0.2ºC before use in the germination tests. The brown:light-brown seed ratio inside mature fruits was around 1.8 ± 0.1.

Germination experiments

Fifty seeds were put into plastic Petri dishes on two Whatman No. 1 filter paper discs moistened with 3 ml of 20 mM KNO_3 (control) supplemented with solutions of gibberellin (GA_{4+7}) and etephon (ET), inhibitors of GAs (PC, paclobutrazol) and ET (AVG, Co_2Cl) synthesis and the inhibitor of ET signalling (NO_3Ag). The germination percentage was determined in a climate room (24ºC, 16 h-light/8 h-dark photoperiod). Seeds were not sterilized in order to avoid influencing their dormancy status; however, by using light microscope fungal infection was not detected. Radicle emergence was taken as evidence of germination. For each experimental condition, three replicates at least were used.

Water uptake

To study the water uptake rate, we took known weights of dark as well as light-brown dry seeds and surface-washed them for 15 s in distilled water. The seeds were then immediately placed onto filter paper dampened with distilled water and kept under conditions identical to those for seed germination. The seeds were rapidly surface dried and weighed hourly, and then immediately replaced to continue the imbibition phase. This process was repeated for 5 h. The water uptake for each lot was considered as fresh weight (FW) minus dry weight (DW) per mg of seeds.

Tetrazolium test

Intact seeds were incubated in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Merck, Germany) at 30ºC in darkness for 2 d. Tetrazolium salts are metabolically reduced to highly coloured end products called formazans by NADH-dependent reductases of the endoplasmic reticulum (Berridge *et al.* 1996).

Ruthenium red staining to seed mucilage

Mucilage was detected in the seed basically as described by Western *et al.* (2000). Whole seeds were allowed to imbibe on moist filter paper for between 5 min and 1 h, before the application of 0.2% (w/v) aqueous ruthenium red solution. Seeds were photographed with an Olympus B061 stereomicroscope.

Statistical treatment

The data obtained here are the mean of at least three replicates. The statistical treatment was based on a variance analysis and the averages/means were compared using the least-significant- difference (LSD) test at P<0.05 (Steel and Torrie 1982).

RESULTS AND DISCUSSION

Seed development is not temporally uniform in any given population, even when mother plants are grown in identical environments. In short, a type of seed heterogeneity develops (Matilla *et al.* 2005). As a result of such heterogeneity, populations of seeds with morphological differences (e.g. colour, size, weight, or shape) appear, and some of these differences can affect physiological properties (e.g. storage capacity or level of dormancy) (Debeaujon *et al.* 2000). Thus, some crucifer seeds do not mature synchronously inside the silique, but sequentially, and some pods shatter before harvesting, resulting in a substantial loss of seeds (Matilla 2007). Given that the heterogeneous character of some seeds can affect germination (Khan *et al.* 1996; Kantar *et al.* 1996; Puga-Hermida *et al.* 2003; Matilla *et al.* 2005; Luzuriaga *et al.* 2006), these heterogeneous lots of seeds constitute a valuable tool in seed physiology to study the endogenous factors that regulate germination. However, the physiological and molecular basis of seed heterogeneity is at present unknown.

Morphological and structural heterogeneity in *S. officinale* seeds

The fruits of *S. officinale*, as with other crucifers described previously (Imbert 2002), contain heterogeneous seeds that we have divided into two lots to study (brown and light-brown) attending to the colour of the outermost part of the seed coat. The colouration of the seed coat in other crucifer seeds studied is due preferentially to the accumulation of flavonoid-type seed compounds (e.g. condensed tannins as proanthocyanidins), which notably influence the seed-coat-imposed dormancy in some species (Debeaujon and Koornneef 2000; Debeaujon *et al.* 2000). The brown seeds of *S. officinale* have a density of approximately 4.7-fold that of the light-brown ones (**Fig. 2B**). Although the volume of the light-coloured seeds is higher than that of the dark ones, the great difference observed in the density is due primarily to different weights between the two lots (i.e. 21.8 ± 0.3 and 13.3 ± 0.6 mg/100 seeds for brown and light-brown, respectively). Once embryogenesis ends, the seed is a completely autonomous organism. Nevertheless, in plant species that produce a large quantity of seeds, this autonomy is not equal in each seed. That is, as seeds are heterogeneous, certain differences affect physiological properties (e.g. storage capacity and dormancy level) that can alter the speed and timing of dispersion (Kigel 1995). The tetrazolium test made in the brown lot of *S. officinale* showed that 100% of the seeds were alive, while in the light-brown lot, only 40 ± 7% were alive. This fact appears to indicate that the light seeds had difficulties in their embryogenic process. Several studies have demonstrated that such difficulty may be determined by, among other factors, a different intensity of nutrient unloading from the mother plant to the embryo (i.e. seed filling) due to a positional effect of the seed in the fruit, thereby affecting seed-size and viability (Fenner 1991; Gutterman 1992; Fenner 1993; Luzuriaga *et al.* 2006). That is, seed-size variability is considered a phenotypical maternal effect (Moles and Westoby 2006).

The brown and light-brown seeds of *S. officinale* are also heterogeneous with respect to each other, given that the former contain much more mucilage in the seed coat than do the light ones. This characteristic (i.e. myxospermy) is manifested in seed imbibition of both lots for a short amount of time in the presence of ruthenium red, a colorant which specifically dyes the mucilage (**Fig. 3**). The mucilage-producing cells are located in epidermal tissue of the seed coat of some species, including those of the family

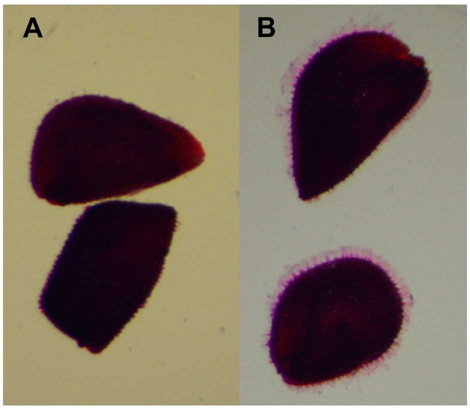

Fig. 3 Structure of light-brown and brown seeds of *S. officinale*. Whole seeds were stained with ruthenium red as described in the Material and Methods. Note red staining capsule of mucilage surrounding dark brown seeds (**B**). No mucilage is visible around light-brown seeds (**A**).

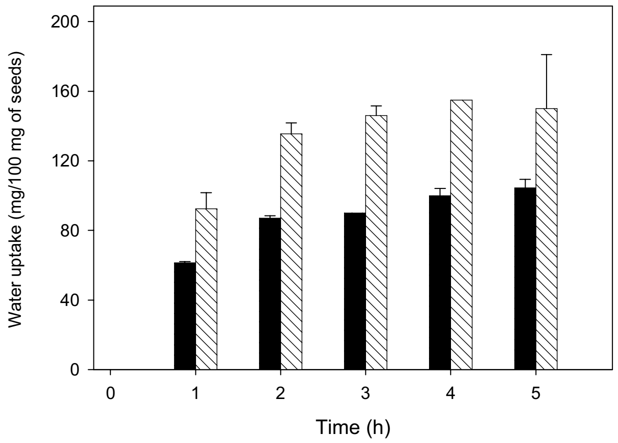

Fig. 4 Hourly water uptake during the first 5 h of imbibition of brown (■) and light-brown (□) lots of *S. officinale* seeds. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between lots were significant (P<0.05) as determined by the LSD test.

Brassicaceae (Boesewinkel and Bouman 1995; Western *et al.* 2000, 2004). When a dry myxospermous seed is placed in an aqueous environment, the mucilage, extremely hydrophilic and pectin-rich (Western *et al.* 2000), is released and completely envelops the seed coat (**Fig. 3**). It was suggested that mucilage extrusion results from the rapid expansion of dried mucilage upon hydration, leading to a local breakage of the cell wall. The nature of mucilage prior to release upon imbibition and its functions when extruded around seed are, at present, unknown aspects. However, mucilage may be related to imbibition, seed dispersal, germination or repair embryo DNA, among other ways (Huang and Gutterman 1998; Huang *et al.* 2000; Western *et al.* 2000, 2004; Huang *et al.* 2007).

The germination capacity of some seeds is altered by seed-coat characteristics (Debeaujon and Koornneef 2000; Debeaujon *et al.* 2000). A controlled imbibitional water-uptake allowed by seed coat components is necessary to initiate the normal germination process. The study of the water-uptake rate in brown and light-brown seeds indicates that the light lot imbibed water more quickly than did the dark lot (**Fig. 4**). The imbibition profile in the brown seeds was linear and increased gradually over the study period (i.e. 5 h); however, the profile of the light seeds was sigmoidal, reaching, at 60 and 180 min, values 1.3- and 1.6-fold greater, respectively, than those obtained for brown seeds (**Fig. 4**). Water uptake profiles from both lot seeds were asintotic after five hours. One of the causes that could favour such a sudden uptake of water in the light seeds could be related to the absence of mucilage. Seed mucilage is a natural example of a hydrogel and is an efficient absorber of water. It was hypothesized that mucilage takes up water to increase and stabilize water potential surrounding the seed, thereby promoting efficient germination and seedling establishment (Penfield *et al.* 2001). If the imbibition behaviour of the brown seeds is as in *A. thaliana* (Penfield *et al.* 2001; Western *et al.* 2000, 2004), the initial hydration of *S. officinale* seeds leads to the immediate release of mucilage, an event correlated with breakage of the outer tangential cell wall of the epidermal cell due to the rapid expansion of dried mucilage upon hydration. This very located cell-wall burst, together with the mucilaginous external envelope, could facilitate a slow and controlled water entry. For demonstrating this, a detailed microscopy study in the seed-coat would be necessary. Besides the presence of mucilage, it is worth mentioning that, although the volume of the light-brown and brown seeds was very similar, the density of the two lots was very different due to the notable mass of the brown seeds (**Fig. 2B**). This flaccidity in light seeds could provoke a quick imbibition in comparison to *S. officinale* brown seeds, and thus the sigmoidal profile of water uptake (**Fig. 4**) should be justified. Very rapid imbibition alters cell permeability and prompts losses of solutes to the external medium, triggering damage that can diminish seed vigour and viability (Legesse and Powell 1992; Puga-Hermida *et al.* 2003).

The heterogeneity in *S. officinale* seeds affects germination and hormonal sensitivity

The experiments to optimise the germination test are shown in **Table 1**. This table reflects the strong dependence on light and the presence of nitrate for rapid radicle emergence. Light cannot be replaced by a stratification period (4ºC), even if nitrate (20 mM) is present (**Table 1**). Nitrate (0.8 mM), in association with light, promotes germination in many species as *Lolium rigidum* Gaud. (Ellery *et al.* 2003). Radicle emergence in *S. officinale* can occur in the absence of nitrate if light and ET are present; however, less time is needed for emergence (**Table 1**). In this work, it has been demonstrated that the ability to germinate under optimal conditions (20 mM KNO_3, 24ºC and 16h light/8h dark photoperiod) differs within two lots of *S. officinale* seeds stu-

Table 1 Effect of several treatments on germination percentage of *S. officinale* seeds. Data are mean values of three replicates ± S.E. Significant differences between values as assessed by LSD test (P<0.05) are shown as different letters.

Treatment	Germination (%)		
	24 h	48 h	72 h
- light			
- nitrate (20 mM)			15 ± 3 a
- nitrate (20 mM) + ET (10 µM)			17 ± 2 a
+ nitrate (20 mM)		50 ± 4 b	100 c
+ nitrate (20 mM) + ET (10 µM)			100 c
+ nitrate (20 mM) + ET (50 µM)			100 c
+ nitrate (20 mM) + ST	n.f.		
+ light			
- nitrate (20 mM)		100 c	
- nitrate (20 mM) + ET (10 µM)		100 c	
+ nitrate (20 mM)	70 ± 8 d		
	13 ± 6 a (LB)		
+ nitrate (20 mM) + ET (10 µM)	95 ± 4 c		
	8 ± 3 a (LB)		
+ nitrate (20 mM) + ACC (10 µM)	98 ± 2 c		
+ nitrate (20 mM) + GA_{4+7} (100 µM)	100 c		15 ± 3 a (LB)
+ nitrate (20 mM) + GA_{4+7} (100 µM) + ET (10 µM)			15 ± 3 a (LB)

LB: light-brown lot; ST: stratification (ed, 4ºC); n.f.: not found.

died. Thus, at 24 h 6-fold more of the brown seeds germinated than the light-brown ones, and the ET and/or the ACC had a positive effect on the brown lot and negative over the light. However, GA_{4+7} strongly stimulated germination in the brown seeds while hardly altering that of the light ones despite that ET was present (**Table 1**). The low percentage of germination in the light lot did not increase by stratification, both in the absence as well as in the presence of GA_{4+7}, nor by a short scarification. Stratification in some seeds affects the expression of genes involved in the synthesis of physiologically active GAs (Yamauchi *et al.* 2004), and also intensifies sensitivity to GAs (Derkx and Karssen 1993; Koornneef *et al.* 2002). The absence of stimulation of the germination of stratified light-brown seeds could imply that GAs did not play a major part in the process or else that these seeds have difficulty synthesising active GAs. By molecular approaches currently under way in our laboratory, we will soon be able to confirm or exclude this possibility.

With the aim of making a thorough study of the effect of GA and ET on the germination rate of brown *S. officinale* seeds, we quantified the radicle emergence in short time periods. The protrusion began to be detectable between 17 and 18 h, reaching 100% at 25-26 h. This demonstrates the speed of the emergence process in this lot. The presence of GA_{4+7} strongly stimulated the germination between 21 and 23 h; in fact, at 23 h the germination was 3.5-fold greater than in control. However, at 26 h, germination reached 100% both in control as well as in the presence of GA_{4+7} (**Fig. 5A**). Conversely brown seeds, light ones have a great positive response to GA_{4+7} reaching in its presence germination percentages of 2.5-8.5–fold higher than the control (**Fig. 5B**). The results of **Figs. 5** and **6** indicate that both lots of seeds present different sensitivity to GA and probably also different hormonal regulation of germination. A very similar profile of germination stimulation found for brown seeds in the presence of GA_{4+7} was also found in the presence of ET (**Fig. 6A**). The fact that the kinetics in the presence of ACC (the immediate precursor of ET) and ET are similar shows that the brown seeds of *S. officinale* have the capacity of oxidizing ACC (i.e. ACC-oxidase). This also occurs in other crucifers (Matilla 2000; Rodríguez-Gacio *et al.* 2004; Matilla *et al.* 2005). On the contrary, ET was not capable of stimulating the germination in light-brown seeds, which had a far lower germination capacity than did the brown seeds (**Fig. 6B**); that is, the results appear to indicate that the ET was strongly inhibitory. Although we cannot discard that the light seeds have difficulties to oxidize their endogenous ACC, the fact that the exogenous ET did not accelerate its germination supports the assumption that this hormone is not required for germination. In heterogeneous turnip-tops seeds, the final step of the ET pathway was altered concomitantly with the changes in germinating capacity affecting the levels and expression of ACC-oxidase (Puga-Hermida *et al.* 2003; Rodríguez-Gacio *et al.* 2004). GA_{4+7} and ET together were not capable, either, of boosting the germination percentage of the light-brown seeds lot.

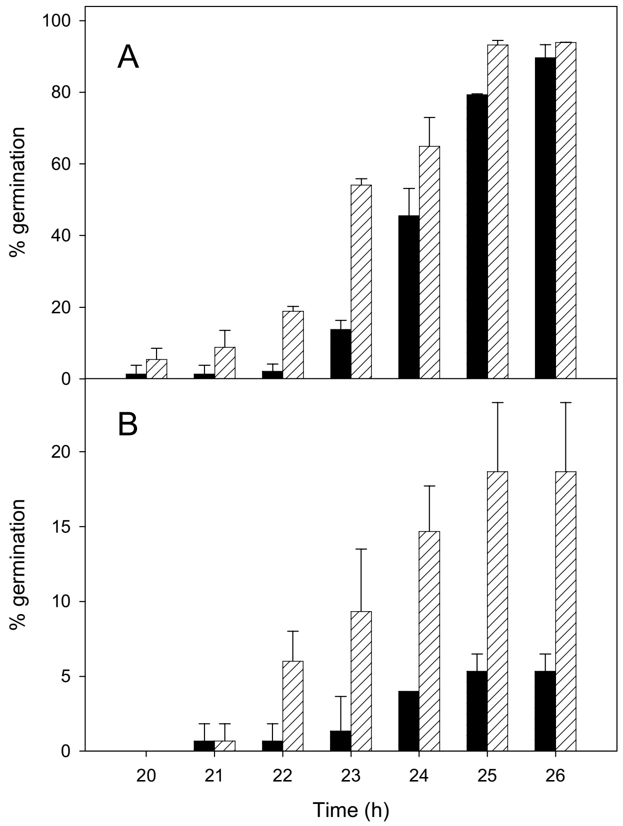

Fig. 5 Germination percentage of brown (A) and light-brown seed lot (B) of *S. officinale* seeds in presence (striped bars) or absence (black bars) of GA_{4+7}. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between GA treated and non-treated seeds in the brown lot were significant (P<0.05) until 25 h; for the light-brown lot, differences were significant except at 21 h (P<0.05) as determined by LSD test.

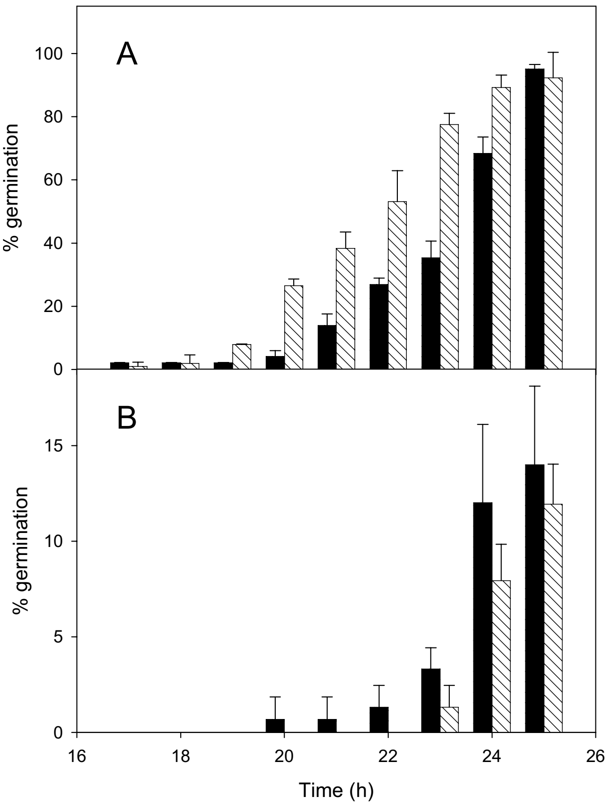

Fig. 6 Germination percentage of brown (A) and light-brown seed lot (B) of *S. officinale* seeds in presence (striped bars) or absence (black bars) of ET. Data are means ± standard error (S.E.) of 3 independent experiments. Differences between ET treated and non-treated seeds in the brown lot were significant (P<0.05) between 19-24 h; for the light-brown lot, differences were significant (P<0.05) as determined by LSD test.

The addition to the germination medium of inhibitors of ET and GA synthesis gives us an idea of the participation of the two hormonal signals in the germination process of the brown seeds of *S. officinale*. Thus, the AVG (inhibitor of ACC-synthetase activity), Co_2Cl (inhibitor of ACC-oxidase activity), and NO_3Ag (inhibitor of the bonding of ET to its receptor) diminished the percentage of germination, particularly when these compounds were added jointly (**Table 2**). The presence of GA_{4+7} partially reversed the inhibition, again demonstrating the preferential action of GAs in the germination process. This last observation is supported by

Table 2 Effect of several treatments on germination percentage (23 h) of *S. officinale* brown-seeds. Data are mean values of three replicates ± S.E. Significant differences between values as assessed by LSD test ($P<0.05$) are shown as different letters.

Treatment	**Germination (%)**
control	40 ± 3 a
ET	75 ± 8 b
GA$_{4+7}$	91 ± 9 c
NO$_3$Ag	27 ± 2 d
AVG	25 ± 4 d
Co$_2$Cl	35 ± 3 e
AVG + Co$_2$Cl	8 ± 2 f
AVG + Co$_2$Cl + NO$_3$Ag	10 ± 3 f
AVG + Co$_2$Cl + NO$_3$Ag + GA$_{4+7}$	52 ± 5 g
NO$_3$Ag + GA$_{4+7}$	58 ± 6 g
AVG + Co$_2$Cl + GA$_{4+7}$	42 ± 5 a
PC	2 ± 1 h
PC + ET	3 ± 1 h

the total inhibition of germination in the presence of paclobutrazol, an inhibitor of GA synthesis (**Table 2**). However, the fact that the reversal caused by GA$_{4+7}$ is not total, leads us to conclude that, apart from the presence of GA, other hormonal signals are needed for right radicle emergence.

CONCLUDING REMARKS

In this work, we have demonstrated several physical and physiological differences between heterogeneous lots of seeds from *Sisymbrium officinale*. The examination of the causes of this different behaviour, concerning the hormonal balance and physical requirements, complemented with a molecular approach, could shed light on the processes involved in germination for these species. Whether or not this heterogeneity reflects an adaptative strategy to control the timing and requirements for germination remains to be investigated; as discussed earlier, it could be also a matter of incomplete embryogenesis/maternal effect. In any case, both these heterogeneous lots are dispersed from wild populations of this weed, and probably this seed heterogeneity has a deep impact on the ecophysiology of this species.

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REFERENCES

Barroco RM, van Poucke K, Bergervoet JHW, de Veylder L, Groot SPC, Inzé D, Engler G (2005) The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiology* **137**, 127-140

Bassel GW, Zielinska E, Mullen RT, Bewley JD (2004) Down-regulation of *DELLA* genes is not essential for germination of tomato, soybean, and Arabidopsis seeds. *Plant Physiology* **136**, 2782-2789

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between ABA and ethylene signalling cascades. *Plant Cell* **12**, 1103-1115

Berridge MV, Tan AS, McCoy KD, Wang R (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4**, 15-20

Bewley JD (1997a) Seed germination and dormancy. *The Plant Cell* **9**, 1055-1066

Bewley JD (1997b) Breaking down the walls: a role for endo-β-mannanase in release from seed dormancy? *Trends in Plant Science* **2**, 464-469

Boesewinkel FD, Bouman F (1995) The seed: structure and function. In: Kigel J, Galili G (Eds) *Seed Development and Germination*, Marcel Dekker, New York, pp 1-24

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE (2006) Gene expression profiles of *Arabidopsis* Cvi seeds during dormancy cycling indicate a common underlaying dormancy control mechanism. *The Plant Journal* **46**, 805-822

da Silva EAA, Toorop PE, van Aelst AC, Hilhorst H (2004) ABA controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination. *Planta* **220**, 251-261

da Silva EAA, Toorop PE, Nijsse J, Bewley JD, Hilhorst H (2005) Exogenous gibberellins inhibit coffee (*Coffea arabica* cv. Rubi) seed germination and cause cell death in the embryo. *Journal of Experimental Botany* **413**, 1029-1038

de la Torre F, Rodríguez-Gacio MC, Matilla AJ (2006) How ethylene works in the reproductive organs of higher plants. *Plant Signaling and Behavior* **1**, 231-242

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P (2006) Proteomic analysis of seed dormancy in *Arabidopsis*. *Plant Physiology* **142**, 1493-1510

Debeaujon I, Koornneef M (2000) GA requirements for *A. thaliana* seed germination in determined both by testa characteristics and embryonic ABA. *Plant Physiology* **122**, 415-424

Debeaujon I, León-Kloosterziel KM, Koornneef M (2000) Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiology* **122**, 403-413

Derkx MPM, Karssen CM (1993) Effects of light and temperature on seed dormancy and gibberellin-stimulated germination in *Arabidopsis thaliana*: Studies with gibberellin-deficient and gibberellin-insensitive mutants. *Physiologia Plantarum* **89**, 360-368

Ellery AJ, Gallagher RS, Dudley SV (2003) Dormancy and germination ecology of annual ryegrass (*Lolium rigidum* Gaud.). In: Nicolás G, Bradford KJ, Côme D, Pritchard HW (Eds) *The Biology of Seeds: Recent Research Advances*, CABI Publishing, Wallingford, Oxon, UK, pp 389-396

Fenner M (1991) The effects of the parent environment on seed germinability. *Seed Science Research* **1**, 75-84

Fenner M (1993) Environmental influences of seed size and composition. *Horticultural Reviews* **13**, 183-213

Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytologist* **171**, 501-523

Forbis TA, Floyd SK, Queiroz A (2002) The evolution of embryo size in angiosperms and other seed plants: implications for the evolution of seed dormancy. *Evolution* **56**, 2112-2125

Frey A, Godin B, Bonnet M, Sotta B, Marion-Poll A (2004) Maternal synthesis of ABA controls seed development and yield in *Nicotiana plumbaginifolia*. *Planta* **218**, 958-964

Friedman WE (1998) The evolution of double fertilization and endosperm: an "historical" perspective. *Sexual Plant Reproduction* **11**, 6-16

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P (2000) Regulation of ABA signaling by the ethylene response pathway in Arabidopsis. *Plant Cell* **12**, 1117-1126

Grossmann K, Hansen H (2001) Ethylene-triggered ABA: a principle in plant growth regulation? *Physiologia Plantarum* **113**, 9-14

Gutterman Y (1992) Maternal effects on seeds during development. In: Fenner M (Ed) *Seeds. The Ecology of Regeneration in Plant Communities*, Wallingford, CAB International, UK, pp 27-59

Hilhorst HWM, Toorop PE (1997) Review on dormancy, germinability, and germination in crop and weed seeds. *Advances in Agronomy* **61**, 111-165

Hilhorst HWM, Downie B (1995) Seed dormancy in tomato (*Lycopersicon esculentum* cv. Moneymaker): Studies with the *sitiens* mutant. *Journal of Experimental Botany* **47**, 89-97

Homrichhausen TM, Hewitt JR, Nonogaki H (2003) Endo-β-mannanase activity is associated with the completion of embryogenesis in imbibed carrot (*Daucus carota* L.) seeds. *Seed Science Research* **13**, 219-227

Huang Z, Gutterman Y (1998) *Artemisia monosperma* achene germination in sand: effects of sand depth, sand/water content, cyanobacterial sand crust and temperature. *Journal of Arid Environments* **38**, 27-43

Huang Z, Huc Z, Gutterman Y (2000) Structure and function of mucilaginous achenes of *Artemisa monosperma* inhabiting the Negev desert of Israel. *Israel Journal of Plant Science* **48**, 255-266

Huang Z, Boubriak I, Osborne DJ, Dong M, Gutterman Y (2007) Possible role of pectin-containing mucilage and dew in reairing embryo DNA of seeds adapted to desert conditions. *Annals of Botany*, in press

Imbert E (2002) Ecological consequences and ontogeny of seed heteromorphism. *Perspectives in Plant Ecology, Evolution and Systematics* **5**, 13-36

Kantar F, Pilbeam CJ, Hebblethwaite PD (1996) Effect of tannin content of faba bean (*Vicia faba*) seed on seed vigour, germination and field emergence. *Annals of Applied Biology* **128**, 85-93

Kepczynski J, Kepzcynska E (1997) Ethylene in seed dormancy and germination. *Physiologia Plantarum* **101**, 720-726

Khan M, Cavers PB, Kane M, Thompson K (1996) Role of the pigmented seed coat of proso millet (*Panicum miliaceum* L.) in imbibition, germination and seed persistence. *Seed Science Research* **7**, 21-25

Kigel J (1995) Seed germination in arid and semiarid regions. In: Kigel J, Galili G (Eds) *Seed Development and Germination*, Marcel Dekker, New York, pp 645-700

Koornneef M, Bentsink L, Hilhorst H (2002) Seed dormancy and germination. *Current Opinion in Plant Biology* **5**, 33-36

Kucera B, Cohn MA, Leubner-Metzger G (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**, 281-307

Lee CS, Chien CT, Lin CH, Chiu YY, Yang YS (2006) Protein changes between dormant and dormancy-broken seeds of *Prunus campanulata* Maxim. *Proteomics* **6**, 4147-4154

Legesse N, Powell AA (1992) Comparisons of water uptake and imbibition damage in eleven cowpea cultivars. *Seed Science Technology* **20**, 173-180

Leubner-Metzger G (2003) Functions and regulation of β-1,3-glucanase during seed germination, dormancy release and after-ripening. *Seed Science Research* **13**, 17-34

Leubner-Metzger G, Petruzzelli L, Waldvogel R, Vögeli-Lange R, Meins F (1998) Ethylene-responsive element binding protein (EREBP) expression and the transcriptional regulation of class I β-1,2-glucanase during tobacco seed germination. *Plant Molecular Biology* **38**, 785-795

Leubner-Metzger G, Kucera B, Müller K (2006) Emerging and established model systems for endosperm weakening. In: Navie S, Adkins S, Ashmore S (Eds) *Seeds: Biology, Development and Ecology*, CAB International, UK, pp 195-204

Liu PP, Koizuka N, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H (2005) Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. *The Plant Journal* **41**, 936-944

Luzuriaga AL, Escudero A, Pérez-García F (2006) Environmental maternal effects on seed morphology and germination in *Sinapis arvensis* (Cruciferae). *Weed Research* **46**, 163-174

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G (2005) Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. *Plant Physiology* **138**, 1538-1551

Matilla AJ (2000) Ethylene in seed formation and germination. *Seed Science Research* **10**, 111-126

Matilla AJ, Gallardo M, Puga-Hermida MI (2005) Structural, physiological and molecular aspects of heterogeneity in seeds: a review. *Seed Science Research* **15**, 63-76

Matilla AJ (2007) How is the silique fruit dismantled over its maturation? *Functional Plant Science and Biotechnology* **1**, 85-93

Moles AT, Westoby M (2006) Seed size and plant strategy across the whole life cycle. *Oikos* **113**, 91-105

Müller K, Tintelnot S, Leubner-Metzger G (2006) Endosperm-limited Brassicaceae seed germination: ABA inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiology* **47**, 864-877

Penfield S. Meissner RC. Shoue DA. Carpita NC. Bevan MW (2001) *MYB61* is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *Plant Cell* **13**, 2777-2791

Petruzzelli L, Müller K, Hermann K, Leubner-Metzger G (2003) Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of Solanaceous seeds. *Seed Science Research* **13**, 139-153

Puga-Hermida MI, Gallardo M, Rodríguez-Gacio MC, Matilla AJ (2003) The heterogeneity of turnip-tops (*Brassica rapa*) seeds incide the silique affects germination, the activity of the final step of the ethylene pathway, and ABA and polyamine content. *Functional Plant Biology* **30**, 767-775

Raghavan V (1986) Developmental Embryogenesis. In: Barlow PW, Green PB, Wylie CC (Eds) *Embryogenesis in Angiosperms. A Developmental and Experimental Study*, Cambridge University Press, Cambridge, UK, pp 13-45

Rodriguez-Gacio MC, Nicolás C, Matilla AM (2004) The final step of the ethylene biosynthesis pathway in turnip tops (*Brassica rapa*): molecular characterization of the 1-aminocyclopropane-1-carboxylate oxidase *BrACO1* throughout zygotic embryogenesis and germination of heterogeneous seeds. *Physiologia Plantarum* **121**, 132-140

Schwachtje J, Baldwin IT (2004) Smoke exposure alters endogenous gibberellin and abscisic acid pools and gibberellin sensitivity while eliciting germination in the post-fire annual *Nicotiana attenuata*. *Seed Science Research* **14**, 51-60

Siriwitayawan G, Geneve RL, Downie AB (2003) Seed germination of ethylene perception mutants of tomato and Arabidopsis. *Plant Cell* **14**, 3133-3147

Steel RGG, Torrie JH (1982) *Principles and Procedures of Statistics*, McGraw-Hill, Tokyo.

Toorop PE, van Aelst AC, Hilhorst H (2000) The second step of the biphasic endosperm cap weakering that mediates tomato (*Lycopersicon esculentum*) seed germination is under control of ABA. *Journal of Experimental Botany* **51**, 1371-1379

Torres-Ruiz RA (1998) Embryogenesis. In: Anderson M, Roberts JA (Eds) *Arabidopsis. Annual Plant Reviews* (Vol 1), CRC Press, Boca Raton, Florida, pp 223-261

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology* **122**, 345-355

Western TL, Diana S, Young DS, Dean GH, Tan WL, Samuels AL, Haughn GW (2004) MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developmentally regulated by APETALA2, TRANSPARENT TESTA GLABRA1, and GLABRA2 in the Arabidopsis seed coat. *Plant Physiology* **134**, 296-306

Wu, CT, Leubner-Metzger G, Meins F Jr., Bradford KJ (2000) Class I β-1,3-glucanase and chitinase are expressed in the micropylar endosperm of tomato seeds prior to radicle emergence. *Plant Physiology* **126**, 1299-1313

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S (2004) Activation of GA biosynthesis and response pathways by low temperature during imbibition of *Arabidopsis thaliana* seeds. *Plant Cell* **16**, 367-378

IV. CAPÍTULO 2:

"The expression pattern of ethylene and gibberellin biosynthesis genes strongly suggest the existence of interactions between both hormonal signalling to achieve the *Sisymbrium officinale* L. seed germination"

Type of article: Original article

Title: "The Expression Pattern of Ethylene and Gibberellin Biosynthesis Genes Strongly Suggest the Existence of Interactions between both Hormonal Signalling to Achieve the *Sisymbrium officinale* L. Seed Germination"

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Short title: "*Ethylene-gibberellin cross-talk in hedge mustard seed germination*"

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- *Background and Aim,* At present, published data indicate that gibberellins (GAs) and ethylene (ET) can individually promote germination in many seeds by interfering with ABA signalling. However, cross-talk between ET and GAs to achieve germination cannot be discarded. The aim of this study was determine if this cross-talk exist in *Sisymbrium officinale* L. seeds, which are endospermic. To this end, we have determined: (i) the expression of genes involved in the ET synthesis (*SoACS7* and *SoACO2*) and GAs synthesis (*SoGA20ox2*, *SoGA3ox2*) and breakdown (*SoGA2ox6*); and (ii) biochemical activity of endo-β-mannanase (MAN). These experiments were done during seed germination in the presence of ET, GA_{4+7} and inhibitors of synthesis and signalling of these hormones.
- *Methods* The structure of the different parts of *Sisymbrium officinale* L. seed was determined by using fixation, sectioning, staining and light microscopy, ACC and MACC levels were done by means of its extraction and oxidation to ethylene, ACC- and GA-oxidases transcript levels were determined by using real-time semiquantitative PCR and activity of endo-β-mannanase (MAN) was spectrophotometrically measured by means of dye release from AZCL-galactomannan.
- *Key Results* The protrusion in *S. officinale* seeds consists of the two sequential phases of seed coat rupture and endosperm rupture. The afterripening (AR) induces a high sensitivity to ET and GA_{4+7}; but, though the germination patterns are similar in the presence of both hormones, the GAs need is higher than of ET during early stage of germination. Both GA_{4+7} and ET increases markedly the ACC and MACC content along germination. At difference of *SoGA2ox6* expression, which is very high around protrusion and hardly inhibited by all studied treatments, *SoGA3ox2* and *SoGA20ox2* expression is mainly noticeable at 6 h of imbibition. Moreover, *SoGA3ox2* and *SoGA20ox2* expression is strongly inhibited by paclobutrazol (PB) and inhibitors of ET synthesis and signalling (IESS). AR induces a remarkable MAN activity in dry seeds and

the peak of MAN is not concomitant to 100% of germination. Both GA_{4+7} and ET stimulates this activity during germination process.

- *Conclussions* The alterations in germination percentage, *SoACS7*, *SoACO2, SoGA3ox2*, *SoGA20ox2* and *SoGA2ox6* expression, and MAN activity induced by GA_{4+7}, ET and inhibitors of synthesis and signalling strongly suggests the existence of an interaction (cross-talk) between ET and GAs to trigger the germination in *S. officinale* seeds. All the results obtained in this work are carefully discussed.

Key words: ACC and MACC, ACC- and GA-oxidases, endo-β-mannanase, endospermic seed, ethylene, germination, gibberellins (GA_{4+7}), mucilage, real-time PCR, *Sisymbrium officinale*.

INTRODUCTION

The seed is the dispersal unit emerged in the course of plant evolution. A mature and viable seed that has overcome the process of dormancy, is prepared to germinate (Bewley, 1997). Seed germination, triggered by gradual water uptake, is achieved once integrated environmental and internal signals coordinate a high transcriptomic and proteomic activity, all this resulting in an optimal growth response (Koornneef *et al*., 2002; Finch-Savage and Leubner-Metzger, 2006). Plant hormones tightly regulate germination (Finkelstein, 2004; Kucera *et al*., 2005). Germination ends with the initial cellular elongation of the embryonic axis, not being essential the cellular division; and it is visible when the radicle has protruded through two covering layers (i.e. seed coat and endosperm single layer in endospermic seeds) at the level of micropylar region (Bewley, 1997; Barroco *et al*., 2005; Kucera *et al*., 2005). Two important forces, growth potential of the radicle and mechanical resistance of the covering layers, play antagonistic roles in the completion of germination. In order for a seed to complete germination, the growth potential of the radicle must overcome the tissue resistance of the micropylar covering layers, this fact also being regulated by hormonal signaling (Kucera *et al*., 2005). Weakening of the micropylar endosperm surrounding the radicle tip appears to be required for radicle protrusion, and is likely to involve cell-wall hydrolytic enzymes (Nonogaki *et al*., 2000; Mo and Bewley, 2003; Nonogaki *et al*., 2007). By contrast, in some hard-seeds the micropylar endosperm presents a lower physical constraint to the completion of germination than the lateral endosperm, and hence its structure is predisposed to permit radicle protrusion (Gong *et al*., 2005). For many endospermic species, seed coat rupture and endosperm rupture are two sequential steps during germination (Petruzzelli *et al*., 2003; Liu *et al*., 2005). Abscisic acid (ABA) inhibits endosperm rupture, but not seed coat rupture (da Silva *et al*., 2004; Leubner-Metzger *et al*., 2006). The increase in the radicle growth potential, and the decrease in the covering layers mechanical resistence, can occur simultaneously (Nonogaki, 2006). Probably, a combination of both possibilities could fully explain the mechanisms of seed germination in *Arabidopsis* and many other endospermic seeds.

Precise and confirmed information is lacking in relation to the possible role of ethylene (ET) in the transition of dormancy to germination and in the germination *sensu stricto* (Kucera *et al*., 2005; Matilla and Matilla-Vázquez, 2008). Although maximum ET production is usually detected after radicle emergence has finished, small amounts of gas are also produced in periods prior to protrusion. Moreover, there are seeds that cannot break dormancy by the administration of ET alone (Kucera *et al*., 2005; Finch-Savage and Leubner-Metzger, 2006; Matilla and Matilla-Vázquez, 2008), while in other cases this gas is sufficient (Matilla, 2000; Mendes-Ribeiro and Santos Barros, 2006). ET synthesis and cellular sensitivity to it are two major factors for the germination in Arabidopsis (Beaudoin *et al*., 2000; Ghassemian *et al*., 2000; Siriwitayawan *et al.*, 2003). Proteomic analysis in Arabidopsis revealed an essential role of endogenous ET only after radicle protrusion (Gallardo *et al*., 2002). ET mutants have been generated, and genetic, physiological, and molecular characterization of these mutants is starting to shed light on the complex process of regulation of the germination by ET. Thus, seeds of *etr1* and *ein2* display enhanced primary dormancy, while *ctr* seeds have slightly reduced dormancy compared to wild type (Beaudoin *et al*., 2000). The *etr1-2* mutation in Arabidopsis confers dominant ET-insensitivity and results in a larger proportion of mature seeds that exhibit hard primary dormancy (Chiwocha *et al*., 2005).

The complexity of hormonal responses and their functional overlap support the presence of an intensive cross-talk between hormone signalling pathways (Brady and McCourt, 2003; Gazzarrini and McCourt, 2003; Razem *et al*., 2006; Weiss and Ori, 2007). The existence of a cross-talk among ET and gibberellins (GAs) seems to exist in *Arabidopsis* (de Grauwe *et al*., 2007, 2008); but their participation during seed germination process need to be confirmed. However, several lines of evidence seem to support its existence: (i) newly synthesized GAs are required for radicle protrusion in endospermic seeds (Ogawa *et al*., 2003) and ET treatment induces the germination of GAs-deficient mutant seeds (Karssen *et al*., 1989); (ii) exogenous GAs induces ACC-oxidase expression in imbibed *ga1-3* seeds (Ogawa *et al*., 2003); (iii) the low germination of *etr1* mutant seeds is reversed by GAs to wild type levels (Bleeker *et al*., 1988); (iv) the absence of ET signaling in *etr1-2* mutation causes an increase in bioactive GAs, which demonstrates that ET negatively regulates the GAs pathway in wild type seeds (Chiwocha *et al*., 2005); (v) GAs stimulate germination via DELLA protein disappearance (Tyler *et al*., 2004) and ET participates in the regulation of DELLA proteins accumulation, which suppress the GA response (de Grauwe *et al*., 2008).

In order to gain insight on the knowledge of hormonal regulation of the germination, in the present work we report some effects of the ET and GA_{4+7} on the percentage of germination, endo-β-mannanase (MAN) activity, and expression of two genes involved in ET synthesis (*SoACS7* and *SoACO2*), two genes involved in bioactive-GA synthesis (*SoGA20ox2*, *SoGA3ox2*) and one involved in GAs breakdown (*SoGA2ox6*). All this work was carried out with seeds of *Sisymbrium officinale* L., which are endospermic, possess a high speed of germination and present a strong dependence of NO_3^- and GAs to germinate (Hilhorst and Karssen, 1988).

MATERIALS AND METHODS

Plant Material

Fruits of wild hedge mustard (*Sisymbrium officinale* L.) were harvested in Galicia (North-west Spain) during July-August of 2007. Dark ripe seeds were separeted from fruits (i.e. pedicel, replum and valve), cleaned and stored dry during 6 months at 21 ± 0.2ºC to break dormancy by after-ripening (AR). Parallely, fruits of *S. officinale* were collected at two developmental stages, the first being whole fruit with early development (named here early fruit; EF) and the second one fruits containing seeds at a very advanced development stage (i.e. late fruit, LF and late seeds, LS).

Light Microscopy

Seeds were fixed in 0.05 M pH 6.8 sodium phosphate buffer containing 2% (w/v) *p*-formaldehyde (Panreac, Spain), 2% (v/v) glutaraldehyde (Merck, Germany) for 2 days at 4ºC. Fixation was followed by an ethanol dilution series. Seeds were embedded in LR White Resin (Sigma-Aldrich, Spain) and cut in 2 μm sections. Staining was carried out in 0.5% (w/v) periodic acid (Merck, Germany), Schiff´s Reagent (Merck, Germany) and 1% (w/v) Naphtol Blue Black (Sigma-Aldrich, Spain). A Phase Contrast Attachment "Ph" for Optiphot-2 microscope (Nikon, Japan) was used for bright field microscopy. Images were taken with Coolpix 8400 camera (Nikon, Japan).

Germination assays

Three replicates of 50 seeds were sown in 90 mm Petri dishes on two layers of filter paper (Whatman No. 1) moistened with 3 ml of 20 mM KNO_3 supplemented with solutions of gibberellin (100 μM $GA_{4+7,}$ Sigma-Aldrich, Spain), ET (10 μM ethrel, Sigma-Aldrich, Spain), inhibitor of GAs synthesis (25 μM paclobutrazol (PB), Sigma-Aldrich, Spain) and a mixture of inhibitors of synthesis (100 μM aminoethoxyvinylglycine (AVG) and 1 mM cobalt chloride (Co_2Cl), Sigma-Aldrich, Spain) and signalling (10 μM silver thiosulphate (STS), Sigma-Aldrich, Spain) of ET called here IESS.

Germination experiments were carried out in a growth chamber at 24ºC in a 16 h photoperiod. Seeds were not surface-sterilized in order to avoid influencing their dormancy status, nevertheless fungal infections were not detected by light microscope. Seeds were considered germinated when radicle protrusion was visible. Germination tests were performed at least twice using three replicates.

Tetrazolium test and ruthenium red staining

Intact seeds were incubated in a 1% (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride (Merck, Germany) at 30ºC in darkness for 2 d. Tetrazolium salts were metabolically reduced to highly coloured end products called formazans by NADH-dependent reductases of the endoplasmic reticulum (Berridge *et al*. 1996). Mucilage was detected in the seed basically as described by Western *et al*. (2000). Whole seeds were allowed to imbibe on moist filter for between 5 min and 1 h, before the application of 0.2% (w/v) aqueous ruthenium red solution. Seeds were photographed with an Olimpus B061 stereomicroscope.

Quantification of ACC and MACC

Frozen at -80ºC tissue (50 mg) was homogenized at 4ºC using mortar and pestle with sterile distilled water in a 1:5 ratio (FW/v). The homogenate was centrifuged at 8000 rpm (Beckman Avanti J-25 minifuge, rotor JA-18.1 for Eppendorf tubes) for 15 min at laboratory temperature and supernatant assayed for ACC analysis using Lizada´s method (Lizada and Yang, 1979). The MACC in supernatant was determined after being hydrolyzed to ACC in 6M HCl at 100ºC for 3 h and quantified as Gallardo *et al*. (1994). The measure of ET from ACC was as described Rodríguez-Gacio and Matilla (2001).

Total RNA isolation from seeds and cDNA synthesis

For RNA extraction, seeds at 0, 6, 12, 18, 20, 22 and 26 h along the germination were collected, immediately frozen in liquid nitrogen and stored at -80ºC until use. Three replicates were taken for each point. Seeds were finely grounded in liquid N_2 using a Mikro-Dismembrator-S

(Sartorius AG, Goettingen, Germany) at 1500 rpm for 2 min. Total RNA was isolated using the phenol extraction/LiCl precipitation method (Verwoerd *et al.* 1989). The integrity and purity of the RNA was checked both electrophoretically and by the 260/280 nm absorbance ratio. Total RNA samples were digested with DNase (DNase I recombinant, RNase-Free, Roche, Germany) following manufacturer´s directions. RNA concentration was estimated by A_{260} measurement, and the samples were stored at -80ºC. Reactives used in this protocol were supplied by Sigma-Aldrich (Spain).The cDNA was synthesised from 1 µg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche, Germany) using oligo-p (dT) as a primer, following manufacturer´s directions. Samples were stored at -20ºC.

Isolation of SoGA3ox2, SoGA20ox2, SoGA2ox6, SoACO2, SoACS7 partial-length cDNA

The cDNA from seeds was used as template for PCR, using pairs of degenerated primers based on regions found to be highly conserved in other species (Supplementary Tab. S1). PCR conditions were as follows: 95ºC for 2 min, 40 cycles of 95ºC for 45 s, 47-55ºC for 45 s, 72ºC for 45 s and a final elongation step of 7 min at 72ºC. PCR reactions were carried out in a 25 µL reaction volume containing 12,5 µL 2X Super Premix, Sapphire (Mbiotech, Seoul, Korea), 1 µL of forward primer (100 µM, final concentration 4 µM), 1 µL of reverse primer (100 µM, final concentration 4 µM) and 9.5 µL of sterelized water and finally 1 µL of cDNA. PCR products were analyzed by electrophoresis and extracted from the agarose gel using MiniElute™ Gel Extraction Kit (Qiagen, Germany), and then sequenced. Sequences were compared to existing sequences in target databases using BLAST (Altschul *et al.*, 1997). They contained the partial-length cDNA of genes with very high similarity to *GA3ox*, *GA20ox*, *GA2oxi*, *ACO*, *ACS* genes of other plant species. Basing on their phylogenetic relationship to the known genes (data not shown), they were called *SoGA3ox2*, *SoGA20ox2*, *SoGA2ox6*, *SoACO2* and *SoACS7* and registered in the GenBank under the accesion numbers EU689111, EU689113, EU689112, EU689115 and EU689114, respectively.

Real-time PCR assay

PCR analysis was performed with the cDNA obtained from different times along the germination as stated above as a template. Specific primer design was performed using the sequences obtained for *SoGA3ox2*, *SoGA20ox2*, *SoGA2ox6*, *SoACO2 and SoACS7* (Supplementary Tab. S2). *18S-RNA* was used as a control for the genes studied since it was found to be expressed at constant levels throughout studied period (data not shown). The PCR was performed in a iCycler iQ^{TM} Real-time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For each 25 µl reaction, 1 µl of sample cDNA was mixed with 12.5 µl of IQ^{TM} SYBR$^{®}$ Green Supermix (Bio-Rad Laboratories), 0.5 µl of forward primer (12 µM, final concentration 240 nM), 0.5 µl of reverse primer (12 µM, final concentration 240 nM) and 10.5 µl of sterilized water. Samples were subjected to thermal cycling conditions of DNA polymerase activation 95ºC for 4 min, 40 cycles of 45 s at 95ºC, 45 s at 52ºC (to *SoGA20ox2*, *SoGA2ox6*) or 55ºC (to *SoGA3ox2*, *SoACO2* and *SoACS7*), 45 s at 72ºC and 45 s at 80ºC; a final elongation step of 7 min at 72ºC was permormed. The melting curve was designed to increase 0.5ºC every 10 s from 62ºC (to *SoGA20ox2*, *SoGA2ox6*) or 65ºC (to *SoGA3ox2*, *SoACO2* and *SoACS7*). Real-time PCR analysis was performed with two different cDNAs from the same time-point (from two different RNAs), and each of them was carried out in triplicate. The amplicon was analyzed by electrophoresis and sequenced once for identity confirmation. Quantification was based on analysis of threshold cycle (Ct) value as described by Pfaffl (2001).

Endo-β-mannanase (EC 3.2.1.78) activity

Triplicate lots of seeds were ground in 1M sodium acetate buffer, pH 4.7 (Sigma Aldrich, Spain). After centrifugation at 20 000 *g* at 4 °C for 45 min, the supernatants were assayed in duplicate for

MAN activity. For enzymatic determination, 100 µl of 0.25% (w/v) AZCL-galactomannan (Megazyme International Ireland Ltd., Wicklow, Ireland) in 100 mM sodium acetate buffer, pH 4.7) were mixed with 25 µl of supernatant and incubated at 28ºC for 3 h, with constant agitation in an orbital shaker. Dye release from AZCL-galactomannan was determined spectrophotometrically by measuring the absorbance at 590 nm in supernatant samples of the reaction mixture. One unit of MAN activity was defined as the amount of enzyme that releases one nanomole of reducing sugar equivalent to D-mannose per minute under the above conditions. A curve relating dye release from AZCL-galactomannan to reducing sugar release from locust bean gum (Sigma Aldrich) as determined by the PAH-BAH method (Lever, 1972) was constructed and used for interconversion of MAN activities.

Statistical analysis

The statistical treatment was based on a variance analysis and averages/means were compared by using the least significant diference (LSD) test at P<0.05 (Steel and Torrie 1982).

RESULTS

Cyto-histological alterations in the micropylar region during imbibition and protrussion of S. officinale seeds

The seeds of *S. officinale*, as with other crucifers described, are endospermic and heterogeneous in colour (i.e. brown and light-brown). We have selected for this work only the brown seeds. The tetrazolium test made in these seeds showed that 100% of the seeds were alive. By contrast, only 40 ± 7% were alive in the light-brown lot. The seed is surrounded by the seed coat wich contains the outer and inner integuments (Fig.1A; general view of *S. officinale* dry seed). The outer integument include: (i) mucilaginous cell layer enriched in columnelles and producer of abundant mucilage wich surrounds the seed within a few minutes of the beginning of imbibition process (this myxospermic character was demonstrated with ruthenium red; Western *et al.*, 2000); and (ii) palisade epidermic cell layer with thickened (i.e. pectin enriched) internal tangential cell walls. On the other hand, the inner integument is composed by a thin endodermic layer without scarce differences in the cell walls thickness (Fig. 1B). Under inner integument is the endosperm composed by only one cell layer. In dry seed, the endospermic cells have abundant proteic bodies and few cell turgor (Fig. 1B). After 4 h of imbibition (Fig. 1C): (i) the density of columnelles decreases; (ii) the degradation of endospermic proteic bodies and the increase of cell turgor pressure in the endospermic micropylar region begins; and (iii) the water uptake triggers the radicle cell elongation and the beginning of the pressure of radicle apex on the surrounding envelops. At final of imbibition (18-19 h) the view is (Fig. 1D; general view): (i) the palisade cell layer corresponding to micropylar zone is dismanteled; (ii) the proteic bodies of both micropylar endosperm and micropylar endospermic adjacent cells are empties and the micropylar endosperm layer is thinner than at 4 h imbibition, into others, by an strong cell wall thinness; (iii) the proteic bodies of radicle lateral cells begins its degradation; and (iv) the seed coat rupture is visible in the lateral region near to micropylar zone (Figs. 1D,E). After the above described events, the cell layer of micropylar endosperm is broken and the protrussion executed (Fig. 1F).

Germination profiles of S. officinale seeds

Before initiating the studies presented here, the freshly harvested *S. officinale* brown seeds (non AR seeds) were stored dry at 21 ± 0.2 ºC and darkness for 6 months (AR seeds). This time was sufficient to complete the AR (data not shown). The radicle emergence of *S. officinale* seeds was strongly dependent of light and the presence of NO_3^- in the germination medium (Hilhorst and Karssen, 1988). In control (20 mM KNO_3), the protrusion began at 19-20 h (2-4 ± 1%) and increased rapidly until 25-26 h (100% germination). In presence of 100 µM GA_{4+7} or 10 µM ethrel, the protrusion became detectable at 15-16 h (4-5 ± 1%) and 100% germination was at 23 h. Quantitatively significative differences were no observed between both hormonal treatments (Tab. 1). The germination percentages in presence of ACC were similar to those obtained with ethrel (data not shown). Together, GA_{4+7} and ethrel were incapable of boosting the germination percentage with respect to individual hormones (data not shown). Maximum germination percentage was not altered by the ACC-synthase inhibitor AVG (10 or 100 µM), while the ACC-oxidase inhibitor Co^{+2} (100 µM) slightly decreased it. All these inhibitions were strongly overcome by GA_{4+7} and ethrel, and the inhibitor of bio-active gibberellin sinthesis paclobutrazol (PB) strongly prevented it (data not shown).

Effect of ethrel and GA_{4+7} pulses on the germination rate of S. officinale

This experiment was conducted to determine whether the time-course of *S. officinale* seed germination was altered by ethrel or GA_{4+7} added to the medium at different times of imbibition phase. The results of the time-course may reflect the need for ET or GAs during imbibition and its effects on germination pattern. To this end, ethrel and GA_{4+7} were applied at 0, 4, 8, 12 y 16 h, and then the kinetics of induced germination quantified. The germination time-course was different depending on the time of application. Thus, ethrel and GA_{4+7} only stimulate the radicle emergence if applied very early (i.e. 0 or 4 h, respectively) (Figs. 2A, B, F, G). Contrary, the hormonal effect was slower or even null when ethrel or GA_{4+7} were added at the middle or late stage of imbibition, respectively (i.e. 8, 12 y 16 h; Figs. 2C-E, H-J).

Alterations in ACC and MACC produced by ethrel and GA_{4+7} during germination

An ACC peak coincident with the onset of radicle emergence was detected in control (Table 1). The protrusion acceleration caused by GA_{4+7} and ethrel also accelerated the appareance of the

ACC peak. However, the presence of GA_{4+7} provocked a sharp decline in ACC after reaching the maximum germination percentage. This last fact was not observed in the presence of ethrel, probably due to the existente of an ACC autocatalytic production. In fact, during all studied period the ACC levels were always greater in presence of ethrel than GA_{4+7} (Table 1). The ACC content significantly decreased with IESS after completing the imbibition phase. However, the PB caused the opposite effect that IESS. With regard to the MACC levels, very abundant in dry seed (two times higher than the ACC): (i) decreased significantly in the control until it starts protrusion (20 h), increasing thereafter; (ii) exogenous ethrel caused an increase in MACC following the same pattern as in control but with minimal value at 18 h; (iii) GA_{4+7} induced higher MACC levels than control decreasing from 12 h; (iv) PB induced MACC values lower than in the control and in the presence of GA_{4+7}, while IESS induced greater amounts of conjugated than the control and in the presence of PB (Table 1).

Effect of ethrel and GA_{4+7} on SoACS7 and SoACO2 transcript levels during germination

Prior to the study of gene expression during germination, we have demonstrated that *SoACS7* and *SoACO2* genes were expressed during seed development. Thus, the level of *SoACS7* transcript was very abundant during all studied development stages, decreasing slightly with the desiccation process. By contrast, *SoACO2* expression was much lower than *SoACS7* in EF, increased concomitantly with the development and also during desiccation. Interestingly, AR caused in dry seeds a decrease in the level of both *SoACS7* and *SoACO2* transcripts (Fig. 3A). The *SoACS7* expression was not detected during imbibition of control AR seeds and those treated with ethrel, GA_{4+7}, PB and IESS. However, notable levels of *SoACS7*-mRNA were found in control when germination reached 50-100 %. The control expression pattern was slightly increased by ethrel and IESS (Figs. 4A, C, D), but was totally eliminated with PB (data not shown). The presence of GA_{4+7} accelerated the *SoACS7* expresión concomitantly with the stimulation of induced radicle emergence (Tab. 1; Fig. 4B). The *SoACO2* transcript was barely detected in control during the first 6 h of imbibition, rose sharply in the final hours of imbibition and during the progress of the radicle emergency (Fig. 5A). The *SoACO2* expression pattern was similar to the control in the presence of GA_{4+7} (Fig. 5B), strongly inhibited by PB (Fig. 5C) and much less inhibited by IESS (Fig. 5E). Finally, ethrel caused a similar *SoACO2* expression pattern than the one found in the control, but with slightly higher values (Fig. 5D).

Effect of ethrel and GA_{4+7} on SoGA3ox2, SoGA20ox2 and SoGA2ox6 transcript levels during seed germination

The level of *SoGA3ox2*-mRNA increased during seed development, was a sharp drop during desiccation and AR process stimulated its expression in dry viable seeds (Fig. 3B). The *SoGA20ox6* expression was very high in the EF phase, while in LF, LS and NAS was approximately 5 times lower. AR slightly rose the *SoGA20ox6* transcript in dry viable seeds (Fig. 3B). However, the *SoGA2ox6* expression was scarce in EF, increased linearly up to the NAS stage and AR process caused a slight decrease in the *SoGA2ox6*-mRNA level (Fig. 3B). During the first 6 h of imbibition occurred a strong stimulation of *SoGA3ox2* expression, while the *SoGA3ox2*-mRNA levels from 12 to 26 h were similar and about half that at 6 h. (Fig. 6A). However, although the *SoGA3ox2* expression profiles were very similar in the presence of ethrel and GA_{4+7}, the ethrel profile was slightly greater than that of GA_{4+7} (Fig. 6B, C). The *SoGA3ox2* expression pattern in control seeds was strongly inhibited by PB and IESS (Figs. 6A, C, E). Ethrel and GA_{4+7} decreased between 3-4 times the *SoGA3ox2* transcript level determined in control at 6 h, increasing gradually over the period studied. The stimulation of *SoGA20ox2* expression observed in control at 6 h of imbibition was identical to the one that was shown for *SoGA3ox2*; but the level of transcripts was 5-6 times lower between 12-26 h (Figs. 6A, 7A). However, as also happened with the *SoGA3ox2* transcript level, exogenous GA_{4+7} and ethrel inhibited the level of *SoGA20ox2*-mRNA at 6 h and slightly increased it from 6 h onwards (Figs. 7A, B, D). On the other hand, the *SoGA20ox2* expression in the presence of GA_{4+7} and ethrel were diminished by PB and IESS (Figs. 7B-E). Interestingly, the *SoGA3ox2* expression pattern in the control was not altered by exogenous PB and IESS between 12-26 h (Figs. 7A, C, E). Contrary to *SoGA3ox2* and *SoGA20ox2*, the *SoGA2ox6* expression was hardly detectable at 6 h and was very high between 12-20 h (Fig. 8A). This expression pattern was markedly inhibited with all treatments used, especially with IESS (Figs. 8B-E).

Alterations in endo-β-mannanase activity in the presence of ethrel and GA_{4+7}

A notable MAN activity was determined in dry AR seeds of *S. officinale* (Fig. 9A) and 3 h imbibed seeds (data not shown). This high MAN decreased dramatically until 12 h and increased sharply again before and during the radicle emergence. The maximum of enzymatic activity did not correspond with the 100% of germination (i.e. 26 h; Tab. 1) (Fig. 9A). The low MAN observed between 6-12 h was strongly stimulated with GA_{4+7} and ethrel, the second being more effective (Figs. 9B, D). Both hormonal treatments increased the MAN between 18-26 h coinciding with the high stimulation of radicle emergence (Tab. 1). The presence of PB, which strongly inhibited the protrusion in *S. officinale* seeds, caused the greatest MAN stimulation observed in this study. However, IESS induced a MAN pattern similar to that observed with GA_{4+7} (Figs. 7C, E).

DISCUSSION

The *S. officinale* seed is composed of the embryo, surrounded by two covering layers (i.e. seed coat and one single layer of endospermic cells), and cotyledons (Fig. 1A-D). The micropylar endosperm surrounding the radicle tip is only composed of one cell layer. In *A. thaliana* also exists one micropylar endospermic cell layer (Liu *et al*., 2005), but one or two exist in *Lepidium sativum* (Müller *et al*., 2006) and a few in *Lepidium virginicum* (Nguyen *et al*., 2000). As occurs in Arabidopsis (Mansfield and Briarty, 1996; Müller *et al*., 2006), the germination of *S. officinale* seed consists of the two sequential phases of seed coat rupture and endosperm rupture (Fig. 1E,F). This temporal sequence was no visible in Solanaceae seeds (Petruzzelli *et al*., 2003). The role of the seed coat as a germination constraint has been studied by using mutants of Arabidopsis (Debeaujon and Koornneef, 2000; Koornneef *et al*., 2002). On the other hand, although the intervention of micropylar endosperm in the rupture of the seed coat is not yet clarified, the secretion of hydrolytic enzymes, whose substrates are structural components of the cell wall (i.e. loosening), or the increase in the cellular expansion to cause the physical rupture of the seed coat, are two possibilities to consider. Thus, the micropylar endosperm is considered as a barrier for radicle protrussion (Leubner-Metzger *et al*., 2006) and play a determinant role in Arabidopsis dormancy (Bethke *et al*., 2007). The endosperm cell wall weakening (Bewley, 1997) seems to be required for lettuce (Toorop *et al*., 1999) and *Lepidium sativum* (Müller *et* al., 2006) endosperm rupture. Moreover, ABA specifically inhibits both weakening and rupture of endosperm (Müller *et* al., 2006) and endosperm rupture is under the control of an ABA-gibberellin antagonism (Kucera *et al*., 2005; Müller *et* al., 2006). Endosperm weakening seems to be biphasic in some seeds: the first phase is ABA insensitive and the second one is inhibited by ABA (Toorop *et al*., 2000; da Silva *et al*., 2004). Here, we have demonstrated that the tissues surrounding to radicle apex are structurally altered during the first hours of imbibition: (i) palisade layer is dismanteled; (ii) in endosperm layer, the volume of cytoplasm increases and the abundant proteic bodies are degradated; and (iii) in radicle, the proteic bodies corresponding to sub-apical space are also degradated. On the other hand, the secretion of mucilage, an extremely hydrophylic polimer and pectin-rich, is very abundant in imbibed *S. officinale* seeds and completely envelops the seed coat (Fig.1A-D). The mucilage-producing cells in mixospermous seeds like *S. officinale* are located in epidermal tissue of the seed coat of several species, including those of the family *Brassicaceae* (Western *et al*., 2004). Because the imbibition process must be tightly regulated in order to start the normal germination process, the mucilage can be involved in a slow and controlled water uptake (Penfield *et al*., 2001). The fruit of *S. officinale* contains two kind of seeds with respect to the colour of their seed coat (i.e. brown seeds used in this work, and light-brown ones). Brown seeds have a greater capacity to secrete mucilage, a slower water uptake rate and a far faster radicle emergency (Iglesias-Fernández *et al*., 2007). That is, the mucilage might work in *S. officinale* to enhance and control the initial imbibition.

The AR *S. officinale* seeds acquire a high sensitivity to ET and GA_{4+7}. So much so that strongly stimulating protrussion, and both germination profiles are very similar in the presence of both phytohormones (Tab. 1). PB and IESS sharply inhibited the effect induced by GA_{4+7} and ET, and this inhibition is overcome by the addition of GA_{4+7} and ET, respectively (data not shown). The intervention of the GAs in the germinative process of endospermic seeds appears to be beyond any doubt. However, the ET seems to act in concert with GAs, and the participation of these appears to be quantitatively and qualitatively more important (for review see Matilla and Matilla-Vázquez, 2008). There are seeds in which the highest production of ET and active GAs temporally coincides (Kucera *et al*., 2005). However, will be very interesting to demonstrate whether ET stimulates germination by altering GAs biosynthesis or sensitivity, or whether it acts through its own production or signalling. Although the germination time-courses in the presence of ET and GA_{4+7} were very similar, a detailed analysis of the germination kinetics shown in figure 2 suggests a greater need for exogenous GAs with respect to ET in the early stages of germination. The obtained time-courses in the presence of PB, an inhibitor of bio-active GAs synthesis, were consistent with this. Thus, its addition at the beginning of imbibition led to a sharp radicular emergence slowdown, and the germination percentages were increasing as the time of application of PB was later. On the other hand, the time-courses in the presence of ISSE were very close between them and very separated regarding to the control minus IEES, suggesting that ET synthesis and signalling during imbibition phase is involved in the germination process.

The functional overlap between different hormonal responses support the presence of an interaction (cross-talk) between theirs signalling pathways (Brady and McCourt, 2003; Chiwocha *et al*., 2005; De Grauwe *et al*., 2007, 2008; Holdsworth *et al*., 2008). We suggest the existence of a relationship between ET and GAs signalling pathways during the germination process since both hormones cause similar pattern of embryonic axis protrusion and this pattern is not altered by the addition of both hormones together. ET and GAs have similar stimulatory effects on Arabidopsis hypocotyl elongation (Smalle *et al*., 1997). Molecular characterization of such and others cross-talk should provide an advance on the picture of seed germination. The approximation to understanding the ET-GAs interaction at the molecular level during germination of *S. officinale* seeds was the main objective of this work. Due to this, we have studied the expression of two genes involved in ET synthesis (*SoACS7* and *SoACO2*) and three genes involved in GAs synthesis (*SoGA20ox2*, *SoGA3ox2*) and breakdown (*SoGA2ox6*). The alteration in the expression of these five genes during zygotic embryogenesis and acquisition of AR shows that: (i) the expression of *SoACS7*, the highest of all transcripts studied, and *SoGA3ox2* decreases sharply with seed desiccation; (ii) the expression of both *SoACO2* and *SoGA2ox6* increases as seed development and desiccation progresses, while the *SoGA20ox2*-mRNA decreases sharply in the transition EF to LF and remains constant until the complete drying, suggesting that, unlike *SoACO2* and *SoGA2ox6*, the expression of *SoGA20ox2* has a very relevant role in the middle and late stages of seed development; (iii) the acquisition of AR causes a significant decrease in the level *of SoACS7*, *SoACO2* and *SoGA2ox6* transcripts or the failure to transcribe them, and on the other hand, a sharp increase in the expression of *SoGA3ox2*. This last result strongly suggests that the mechanism of action of AR to overcome the *S. officinale* seed dormancy directly affects the GAs synthesis *via SoGA3ox2*. However, it seems that the ET pathway is not involved in this post-harvest process. Although the molecular mechanisms involved in the acquisition of AR are not clearly understood, there are convincing indications that processes related to dormancy release occur in dry seeds. Thus, recent results from cDNA-AFLP (Bove *et al*., 2005; Leymarie *et al*., 2007), microarrays (Finch-Savage *et al*., 2007; Carrera *et al*., 2008) and gen- and protein-omic (Johnson and Dyer, 2000; Chibani *et al*., 2006) analyses show that gene expression is altered in AR dry seeds. To try to explain that a seed with a very low hydration could carry out a measurable gene activity, Manz *et al*. (2005) demonstrated the existence of areas with high moisture in tobacco viable dry seeds.

Bioactive-GAs are required for seed germination as demonstrated by the non-germinating phenotype of the *gai1-3* Arabidopsis mutant (Koornneef and van der Veen, 1980; Ogawa *et al*., 2003). GA biosynthesis takes place during early seed imbibition because germination can be inhibited at that time by GA-biosynthesis inhibitors (Pen *et al*., 2002; Ogawa *et al*., 2003; Fig. 2 of this work). The fact that *SoGA3ox2*-mRNA increases during the acquisition of AR in *S. officinale* suggests the involvement of GAs synthesis in the preparation for germination. However, the levels of bioactive-GAs produced during AR does not appear to be sufficient to start the germination process, since at 6 h of imbibition: (i) the *SoGA3ox2* and *SoGA20ox2* expression is markedly increased; and (ii) the *SoGA2ox6* expression, involved in GA deactivation, is very scarce regarding to the rest of the germination period. Nevertheless, it is probable that *SoGA3ox2* is more involved than *SoGA20ox2* in the synthesis of bioactive-GAs during *S. officinale* seed germination since the levels of *SoGA20ox2* transcripts are less abundant. On the other hand, given the wide expression of *SoGA2ox6* that takes place during the progression of radicle emergence, we can conclude that the level of bioactive-GAs during germination of *S. officinale* seeds is tightly controlled by modulating both their synthesis and catabolism.

Cross-talk between ET/GAs has been demonstrated to control several pathways of plant growth and development in *Arabidopsis* (Ogawa *et al.* 2003; Chiwocha *et al.*, 2005; Weis and Ori, 2007; De Grauwe *et al.*, 2008; Dugardeyn *et al.*, 2008). Recently, hypocotyl elongation induced by ET was found to be dependent but not mediated by GAs (Vandendussche *et al.*, 2007). Yet, there are few studies at molecular level on the ET/GAs cross-talk during the germination of seeds different to Arabidopsis. The Chiwocha´s results interestingly suggest that the ET signalling modulates the metabolism of several plant-hormone pathways in seeds (v.e. those of GAs and ABA) (Chiwocha *et al.*, 2005). In *S. officinale* seeds, the results obtained using pulses of hormones and inhibitors (v.e. PB and IESS; Fig. 2) suggest that ET seems less important than the GAs to trigger the protrusion. Moreover, *SoACS7* is expressed very late in the germination process, perhaps because the endogenous ACC levels are sufficient to supply the *SoACO2*, whose expression is very noticeable around the protrusion. It is very possible that *SoACS7* is not the only member of *SoACS* family related to germination; but we failed to isolate others. On the other hand, the presence of ethrel or GA_{4+7}, which accelerate the radicle emergence in *S. officinale*, alter the ACC levels before protrusion and the *SoACO2* expression pattern. By contrast, in the presence of PB and IESS, the germination percentage, ACC content and *SoACO2* expression are markedly inhibited. The addition of ET triggers a positive feedback that raises the *PsACO1* expression in pea seeds (Petruzzelli *et al.*, 2000). Likewise, the application of GA_3 also produced an increase in ACC content, ACC-oxidase, *FsACO1* expression and ET synthesis in *Fagus sylvatica* seeds, suggesting some ET/GAs cross-talk (Calvo *et al.*, 2004a). During GA_4 treatment of *ga1-3* seeds, which stimulates germination, expression of *AtACO* was also increased (Ogawa *et al.*, 2003).

By comparing the levels of *SoGA3ox2* and *SoGA20ox2*-mRNA in the control we can conclude that the expression and regulation *SoGA3ox2* must be of great importance during germination since their expression is higher than that of *SoGA20ox2*. Several results presented in this work referred to *SoGA3ox2*, *SoGA20ox2* and *SoGA2ox6* expression point out an ET/GAs cross-talk regulation during *S. officinale* seed germination. Thus, (i) *SoGA3ox2* expression is strongly inhibited by PB and IESS, and by GA_{4+7} and ethrel during germination and imbibition, respectively; (ii) ethrel and GA_{4+7} diminish two times the levels of *SoGA20ox2*-mRNA during the first 6 h of imbibition and increase the *SoGA20ox2* expression along germination, while PB markedly inhibits the level of *SoGA20ox2*-mRNA at 6 h and the levels of *SoGA20ox2* transcrips in presence of IESS and PB are lightly higher than control between 18-26 h; (iii) *SoGA2ox6* expression is severely inhibited by inhibitors PB and IESS during the germinative process, and by GA_{4+7} and ethrel between 18-26 h; but these hormone stimulate the *SoGA2ox6*-mRNA at onset of imbibition. Taking all this into account, the results summarized in this paragraph suggest the hypothesis that the regulation of the synthesis of bioactive-GAs involved in the germination of *S. officinale* seeds is subject to strong control by ET and GAs (i.e. ET/GAs cross-talk regulation), and if the threshold level of GAs necessary to prompt germination is surpassed, a destruction mechanism exists (v.e. *SoGA2ox6*) to respond at this unnecessary synthesis. As an alternative explanation, and based on the study of the impact of the *etr1-2* mutation during germination of *A. thaliana* (Chiwocha *et al*., 2005), it is possible that the lack of GAs synthesis (i.e. PB) or ET synthesis and signalling (i.e. IESS) in *S. officinale* seeds causes compensatory responses in other hormonal pathways or that GAs and ET normally regulate these hormonal pathways in a positive or negative way, either directly or indirectly. Recently, a cross-talk of *FsGA20ox1* by GAs and ET in germinating beechnuts seeds was evidenced (Calvo *et al*., 2004b); and more recently an up- and down-regulation of different GA metabolism genes by ET have been demonstrated, this finding being also related to ET/GA cross-talk (Dugardeyn *et al.*, 2008).

To conclude this study on some interactions between ET and GAs in *S. officinale*, we have determined the effect of both hormones on MAN activity. In tomato seeds, MAN activities are expressed sequentially in different parts of endosperm, initially in the micropylar area (germinative expression) and subsequently in the surrounding lateral area (postgerminative expression) (Nonogaki *et al.*, 2000; Gong and Bewley, 2007 and references therein). At present, the consensus appears to be that, while MAN activity is required for endosperm weakening, it is not, by itself, sufficient to allow germination to be completed (Gong *et al.*, 2005). In *S. officinale*, a noticeable *in vitro* MAN activity is present in dry seeds, and its activity strongly decrease until 12 h of imbibition. Presumably this enzyme protein was synthesized during zygotic embryogenesis or alternativelly their gene(s) translated in the own dry seed during AR process since MAN activity is scarcely detected in not AR seeds. By means of western blot analysis, a MAN protein was recently found in an inactive form in dry rice seeds (Ren *et al.*, 2008). After 12 h imbibition, MAN activity increases in *S. officinale* seeds and reaches a maximum between 20-22 h (9-51% of germination). Previous studies in rice have shown that there is an increase in MAN activity mostly after the completion of germination, this increase being positively affected by GAs (Wang *et al.*, 2005). Interestingly, (i) GA_{4+7}, ethrel and IESS augments in *S. officinale* seeds the MAN activity between 6-12 of imbibition. However, GA_{4+7} do not affect this activity along protrussion, while ethrel does; and (ii) the MAN activity is strongly risen in the presence of PB along germination process, apparently indicating that a fall in the content of bioactive-GAs triggers a rapid desynchronization in the seed, whereupon a non-specific enzymatic stimulation takes place without provoking protrussion due to the absence of GAs. All data presented here on MAN activity during germination process of *S. officinale* seeds point to an ET/GAs cross-talk regulation, as discussed for the expression of the genes studied. To shed light on this approach, we are carrying out an *in situ* hybridization to know the spatial and temporal localization of *SoMAN* in hedge mustard seeds.

SUPPLEMENTARY DATA

Supplementary information is available online at www.aob.oxfordjournal.org and includes the following. Table S1: List of degenerated primers used for PCR assay in the isolation of partial-length cDNAs. Table S2: List of primers used for the Real-time PCR assay.

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LITERATURE CITED

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389-402.

Barroco RM, Van Poucke K, Bergervoet JHW, De Veylder L, Groot SPC, Inzé D, Engler G. 2005. The role of the cell cycle machinery in resumption of postembryonic development. *Plant Physiology* **137**: 127-140.

Beaudoin N, Serizet C, Gosti F, Giraudat J. 2000. Interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12**: 1103-1115.

Berridge MV, Tan AS, McCoy KD, Wang R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica* **4,** 15-20.

Bethke PC, Libourel IGL, Aoyama N, Chung YY, Still DW, Jones RL. 2007. The Arabidopsis aleurone layer responds to nitric oxide, gibberellin, and abscisisc acid and is sufficient and necessary for seed dormancy. *Plant Physiology* **143**: 1173-1188.

Bewley JD. 1997. Seed germination and dormancy. *Plant Cell* **9**: 1055-1066.

Bleeker AB, Estelle MA, Somerville C, Kende H. 1988. Intensivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. *Science* **241**: 1086-1089.

Bove J, Lucas P, Godin B, Oge L, Jullien M, Grappin P. 2005. Gene expression analysis by cDNA-AFLP highlights a set of new signalling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Molecular Biology* **57**: 593-612.

Brady SM, McCourt P. 2003. Hormone cross-talk in seed dormancy. *Journal of Plant Growth Regulation* **22**: 25-31.

Calvo AP, Nicolás C, Lorenzo O, Nicolás G, Rodríguez D. 2004a. Evidence for positive regulation by gibberellins and ethylene of ACC oxidase expresión and activity during transition from dormancy to germination in *Fagus sylvatica* L. seeds. *Journal of Plant Growth regulation* **23**: 44-53.

Calvo AP, Nicolás C, Nicolás G, Rodríguez D. 2004b. Evidence for positive cross-talk regulation of a GA20-oxidase (*FsGA20ox1*) by gibberellins and ethylene during breaking of dormancy in *Fagus silvatica* seeds. *Physiologia Plantarum* **120**: 623-630.

Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL, Holdsworth MJ. 2008. Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *The Plant Journal* **53**: 214-224.

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P. 2006. Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiology* **142**: 1493-1510.

Chiwocha SDS, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, Ross ARS, Kermode A. 2005. The *etr1*-2 mutation in *Arabidopsis thaliana* affects the ABA, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. *The Plant Journal* **42**: 35-48.

Da Silva EAA, Toorop PE, van Aelst AC, Hilhorst HWM. 2004. Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea Arabica* cv. Rubi) seed germination. *Planta* **220**: 251-261.

Debeaujon I, Koornneef M. 2000. Gibberellin requirement for *Arabidopsis* seed germination is determined both by testa characteristics and embryonic abscisic acid. *Plant Physiology* **122**: 415-424.

De Grauwe, L., Vriezen, W., Bertrand, S., Phillips, A., Vidal, A., Hedden, P., van der Straeten, D. 2007. Interactions between gibberellin and ethylene signalling pathways in *Arabidopsis thaliana*. *Planta* **226**: 485-498.

De Grauwe L, Chaerle L, Dugardeyn J, Decat J, *et al.* 2008. Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the Arabidopsis *gai eto 2-1* double mutant. *New Phytology* **177**: 128-141.

Dugardeyn J, Vandenbussche F, Van Der Straeten D. 2008. To grow or not grow: what can we learn on ethylene-gibberellin cross-talk by *in silico* gene expression analysis?. *Journal Experimental Botany* **59**: 1-16.

Finch-Savage WE, Leubner-Metzger L. 2006. Seed dormancy and the control of germination. *New Phytol*ogy **171**: 501-523.

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW. 2007. Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows commun quantitative patterns of gene expression directly by environment specific sensing. *The Plant Journal* **51**: 60-78.

Finkelstein RR. 2004. The role of hormones during seed development and germination. In: Davies, P.J. (ed.) *Plant Hormones-Biosynthesis, signal transduction, action!*. Dordrecht, Kluwer Academic. pp. 513-537.

Gallardo M, Muñoz de Rueda P, Matilla AJ, Sánchez-Calle IM. 1994. Effect of short-chain fatty acids on the ethylen pathway in embryonic axes of *Cicer arietinum* during germination. *Physiologia Plantarum* **92,** 629-635.

Gallardo K, Job C, Groot SPC, Puype M, Demol H, Vandekerckhove J, Job D. 2002. Importance of methionine biosynthesis for *Arabidopsis* seed germination and seedling growth. *Physiologia Plantarum* **116,** 238-247.

Gazzarrini S, McCourt P. 2003. Cross-talk in plant hormone signalling: what Arabidopsis mutants are telling us. *Annals of Botany* **91**: 605-612.

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, Mc Court P. 2000. Regulation of ABA signaling by the ethylene response pathway in *Arabidopsis*. *Plant Cell* **12**: 1117-1126.

Gong X, Bassel G, Wang A, Greewood JS, Bewley JD. 2005. The emergence of embryos from hard seeds is related to the structure of the cell walls of the micropylar endosperm, and not to endo-ß-mannanase activity. *Journal of Experimental Botany* **96**, 1165-1173.

Gong X, Bewley JD. 2007. Sorting out the *LeMANs*: endo-β-mannanase genes and their encoded proteins in tomato. *Seed Science Research* **17**: 143-154.

Hilhorst H, Karssen CM. 1988. Dual effect of light on the gibberellin- and nitrate-stimulated seed germination of *S. officinale* and *A. thaliana*. *Plant Physiology* **86**: 591-597.

Holdsworth MJ, Bentsink L, Soppe WJJ. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179**: 33-54.

Iglesias-Fernández R, Matilla AJ, Pulgar I, de la Torre F. 2007. Ripe fruits of *Sisymbrium officinale* L. contain heterogeneous endospermic seeds with different germination rates. *Seed Science and Biotechnology* **1**: 18-24.

Johnson RR, Dyer WE. 2000. Degradation of endosperm mRNAs during dry afterripening of cereal grains. *Seed Science Research* **10**: 233-241.

Karssen CM, Zaórski S, Kepczynski J, Groot SPC. 1989. Key role for endogenous gibberellins in the control of seed germination. *Annals of Botany* **63**: 71-80.

Koornneef M, van der Veen JH. 1980. Induction and analysis of gibberellin sensitivity mutants in *Arabidopsis thaliana* (L.) Heynh. *Theoretical Applied of Genetic* **58**: 257-263.

Koornneef M, Bentsink L, Hilhorst H. 2002. Seed dormancy and germination. *Current Opinion in Plant Biology* **5**: 33-36.

Kucera B, Cohn MA, Leubner-Metzger L. 2005. Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15**: 281-307.

Leubner-Metzger G, Kucera B, Müller K. 2006. Emerging and established model system for endosperm weakening. In: S. Navie, S. Adkins, S. Ashmore (eds) Seeds: Biology, Development and Ecology. CAB International. England.

Lever M. 1972. A new reaction for colorimetric determination of carbohydrates. *Analytical Biochemistry* **47:** 273–279.

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F. 2007. Identification of transcripts potentially involved in barley seed germination: and dormancy using cDNA-AFLP. *Journal of Experimental Botany* **58**: 425-437.

Liu P-P, Koizuka N, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H. 2005. Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. *The Plant Journal* **41**: 936-944.

Lizada MCC, Yang SF. 1979. A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. *Analytical Biochemistry* **100:** 140-145.

Mansfield SG, Briarty LG. 1996. The dynamics of seedling and cotyledon cell development in *Arabidopsis thaliana* during reserve mobilization. *International Journal of Plant Sciences* **157**: 280-295.

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G. 2005. Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. *Plant Physiology* **138**: 1538-1551.

Matilla AJ. 2000. Ethylene in seed formation and germination. *Seed Science Research* **10**: 111-126.

Matilla AJ, Matilla Vázquez MA. **2008.** Involvement of ethylene in seed physiology. *Plant Science* **175**: 87-97.

Mendes-Ribeiro D, Santos Barros R. 2006. Sensitivity to ethylene as a major component in the germination of seeds of *Stylosanthes humilis*. *Seed Science Research* **16**: 37-45.

Mo B, Bewley JD. 2003. The relationship between β-mannosidase and endo-β-mannanase activities in tomato seeds during and following germination: a comparison of seed populations and individual seeds. *Journal of Experimental Botany* **54**: 2503-2510.

Müller K, Tintelnot S, Leubner-Metzger G. 2006. Endosperm-limited Brassicaceae seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiology* **47**: 864-877.

Nguyen H, Brown RC, Lemmon BE. 2000. The specialized chalazal endosperm in *Arabidpsis thaliana* and *Lepidium virginicum* (Brassicaceae). *Protoplasm* **212**: 99-110.

Nonogaki H, Gee OH, Bradford KJ. 2000. A germination-specific endo-β-mannanase gene is expressed in the micropylar endosperm cap of tomato seeds. *Plant Physiology* **123**: 1235-1246.

Nonogaki H. 2006. Seed germination – The biochemical and molecular mechanisms. *Breeding Science* **56**: 93-105.

Nonogaki H, Chen F, Bradford KJ. 2007. Mechanisms and genes involved in germination *sensu stricto*. In: K.J. Bradford and H. Nonogaki (eds.) Seed development, dormancy and germination. *Ann. Plant Rev.* **27**, 264-304. Blackwell Pub Ltd, UK.

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S. 2003. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell* **15**, 1591-1604.

Pen JR, Harberd NP. 2002. The role of GA-mediated signalling in the control of seed germination. *Current Opinion of Plant Biology* **5**: 376-381.

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW. 2001. *MYB61* is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *The Plant Cell* **13**: 2777-2791.

Petruzzelli L, Coraggio I, Leubner-Metzger G. 2000. Ethylene promotes ethylene biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclo-propane-1-carboxylic acid oxidase. *Planta* **211**: 144-149.

Petruzzelli L, Müller K, Hermann K, Leubner-Metzger G. 2003. Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of solanaceous seed. *Seed Science Research* **13**: 139-153.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acid Research* **29**: 2002-2007.

Rodríguez-Gacio MC, Matilla AJ. 2001. The last step of the ethylene biosynthesis pathway in turnip tops (*Brassica rapa*) seeds: Alterations related to development and germination and its inhibition during desiccation. *Physiologia Plantarum* **112**: 273-279.

Razem FA, Baron K, Hill RD. 2006. Turning on gibberellin and ABA signalling. *Current Opinion in Plant Biology* **9**: 454-459.

Ren Y, Bewley JD, Wang X. 2008. Protein and gene expression patternss of endo-β-mannanase following germination of rice. *Seed Science Research* **18**: 139-149.

Siriwitayawan G, Geneve RL, Downie AB. 2003. Seed germination of ethylene perception mutants of tomato and Arabidopsis. *Seed Science Research* **13**: 303-314.

Smalle J, Haegman M, Kurepa J, Van Montagu M, Van Der Straeten D. 1997. Ethylene can stimulate *Arabidospsis* hypocotyl elongation in the light. *Proceedings of the Nacional Academy of Sciences, USA*. **94**: 2756-2761.

Steel RG, Torrie JH. 1982. Principles and procedures of statistics. Mc Graw-Hill, Tokyo.

Toorop PE, Bewley JD, Abrams SR, Hilhorst HWM. 1999. Structure-activity studies with ABA analogs on germination and endo-β-mannanase activity in tomato and letttuce seeds. **154**: 679-685.

Toorop PE, van Aelst AC, Hilhorst HWM. 2000. The second step of the biphasic endosperm cap weakening that mediates tomato (*Lycopersicom esculentum*) seed germination is under control of ABA. *Journal of Experimental Botany* **51**: 1371-1379.

Tyler L, Thomas SG, Hu J, Dill A, Alonso JM, Ecker JR, Sun T. 2004. DELLA proteins and gibberellin-regulated seed germination and floral development in Arabidopsis. *Plant Physiology* **135**: 1008-1019.

Vandenbussche F, Vancompernolle B, Rieu I, Ahmad M, Phillips A, Hedden P, Moritz T, Van Der Straeten D. 2007. Ethylene-induced *Arabidopsis* hypocotyl elongation is dependent on but not mediated by gibberellins. *Journal of Experimental Botany* **58**: 4269-4281.

Verwoerd TC, Dekker BMM, Koekema A. 1989. A small-scale procedure for the rapid isolation of RNAs. *Nucleics Acids Research* **17**: 2362-2368.

Wang A, Wang X, Ren Y, Gong X, Bewley JD. 2005. Endo-β-mannanase and β-mannosidase activities in rice grains during and following germination, and the influence of gibberellin and abscisic acid. *Seed Science Research* **15**: 219-227.

Weis D, Ori N. 2007. Mechanism of cross talk between gibberellin and other hormones. *Plant Physiology* **144**: 1240-1246.

Western TL, Skinner DJ, Haughn GW. 2000. Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology* **122**: 345-355.

LEGENDS

Fig. 1. Structure of *Sisymbrium officinale* L. mature seed. Bright field microscopy of longitudinal sections of seeds stained with PAS-Naphthol Blue Black. (A) Entire dry seed, showing the mature and fully differentiated embryo, the endosperm (aleurone layer) and the testa (seed coat). (B) Structure of micropylar zone enclosing the radicle tip in dry seed. (C) Structure of micropylar zone enclosing the radicle tip at 4 h of imbibition. (D) General view of a seed at 18- 19 h of imbibition. (E) Detail of testa rupture in germinating seed (18- 19 h). (F) Radicle protrusion in germinating seed (22 h). **AL**, aleurone layer; **C**, cotyledon; **ChE**, chalazal endosperm; **Col**, columella; **II**, inner integument; **ME**, micropylar endosperm; **PL**, palisade layer; **R**, radicle; **SAM**, stem apical meristem; **SC**, seed coat. Arrows show empty proteic bodies. The letter refers to the position of the close-up section. Size bars are given for each panel.

Fig. 2. Germination time-course of *Sisymbrium officinale* seeds under indicated pulses of 10 µM ethrel (■); 100 µM AVG, 1 mM Cl_2Co, 1 mM Ts-Ag (▨)(pannels A-E). 100 µM GA_{4+7} (■); 25 µM PB (▨) (pannels F-J). Control (20 mM KNO_3) (▣). Data are means ± standard error (S.E) of 3 independent experiments.

Fig. 3. Transcript analysis by real-time PCR of ethylene and gibberellin biosynthesis genes during the development of the fruit and seed of *S. officinale*. (A) Expression profile of *SoACO2* and *SoACS7*. (B) Expression profile of *SoGA3ox2*, *SoGA20ox2, SoGA2ox6*. **EF**: early fruit; **LF**: late fruit; **LS**: late seed; **NAS**: not after-ripened dry seed; **AS**: after-ripened dry seed. Error bars represent the standard error of three independent experiments.

Fig. 4. Expression profile of *SoACS7* during germination time course of *S. officinale* seeds analyzed by real-time PCR. (A) Control (20 mM KNO_3); (B) 100 µM GA_{4+7}; (C) 10 µM ethrel; (D) 100 µM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Fig. 5. Expression profile of *SoACO2,* during germination time course of *S. officinale* seeds analyzed by real-time PCR. (A) Control (20 mM KNO_3); (B) 100 μM GA_{4+7}; (C) 25 μM PB; (D) 10 μM ehtrel; (E) 100 μM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Fig. 6. Expression profile of *SoGA20ox2* during germination time course of *S. officinale* seeds analyzed by real-time PCR. (A) Control (20 mM KNO_3); (B) 100 μM GA_{4+7}; (C) 25 μM PB; (D) 10 μM ethtrel; (E) 100 μM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Fig. 7. Expression profile of *SoGA3ox2* during germination time course of *S. officinale* seeds analyzed by real-time PCR. (A) Control (20 mM KNO_3); (B) 100 μM GA_{4+7}; (C) 25 μM PB; (D) 10 μM ethrel; (E) 100 μM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Fig. 8. Expression profile of *SoGA2ox6* during germination time course of *S. officinale* seeds analyzed by real-time PCR. (A) Control (20 mM KNO_3); (B) 100 μM GA_{4+7}; (C) 25 μM PB; (D) 10 μM ethrel; (E) 100 μM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Fig. 9. Profile of endo-β-mannanase activity during germination time course of *S. officinale* seeds. (A) Control (20 mM KNO_3); (B) 100 μM GA_{4+7}; (C) 25 μM PB; (D) 10 μM ethrel; (E) 100 μM AVG, 1 mM Cl_2Co, 1 mM STS. Error bars represent the standard error of three independent experiments.

Table 1. *Effect of GA_{4+7} and ethrel on ACC and MACC levels during the S. officinale seed germination*

Treatment		6h	12h	15h	18h	20h	22h	26h
control	not AR	-	-	-	-	-	2±1a	7±2a*
	AR	-	-	-	-	9±2b	51±5f	100±4i*
		68±3c	70±2c	73±1d	80±7d	90±6g	80±2d	79±5d**
		301±16f	305±21f	240±28e	206±12d	175±11b	254±18e	303±18f***
GA_{4+7}	not AR	-	-	-	-	-	7±2a	14±5b
	AR	-	-	4±2a	10±3b	25±4d	92±7h	100±2i
		70±7c	76±5d	79±3d	84±6e	75±3d	47±3b	30±6c
		276±24e	392±31j	364±14h	341±27h	332±18g	231±16e	255±24e
PB		-	-	-	2±1a	25±4d	92±7h	100±2i
		64±4c	65±3c	67±5c	70±2c	87±4e	96±5g	102±8g
		254±21e	123±9a	169±11b	198±17c	328±23g	142±12a	273±31e
Ethrel	not AR	-	-	-	-	-	5±1a	12±3b
	AR	-	-	-	8±2b	24±3d	89±6h	100±3i
		80±6e	89±6f	97±6g	118±7g	105±6g	100±4g	95±2g
		329±21g	382±27j	325±19g	271±8e	332±14g	359±12h	343±11h
IESS		-	-	-	-	-	7±2b	61±1g
		76±4d	79±5d	76±3d	75±3d	71±3c	69±5c	35±2a
		254±20e	281±26e	312±11f	338±17g	388±14g	359±12h	343±11h

*Percentage of germination; **ACC (nmol g^{-1} FW); *** (nmol g^{-1} FW). Data are mean values of three replicates ± SE. The ACC and MACC values in dry seed were 320±15 and 619±31, respectively. Significant differences between values as assessed by LSD test (P>0.05) are shown as different letters.

Figure 1:

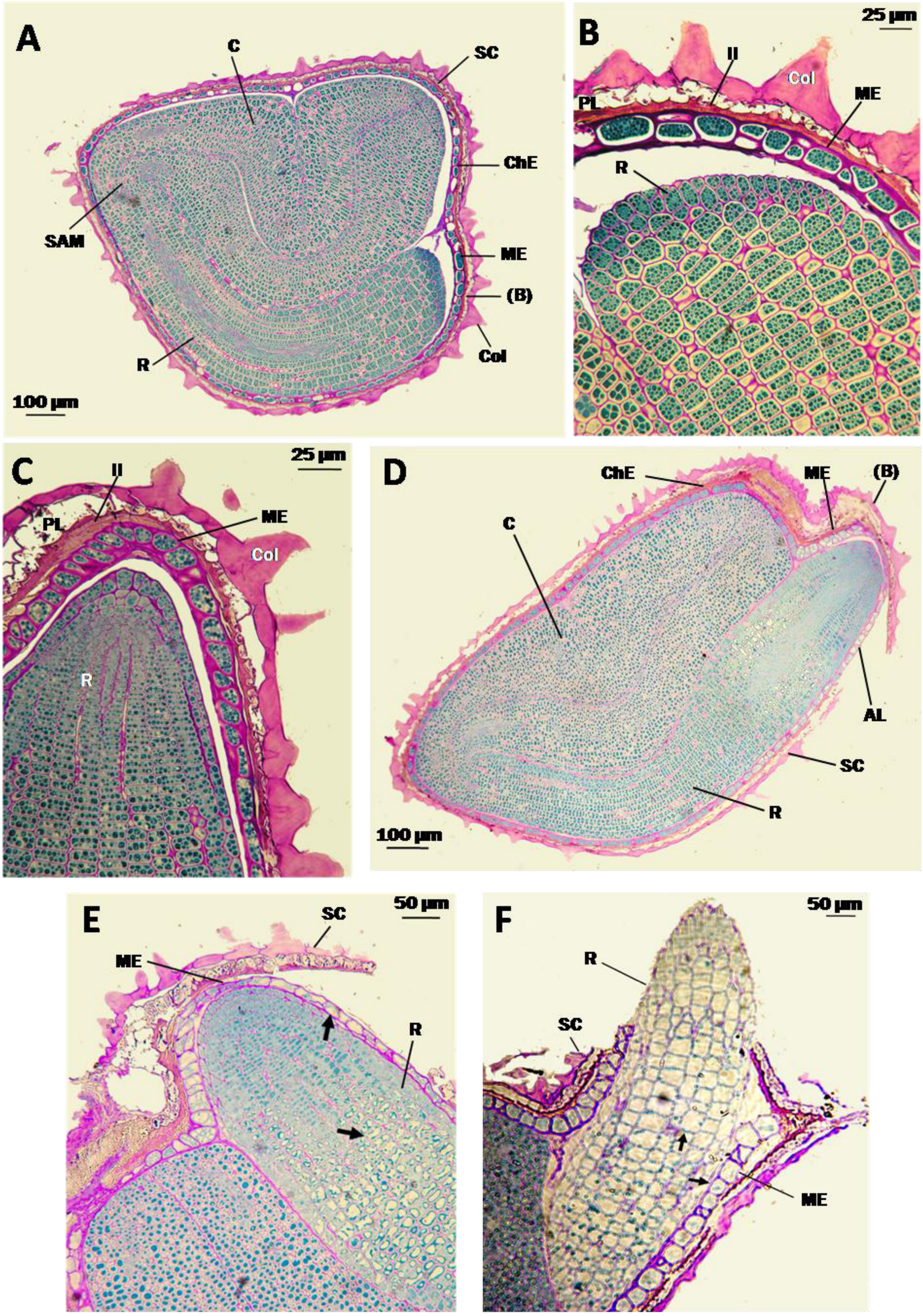
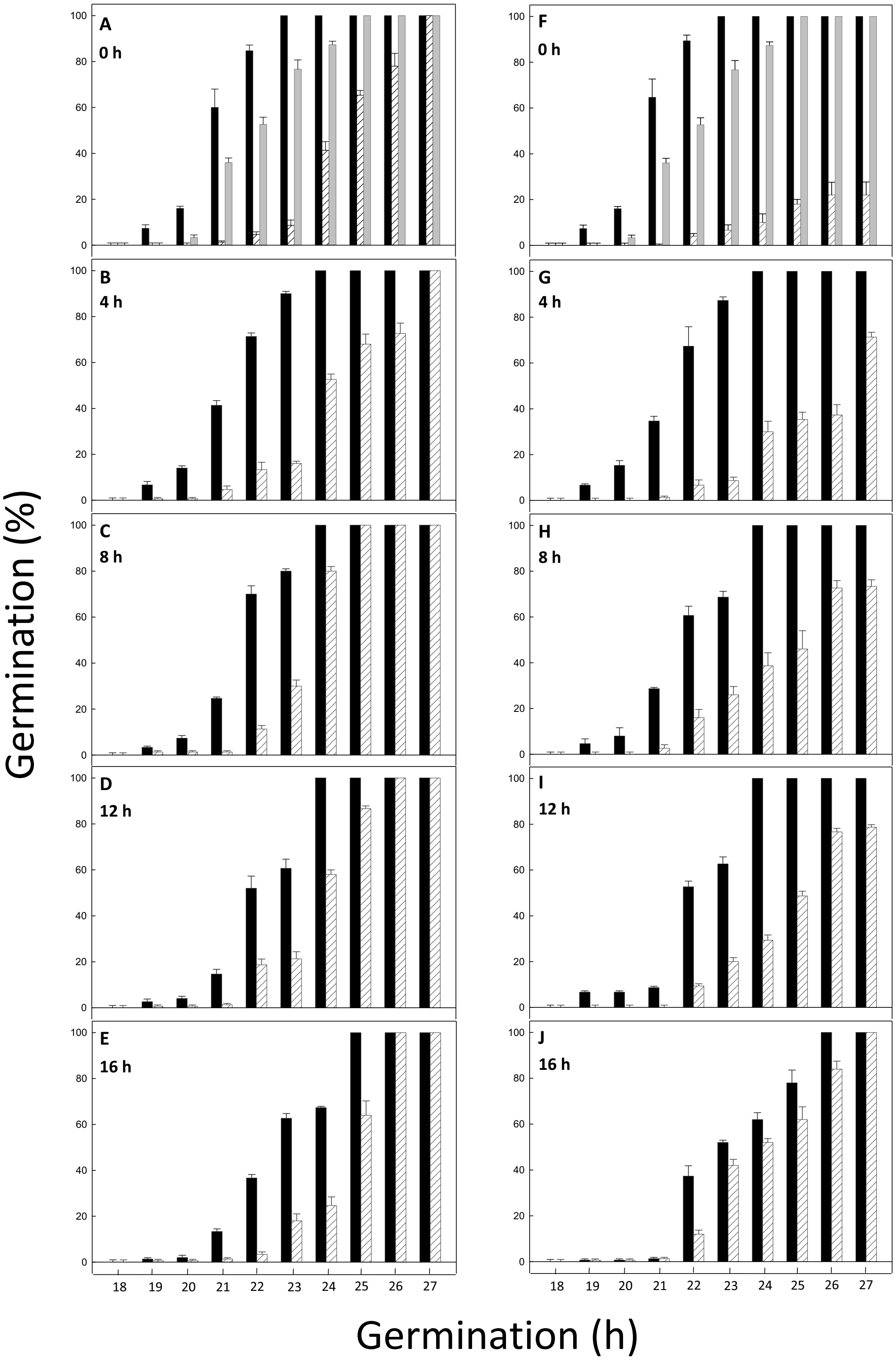
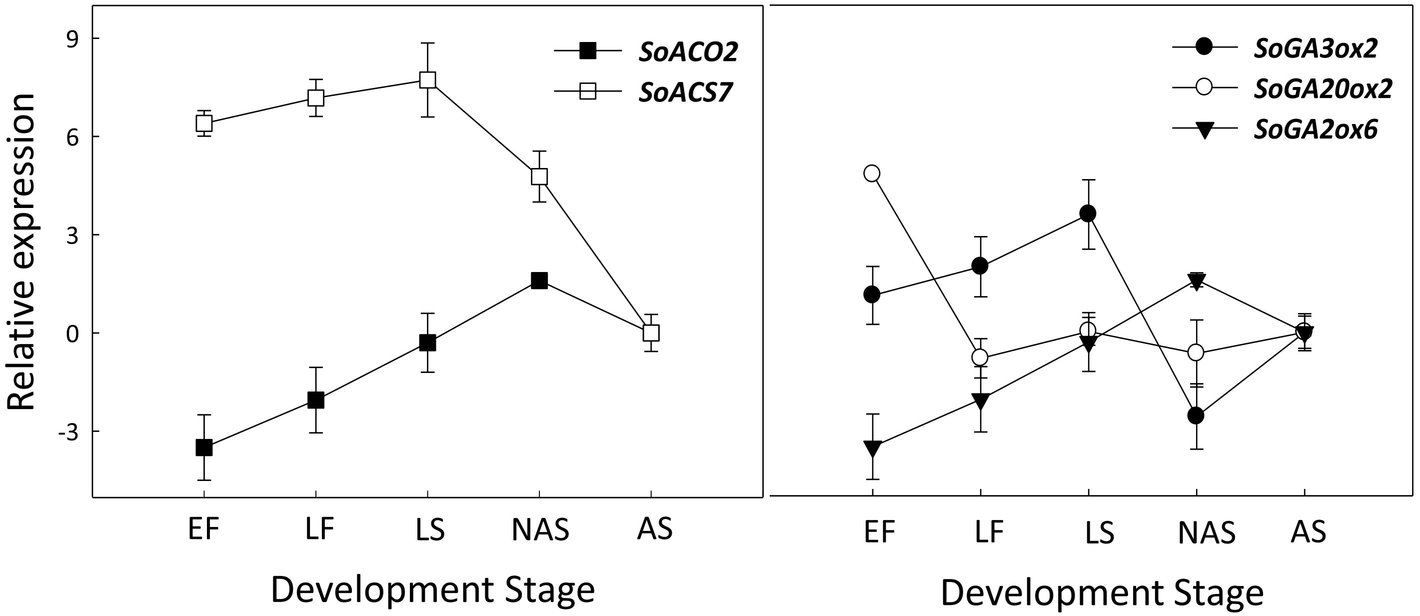

A
c
SC
ChE
SAM
ME
(B)
R
Col
100 µm
B
25 µm
II
Col
PL
ME
R
C
II
25 µm
PL
ME
Col
R
D
ChE
ME
(B)
c
AL
SC
R
100 µm
E
50 µm
SC
ME
R
F
50 µm
R
SC
ME

Figure 2:



A
0 h
B
4 h
C
8 h
D
12 h
E
16 h
F
0 h
G
4 h
H
8 h
I
12 h
J
16 h
0
20
40
60
80
100
18
19
20
21
22
23
24
25
26
27
Germination (%)
Germination (h)

Figure 3:



Relative expression
9
6
3
0
-3
SoACO2
SoACS7
SoGA3ox2
SoGA20ox2
SoGA2ox6
EF
LF
LS
NAS
AS
Development Stage
EF
LF
LS
NAS
AS
Development Stage

Figure 4:

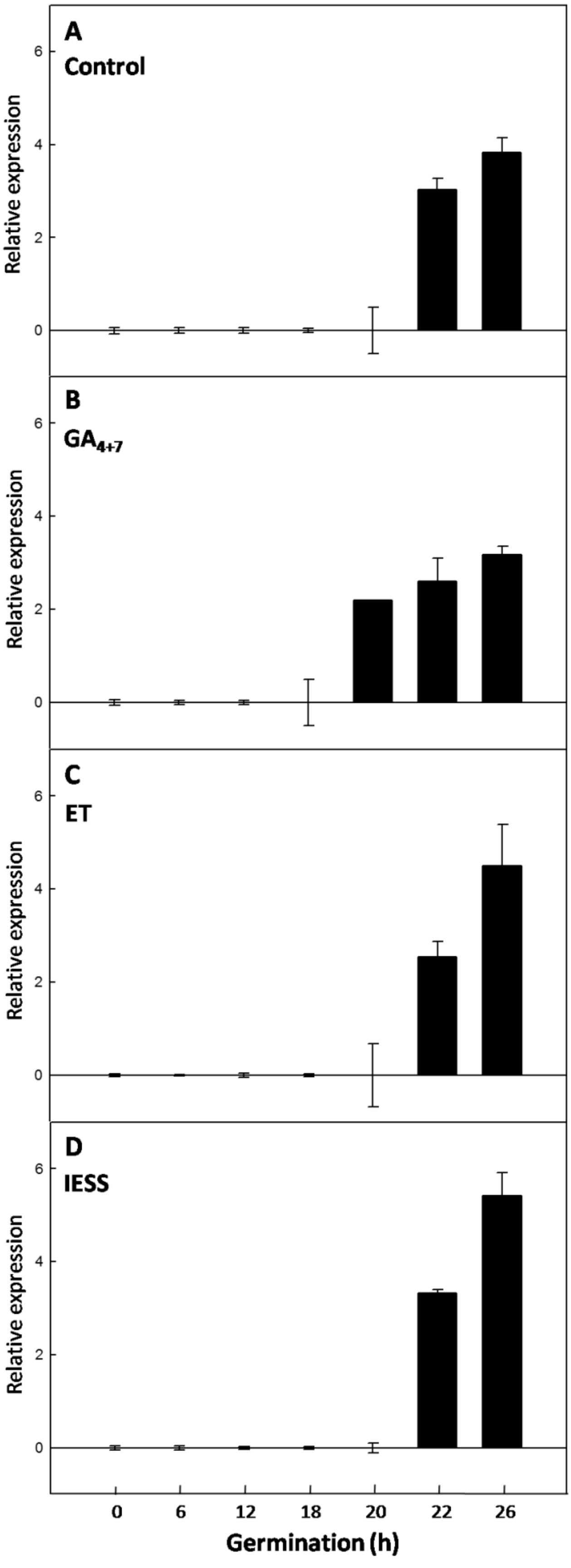

A
Control
B
GA_{4+7}
C
ET
D
IESS
Relative expression
0
2
4
6
0
6
12
18
20
22
26
Germination (h)

Figure 5:

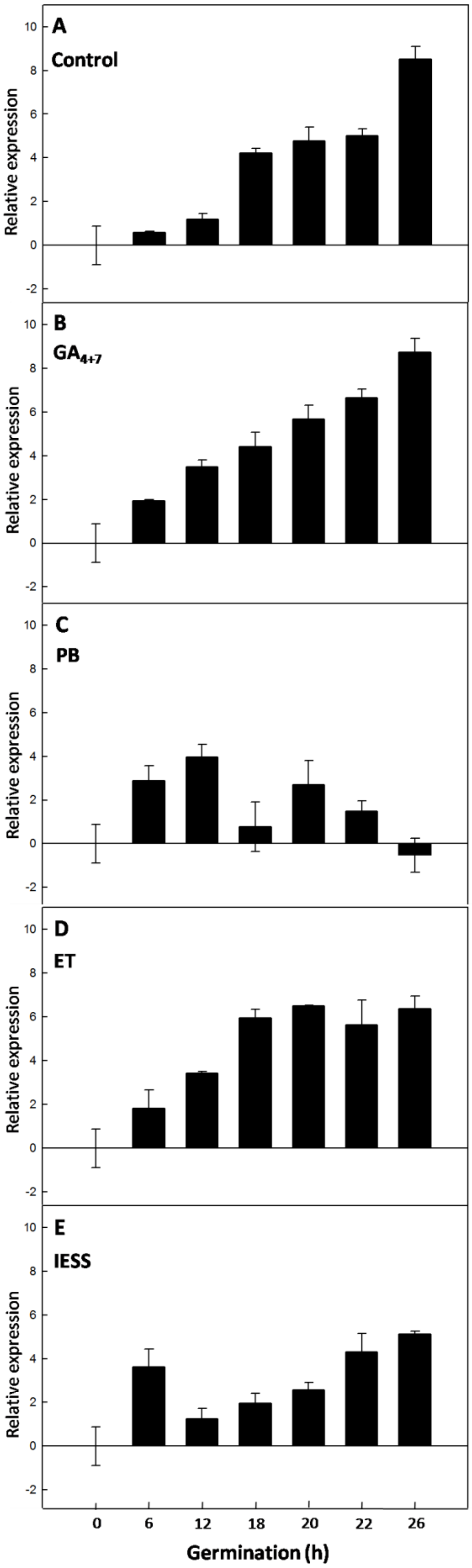

A
Control
B
GA4+7
C
PB
D
ET
E
IESS
Relative expression
10
8
6
4
2
0
-2
0
6
12
18
20
22
26
Germination (h)

Figure 6:

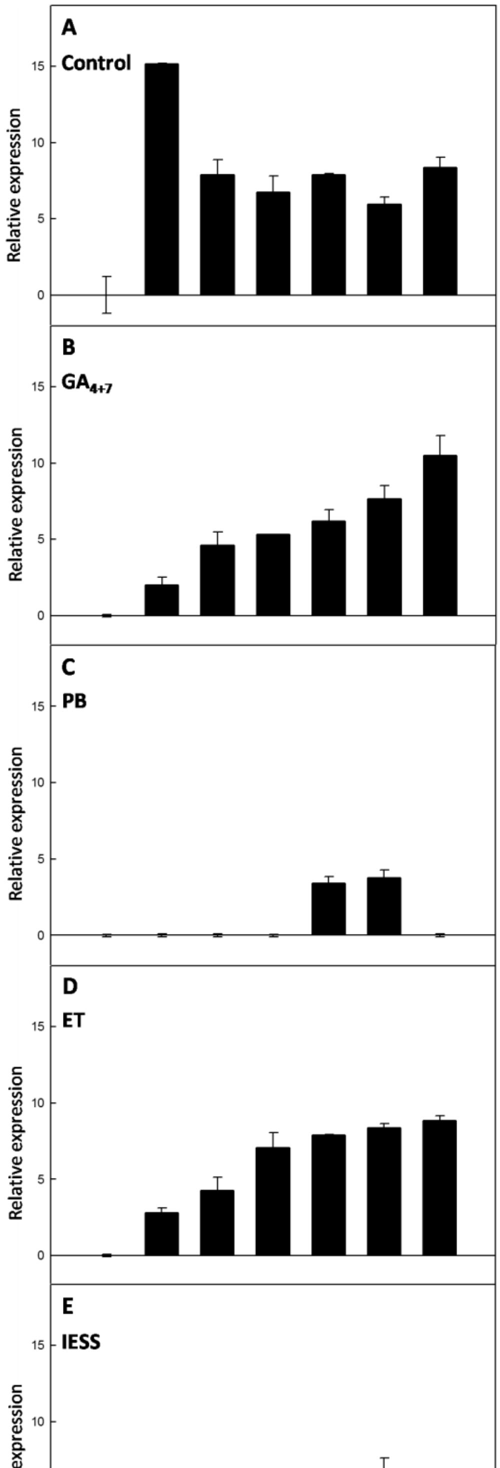

A
Control
B
GA4+7
C
PB
D
ET
E
IESS
Relative expression
0
5
10
15
0
6
12
18
20
22
26
Germination (h)

Figure 7:

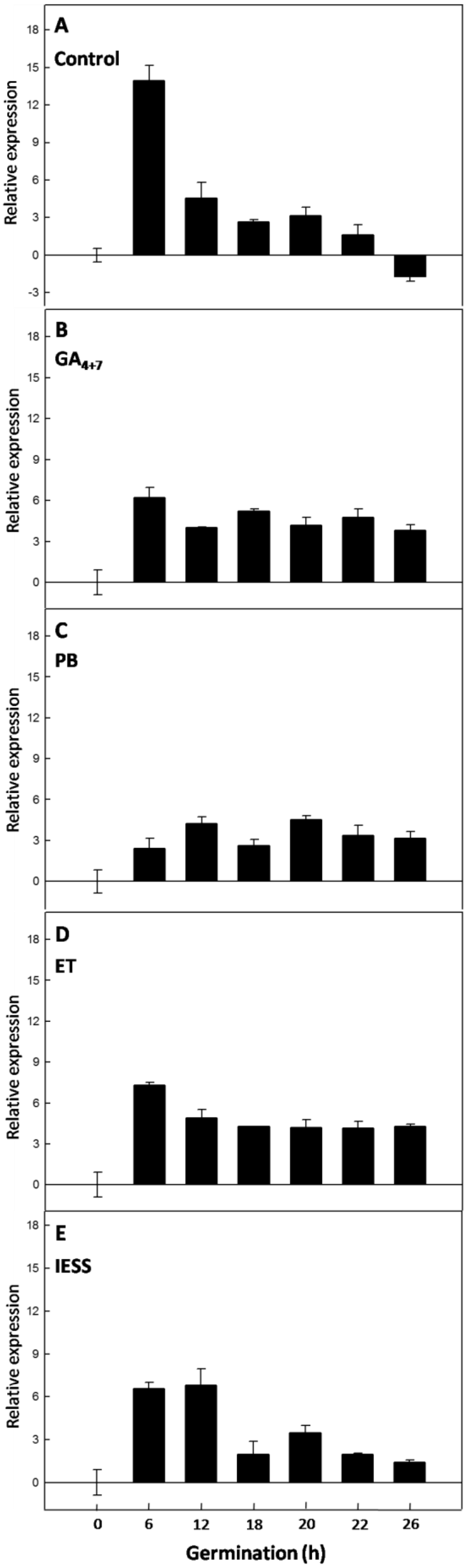

A
Control
B
GA_{4+7}
C
PB
D
ET
E
IESS
Relative expression
18
15
12
9
6
3
0
-3
0
6
12
18
20
22
26
Germination (h)

Figure 8:

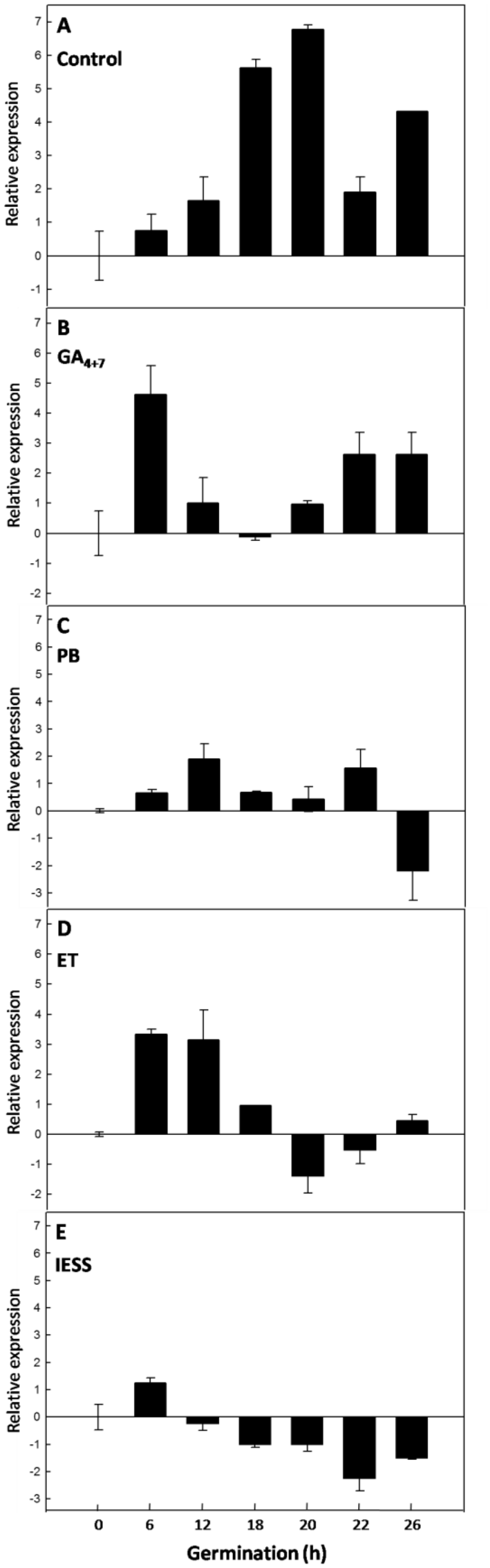

A
Control
B
GA4+7
C
PB
D
ET
E
IESS
Relative expression
7
6
5
4
3
2
1
0
-1
-2
-3
0
6
12
18
20
22
26
Germination (h)

Figure 9:

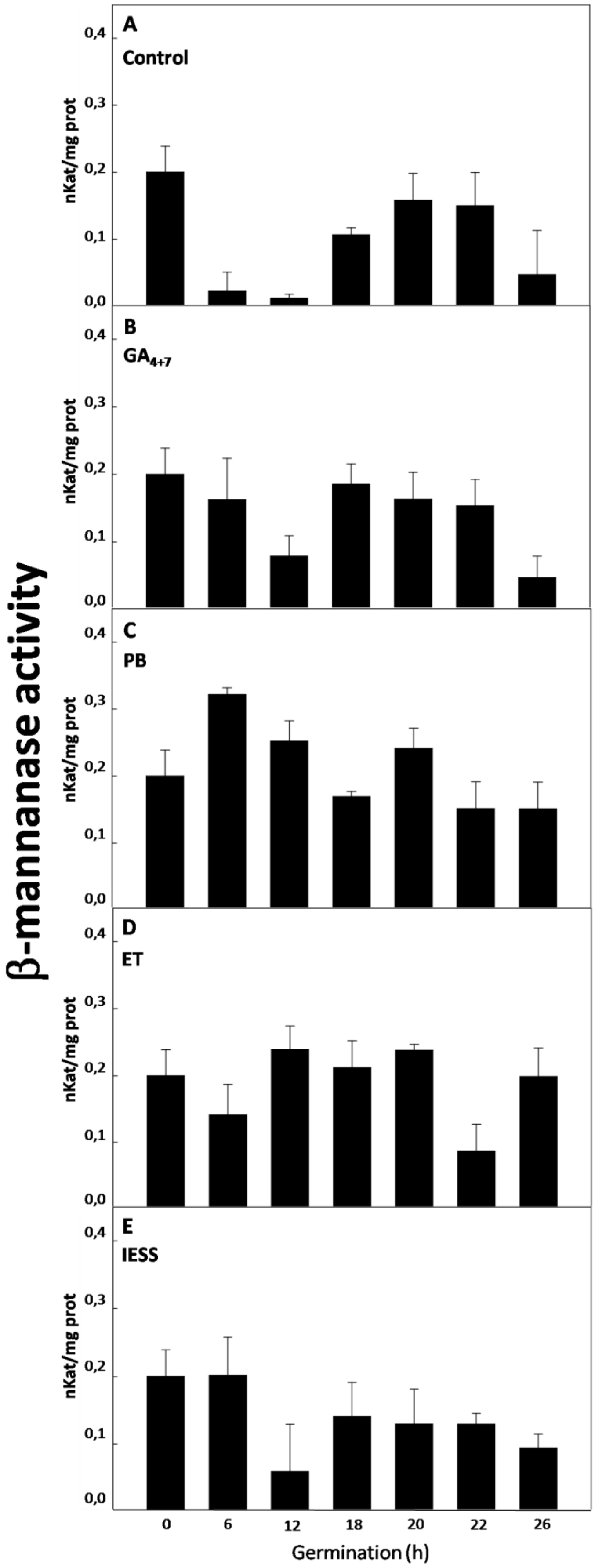

β-mannanase activity
A
Control
B
GA4+7
C
PB
D
ET
E
IESS
nKat/mg prot
0,0
0,1
0,2
0,3
0,4
0
6
12
18
20
22
26
Germination (h)

Table S1. *List of degenerated primers used for PCR assay in the isolation of partial-length cDNAs.*

Gene	**Primer name**	**Primer sequence (5´-3´)**
SoGA3ox		
	FwConGA3ox	ATGTGGTCNGAAGGNTTCAC
	RvConGA3ox	ATGTGNAANAAGTCACC
SoGA20ox		
	FwConGA20ox	AADCTNCCNTGGAARGAGAC
	RvConGA20ox	TGBAARCARCTCTTGTA
SoGA2ox		
	FwConGA2ox	GGNTTYGGAGARCAYWCWGACCC
	RvConSoGA2ox	CACTNNTAAAYCTYCCATTNGTCA
SoACO		
	FwConACO	ATGGAGAGAACATCAAGYTTYCTVTT
	RvConACo	TTAGAATGTCTCCTCVGTNGCCA
SoACS		
	FwConACS	CCAGGGTTTGATAGAGATTTGAG
	RvConACS	GCAGNSGACGCAAATYCATCC

Table S2. *List of primers used for the Real-time PCR assay*

Gene	Primer name	Primer sequence (5´-3´)	Amplicon size (bp)
SoGA3ox2			
	FwSoGA3ox	CTGTGGTTGGCATTAGGTTC	168
	RvSoGA3ox	GAGAGTTGAGTCGGTATGGG	
SoGA20o2x			
	FwSoGA20ox	GGTCTTGGTGAAGGATGG	180
	Rv SoGA20ox	AAGATCATGGAGCTTCTGG	
SoGA2ox6			
	Fw SoGA2ox	GTAGATGGACTTGAGATTTGC	89
	Rv SoGA2ox	CAGTCACCGACCAATACG	
SoACO2			
	FwSoACO	GGTGATAACCAACGGCAAGT	89
	RvSoACo	TGTAGAACGAGGCAATGGAC	
SoACS7			
	FwSoACS	CGATCCCTGCCTTCTTA	113
	RvSoACS	TAAGAAGGCAGGGATCG	
18SRNA			
	Fw18S-RNA	GGCTCGAAGACGATCAGATA	87
	Rv18S-RNA	TCATAAGGTGCCGGCGGAGT	

V. CAPÍTULO 3:

"After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during early imbibition of *Sisymbrium officinale* L. seeds" (2009) *J Exp Bot* **60**: 1645-61

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RESEARCH PAPER

After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during early imbibition of *Sisymbrium officinale* L. seeds

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Abstract

After-ripening (AR) in *Sisymbrium officinale* seeds altered *SoACS7*, *SoACO2*, *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6* gene expression. Except for *SoGA20ox2* expression, which sharply diminished, the expression of the other genes rose during development, particularly that of *SoACS7*. In contrast, only the *SoACO2* and *SoGA2ox6* transcripts increased with seed desiccation; the others decreased. AR increased the *SoGA3ox2* transcript in dry seed, but dramatically decreased the *SoACS7* transcript. At the onset of imbibition, AR inhibited *SoACS7* and *SoACO2* expression and stimulated that of *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6*, demonstrating that the participation of ethylene (ET) and gibberellins (GAs) differs in after-ripened and non-after-ripened seeds. The inhibition of *SoACO2* expression in the presence of GA$_{4+7}$, paclobutrazol (PB), inhibitors of ET synthesis and signalling (IESS), and notably ET+GA$_{4+7}$ indicated ET–GA cross-talk in non-after-ripened seeds. A positive effect of AR in reversing this inhibition was found. The idea of ET–GA cross-talk is also supported by the effect of ET on *SoGA3ox2* expression, notably induced by the AR process. In contrast, *SoGA20ox2* expression did not appear to be susceptible to AR. *SoGA2ox6* expression, poorly known in seeds, suggests that AR prompted an up-regulation under all treatments studied, whereas in non-after-ripened seeds expression was down-regulated. On the other hand, the β-mannanase (MAN) activity dramatically increased in dry after-ripened seed, being significantly boosted by ET. The absence of MAN inhibition by IESS suggests that although ET seems to be one of the factors controlling MAN, its presence did not appear to be essential. GA$_{4+7}$ only increased MAN in seeds wich were after-ripened. Here, it is proposed that ET and GAs participate actively in establishing the AR process.

Key words: After-ripening, ethylene, endospermic seed, germination, gibberellin, inhibitors of ethylene synthesis and signalling (IESS), β-mannanase, paclobutrazol, *SoACS7*, *SoACO2*, *SoGA3ox2*, *SoGA20ox2*, *SoGA2ox6*.

Introduction

The seed, which is the dispersal unit in angiosperms and ensures the survival and perpetuation of the mother plant, is formed by zygotic embryogenesis. This complex process, regulated by hormones and a developmental programme (Yamaguchi and Nambara, 2006, and references therein), is divided into two extensive phases called morphogenesis and maturation. During seed maturation, the cell cycle ceases, molecular dependence on the mother plant disappears, water content diminishes, storage products are synthesized, abscisic acid (ABA) accumulates, and primary dormancy is established (reviewed in Hilhorst and Toorop, 1997; Raz *et al.*, 2001; Finkelstein *et al.*, 2002; Kermode, 2005; Weber *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). Dormancy, defined as the failure of an intact viable seed to germinate under favourable conditions, is an adaptive trait optimizing germination to the most suitable time for the seed to complete its life cycle (Finch-Savage and Leubner-Metzger, 2006; Bentsink *et al.*, 2007). Thus, in order for germination to begin, seed dormancy must be lost (Filkelstein *et al.*, 2008).

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After-ripening (AR) is one way to overcome seed dormancy. A recent concept of seed AR suggested that this process triggers a widening or increasing sensitivity of seeds to environmental conditions, promoting germination, at the same time as it narrows or decreases sensitivity to conditions that repress germination (Finch-Savage and Leubner-Metzger, 2006). Seed AR is determined by moisture and oil contents, seed covering structures, and temperature, and requires seed moisture contents above a threshold value (Manz *et al.*, 2005, and references therein). The main seed AR effects can be grouped as: (i) a widening of the temperature range for germination (Oracz *et al.*, 2007); (ii) a lowering of the ABA level and sensitivity plus a rise in sensitivity to gibberellins (GAs) or loss of requirement for GAs (Grappin *et al.*, 2000; Ali-Rachedi *et al.*, 2004; Cadman *et al.*, 2006); (iii) a loss of light requirement for germination of seeds that do not germinate in darkness (Derks and Karssen, 1993) and an increase in seed sensitivity to light in seeds that do not germinate even with light (Derks and Karssen, 1993; Batlla and Benech-Arnold, 2005, and references therein); (iv) a loss of the nitrate requirement (Derks and Karssen, 1993; Alboresi *et al.*, 2005, and references therein); and (v) an accelerated germination velocity (reviewed by Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). However, although the need for AR is well known in several species, it has been hardly studied at the molecular level with respect to changes induced by AR signals in the dry viable seed and their impact during imbibition (Kucera *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008). Recent results indicate that non-imbibed seeds (i.e. dry seeds), characterized by a low moisture level, are competent for both transcription and translation. Thus, the AR process in viable dry seeds can positively or negatively alter the level of several transcripts (Bove *et al.*, 2005; Finch-Savage *et al.*, 2007; Leymarie *et al.*, 2007) and proteins (Chibani *et al.*, 2006). It is probable that there are zones in the dry seed where the moisture level is relatively high (i.e. above the threshold) to allow these alterations (Manz *et al.*, 2005). This partial and localized imbibition environment was called 'low hydration' by Holdsworth *et al.* (2008). The conditions that generate optimal 'low hydration' values for seed AR have been determined (Leubner-Metzger, 2005). Likewise, complex and specific gene networks related to seed AR were recently updated (Finch-Savage and Leubner-Metzger, 2006; Holdsworth *et al.*, 2008, and references therein).

Many plant hormones have been shown to be involved in germination (Kucera *et al.*, 2005). Among these, GAs have long been known as stimulators and ABA as an inhibitor (Finkelstein *et al.*, 2002, 2008; Yamaguchi and Kamiya, 2002; Yamaguchi and Nambara, 2006). However, the role of ethylene (ET) seem less obvious than that of ABA and GAs, since the intervention of ET during the maintenance of seed dormancy and during the transition from dormancy to germination involves a complex network with many steps still to be clarified (Vandenbussche and Van der Straeten, 2007). Thus, opinions vary concerning the developmental stage during which ET regulates dormancy. Some suggest that ET acts minimally during dormancy inception and that its major action is during imbibition to terminate dormancy and/or initiate germination (Matilla and Matilla-Vázquez, 2008, and references therein). In studies using ET response mutants of *Arabidopsis*, endogenous ET promoted seed germination by decreasing sensitivity to endogenous ABA (Beaudoin *et al.*, 2000). ET appears to be a negative regulator of ABA during germination (Ghassemian *et al.*, 2000). In short, ET seems to act antagonistically against ABA during dormancy termination but acts in concert with GAs to promote these transitional changes. Although ET and GAs work together in the process of radicle emergence, the participation of GAs appears to be quantitatively and qualitatively more important. To date, published data indicate that ET is not the hormone that triggers the decisive steps during the appearance and elimination of dormancy in seeds, but rather is part of a complex network of interacting signals involved in dormancy, the details of which are currently difficult to assess with precision. The *etr1-2* mutation confers dominant ET insensitivity and as a consequence results in mature seed populations that exhibit more pronounced primary dormancy (Chiwocha *et al.*, 2005). Moreover, *etr1-2* mutation disrupts ABA homeostasis, and auxin, cytokinin, and GA pathways are all affected in mutant seeds (Chiwocha *et al.*, 2005).

Although the signs of seed germination become visible with radicle emergence, it is unquestionable that during the maturation period (Nakabayashi *et al.*, 2005; Holdsworth *et al.*, 2008), imbibition (Yamaguchi *et al.*, 2004; Finch-Savage and Leubner-Metzger, 2006), and dry storage (Grappin *et al.*, 2000; Holdsworth *et al.*, 2008) of the seed a series of preparatory processes occur to break seed coats. However, the identity of these processes and their hormonal regulation is far from being known in detail at the molecular level (Kucera *et al.*, 2005). Similarly, there are major gaps in our knowledge of the control of the molecular mechanisms that participate in the reduction and elimination of dormancy, as in the case of AR, a temporally and environmentally regulated process in the dry seed, which determines the germination potential and the loss of dormancy (Carrera *et al.*, 2008). At present, most of the information on AR has been provided by studies on tobacco and *Arabidopsis*. However, the *Arabidopsis thaliana* accessions Ler and Col have a weak dormancy that is eliminated by short periods of AR (van der Schaar *et al.*, 1997), making these species less suitable for dormancy studies. In contrast, the Cvi accession, which is considered profoundly dormant because it requires several months of AR, is currently used for genetic and molecular studies of dormancy and AR (Alonso *et al.*, 2003; Ali-Rachedi *et al.*, 2004; Bentsink *et al.*, 2006; Carrera *et al.*, 2008; Holdsworth *et al.*, 2008). In this work, using endospermic seeds of the nitrophilous species hedge mustard, *Sisymbrium officinale* L., in which dormancy is overcome by a long AR, it is demonstrated that the expression pattern of genes involved in ET synthesis (*SoACS7* and *SoACO2*) and in GA synthesis (*SoGA20ox2* and *SoGA3ox2*) and breakdown (*SoGA2ox6*) is notably altered during the imbibition period of after-ripened seeds; these alterations are strongly affected by the presence of ET and/or GA_{4+7}. It is proposed that ET–GA cross-talk exists to overcome seed dormancy by AR.

Materials and methods

Plant material and seed after-ripening treatment

Ripe fruits of hedge mustard, *S. officinale* (L.), were collected in the field in Galicia (north-western Spain) at the time of their natural dispersal (July–August 2006). After harvest, the fruits were dried at room temperature for 1 month to allow separation of seeds from the rest of the fruit (i.e. valves, replum, and pedicel) by hand. After collection, seeds were air-dried for 7 d and mature dark seeds were separated from light ones, which were discarded. Freshly harvested dark seeds (non-after-ripened seeds) were stored dry at 21±0.2 °C for 6 months (after-ripened seeds) until the experiment began. The loss of seed dormancy by AR was demonstrated by means of a germination test. In parallel, fruits of *S. officinale* were collected at two developmental stages: the first involved whole fruits with early development (early fruits; EF) while the second one included both whole fruits and seeds with very advanced development [late fruits (LF) and late seeds (LS)] .

Germination assays

Three replicates of 50 seeds were sown in 90 mm Petri dishes on two layers of filter paper (Whatman No. 1) moistened with 3 ml of sterile 20 mM KNO_3, pH 7.0 (control) supplemented with solutions of gibberellin (100 μM GA_{4+7}, Sigma-Aldrich, Spain), ET (10 μM etephon, Sigma-Aldrich, Spain), an inhibitor of GA synthesis [25 μM paclobutrazol (PB), Sigma-Aldrich, Spain], and a mixture of inhibitors of synthesis [100 μM aminoethoxyvinylglycine (AVG) and 1 mM cobalt chloride (Co_2Cl), Sigma-Aldrich, Spain] and signalling of ET [1 mM silver thiosulphate (STS), Sigma-Aldrich, Spain] called IESS (inhibitors of ET synthesis and signalling).

Germination experiments were conducted in a growth chamber at 24 °C with a 16 h photoperiod (photosynthetic photon flux density of 55 μmol m^{-2} s^{-1}). Seeds were not surface-sterilized in order to avoid influencing their dormancy status; in any case, fungal infections were not detected by light microscopy. Seeds were considered germinated when radicle protrusion was visible. Germination tests were performed at least twice using three replicates. The imbibition period in this study ended immediately before the onset of radicle protrusion. The specificity of the ethephon effects in this study was checked as described in Calvo *et al.* (2004*a*).

Total RNA isolation from seeds and cDNA synthesis

After-ripened and non-after-ripened seeds were imbibed for 0, 3, 6, 12, and 15 h; three replicates of 50 seeds were collected in 2 ml tubes from the Petri dishes, immediately frozen in liquid N_2, and stored at –80 °C until RNA extraction. A grinding ball (stainless steel, 0.7 mm) was added to the tubes, and seeds were finely ground in liquid N_2 using a Mikro-Dismembrator-S (Sartorius AG, Goettingen, Germany) for 2 min at 1500 rpm. For each point, three replicates were taken. Total RNA was isolated using the phenol extraction/LiCl precipitation method (Verwoerd *et al.*, 1989). The integrity and purity of the RNA were checked both electrophoretically and by the 260/280 nm absorbance ratio. Total RNA samples were digested with DNase (DNase I recombinant, RNase-Free, Roche, Switzerland) following the manufacturer's directions. The RNA concentration was estimated by A_{260} measurement, and the samples were stored at –80 °C. Reagents used in this protocol were supplied by Sigma-Aldrich (Spain). The cDNA was synthesized from 1 μg of total RNA using the First-Strand Synthesis kit for RT-PCR (Roche, Switzerland), using oligo-p (dT) as a primer and following the manufacturer's directions. Samples were stored at –20 °C until used.

Isolation of SoACS7, SoACO2, SoGA3ox2, SoGA20ox2, *and* SoGA2ox6 *partial-length cDNA*

The cDNA sequences were obtained from seed RNA using degenerate primer pairs based on highly conserved regions of corresponding genes from other species (Table 1). That is, primers were designed in such a way that they would pick up any *SoACS*, *SoACO*, *SoGA20ox*, *SoGA3ox*, or *SoGA2ox*, respectively. PCR conditions were as follows: 95 °C for 2 min, 40 cycles of 95 °C for 45 s, 47–55 °C for 45 s, 72 °C for 45 s, and a final elongation step of 7 min at 72 °C. PCRs were performed in a 25 μl reaction volume containing 12.5 μl of 2× Super Premix, Sapphire (Mbiotech, Seoul, Korea), 1 μl of forward primer (100 μM, final concentration 4 μM), 1 μl of reverse primer (100 μM, final concentration 4 μM), and 9.5 μl of sterilized water, and finally 1 μl of cDNA. PCR products were analysed electrophoretically and the bands of the expected size were excised and extracted from the agarose gel using a MiniElute™ Gel Extraction Kit (Qiagen, Hilden, Germany), and then

Table 1. List of degenerate primers used for PCR assay in the isolation of partial length cDNAs

Gene	Primer name	Primer sequence (5′–3′)
SoGA3ox2	*FwConGA3ox*	ATGTGGTCNGAAGGNTTCAC
	RvConGA3ox	ATGTGNAANAAGTCACC
SoGA20ox2	*FwConGA20ox*	AADCTNCCNTGGAARGAGAC
	RvConGA20ox	TGBAARCARCTCTTGTA
SoGA2ox6	*FwConGA2ox*	GGNTTYGGAGARCAYWCWGACCC
	RvSoGA2ox	CACTNNTAAAYCTYCCATTNGTCA
SoACO2	*FwConACO*	ATGGAGAGAACATCAAGYTTYCTVTT
	RvConACo	TTAGAATGTCTCCTCVGTNGCCA
SoACS7	*FwConACS*	CCAGGGTTTGATAGAGATTTGAG
	RvConACS	GCAGNSGACGCAAATYCATCC

sequenced. Sequences were compared with existing sequences in target databases using BLAST (Altschul *et al.*, 1997). They contained cDNA sequences of genes with vey high similarity to *GA3ox*, *GA20ox*, *GA2ox*, *ACO*, and *ACS* genes of other plant species (GenBank databases). They were named *SoACS7*, *SoACO2*, *SoGA3ox2*, *SoGA20ox2,* and *SoGA2ox6*, and registered in GenBank under the accession numbers EU689114, EU689115, EU689111, EU689113, and EU689112, respectively.

Real-time semi-quantitative PCR assay

Semi-quantitative PCR analysis was performed with the cDNA obtained as described above as a template. Specific primer design was performed using the sequences found for *SoGA3ox2*, *SoGA20ox2*, *SoGA2ox6*, *SoACO2*, and *SoACS7* (Table 2). Meanwhile, *18S RNA* was used as a control for the genes studied, since it was found to be expressed at constant levels throughout the study period (Supplementary Figs S1, S2 available at *JXB* online). The PCR was performed in an iCycler iQ™ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA, USA). For each 25 μl reaction, a 1 μl cDNA sample was mixed with 12.5 μl of IQ™ SYBR® Green Supermix (Bio-Rad Laboratories), 0.5 μl of forward primer (12 μM, final concentration 240 nM), 0.5 μl of reverse primer (12 μM, final concentration 240 nM), and 10.5 μl of sterilized water. Samples were subjected to thermal cycling conditions of DNA polymerase activation at 95 °C for 4 min, 40 cycles of 45 s at 95 °C, 45 s at 52 °C (for *SoGA20ox2* and *SoGA2ox6*) or 55 °C (for *SoGA3ox2*, *SoACO2*, and *SoACS7*), 45 s at 72 °C, and 45 s at 80 °C; a final elongation step of 7 min at 72 °C was performed. The melting curve was designed to increase 0.5 °C every 10 s from 62 °C (for *SoGA20ox2* and *SoGA2ox6*) or 65 °C (for *SoGA3ox2*, *SoACO2*, and *SoACS7*). Real-time PCR analysis was performed with two different cDNAs from the same time point (from two different RNAs), and each was made in triplicate. The amplicon was analysed by electrophoresis and sequenced once for identity confirmation. Real-time PCR efficiency was estimated via a calibration dilution curve and slope calculation. Expression levels were determined as the number of cycles needed for the amplification to reach a threshold fixed in the exponential phase of the PCR (C_T). The $\Delta\Delta C_T$ method was used to analyse data (Pfaffl, 2001). In order to observe the alterations in the transcript levels, the expression in dry seeds was used to relativize data (Finch-Savage *et al.*, 2007).

Table 2. List of primers used for the real-time PCR assay

Gene	Primer name	Primer sequence (5′–3′)	Amplicon size (bp)
SoGA3ox2	*FwSoGA3ox2*	CTGTGGTTGGCATTAGGTTC	168
	RvSoGA3ox2	GAGAGTTGAGTCGGTATGGG	
SoGA20ox2	*FwSoGA20ox2*	GGTCTTGGTGAAGGATGG	157
	Rv SoGA20ox2	AAGATCATGGAGCTTCTGG	
SoGA2ox6	*Fw SoGA2ox6*	GTAGATGGACTTGAGATTTGC	89
	Rv SoGA2ox6	CAGTCACCGACCAATACG	
SoACO2	*FwSoACO2*	GGTGATAACCAACGGCAAGT	89
	RvSoACO2	TGTAGAACGAGGCAATGGAC	
SoACS7	*FwSoACS7*	GGCTTCTATGTTGTCGGA	113
	RvSoACS7	CGATCCCTGCCTTCTTA	
18S RNA	*Fw18S-RNA*	GGCTCGAAGACGATCAGATA	87
	Rv18S-RNA	TCATAAGGTGCCGGCGGAGT	

Endo-β-mannanase (EC 3.2.1.78) activity

Triplicate lots of after-ripened and non-after-ripened seeds were ground in 1 M sodium acetate buffer, pH 4.7 (Sigma Aldrich, Spain). After centrifugation at 20 000 *g* at 4 °C for 45 min, the supernatants were assayed in duplicate for endo-β-mannanase (MAN) activity. For enzymatic determination, 100 μl of 0.25% (w/v) AZC L-galactomannan (Megazyme International Ireland Ltd, Wicklow, Ireland) in 100 mM sodium acetate buffer, pH 4.7) were mixed with 25 μl of supernatant and incubated at 28 °C for 3 h, with constant agitation in an orbital shaker. Dye release from AZC L-galactomannan was determined spectrophotometrically by measuring the absorbance at 590 nm in supernatant samples of the reaction mixture. One unit of MAN activity was defined as the amount of enzyme that releases 1 nmol of reducing sugar equivalent to D-mannose per minute under the above conditions. A curve relating dye release from AZC L-galactomannan to reducing sugar release from locust bean gum (Sigma Aldrich) as determined by the PAH–BAH method (Lever, 1972) was constructed and used for interconversion of mannanase activities.

Results

Germination characteristics in after-ripened seeds

Freshly harvested and mature dark *S. officinale* seeds hardly germinated. However, the germination rate (i.e. percentage of seeds that are likely to germinate) increased with the time of dry storage at 21±0.2 °C (Table 3), implying that AR was strongly involved in breaking dormancy of these endospermic seeds. On the other hand, the absence of nitrate strongly delayed the germination, and complete germination was reached after 73 h in after-ripened seeds, as opposed to only 5±1% without AR (Fig. 1). Likewise, AR in *S. officinale* seeds broadened the range of optimal germination temperatures. Hence, the highest germination percentage was reached between 20 °C and 30 °C in after-ripened seeds, and emergence of the radicle occurred far earlier than in the seed lot without AR, in which germination peaked at 30 °C (Fig. 2). The water uptake rate during imbibition, which was sigmoidal in *S. officinale* seeds, was also affected by AR. Non-after-ripened seeds imbibed hardly any more than did seeds after AR was fully established (Fig. 3). This varying imbibition pattern does not appear to be related to mucilage production by epidermal tissue of the seed coat, since both after-ripened and non after-ripened seeds showed the same secretion

Table 3. Effect of GA_{4+7} and ethephon on germination percentage of *S. officinale* seeds with (6 months) and without (freshly harvested seeds) after-ripening

IESS, inhibitors of ET synthesis and signalling; PB: paclobutrazol; 0, not found

Treatment		**Germination (%)***						
		15 h	**18 h**	**19 h**	**20 h**	**21 h**	**22 h**	**26 h**
Control	Not after-ripened	0	0	0	0	0	2±1 a	8±1 b
	After-ripened[†]	0	0	0	3±1 a	16±2 c	27±5 d	53±4 f
	After-ripened	0	0	2±1 a	9±2 b	40±3 e	51±5 f	100±4 i
GA_{4+7}	Not after-ripened	0	0	0	0	5±2 a	7± 2 a	14±3 b
	After-ripened	4±2 a	10±3 b	15±2 c	25±4 d	64±3 g	92±7 h	100±2 i
PB	Not after-ripened	0	0	0	0	0	1±1 a	2±1 a
	After-ripened	0	2±1 a	3±1 a	4±1 a	4±1 a	8±2 b	19±4 c
PB+GA_{4+7}	Not after-ripened	0	0	0	0	2±1 a	6±1 a	12±1 b
	After-ripened	1±1 a	8±1 b	16±2 c	23±3 d	60±5 g	91±4 h	100±2 i
ET	Not after-ripened	0	0	0	0	2±1 a	5±1 a	12±3 b
	After-ripened	0	8±2 b	11±3 b	24±3 d	62±4 g	89±6 h	100±3 i
IESS	Not after-ripened	0	0	0	0	0	4±2 a	4±2 a
	After-ripened	0	0	0	0	2±1 a	7±2 b	61±1 g
IESS+ET	Not after-ripened	0	0	0	0	0	4±1 a	10±2 b
	After-ripened	0	5±1 a	10±2 b	21±1 c	59±2 g	85±3 h	100±1 i

[*] Data are mean values of three replicates ±SE. Significant differences between values as assessed by LSD test ($P < 0.05$) are shown as different letters (Steel and Torre, 1982).

[†] Dry seeds stored at 21±0.2 °C for 3 months.

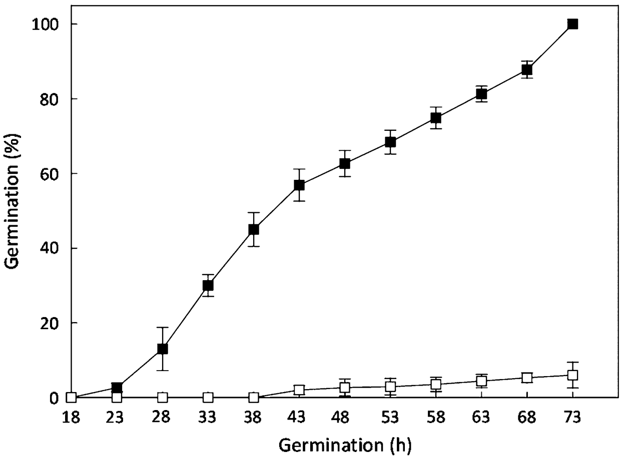

Fig. 1. Germination percentage at 24 °C of *Sisymbrium officinale* seeds in the absence of 20 mM KNO_3. After-ripened seeds (filled squares); non-after-ripened seeds (open squares). Data are means ±SE of three independent experiments.

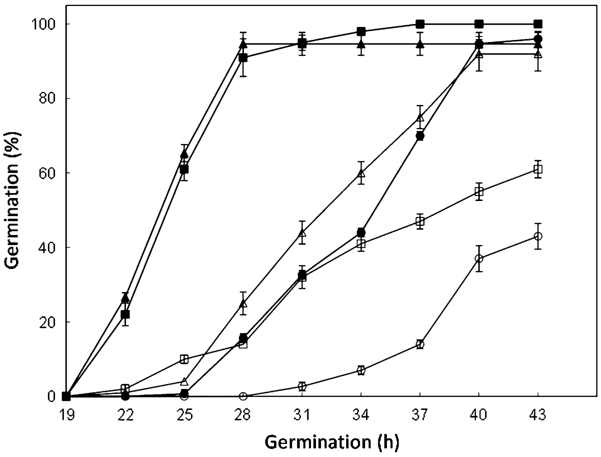

Fig. 2. Germination percentage of *Sisymbrium officinale* seeds at different germination temperatures in the presence of 20 mM KNO_3. Filled symbols, after-ripened seeds; open symbols, non-after-ripened seeds. Circles, 20 °C, squares, 24 °C; triangles, 30 °C. Data are means ±SE of three independent experiments.

capacity (data not shown). Moreover, the water uptake rate was altered by the imbibition temperature, increasing with temperature (i.e. 20, 24, or 30 °C) in non-after-ripened seeds, but peaking at 24 °C in after-ripened seeds (Fig 3).

Prior to the investigation of the molecular effects of GAs and ET during imbibition of after-ripened seeds, a thorough study of the effect of both hormones on germination was performed by quantifying the radicle emergence over short time periods (Table 3). In control seeds (20 mM KNO_3), radicle protrusion began to be detectable at 19 h, and reached 100% after 26 h. At this time point, only 8% of the non-after-ripened seeds germinated. The presence of GA_{4+7} advanced and strongly stimulated germination in the after-ripened seeds between 18 h and 22 h, whiles carcely affecting non-after-ripened seeds (Table 3). A similar profile was found in the presence of exogenous ethephon (Table 3) or the ET immediate precursor ACC (data not shown). Together, GA_{4+7} and ethephon were incapable of boosting the germination percentage with respect to individual hormones (data not shown). The addition of PB, a widely used inhibitor of GA synthesis, or a mixture of IESS (i.e.

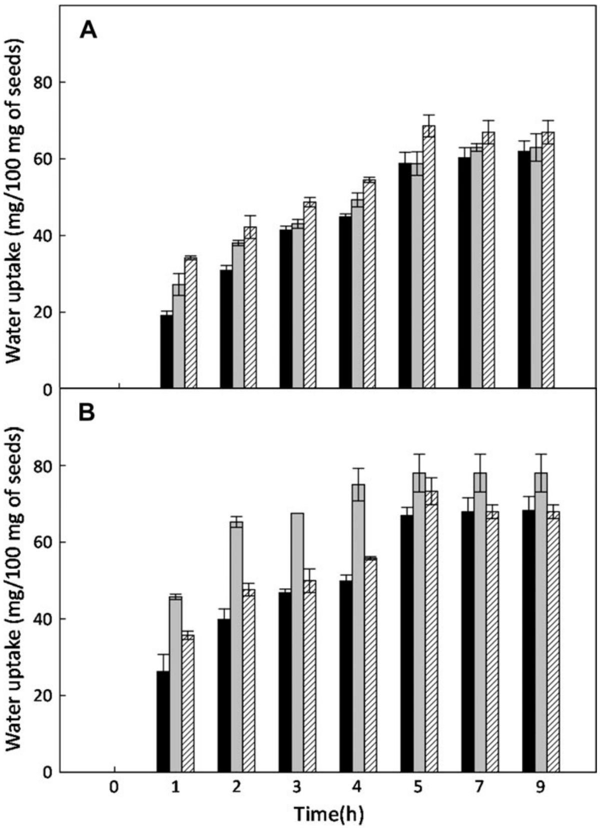

Fig. 3. Water uptake of *Sisymbrium officinale* seeds during the first 9 h of imbibition at different temperatures in the presence of 20 mM KNO_3. (A) Non-after-ripened seeds. (B) After-ripened seeds. Black bars, 20 °C; grey bars, 24 °C; striped bars, 30 °C. Data are means ±SE of three independent experiments.

AVG+$CoCl_2$+STS) strongly depressed radicle emergence in both after-ripened and non-after-ripened seeds (Table 3). In contrast, the inhibition provoked by PB and IESS was overcome by the addition of GA_{4+7} and ethephon, respectively (Table 3).

Alterations in SoACS7*,* SoACO2*,* SoGA20ox2*,* SoGA3ox2*, and* SoGA2ox6 *expression in late embryogenesis and dry seed with and without AR*

Prior to the study of the alterations of the transcripts provoked by AR during the imbibition of *S. officinale* seeds, the expression of *SoACS7*, *SoACO2*, *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6* was evaluated during embryogenesis. The objective of this experiment was to determine whether these genes were active during seed formation and whether the level of their transcripts was altered by AR in ripe seed. For this, whole seeds showing early development (early fruits, EF) and full development (late fruits, LF) were collected, as well as seeds in the desiccation phase (LS), seeds submitted to AR (after-ripened seeds, AS), and those which were not (non-after-ripened seeds, NAS).

With the gene expression in AS as control, the following results were found. (i) The level of *SoACO2* mRNA increased concomitantly with development and during seed desiccation; however, AR lowered the level of this transcript (Fig. 4A). (ii) The expression of *SoACS7* was very abundant during embryogenesis, diminishing with the desiccation process and very strongly with AR (Fig. 4B). (iii) The *SoGA3ox2* transcript level increased with development and strongly decreased with desiccation; AR induced a notable expression of this gene related to the synthesis of bioactive GAs (Fig. 4C). (iv) *SoGA20ox2* expression was quantitatively very strong only in the early developmental phases, and AR hardly affected the level of this transcript (Fig. 4D). (v) The transcription of *SoGA2ox6* increased with development and desiccation, negatively affecting AR at the level of *SoGA2ox6* mRNA (Fig. 4E).

After-ripening alters SoACS7*,* SoACO2*,* SoGA20ox2*,* SoGA3ox2*, and* SoGA2ox6 *expression patterns during early imbibition*

In order to study the effect of AR on the expression of genes that are involved in ET synthesis and in GA synthesis and breakdown, homologues of *ACS*, *ACO*, *GA20ox*, *GA3ox*, and *GA2ox* were isolated. They were cloned by means of the primer strategy mentioned in Materials and methods. In total, five partial cDNAs (*SoACS7*, *SoACO2*, *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6*) were isolated, and their phylogenetic relationships to the known genes are shown in Supplementary Figs S3–S7 at *JXB* online. The molecular mechanism operating during the imbibition phase of after-ripened seeds is at present largely unknown. The notable differences observed in the germination rate of after-ripened and non-after-ripened seeds in the presence of ethephon, GA_{4+7}, or inhibitors (Table 3) led to the analysis of the effect of AR on alterations in the accumulation of five transcripts involved in the synthesis of ET (*SoACS7* and *SoACO2*) and GAs (*SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6*) during the early imbibition period (0–15 h). In the control (20 mM KNO_3), the following results were recorded. (i) The *SoACS7* transcript was expressed only at the beginning of imbibition (3 h) in seeds that were not after-ripened, and the AR process eliminated this expression (Fig. 5A). (ii) The level of *SoACO2* transcript was very high in non-after-ripened seeds at 3 h and strongly diminished up to 12 h, increasing afterwards; AR reduced transcript accumulation during the first 6 h of imbibition (Fig. 5C). (iii) *SoGA20ox2* mRNA levels were almost similar at 3, 12, and 15 h, hardly being affected by the AR process; however, AR strongly increased the lowest transcript level found at 6 h (Fig. 6A). (iv) The transcript accumulation pattern found for the *SoGA3ox2* gene (Fig. 6G) was similar to that of *SoGA20ox2*. (v) The expression of *SoGA2ox6* was the lowest of all GA-oxidases studied in this work, notably at 6 h in non-after-ripened seeds; and the expression levels at 12 h and 15 h imbibition were slightly increased by AR (Fig. 6M).

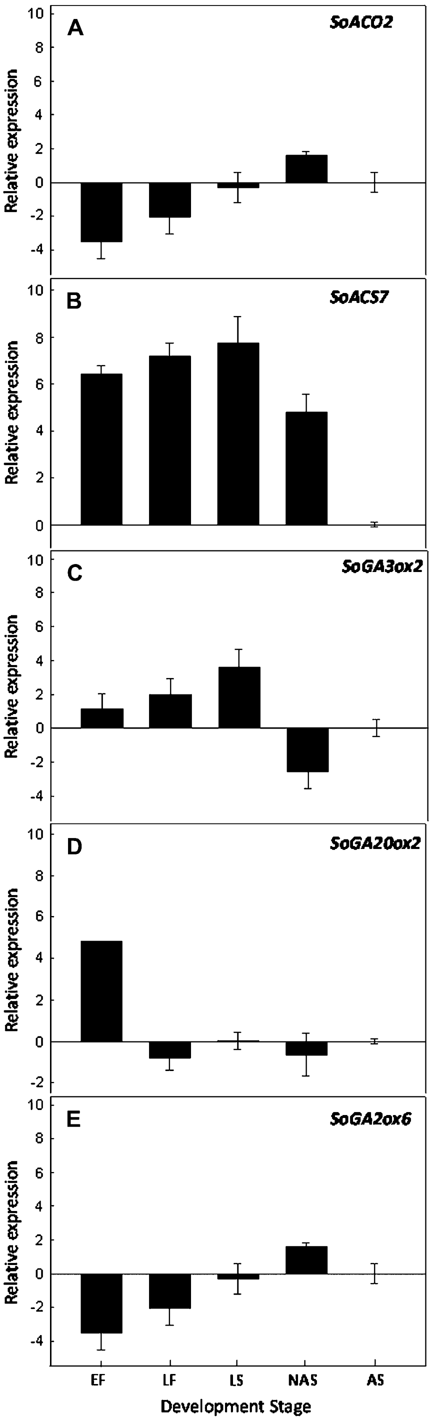

Fig. 4. Transcript analysis by real-time PCR of *SoACO2*, *SoACS7*, *SoGA3ox2*, *SoGA20ox2*, and *SoGA2ox6* (A–E) during the development of the fruit and seed of *S. officinale*. EF, early fruit; LF, late fruit; LS, late seed; NAS, non-after-ripened dry seed; AS, after-ripened dry seed. Error bars indicate the standard deviations of three independent experiments.

The GA$_{4+7}$ treatments, compared with the control, strongly reduced the expression of *SoACO2* in non-after-ripened seeds, this expression being stimulated by AR (Fig. 5E). PB induced a *SoACO2* expression pattern resembling that produced by GA$_{4+7}$, but quantitatively higher (Fig. 5G), the stimulation of expression surpassing that of the control in after-ripened seeds (Fig. 5C, G). No *SoACS7* transcription was detected in the presence of GA$_{4+7}$ or PB in either seed lot. GA$_{4+7}$ provoked a notable accumulation of *SoGA20ox2* transcript in non-after-ripened seeds at 6 h and 15 h imbibition, and AR diminished this accumulation compared with the control and GAs treatments (Fig. 6A, B). The expression of *SoGA20ox2* increased during imbibition in the presence of PB and, even though hardly any differences were found in after-ripened seeds, the AR process induced more *SoGA20ox2* transcripts than in the control at 12 h and 15 h (Fig. 6A, C). At the beginning of imbibition, AR caused the absence of *SoGA3ox2* expression in the presence of GA$_{4+7}$ and PB (Fig. 6H, I). However, in the presence of GA$_{4+7}$ and PB, the accumulation of *SoGA2ox6* transcripts was strongly stimulated by AR (Fig. 6N, O).

In the presence of ethephon-derived ET, the following results were obtained. (i) *SoACS7* transcript accumulation was strongly inhibited, and, as in the control, no transcripts were detected in after-ripened seeds (Fig. 5B); on the other hand, no transcription was found in the presence of either IESS or ethephon+GA$_{4+7}$. (ii) In non-after-ripened seeds, a significant decline with respect to control was found in the level of *SoACO2* transcripts during the first 6 h of imbibition, then rising until the end of the imbibition, unlike those of the control. AR consistently induced lower transcript accumulation (Fig. 5D). The *SoACO2* expression pattern in the presence of IESS was very similar to that in the presence of GA$_{4+7}$ (Fig. 5H, E), whereas ethephon and GA$_{4+7}$ added together registered the lowest *SoACO2* transcript accumulation of all treatments studied (Fig. 5F). (iii) The *SoGA20ox2* expression pattern was qualitatively similar to that of the control, but ethephon notably lowered the transcript level during the first 12 h of imbibition in after-ripened seeds (Fig. 6D). (iv) The presence of IESS strongly decreased the *SoGA20ox2* transcript accumulation at the beginning of imbibition of non-after-ripened seeds, this treatment being the only one in which AR stimulated transcripts during imbibition (Fig. 6E). When ethephon and GA$_{4+7}$ were added together, the *SoGA20ox2* transcript levels were quantitatively lower than when both hormones were added separately (Fig. 6F). (v) Treatments with ethephon, IESS, and ethephon+GA$_{4+7}$ strongly inhibited the *SoGA3ox* expression in after-ripened and non-after-ripened seeds (Fig. 6J–L). (vi) The *SoGA3ox2* mRNA accumulation in the presence of ethephon was similar to that of seeds treated with PB (Fig. 6I, J), whereas this *SoGA3ox2* transcript accumulation was similar in the presence of IESS and ethephon+GA$_{4+7}$, showing strong inhibition, between 6 h and 15 h, compared with the ethephon treatment in after-ripened seeds (Fig. 6J–L). (vii) In the presence of ethephon, the accumulation of *SoGA2ox6* transcripts was strongly stimulated by AR (Fig. 6P), this

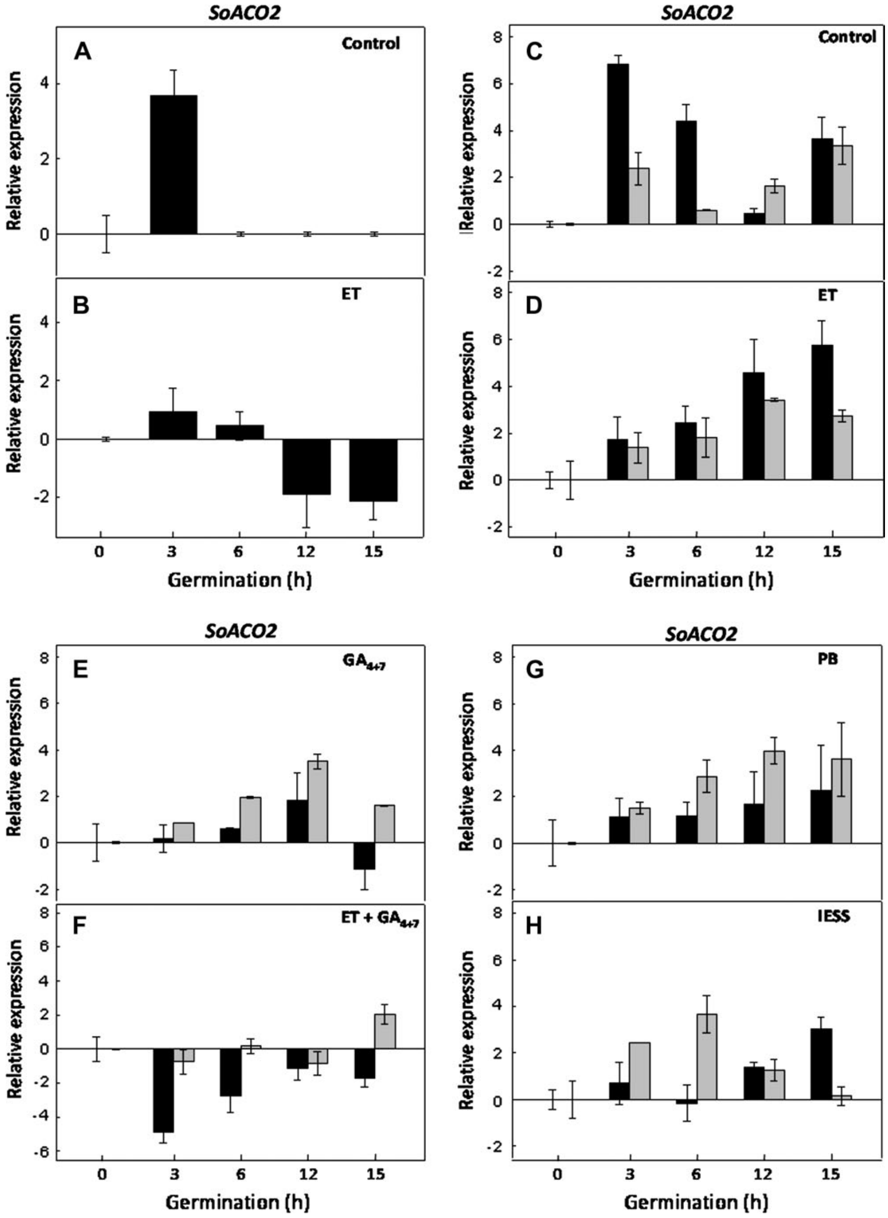

Fig. 5. Transcript analysis by real-time PCR of *SoACS7* and *SoACO2* during the time course of imbibition at 24 °C of *S. officinale* seeds. *SoACS7*: (A) control and (B) ethephon. Transcription in after-ripened seeds was not detected. *SoACO2*: (C) control; (D) ethephon; (E) GA$_{4+7}$; (F) ethephon+GA$_{4+7}$; (G) PB; (H) IESS. Non-after-ripened seed (black bars); after-ripened seed (grey bars). Error bars indicate the standard deviations of three independent experiments.

accumulation being strongly inhibited by IESS (Fig. 6Q), and the presence of ethephon+GA$_{4+7}$ was not capable of overcoming this inhibition (Fig. 6R).

Alterations in β-mannanase activity induced by AR in the presence of ET and GA$_{4+7}$

AR strongly altered MAN activity both in dry seeds and during imbibition. In control seeds, the main difference took place in dry seeds, where AR provoked the enzymatic activity ~12-fold more than in non-after-ripened seeds, in which the MAN activity increased slightly as imbibition progressed (Fig. 7A). In addition, the AR notably boosted enzymatic activity during the first 3 h of imbibition, but clearly depressed it between 6 h and 12 h compared with the non-after-ripened seeds (Fig. 7A). In both seed lots, MAN activity was considerable prior to radicle emergence (Fig. 7A). The ethephon treatment substantially affected the MAN activity, so that, in non-after-ripened seeds, ethephon caused strong stimulation in the initial (0–3 h) and final imbibition phases (12–15 h). However, in after-ripened seeds, ethephon stimulated the enzymatic activity throughout the entire study

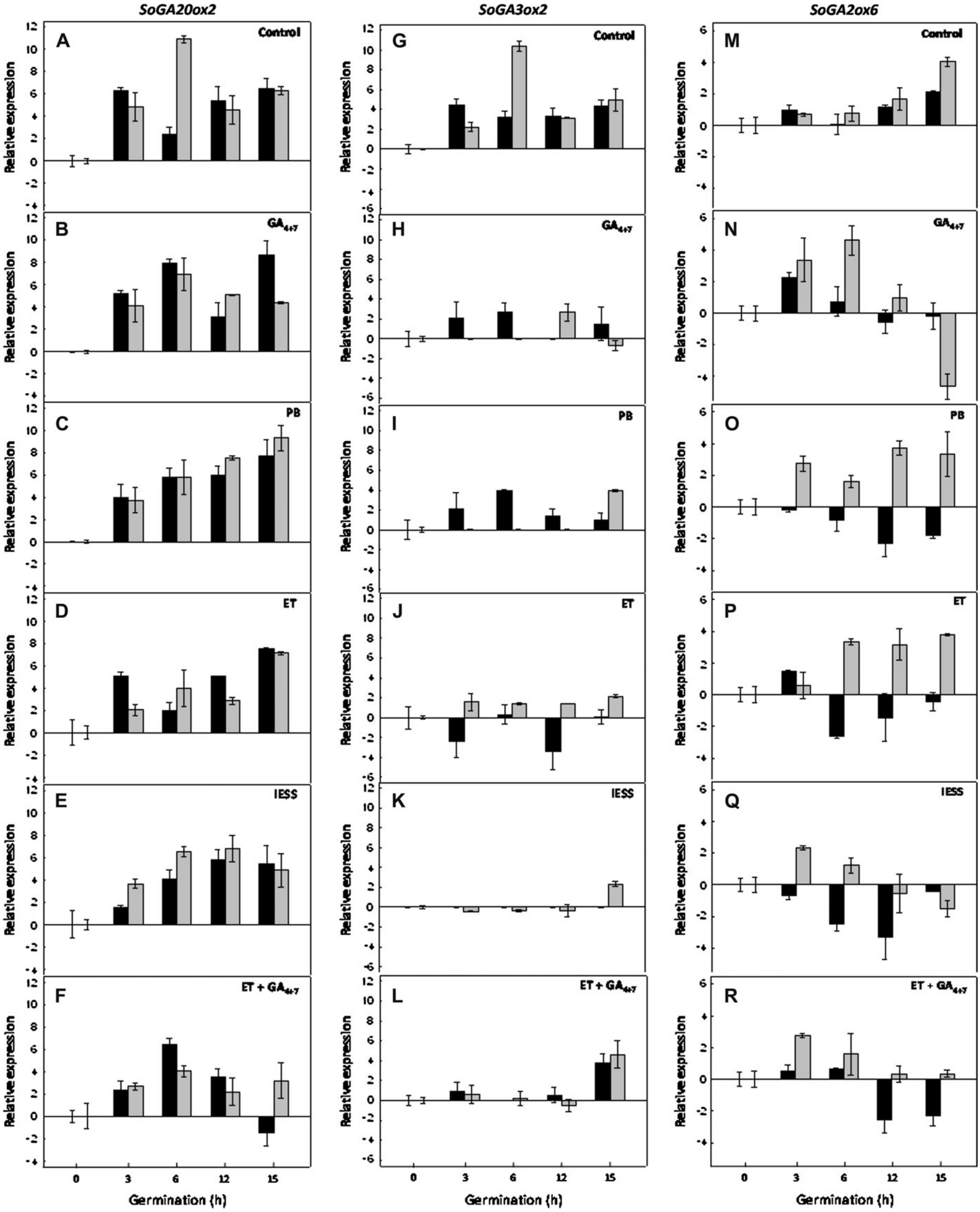

Fig. 6. Transcript analysis by real-time PCR of *SoGA20ox2, SoGA3ox2,* and *SoGA20x6* during the time course of imbibition at 24 °C of *S. officinale* seeds. *SoGA20ox2*: (A) control; (B) GA_{4+7}; (C) PB; (D) ethephon; (E) IESS; (F) ethephon+GA_{4+7}. *SoGA3ox2*: (G) control; (H) GA_{4+7}; (I) PB; (J) ethephon; (K) IESS; (L) ethephon+GA_{4+7}. *SoGA2ox6*: (M) control; (N) GA_{4+7}; (O) PB; (P) ethephon; (Q) IESS; (R) ethephon+GA_{4+7}. Non-after-ripened seed (black bars); after-ripened seed (grey bars). Error bars indicate the standard deviations of three independent experiments.

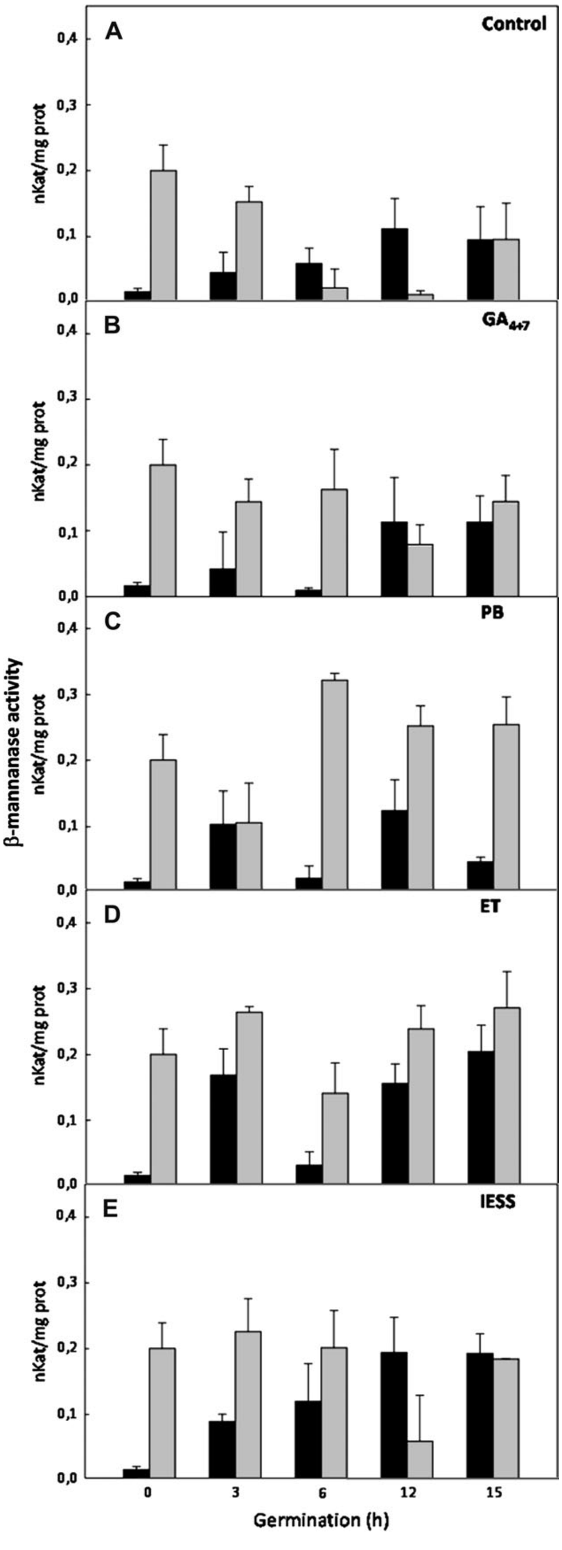

Fig. 7. Analysis of endo-β-mannanase activity during the time course of imbibition at 24 °C of *S. officinale* seeds. (A) Control; (B) GA_{4+7}; (C) PB; (D) ethephon; (E) IESS. Non-after-ripened seed (black bars); after-ripened seed (grey bars). Error bars indicate the standard deviations of three independent experiments.

period compared with the control (Fig. 7A) and the non-after-ripened seeds, as well as those treated with ethephon (Fig. 7D). The presence of IESS in after-ripened and non-after-ripened seeds sharply boosted MAN activity throughout the study period (Fig. 7E). The presence of GA_{4+7} in the non-after-ripened seeds resulted in a profile of MAN which was qualitatively and quantitatively similar to that of the control (Fig. 7A, B) but quantitatively far lower than in the presence of ethephon (Fig. 7B, D). However, the enzymatic stimulation by GA_{4+7} in after-ripened seeds was quantitatively lower than that found with ethephon (Fig. 7B, D). Finally, after the first 3 h of imbibition of after-ripened seeds, PB induced the greatest stimulation in the enzymatic activity of all the treatments studied, but hardly altered the activity in non-after-ripened seeds (Fig. 7C).

Discussion

AR affects germination and some related parameters

In this study, it is shown that the AR process affects the germination of *S. officinale* seeds in four ways. First, AR was incapable of replacing or preventing the presence of NO_3^- in the germination medium. The role of NO_3^- in the alleviation of dormancy by low temperatures (i.e. stratification) is strongly supported by previous results in *Arabidopsis*, suggesting a notable role for NO_3^- transported by the mother plant to the seed to promote germination through a complex signalling network in which the ABA and GA pathways may be involved (Ali-Rachedi *et al.*, 2004; Alboresi *et al.*, 2005). Recently, it was demonstrated in the Cvi accession that the seeds first become sensitive to NO_3^-, then to cold, and finally to light (Finch-Savage *et al.*, 2007). It was tentatively concluded that increased NO_3^- accumulation and reduction convey a signal to break dormancy rather than to function as a nitrogen source for nutrition (Finch-Savage *et al.*, 2007). However, little is known about the role of NO_3^- in dry AR at moderate temperatures. The presence of a signalling pathway for the NO_3^- in *S. officinale* seeds was initially suggested due to the possible presence of receptors and because the effect of the NO_3^- in promoting germination was independent of its reduction in the plant (Hilhorst and Karssen, 1989; Hilhorst, 1990). The results found in this study point to the idea that the NO_3^- signalling networks and AR engage in cross-talk, given the strongly positive effect of AR on the stimulation of radicle emergence in medium without NO_3^- compared with medium with NO_3^- (i.e. protrusion 50 h ahead, comparing Fig. 1 and Fig. 2). That is, it cannot be ruled out that NO_3^- affects a very early stage of the imbibition of after-ripened seeds, provoking greater effectiveness in the breaking of dormancy. In support of this hypothesis, it was previously demonstrated that NO_3^- positively altered other signalling pathways and levels of hormones involved in the germination of other species. Thus, in seeds of the *Arabidopsis* accession Ler, NO_3^- provoked a reduction in the light requirement (Batak *et al.*, 2002), and altered the ABA levels in seeds of Cvi during early imbibition (Ali-Rachedi *et al.*, 2004). Other nitrogenous molecules (e.g. nitric oxide and nitrite) stimulate germination in *Arabidopsis* (Bethke *et al.*, 2004). However, it is not known whether they do so *per se* or whether this happens because they are metabolic derivatives of NO_3^-. Secondly, in the presence of NO_3^-, AR in *S. officinale* broadened the optimal temperature range for germination (i.e. 24–30 C), accelerating the protrusion compared with non-ripened seeds. This widening of the temperature range compatible with good germination was also described in *Avena sativa* and *Bromus tectorum* after-ripened seeds [Corbineau *et al.*, 1986; Bair *et al.*, 2006; and updated by Leubner-Metzger (http://www.seedbiology.de)]. Dry AR may represent a natural mechanism for controlling dormancy release in dry climates (Probert, 2000) and it is widely accepted that the temperature is the greatest regulator of dormancy cycles in the soil (Probert, 2000; Baskin and Baskin, 2004).

Thirdly, the AR in *S. officinale* induced a notable sensitivity to ET and GA_{4+7}, both hormones strongly stimulating germination. The germination profiles in the presence of ET and GA_{4+7} are very similar. PB and IESS strongly inhibited the effect induced by ET and GA_{4+7}. Although the intervention of the GAs in the avoidance of dormancy in endospermic seeds appears to be beyond any doubt, the role of ET is far from being known in detail. Briefly, ET seems to act in concert with GAs to promote germination; however, the participation of GAs appears to be quantitatively and qualitatively more important (reviewed in Matilla and Matilla-Vázquez, 2008, and references therein). Fourthly, AR quantitatively altered the initial seed water uptake rate, with 24 °C (used in this work) being the temperature at which the seed is most rapidly imbibed. However, although the entry of water is more rapid in after-ripened seeds, this imbibition must be tightly controlled in order to initiate the normal germination process. Currently, there are no scientific data to explain this difference in water uptake rate between after-ripened and non-after-ripened seeds. The secretion of mucilage by the seed coat during hydration could act as a mechanism to control the entry of water, affecting the seed viability and germination (Western *et al.*, 2000; Penfield *et al.*, 2001; Rautengarten *et al.*, 2008). The fruit of *S. officinale* contains mixospermous seeds that are heterogeneous with respect to the colour of their seed coat, and the dark seeds (used in this work) have: (i) a greater capacity to secrete mucilage; (ii) a slower and controlled water uptake rate; and (iii) a far faster protrusion of the radicle than in the other population (Iglesias-Fernández *et al.*, 2007). However, the AR process does not alter mucilage production (data not shown), apparently ruling out that this hygroscopic compound may function to enhance and control the water uptake during *S. officinale* seed imbibition.

AR alters the expression patterns of SoACS7, SoACO2, SoGA20ox2, SoGA3ox2, *and* SoGA2ox6 *genes during the imbibition period*

To gain knowledge at the molecular level concerning hormonal regulation of AR in *S. officinale* seeds, the expression patterns of genes involved in ET synthesis (i.e. *SoACS* and *SoACO*) and GA metabolism (i.e. *SoGA3ox*, *SoGA20ox*, and *SoGA2ox*) were studied. Previously, it was shown that the level of *SoACO2*, *SoGA3ox2*, and *SoGA2ox6* transcripts rose during development. Notably, the content in the transcripts corresponding to *SoACO2* and *SoGA2ox6* increased with the seed desiccation process, decreasing *SoACS7*, *SoGA20ox2*, and *SoGA3ox2*. However, except for the expression of *SoGA3ox2* which was markedly increased, and *SoGA20ox2*, AR triggered a major fall in the level of the rest of the transcripts studied, above all *SoACS7*, a gene strongly expressed during embryogenesis. Taking into account that the expression in after-ripened dry seeds was used to normalize data, the existence of transcription in dry *S. officinale* seeds is evident. Transcriptional activity in environments which are hardly hydrated, such as that in dry seeds, is under debate. However, the discovery of zones with high hydration in after-ripened tobacco seeds has led to strong expectations that this enigma can be deciphered (Leubner-Metzger, 2005; Manz *et al.*, 2005). The cDNA-AFLP analysis of *Nicotiana plumbaginifolia* (Bove *et al.*, 2005) and barley (Leymarie *et al.*, 2007) demonstrates that the great majority of the transcripts studied declined in abundance during AR. Global transcript analysis in *Arabidopsis* using microarrays also showed that the expression level of 30 genes, including *DOG1*, decreased during AR (Finch-Savage *et al.*, 2007). Although the dry seeds may contain stored mRNAs from the final phases of embryogenesis with a function far from being known, the present results also suggest that after-ripened and non-after-ripened dry seeds have the capacity for transcription. The confirmation of this capacity will be important in order to delve into the mechanism of AR in *S. officinale*.

Although the alteration in the expression of various gene groups has been studied in *Arabidopsis* during the breaking of dormancy by stratification and AR (Yamaguchi *et al.*, 2004; Finch-Savage *et al.*, 2007; Holdsworth *et al.*, 2008, and references therein), there are no detailed studies at the molecular level on the hormonal regulation of the mechanisms induced by the AR process at the onset of germination. It has been demonstrated here that in the very early phase of imbibition (i.e. the first 6 h), AR strongly inhibits the expression of *SoACS7* and *SoACO2*, whereas it stimulates the expression of *SoGA20ox2*, *SoGA3ox2*, and *SoGA2ox6*. This indicates that the preparation for radicle protrusion during the imbibition phase under the AR process requires strong stimulation of GA synthesis and has less need for the stimulation of ET synthesis. That is, the need for and participation of GAs and ET appear to differ in after-ripened and non-after-ripened seeds in early imbibition. As occurs in *Arabidopsis* (De Grauwe *et al.*, 2007; Weiss and Ori, 2007), cross-talk clearly takes place between ET and GAs in *S. officinale*. Major germination-associated changes in the transcriptome of *A. thaliana* were evident within 6 h of the initiation of imbibition (Nakabayashi *et al.*, 2005; Holdsworth *et al.*, 2008). The expression of *SoACO2* and *SoACS7* is inhibited very rapidly by ethephon (i.e. the first 3 h). However, while

SoACO2 expression increased during the progression of imbibition and was inhibited by the AR process, the expression of *SoACS7* sharply diminished and was not detectable in after-ripened seeds. Petruzzelli *et al.* (2000) have reported that in pea seeds ET provokes a positive feedback that raises the *Ps-ACO1* mRNA level; and Hermann *et al.* (2007) have shown that the ACO transcript is accumulated in *Beta vulgaris* seeds upon imbibition. On comparing after-ripened and non-after-ripened seeds when *SoACO2* expression was stimulated by ethephon, it was again concluded that the intensity of *SoACO2* expression was lower in after-ripened seeds. The strong *SoACO2* expression observed during the first 6 h in seeds not treated with ethephon was probably related to the production of ET involved in the protrusion. This assumption is supported by the fact that, in the presence of ethephon, *SoACO2* expression was inhibited compared with seeds not treated with ethephon. The role of ET during the imbibition phase is not known and its role in the removal of dormancy is debated (Matilla and Matilla-Vázquez, 2008), but it has been demonstrated that both ET biosynthesis and sensitivity are important for seed germination of *Arabidopsis* (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Kucera *et al.* 2005). Thus, there are seeds in which ET is not required for dormancy maintenance or release, nor is it needed for germination to start (Matilla, 2000; Kucera *et al.* 2005; Gianinetti *et al.*, 2007). The inhibition of *SoACO2* expression in non-after-ripened seeds in the presence of GA_{4+7}, PB, or ISSE, and notably with ET+GA_{4+7}, as well as the clear effect of AR in reversing this inhibition, strongly indicated the presence of cross-talk between the two hormone signalling pathways. The existence of some cross-talk between ET and GAs to regulate *FsACO1* gene expression during the breaking of dormancy in stratified *Fagus silvatica* seeds has previously been shown (Calvo *et al.*, 2004*a*).

Previous works have demonstrated that the synthesis and perception of GAs are essential for seed germination in *Arabidopsis* (Ogawa *et al.*, 2003). Central players are the *GA3ox* and *GA2ox* gene families involved in GA biosynthesis and breakdown, respectively (Yamaguchi *et al.*, 2004; Mitchum *et al.*, 2006). Thus, the *GA3ox1* gene, but not the *GA3ox2* gene, is induced by stratification during seed imbibition (Yamaguchi *et al.*, 2004; Mitchum *et al.*, 2006). In this study, it was demonstrated that the AR process provokes a strong expression of *SoGA3ox2* and *SoGA20ox2* genes at the onset of imbibition, *SoGA3ox2* expression being fully inhibited by GA_{4+7}, PB, ethephon+GA_{4+7}, and ISSE. However, exogenous ethephon, which considerably lowers the level of *SoGA3ox2* transcripts in non-after-ripened seeds, can slightly raise *SoGA3ox2* expression in after-ripened seeds. This fact again leads to the assumption of the existence of cross-talk between ET and GAs during the transition from seed dormancy to germination induced by AR. On the other hand, the fact that exogenous GA_{4+7} strongly inhibits *SoGA3ox2* expression during imbibition agrees with previous evidence indicating that bioactive GAs may control their own synthesis through a negative feedback regulation of the expression genes of GA biosynthesis (Olszewski *et al.*, 2002). However, this feedback regulation does not appear to be identical in after-ripened and non-after-ripened seeds, since the inhibition by exogenous GA_{4+7} of *SoGA3ox2* expression compared with the control is not quantitatively similar in the two seed lots. In support of this hypothesis are the results of *SoGA3ox2* expression in after-ripened and non-after-ripened seeds in the presence of the PB, which strongly alters its expression pattern.

Taking into account the results of *SoGA3ox2* and *SoGA20ox2* expression and those of percentage germination, it is concluded that the requirement for ET and GAs for radicle emergence preparation involves, apart from the two hormone signalling pathways, the strict control of the level of ET and GAs in the appropriate tissue, and the regulation of *SoGA20ox2* whose expression profiles are qualitatively and quantitatively different from those observed for *SoGA3ox2*. Thus, it is proposed that *SoGA20ox2* expression and regulation must be of great importance during the imbibition of *S. officinale* seeds, since their expression is considerably higher than that of *SoGA3ox2* in all the treatments studied, and does not appear to be as susceptible to AR, as has been demonstrated here for *SoGA3ox2*. It has been postulated that cross-talk between ET and GA signalling regulates *FsGA20ox* gene expression during the breaking of dormancy in stratified *F. silvatica* seeds (Calvo *et al.*, 2004*b*). The results of *SoGA20ox2* expression in the presence of ethephon, GA_{4+7}, and ethephon+GA_{4+7} also appear to support ET–GA cross-talk. Taking together the results of *SoGA3ox* and *SoGA20ox2* expression during imbibition, it is concluded that: (i) GA biosynthesis is indispensable to overcome hedge mustard seed dormancy; (ii) both genes are regulated by both ET and GAs to carry out the transition from dormancy to germination induced by AR; and (iii) AR strongly inhibits *SoGA3ox2* expression in the presence of GA_{4+7}, PB, and ethephon+GA_{4+7}, indicating that this gene is subjected to a tight feedback regulation, possibly to prevent accumulation of GAs after the signal for AR has been decided.

On the other hand, it is demonstrated here that, at the onset of imbibition of *S. officinale* seeds, *SoGA2ox6* is expressed at basal levels, confirming the need for the synthesis of bioactive GAs in both the presence and absence of AR (i.e. high expression of *SoGA3ox2* and *SoGA20ox2*). Moreover, it was also demonstrated that the AR process up-regulated *SoGA2ox6* expression under all the treatments studied, as opposed to the down-regulation observed in non-after-ripened seeds. Due to the scant information on *SoGA2ox6* physiology in seeds, the alterations found in *SoGA2ox6* expression in *S. officinale* are very complex to explain and relate to the breaking of dormancy induced by the AR process. Nevertheless, it is worth emphasizing that although the AR does not appear to affect *SoGA2ox6* expression in controls, the expression in after-ripened seeds tended to be greater than in non-after-ripened seeds, in the treatments studied. Previous results demonstrate that

AtGA2ox6 is down-regulated by stratification in dark, imbibed *Arabidopsis* seeds (Yamaguchi *et al.*, 2004). Also, up- and down-regulation of different GA metabolism genes by ET in *Arabidopsis* seedlings have recently been demonstrated, this finding being related to ET–GA cross-talk (Vandenbussche *et al.*, 2007; Dugardeyn *et al.*, 2008). The present results, considered overall, suggest the hypothesis that the regulation of the synthesis of bioactive GAs involved in the AR process is subject to strong control. Consequently, if the threshold level of GAs necessary to prompt germination is surpassed because of any endogenous or exogenous agent, the seed responds with the destruction of the unnecessary bioactive GAs, and *SoGA2ox6* would be involved in this destruction.

ET and GAs alter β-mannanase activity during imbibition of after-ripened seeds

In the seeds of a number of plant species, MAN activity shows a sharp surge during germination. However, the timing of the highest enzymatic activity depends on the species. The MAN activity increases in the micropylar endosperm prior to the completion of seed germination (Toorop *et al.*, 1996; Nonogaki *et al.*, 2000), or it increases afterwards (Bewley *et al.*, 1997, and references therein; Marraccini *et al.*, 2001; Gong *et al.*, 2005). Controversy persists as to whether the rise in MAN activity in the endosperm during germination is sufficient to permit radicle emergence, and the consensus appears to be that, while this enzyme is required for endosperm weakening, it is not, by itself, sufficient to allow germination to be completed (Gong *et al.*, 2005). In contrast to the extensive investigation of MAN before and after the protrusion process, there is no information on the evolution of this cell wall-loosening enzyme in AR seeds. Here, it is shown for the first time that the specific activity of MAN is much higher in dry after-ripened seeds that in dry non-after-ripened seeds, and MAN activity remains high in after-ripened seeds over the first 3 h of imbibition, abruptly declining afterwards. The cause of this high enzymatic activity early on is unknown. However, while this manuscript was in preparation, Ren *et al.* (2008), using anti-MAN antibodies of tomato in rice seeds, reported that a MAN protein is present in an inactive form in dry rice grains. If these results are applicable to *S. officinale*, possibilities to explain the presence of MAN activity in dry seed can be proposed. (i) This protein forms part of a pool of proteins stored from zygotic embryogenesis. (ii) AR promotes *MAN* gene expression in dry seeds. Leubner-Metzger (2005) demonstrated a transient low level transcription and translation of the β-1,3-glucanase gene during tobacco seed AR, leading to the release of dormancy, whereas in *Arabidopsis* the expression profiling revealed that transcripts of a number of genes exhibited a transient accumulation within 6 h after imbibition (Nakabayashi *et al.*, 2005). Ren *et al.* (2008) do not present data on the expression of *OsMAN1*, *OsMAN2*, *OsMAN6*, and *OsMANP* in the very early phases of rice seed imbibition. (iii) The enzymatic activity is high in dry seed and after 3 h of imbibition as a consequence of the fact that the enzyme is studied *in vitro* under optimal conditions that do not exist *in vivo*. (iv) If the present results reflect *in vivo* events, the AR may soften the tissue very early with unknown implications in the germination process; and (v) as an alternative to (iv), MAN may be involved in the production of sugars from the degradation process of the cell wall, these sugars serving to nourish the embryo. The MAN activity in lettuce endosperm is assumed to be associated with reserve mobilization closely following radicle emergence rather than with prior endosperm weakening (Wang *et al.*, 2004). It bears mentioning in relation to proposal (iv) that the MAN activity increased notably in after-ripemed *S. officinale* seeds immediately before radicle emergence (data not shown). On the other hand, MAN activity was significantly increased by ethephon treatment both at the onset of imbibition and in the period near radicle emergence. Nevertheless, IESS did not inhibit MAN activity in either after-ripened or non-after-ripened seeds, suggesting that ET is one of the factors contributing to, but not indispensable for, the regulation of MAN activity during imbibition. ET increases MAN activity in germinating thermotolerant lettuce seeds (Nascimento *et al.*, 2000), and the authors hypothesize that the endosperm weakening is a result of elevated enzymatic activity. However, this hypothesis does not agree with the findings of Wang *et al.* (2004) who point out that some members of the MAN family may be ET responsive and may be associated with sugar reserve mobilization from the cell wall rather than with endosperm weakening prior to protrusion. It is noteworthy that exogenous GA_{4+7} increases MAN activity during imbibition of *S. officinale* after-ripened seeds, but not in non-after-ripened seeds. In contrast, the MAN activity is dramatically increased in the presence of the GA synthesis inhibitor PB, apparently indicating that a fall in the level of bioactive GAs triggers a rapid desynchronization in the seed, whereupon a non-specific enzymatic stimulation takes place without provoking radicle emergence due to the absence of GAs. A search at http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl revealed ABA, GAs, and dehydration motifs/transcription factors in the promoters of the MAN of *Arabidopsis*. However, no motif for ET was found. Nevertheless, the expression of the *LeMAN2* gene is up-regulated in *Sl-ERF2*-overexpressing seeds, suggesting that *Sl-ERF2* (an ET response-factor gene) stimulates seed germination through the induction of *LeMAN2* (Pirello *et al.*, 2006). Taken together, the data presented on MAN activity during early imbibition of *S. officinale* after-ripened seeds point to ET–GA cross-talk, as discussed for the expression of the genes studied.

Is ET–GA cross-talk required for AR?

The complexity of hormonal responses and their functional overlap support the presence of an intensive cross-talk between hormone signalling pathways (Brady and McCourt, 2003). Although the influence of ET on expression of GA response and synthesis genes provided evidence for the existence of an interaction between both hormones

(De Grauwe *et al.*, 2008, and references therein), it was not clear at which level this cross-talk appeared. DELLA proteins, which act as nuclear repressors of GA signalling, appear to be key integrators in the ET–GA cross-talk (Jiang and Fu, 2007; Steber, 2007, and references therein). The current tendency is to suggest that the ET–GA cross-talk is multiple, depending on the process and the state of development. Recent reviews have dealt extensively with these interactions (Kucera *et al.*, 2005; De Grauwe *et al.*, 2007, 2008; Dugardeyn *et al.*, 2008; Holdsworth *et al.*, 2008). On the other hand, ET promotes dormancy breaking through interactions with ABA signalling. Seeds of *etr1* and *ein2/era3* mutants display increased dormancy correlated with increased sensitivity to ABA in seed germination. In contrast, the *ctr1* mutation and treatment of *A. thaliana* wild-type seeds with ACC result in decreased sensitivity to ABA. Thus, ET stimulation of seed germination may occur via antagonism of ABA signalling (Filkenstein *et al.*, 2008, and references therein). Having said all this, and taking into account the results presented here, it is proposed that the intervention of ET–GA cross-talk seems probable in the *S. officinale* AR process. However, whether this intervention is direct or indirect is at present unclear. Obviously, any hormonal interaction results in an alteration of hormone levels (Chiwocha *et al.*, 2005). It is probable that the effect of ET on GA, and vice versa, might be indirect, possibly via ABA. The ET–ABA cross-talk is under study at present by our group.

Supplementary data

Supplementary data are available at *JBX* online.

Figure S1. Transcription levels of a housekeeping gene (*18S RNA*), presented as C_T mean values, during the development of the fruit and seed of *S. officinale*. EF, early fruit; LF, late fruit; LS, late seed; NAS, not after-ripened dry seed; AS,after-ripened dry seed. Error bars indicate the SDs of 10 independent experiments.

Figure S2. Transcription levels of a housekeeping gene (*18S RNA*), presented as C_T mean values, during the time course of imbibition at 24 °C of *S. officinale* seeds. (A) Control; (B) GA4+7; (C) PB; (D) etephon; (E) IESS; (F) etephon+GA4+7. Non-after-ripened seed (open circles); after-ripened seed (filled circles). Error bars indicate the SDs of 10 independent experiments.

Figure S3. Phylogenetic tree (cladogram) including *SoACS7* and other plant *ACS* genes. Accession numbers are given in parentheses. The aLRT statistical test of branch support was used (numerical values in branch).

Figure S4. Phylogenetic tree (cladogram) including *SoACO2* and other plant *ACO* genes. Accession numbers are given in parentheses. The aLRT statistical test of branchsupport was used (numerical values in branch).

Figure S5. Phylogenetic tree (cladogram) including *SoGA20ox2* and other plant *GA20ox* genes. Accession numbers are given in parentheses. The aLRT statistical test of branch support was used (numerical values in branch).

Figure S6. Phylogenetic tree (cladogram) including *SoGA3ox2* and other plant *GA3ox* genes. Accession numbers are given in parentheses. The aLRT statistical test of branch support was used (numerical values in branch).

Figure S7. Phylogenetic tree (cladogram) including *SoGA2ox6* and other plant *GA2ox* genes. Accession numbers are given in parentheses. The aLRT statistical test of branch support was used (numerical values in branch).

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References

Alboresi A, Gestin C, Leydecker M-T, Bedu M, Meyer C, Truong HN. 2005. Nitrate, a signal relieving seed dormancy in Arabidopsis. *Plant, Cell and Environment* **28,** 500–512.

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P, Jullien M. 2004. Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta* **219,** 479–488.

Alonso-Blanco C, Bentsink L, Hanhart CJ, Blankestijn de Vries H, Koornneef M. 2003. Analysis of natural allelic variation at seed dormancy loci of *Arabidopsis thaliana*. *Genetics* **164,** 711–729.

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25,** 3389–3402.

Bair NB, Meyer SE, Allen PS. 2006. A hydrothermal after-ripening time model for seed dormancy loss in *Bromus tectorum* L. *Seed Science Research* **16,** 17–28.

Baskin JM, Baskin CC. 2004. A classification system for seed dormancy. *Seed Science Research* **14,** 1–16.

Batak I, Devi M, Giba Z, Grubisi D, Poff KL, Konjevic R. 2002. The effects of potassium nitrate and NO-donors on phytochrome A- and phytochrome B-specific induced germination of *Arabidopsis thaliana* seeds. *Seed Science Research* **12,** 253–259.

Batlla D, Benech-Arnold RL. 2005. Changes in the light sensitivity of buried *Polygonum aviculare* seeds in relation to cold-induced dormancy loss: development of a predictive model. *New Phytologist* **165,** 445–452.

Beaudoin N, Serizet C, Gosti F, Giraudat J. 2000. Interactions between abscisic acid and ethylene signaling cascades. *The Plant Cell* **12,** 1003–1115.

Bentsink L, Jowett J, Hanhart CJ, Koornneef M. 2006. Cloning of *DOG1*, a quantitative trait locus controlling seed dormancy in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **103,** 17042–17047.

Bentsink L, Soppe W, Koornneef. 2007. Genetic aspects of seed dormancy. *Annual Plant Reviews* **27,** 176–223.

Bethke PC, Gubler F, Jacobsen JV, Jones RL. 2004. Dormancy of Arabidopsis seeds and barley grains can be broken by nitric oxide. *Planta* **219,** 847–855.

Bewley JD. 1997. Breaking down the walls—a role for endo-β-mannanase in release from seed dormancy? *Trends in Plant Science* **2,** 464–469.

Bove J, Lucas P, Godin B, Oge L, Jullien M, Grappin P. 2005. Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Molecular Biology* **57,** 593–612.

Brady SM, McCourt P. 2003. Hormone cross-talk in seed dormancy. *Journal of Plant Growth Regulation* **22,** 25–31.

Cadman CSC, Toorop PE, Hilhorst HWM, Finch-Savage WE. 2006. Gene expression profiles of Arabidopsis Cvi seed during cycling through dormant and non-dormant states indicate a common underlying dormancy control mechanism. *The Plant Journal* **46,** 805–822.

Calvo AP, Nicolás C, Lorenzo O, Nicolás G, Rodríguez D. 2004*a*. Evidence for positive regulation by gibberellins and ethylene of ACC oxidase expression and activity during transition from dormancy to germination in *Fagus silvatica* L. seeds. *Journal of Plant Growth Regulation* **23,** 44–53.

Calvo AP, Nicolás C, Nicolás G, Rodríguez D. 2004*b*. Evidence of a cross-talk regulation of a GA20-oxidase (*FsGA20ox1*) by gibberellins and ethylene during the breaking of dormancy in *Fagus silvatica* seeds. *Physiologia Plantarum* **120,** 623–630.

Carrera E, Holman T, Medhurst A, Dietrich D, Footitt S, Theodoulou FL, Holdsworth MJ. 2008. Seed after-ripening is a discrete developmental pathway associated with specific gene networks in Arabidopsis. *The Plant Journal* **53,** 214–224.

Chibani K, Ali-Rachedi S, Job C, Job D, Jullien M, Grappin P. 2006. Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiology* **142,** 1493–1510.

Chiwocha SDS, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, Kermode AR. 2005. The *etr1-2* mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. *The Plant Journal* **42,** 35–48.

Corbineau F, Lecat S, Côme D. 1986. Dormancy of three cultivars of oat seeds (*Avena sativa* L.). *Seed Science and Technology* **14,** 725–735.

De Grauwe L, Chaerle L, Dugardeyn J, *et al*. 2008. Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the Arabidopsis *gai eto2-1* double mutant. *New Phytologist* **177,** 128–141.

De Grauwe L, Vriezen WH, Bertrand S, Phillips A, Vidal AM, Hedden P, Van der Straeten D. 2007. Reciprocal influence of ethylene and gibberellins on response-gene expression in *Arabidopsis thaliana*. *Planta* **226,** 485–498.

Derks MPM, Karssen CM. 1993. Changing sensitivity to light and nitrate but not to gibberellins regulates seasonal dormancy patterns in *Sisymbrium officinale* seeds. *Plant, Cell and Environment* **16,** 469–479.

Dugardeyn J, Vandenbussche F, Van Der Straeten D. 2008. To grow or not to grow: what can we learn on ethylene–gibberellin cross-talk by *in silico* gene expression analysis? *Journal of Experimental Botany* **59,** 1–16.

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW. 2007. Seed dormancy release in Arabidopsis Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directly by environment specific sensing. *The Plant Journal* **51,** 60–78.

Finch-Savage WE, Leubner-Metzger G. 2006. Seed dormancy and the control of germination. *New Phytologist* **171,** 501–523.

Finkelstein R, Reeves W, Ariizumi T, Steber C. 2008. Molecular aspects of seed dormancy. *Annual Review of Plant Biology* **59,** 387–415.

Finkelstein RR, Srinivas SL, Gampala SSL, Rock CD. 2002. Abscisic acid signaling in seeds and seedlings. *The Plant Cell* **14,** S15–S45.

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, McCourt P. 2000. Regulation of abscisic acid signaling by ethylene pathway in Arabidopsis. *The Plant Cell* **12,** 1117–1126.

Gianinetti A, Laarhoven LJJ, Persijn ST, Harren FJM, Petruzzelli L. 2007. Ethylene is associated with germination but not seed dormancy in red rice. *Annals of Botany* **99,** 735–745.

Gong X, Bassel GW, Wang AX, Greenwood J, Bewley JD. 2005. The emergence of embryos from hard seeds is related to the structure of the cell walls of the micropilar endosperm, and not to endo-β-mannanase activity. *Annals of Botany* **96,** 1165–1173.

Grappin P, Bouinot D, Sotta B, Miginiac E, Julien M. 2000. Control of seed dormancy in *Nicotiana plumbaginifolia*: post-imbibition abscisic acid synthesis imposes dormancy maintenance. *Planta* **210,** 279–285.

Hermann K, Meinhard J, Dobrev P, Linkies A, Pesek B, Hess B, Machácková I, Fischer U, Leubner-Metzger G. 2007. 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds. *Journal of Experimental Botany* **58,** 3047–3060.

Hilhorst HWM. 1990. Dose–response analysis of factors involved in germination and secondary dormancy of seeds of *Sisymbrium officinale*. *Plant Physiology* **94,** 1096–1102.

Hilhorst HWM, Karssen CM. 1989. Dual effect of light on gibberellin- and nitrate-stimulated seed germination of *Sisymbrium officinale* and *Arabidopsis thaliana*. *Plant Physiology* **86,** 591–597.

Hilhorst HWM, Toorop PE. 1997. Review on dormancy, germinability, and germination in crop and weed seeds. *Advances in Agronomy* **61,** 111–165.

Holdsworth MJ, Bentsink L, Soppe WJJ. 2008. Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytologist* **179,** 33–54.

Iglesias-Fernández R, Matilla AJ, Pulgar I, de la Torre F. 2007. Ripe fruits of *Sisymbrium officinale* L. contain heterogeneous endospermic seeds with different germination rates. *Seed Science and Biotechnology* **1,** 18–24.

Jiang C, Fu X. 2007. GA action: turning on de-DELLA repressing signaling. *Current Opinion in Plant Biology* **10,** 461–465.

Kermode AR. 2005. Role of abscisic acid in seed dormancy. *Journal of Plant Growth Regulation* **24,** 319–344.

Kucera B, Cohn MA, Leubner-Metzger G. 2005. Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* **15,** 281–307.

Leubner-Metzger G. 2005. Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Ther Plant Journal* **41,** 133–145.

Lever M. 1972. A new reaction for colorimetric determination of carbohydrates. *Analytical Biochemistry* **47,** 273–279.

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F. 2007. Identification of transcripts potentially involved in barley seed germination: and dormancy using cDNA-AFLP. *Journal of Experimental Botany* **58,** 425–437.

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G. 2005. Water uptake and distribution in germinating tobacco seeds investigated *in vivo* by nuclear magnetic resonance imaging. *Plant Physiology* **138,** 1538–1551.

Marraccini P, Rogers WJ, Allard C, Andrè ML, Caillet V, Lacoste N, Lausanne F, Michaux S. 2001. Molecular and biochemical characterization of endo-β-mannanases from germinating coffee (*Coffea arabica*) grains. *Planta* **213,** 296–308.

Matilla AJ, Matilla-Vázquez MA. 2008. Involvement of ethylene in seed dormancy. *Plant Science* **175,** 87–97.

Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T, Tabata S, Kamiya Y, Sun TP. 2006. Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. *The Plant Journal* **45,** 804–818.

Nakabayashi K, Okamoto M, Koshiba T, Nambara E. 2005. Genome-wide profiling of stored mRNA in *Arabidopsis thaliana* seed germination: epigenetic and genetic regulation of transcription in seed. *The Plant Journal* **41,** 697–709.

Nascimento WM, Cantliffe DJ, Huber DJ. 2000. Thermotolerance in lettuce seeds: association with ethylene and endo-β-mannanase. *Journal of the American Society of Horticultural Sciences*, 125. .518–524.

Nonogaki H, Gee OH, Bradford KJ. 2000. A germination-specific endo-β-mannanase gene is expressed in the micropilar endosperm cap of tomato seeds. *Plant Physiology* **123,** 1235–1245.

Ogawa M, Hanada A, Yamauchi Y, Kuwalhara A, Kamiya Y, Yamaguchi S. 2003. Gibberellin biosynthesis and response during Arabidopsis seed germination. *The Plant Cell* **15,** 1591–1604.

Olszewski N, Sun TP, Gubler F. 2002. Gibberellin signaling: biosynthesis, catabolism and response pathways. *The Plant Cell* **14,** S61–S80.

Oracz K, Bouteau H, Farrant JM, Cooper K, Belghazi M, Job C, Job D, Corbineau F, Ch Bailly. 2007. ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. *The Plant Journal* **50,** 452–465.

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW. 2001. MYB61 is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *The Plant Cell* **13,** 2777–2791.

Petruzzelli L, Coraggio I, Leubner-Metzger G. 2000. Ethylene promotes ethylene biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclo-propane-1-carboxylic acid oxidase. *Planta* **211,** 144–149.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29,** 2002–2007.

Pirello J, Jaimes-Miranda F, Sánchez-Ballesta MT, Tournier B, Khalil-Ahmad, Regad F, Latché A, Pech JC, Bouzayen M. 2006. Sl-ERF2, a tomato ethylene response factor involved in ethylene response and seed germination. *Plant and Cell Physiology* **47,** 1195–1205.

Probert RJ. 2000. The role of temperature in the regulation of seed dormancy and germination. In: Fenner M, ed. *Seeds: the ecology of regenation in plant communities*. Wallingford, Oxon: CAB International, 26–292.

Rautengarten C, Usadel B, Neumetzler L, Hartmann J, Büssis D, Altmann T. 2008. A subtilisin-like serine protease essential for mucilage release from Arabidopsis seed coats. *The Plant Journal* **54,** 466–480.

Raz V, Bergervoet JHW, Koorneef M. 2001. Sequential steps for development arrest in Arabidopsis seeds. *Development* **128,** 243–252.

Ren Y, Bewley JD, Wang X. 2008. Protein and gene expression patterns of endo-β-mannanase following germination of rice. *Seed Science Research* **18,** 139–149.

Steber CM. 2007. De-repression of seed germination by GA signaling. *Annual Plant Reviews* **27,** 248–263.

Steel RG, Torrie JH. 1982. *Principles and procedures of statistics*. Tokyo: Mc Graw-Hill.

Toorop PE, Bewley JD, Hilhorst HWM. 1996. Endo-β-mannanase isoforms are present in the endosperm and embryo of tomato seeds, but are not essentially linked to the completion of germination. *Planta* **200,** 153–158.

Vandenbussche F, Vancompernolle B, Rieu I, Ahmad M, Phillips A, Hedden P, Moritz T, Van Der Straeten D. 2007. Ethylene-induced *Arabidopsis* hypocotyl elongation is dependent on but not mediated by gibberellins. *Journal of Experimental Botany* **58,** 4269–4281.

Vandenbussche F, Van Der Straeten D. 2007. One for all and all for one: cross-talk of multiple signals controlling the plant phenotype. *Journal Plant Growth Regulation* **26,** 78–187.

Van der Schaar W, Alonso-Blanco C, Léon-Kloosterziel KM, Cansen RC, van Ooijen JW, Koornneef M. 1997. QTL analysis of seed dormancy in *Arabidopsis* using recombinant inbred lines and MQM mapping. *Heredity* **79,** 190–200.

Verwoerd TC, Dekker BMM, Hoekema A. 1989. A small-scale procedure for the rapid isolation of RNAs. *Nucleic Acids Research* **17,** 2362–2368.

Wang AX, Li JR, Bewley JD. 2004. Molecular cloning and characterization of an endo-β-mannanase gene expressed in the lettuce endosperm following radicle emergence. *Seed Science Research* **14,** 267–276.

Weber H, Borisjuk L, Wobus U. 2005. Molecular physiology of legume seed development. *Annual Review of Plant Biology* **56,** 253–279.

Weiss D, Ori N. 2007. Mechanism of cross talk between gibberellin and other hormones. *Plant Physiology* **144,** 1240–1246.

Western TL, Skinner DJ, Haughn GW. 2000. Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiology* **122,** 345–356.

Yamaguchi S, Kamiya Y. 2002. Gibberellins and light-stimulated seed germination. *Journal of Plant Growth Regulation* **20,** 369–376.

Yamaguchi S, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S. 2004. Activation of gibberellin biosynthesis and response pathways by low temperatura during imbibition of *Arabidopsis thaliana* seeds. *The Plant Cell* **16,** 367–378.

Yamaguchi S, Nambara N. 2006. Seed development and germination. In: Hedden P, Thomas SG, eds. *Plant hormone signaling*. Oxford: Blackwell Publishing, 311–338.

After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during the early imbibition of *Sisymbrium officinale* L. seeds

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Supplementary material:

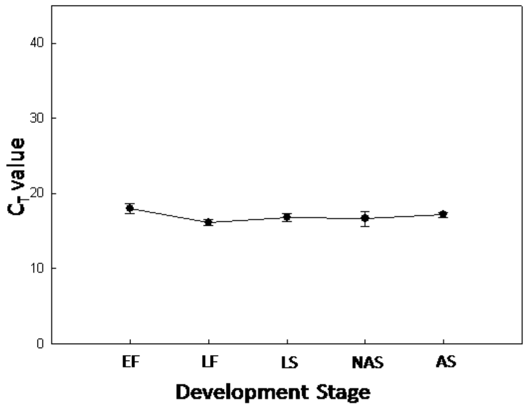

Figure S1. Transcription levels of housekeeping gene (18S-RNA), presented as C_T mean values, during the development of the fruit and seed of *S. officinale*. EF: early fruit; LF: late fruit; LS: late seed; NAS: not after-ripened dry seed; AS: after-ripened dry seed . Error bars indicate standard deviations of ten independent experiments.

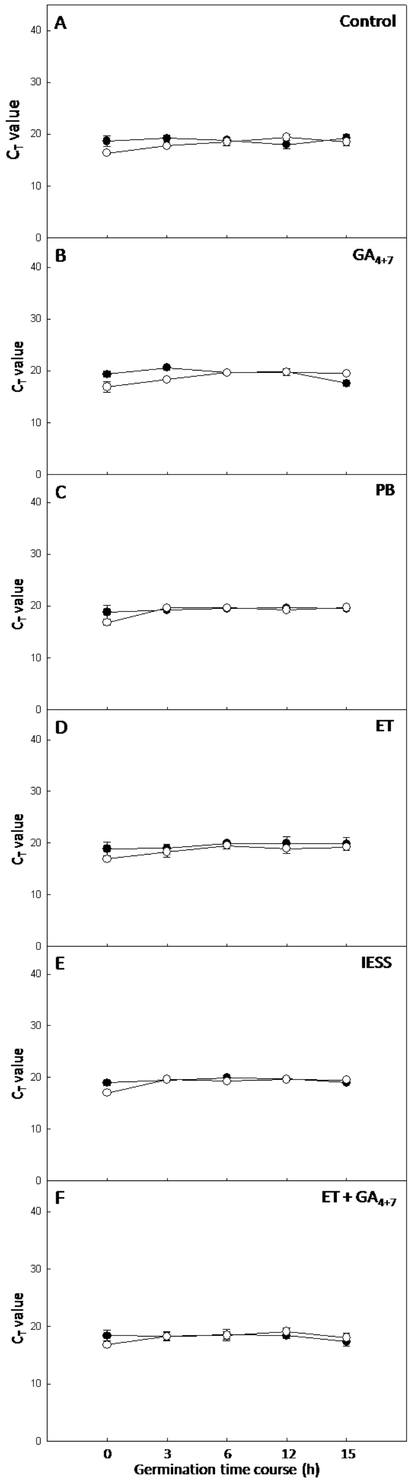

Figure S2. Transcription levels of housekeeping gene (18S-RNA), presented as C_T mean values, during imbibition time course at 24°C of *S. officinale* seeds. (A) Control; (B) GA_{4+7}; (C) PB; (D) etephon; (E) IESS; (F) etephon + GA_{4+7}. Not after-ripened seed (○); after-ripened seed (●). Error bars indicate standard deviations of ten independent experiments.

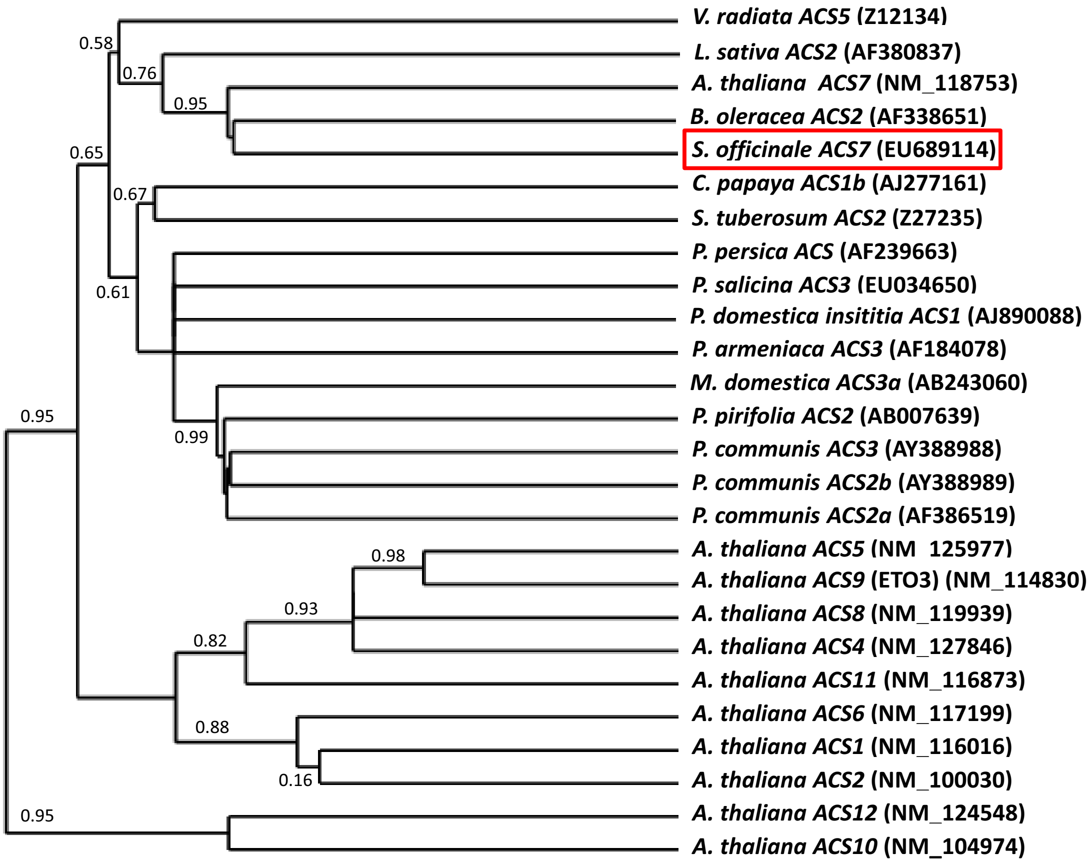

Figure S3. Phylogenetic tree (cladogram) including *SoACS7* and other plant *ACS*genes. Accession numbers are given between brackets. aLRT statistical test of branch support was used (numerical values in branch).

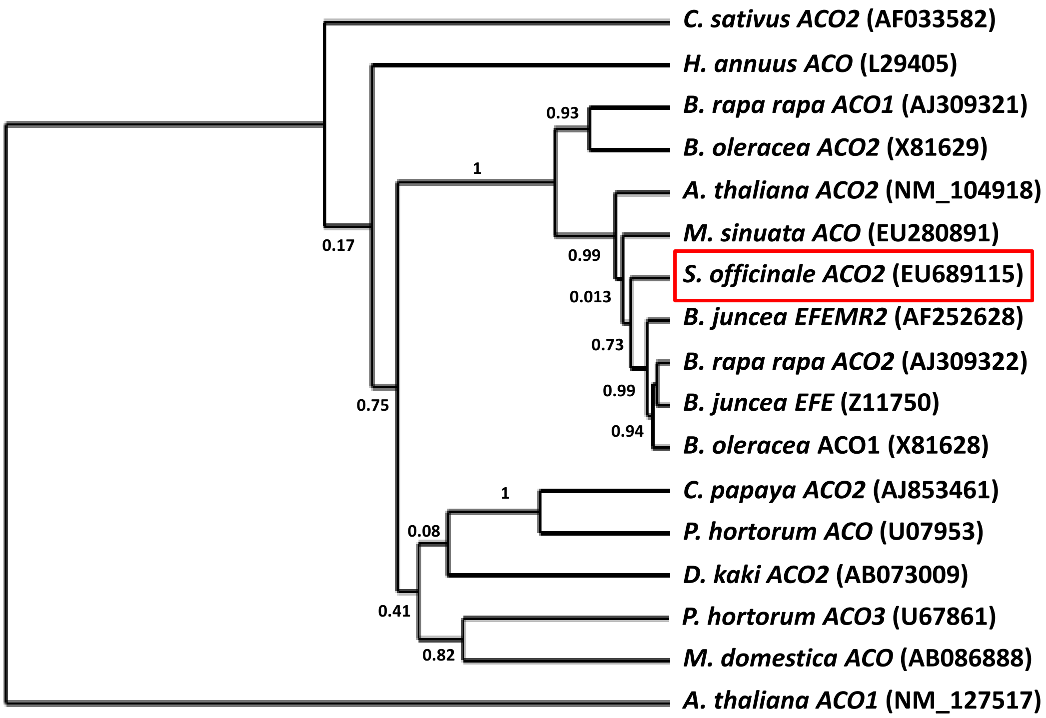

Figure S4. Phylogenetic tree (cladogram) including *SoACO2* and other plant *ACO* genes. Accession numbers are given between brackets. aLRT statistical test of branchsupport was used (numerical values in branch).

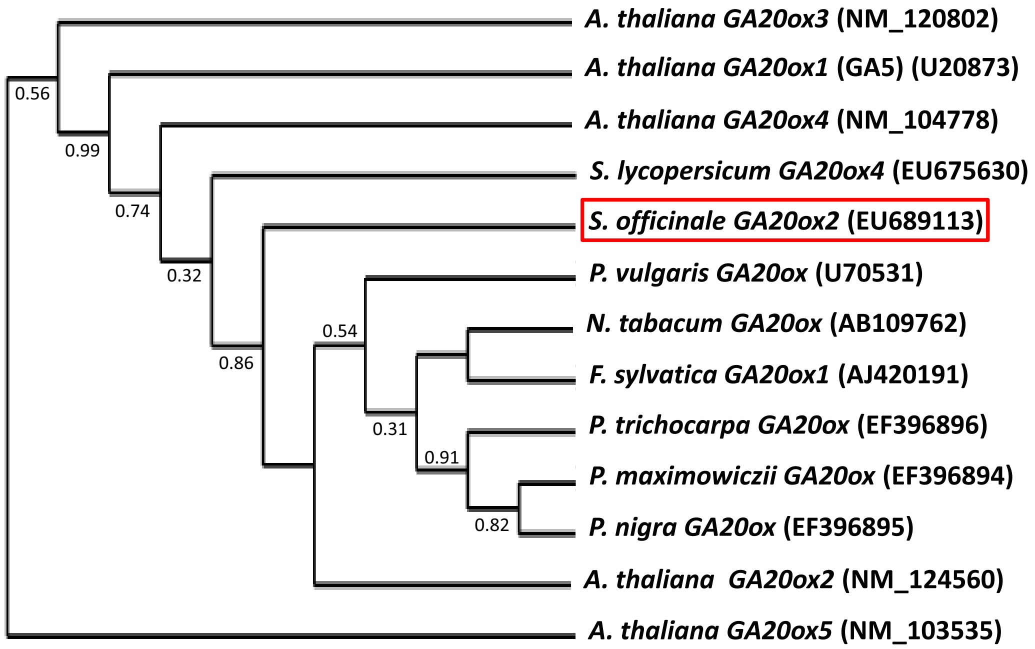

Figure S5. Phylogenetic tree (cladogram) including *SoGA20ox2* and other plant *GA20ox* genes. Accession numbers are given between brackets. aLRT statistical test of branch support was used (numerical values in branch).

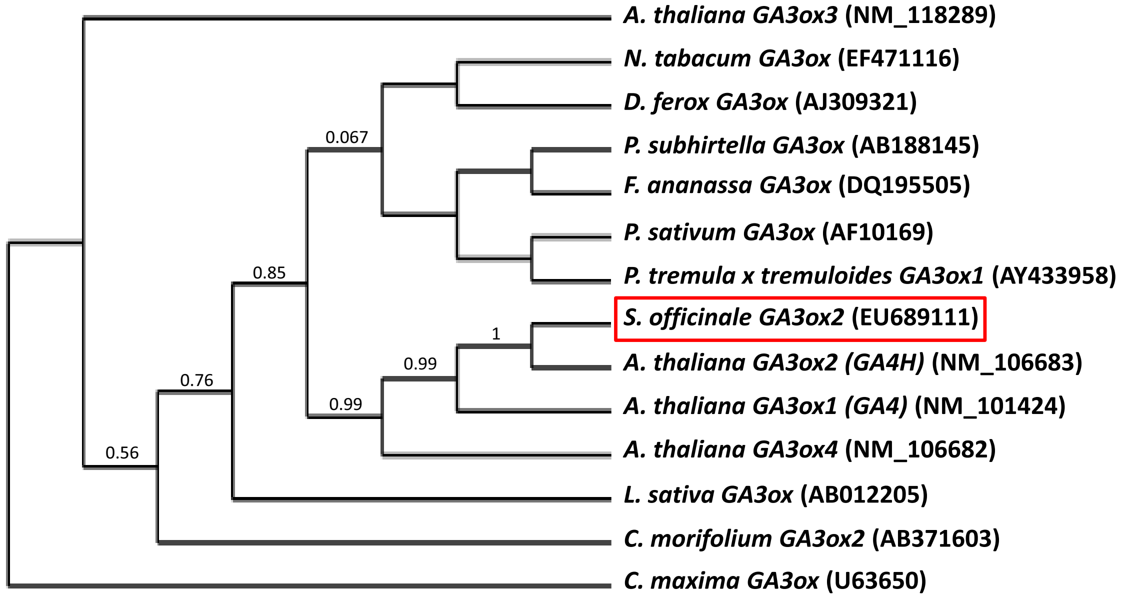

Figure S6. Phylogenetic tree (cladogram) including *SoGA3ox2* and other plant *GA3ox* genes. Accession numbers are given between brackets. aLRT statistical test of branch support was used (numerical values in branch).

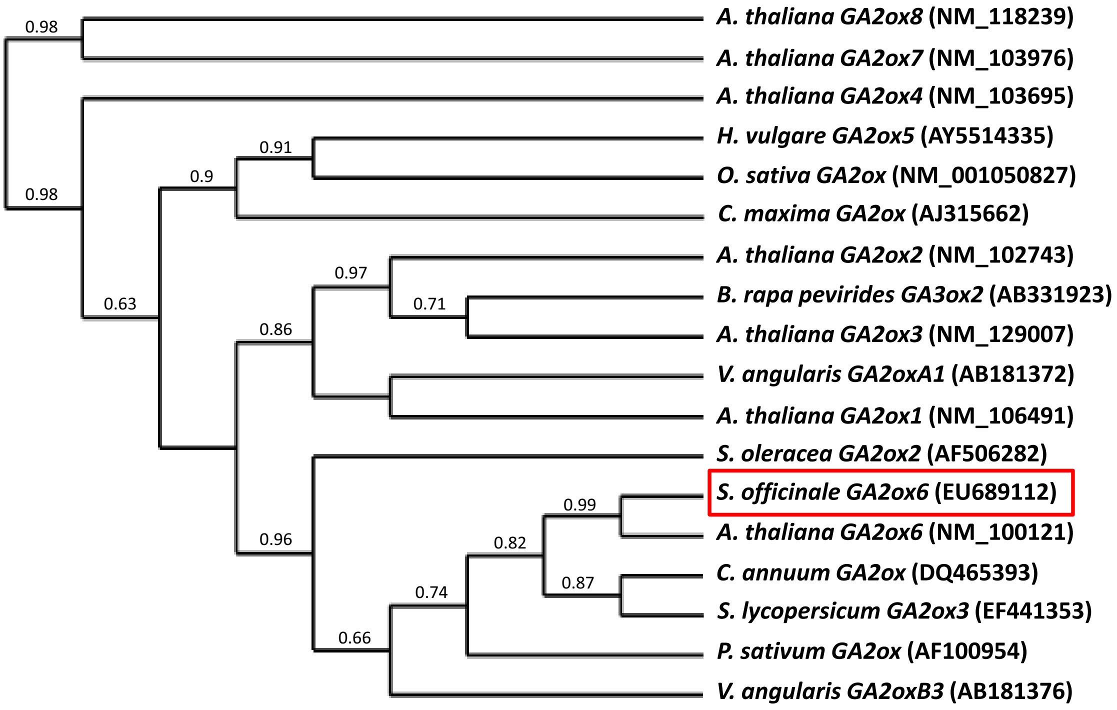

Figure S7. Phylogenetic tree (cladogram) including *SoGA2ox6* and other plant *GA2ox* genes. Accession numbers are given between brackets. aLRT statistical test of branch support was used (numerical values in branch).

VI. CAPÍTULO 4

Discusión General

VI. – Discusión General

La biodiversidad tan amplia que existen en la familia *Cruciferae*, con más de 3000 especies, se ve reflejada en la gran variedad de frutos que poseen (revisado en Bailey *et al.*, 2006). Tanto es así que este rasgo se utiliza como una de las características fundamentales para la identificación y clasificación interespecífica en Crucíferas. Dentro de esta gran variabilidad, es de interés la agrupación de sus frutos según el tipo de compartimentos en que se alojan las semillas. Es decir, existen frutos tipo silicua con un único compartimento llamado valva, cuyas semillas están unidas a un tabique central mediante un pedúnculo denominado pedicelo (ej. *Arabidopsis* y *Sisymbrium officinale*). La silicua es un fruto dehiscente cuyo mecanismo de apertura está finamente controlado por multitud de factores (revisado en Dinneny y Yanofsky, 2005; Matilla y Matilla-Vázquez, 2008 y algunas referencias citadas). Por otra parte, hay frutos en silicua que poseen varias semillas (2-3) en un compartimento anexo a las valvas y embriológicamente diferente llamado rostro, el cual es indehiscente (ej. *Brassica* y *Synapis*). Finalmente, otras crucíferas producen frutos sin valvas, y sus semillas escasas están encerradas en un solo rostro que es indehiscente (ej. *Raphanus* y *Cakile*). A nivel evolutivo, se desconoce el porqué de esta enorme variabilidad en el tipo de frutos de crucíferas, así como si las semillas que encierran los compartimentos son homogéneas a nivel morfológico y fisiológico, o si por el contrario presentan algún tipo de heterogeneidad y cual(es) son las razones para que lo sean (revisado en Imbert, 2002; Matilla *et al.*, 2005 y referencias citadas). Es muy posible que varias causas implicadas en asegurar la dispersión temporal justifique la existencia del carácter heterogéneo descrito en las semillas de más de 200 especies de Angiospermas (Imbert, 2002). Al margen de razones eminentemente evolutivas y a veces morfológicas, apenas existen estudios serios sobre el impacto de la heterogeneidad de las semillas a nivel fisiológico y -ómico.

Nuestro Grupo de Investigación inició con semillas de *Brassica rapa* en el año 2003 (Proyectos BFI2000-0305; PGIDT00AGR203010PR; CGL2004-01996/BOS) un estudio a nivel fisiológico y molecular sobre la heterogeneidad en Crucíferas. Primeramente, se demostró que estas semillas poseen diferente capacidad germinativa dependiendo del color de la cubierta seminal (Puga-Hermida *et al.*, 2003b; Rodríguez-Gacio *et al.* 2003). Algunos autores opinan que la heterogeneidad germinativa es debida a diferentes grados de dormición y puede estar relacionada con aspectos ecológicos de la reproducción y supervivencia de la especie en cuestión (revisado en Baskin y Baskin, 2004). Asimismo, existen datos que atribuyen a las características externas de la cubierta seminal (ej. dureza y color) el grado de dormición de la semilla. Es lo que hemos definido como dormición física (DF) y como se explicitó en la Introducción General, la intensidad en la toma de agua (imbibición) e intercambio de gases están implicados en la misma (Baskin y Baskin, 2004). Experimentos de imbibición llevados a cabo con semillas mutantes que poseen diferentes tonalidades en la testa indican que la toma de agua es más lenta y poseen menor grado de dormición que el WT (Debeaujon y Koornneef, 2000). Las semillas oscuras de *Brassica rapa* se imbiben más lentamente que las claras y su germinación es más rápida (Puga-Hermida *et al.*, 2003a). Este hecho diferencial en la imbibición también se demostró en esta Memoria con las semillas de *S. officinale* (Iglesias-Fernández *et al.*, 2007). Por otra parte, la sensibilidad al ET en semillas heterogéneas de *B. rapa* no parece ser la misma (Rodríguez-Gacio *et al.*, 2003), y la activad ACC-oxidasa (ACO) y la expresión del gen *BrACO1* son más intensas en el lote oscuro que en el claro durante todo el proceso germinativo (Puga-Hermida *et al.*, 2003a). Además, los niveles de ABA-libre y conjugado son muy diferentes en ambos lotes (Puga-Hermida *et al.*, 2003a, b). Todos nuestros resultados tomados en conjunto nos sugieren la hipótesis de que uno de los factores que desencadenan diferencias en la germinación de las semillas heterogéneas de *B. rapa* puede ser una alteración en el mecanismo de acción del ET y ABA. Las interacciones entre ambas señalizaciones y acciones fisiológicas fueron demostradas en *Arabidopsis* (Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000; Chiwocha *et al.*, 2005) y posteriormente revisadas (Matilla *et al.*, 2005; Matilla y Matilla-Vázquez, 2008 y algunas referencias citadas).

En esta Memoria se demuestra que las semillas endospérmicas de *S. officinale* son heterogéneas, aunque la planta madre se haya desarrollado en idénticas condiciones ecológicas. Esta heterogeneidad, que se demuestra por la presencia de semillas con diferente color en la capa externa de la testa, afecta, al igual que en *B. rapa*, a la fisiología de la semilla y también constituye una herramienta fisiológica muy valiosa ya que la planta madre produjo semillas con diferentes grados de capacidad germinativa; no sabemos si también de dormición. Semillas que pueden ser estudiadas en aras a determinar que factores están alterados con respecto a las semillas que tienen el máximo poder germinativo. La aparición de semillas diferentes en el interior de la silicua o bien la existencia de maduración no sincrónica dentro de una población no es un hecho novedoso. Así por ejemplo, son bien conocidas las pérdidas millonarias ocasionadas por el *shattering* (rozamiento de frutos maduros) en cultivos de crucíferas. El *shattering* está siendo estudiado en detalle y contempla una amplia variedad de interacciones de diversas señalizaciones hormonales en la que están implicados multitud de genes con una expresión espacial y temporal en la silicua altamente compleja (Dinneny y Yanofsky, 2005; Lewis *et al.*, 2006; Li *et al.*, 2006; Lin *et al.*, 2007; Matilla y Matilla-Vázquez, 2008). Finalmente, la aparición de la heterogeneidad en el interior de una misma silicua puede deberse también al posicionamiento de las semillas, lo cual provocaría quizás una diferente capacidad para importar fotoasimilados desde los sumideros de la planta madre. Pero esta posibilidad no está muy estudiada en la actualidad (revisado en Fenner, 1993; Luzuriaga *et al.*, 2006). El hecho de que el 100% de las semillas de *S. officinale* pertenecientes al lote oscuro estén vivas (test del tetrazolium) y solamente el 40 ± 7% lo estén en el lote claro puede apoyar esta hipótesis (Iglesias-Fernández *et al.*, 2007). Sin embargo, no hemos estudiado la posición de ambos tipos de semillas en el tabique central de la silicua.

El embrión de la semilla de *S. officinale* está rodeado por una cubierta seminal y una capa monoestratificada de carácter endospérmico. Esto mismo ocurre en *A. thaliana* (Liu *et al.*, 2005); pero no en otras semillas como *Lepidium sativum* (Müller *et* al., 2006) y *L. virginicum* (Nguyen *et al.*, 2000) las cuales, si bien tienen una única capa endospérmica rodeando la mayor parte del embrión, la zona correspondiente al micropilo posee dos o más capas de endospermo, respectivamente. La existencia de más de una capa endospérmica sobre el area micropilar implica que se genera una mayor resistencia para que se produzca la emergencia radicular. Es decir, el proceso de ablandamiento (*loosening*) en esta zona será más intenso que en *Arabidopsis* y *S. officinale.* Sin embargo, todavía no está aclarado el mecanismo que opera a nivel del micropilo para que el ápice radicular emerja y el eje radicular transforme su metabolismo en aerobio. Se barajan dos posibilidades: (i) las paredes celulares del endospermo micropilar se degradan parcialmente; y (ii) la estructura de estas paredes se modifica y se hace menos resistente. Sin embargo, y al margen del conocimiento del mecanismo de ablandamiento, existen semillas que poseen las paredes celulares del endospermo micropilar de menor grosor que en las células del endospermo lateral; lo que sugiere una menor resistencia ante el empuje del ápice radicular. Este hecho, que se describió recientemente (Williams *et al.*, 2001; da Silva *et al.*, 2005; Gong *et al.*, 2005), no hemos podido constatarlo en *S. officinale*.

El proceso de germinación se inicia con la toma de agua y finaliza cuando la radícula atraviesa las envueltas que la rodean (revisado en Kucera *et al.*, 2005). En muchas especies, incluyendo *Arabidopsis*, la rotura de la testa y el endospermo son dos pasos secuenciales (Petruzzelli *et al.*, 2003; Liu *et al.*, 2005; Leubner-Metzger *et al.*, 2006) y regulados de forma diferente por el *ABA* (Müller *et al.*, 2006; revisado por Nambara y Marion-Poll, 2003; Leubner-Metzger *et al.*, 2006). Estos pasos secuenciales no se observaron en *Solanaceae* (Petruzzelli *et al.*, 2003). El ABA inhibe específicamente la rotura del endospermo y las GAs producidas por el embrión contrarrestan el efecto. Es decir, el proceso de emergencia radicular en *Brassicaceae* está regulado por la relación GAs/ABA y muy probablemente también por el ablandamiento de la capa endospérmica que rodea el micropilo (Yamaguchi y Kamiya, 2002; Yamauchi *et* al., 2004; Müller *et al.*, 2006). La rotura de la cubierta seminal no parece estar afectada por ambas hormonas antagónicas (Kucera *et al.*, 2005). Es más, en experimentos antiguos llevados a cabo con semillas de *Brassicaceae* carentes de la capa endospérmica, el ABA no inhibe la emergencia radicular (Schopfer y Plachy, 1984). Estudios efectuados en varias semillas endospérmicas y que han sido revisados cuidadosamente por Leubner-Metzger, parecen sugerir que la evolución mantuvo en *Brassicaceae* el mecanismo molecular relacionado con la inhibición promovida por ABA sobre el ablandamiento del endospermo; y eliminó la fase del ablandamiento insensible al ABA (da Silva *et al.*, 2004; Kucera *et al.*, 2005; Leubner-Metzger *et al.*, 2006; Müller *et al.*, 2006). Recientemente se ha demostrado que la capa celular correspondiente al endospermo micropilar juega un papel determinante en la dormición de *Arabidopsis* (Bethke *et al.*, 2007).

En esta Memoria hemos demostrado que para completarse la emergencia radicular en *S. officinale* es preciso que la barrera correspondiente a la testa se rompa antes que la endospérmica (cap II, Fig. 1). Previamente, la capa de empalizada se desmantela y los cuerpos proteicos de la zona sub-apical de la radícula y del endospermo micropilar se vacían. No conocemos, si es que sucede, el grado de alteración de las paredes celulares en la zona micropilar y anejas. Sin embargo, y al igual que proponen algunos autores (Gong *et al.*, 2005; Gong y Bewley, 2007; Ren *et al.*, 2008), también especulamos que la activad β-mananasa (MAN) esté implicada. Mediante el uso de mutantes de *Arabidopsis thaliana* con alteraciones en genes *MAN* y el análisis por hibridación *in situ*, pretendemos conocer si alguno de los transcritos *MAN* se ubica específicamente en el area micropilar y cómo afecta esta mutación a la germinación en semillas heterogéneas de *S. officinale*. Este protocolo lo está llevando a cabo actualmente nuestro Grupo.

Muchas semillas de *Brassicaceae* producen y excretan mucílago (*mixospermia*) durante los primeros instantes de la imbibición (Western *et al.*, 2000), para lo que se requiere la expresión del gen *MIB61* (Penfield *et al.*, 2001). La función de este polisacárido péctico en el proceso germinativo no se conoce con exactitud, aunque se especula que interviene en la toma controlada de agua (Penfield *et al.*, 2001). El estudio de mutantes con dificultades en la biosíntesis del mucílago son una excelente herramienta para el progreso en el conocimiento de su papel fisiológico (Wester *et al.*, 2001; 2004). Muy recientemente se obtuvieron, con la ayuda de mutantes, interesantes resultados que parecen confirmar el papel de este polímero hidrofílico en la imbibición eficaz de la semilla de *A. thaliana* (Arsovski *et al.*, 2009). Las semillas de *S. officinale* son mixospérmicas y las implicaciones del mucílago en la germinación vienen avaladas por el hecho de que las del lote oscuro, muy productoras de mucílago, se imbiben más lentamente y germinan con mayor velocidad que las del lote claro, las cuales apenas producen y excretan este polisacárido (Iglesias-Fernández *et al.*, 2007). Está descrito que una toma de agua muy rápida altera la permeabilidad celular y desencadena la pérdida de solutos al medio externo provocando un daño en la semilla que puede disminuir su vigor y viabilidad (Legesse y Powel, 1992; Puga-Hermida *et al.*, 2003b) Sin embargo, no debemos de descartar otro papel alternativo del mucílago previo a su excrección. Así, cuando este polímero hidrofílico se hidrata, antes de ser excretado al apoplasto celular y a la superficie externa de la cubierta seminal, aumenta de tamaño y produce roturas en las paredes celulares primarias de las células de la cubierta seminal que lo producen. La expansión del mucílago en *Arabidopsis* depende de una β-galactosidasa (Dean *et al.*, 2007). Estas roturas en la pared podrían favorecer el ablandamiento de la testa y contribuir de esta manera a la emergencia radicular. Hipótesis que está pendiente de confirmación.

Independientemente del mecanismo que opere para desencadenar la emergencia radicular, las semillas de *S. officinale* son dependientes de un fotoperíodo y NO_3^- para ejecutarlo. Un caso similar tiene lugar en semillas de *Lolium rigidum* (raigrás) (Ellery *et al.*, 2003). El NO_3^- promueve la germinación mediante una complicada red de señalización en la que parecen implicados el ABA y GAs (Ali-Rachedi *et al.*, 2004; Alboresi *et al.*, 2005). En presencia de luz y NO_3^- las semillas del lote oscuro tienen un elevado poder germinativo y la emergencia radicular es fuertemente estimulada por ET. Con las semillas del lote claro sucede todo lo contrario. Estos resultados demuestran que las semillas de *S. officinale* son heterogéneas con respecto a la sensibilidad al ET (Iglesias-Fernández *et al.*, 2007). Curiosamente, todas las semillas de *S. officinale* responden de igual forma al ET que a su inmediato precursor ACC (datos no mostrados); lo cual demuestra que tienen la capacidad de oxidar el ACC (ACC-oxidasa), pero no poseen la misma sensibilidad hacia el ET. Sin embargo, no descartamos que las semillas claras no requieran ET para llevar a cabo su escasa germinación. Por el contrario, GA_{4+7} estimula la germinación en ambos lotes, siendo las semillas claras mucho más sensibles (Iglesias-Fernández *et al.*, 2007). La presencia de GA_{4+7} y ET juntos no provoca efecto sumatorio en el porcentaje de germinación; ni tan siquiera una estimulación mayor que cada una de las hormonas por separado (datos no mostrados). Los resultados de germinación en presencia de inhibidores de la síntesis y señalización de GAs y ET demuestran una fuerte inhibición que fue revertida con la adición de las hormonas correspondientes. Estos resultados, como veremos más adelante, sugieren fuertemente la existencia de un cross-talk entre las señalizaciones de GAs/ET. Sea como fuere, el papel del ET durante la imbibición y emergencia radicular no se conoce con exactitud, y su función en la eliminación de la dormición está siendo debatida en la actualidad (revisado en Matilla y Matilla-Vazquez, 2008). No obstante, se ha demostrado que, tanto la síntesis de ET como la sensibilidad al mismo, son dos factores importantes en la germinación de semillas de *Arabidopsis* (Beaudoin *et al.,* 2000; Ghassemian *et al.*, 2000; revisado en Kucera *et al.*, 2005). Pero también se demostró que existen semillas en las que no se requiere ET para mantener el estatus de dormición, eliminarlo y desencadenar el proceso germinativo (Matilla, 2000; Kucera *et al.*, 2005; Gianinetti *et al.*, 2007).

Dada la respuesta tan compleja de ambos lotes de semillas de *S. officinale* al ET y GAs, hemos decidido estudiar en profundidad solamente las oscuras. Este estudio abarca las alteraciones en la expresión de dos genes pertenecientes a las familias ACS (*SoACS7*) y ACO (*SoACO2*), y tres de las familias GA20ox (*SoGA20ox2*), GA3ox (*SoGA3ox2*) y GA2ox (*SoGA2ox6*) y engloba la embriogénesis zigótica, la germinación y el alfer-ripening (AR). Los resultados de expresión obtenidos durante el desarrollo y desecación de las semillas se pueden resumir del siguiente modo: (i) la expresión de *SoACS7*, la más elevada de todos los transcritos estudiados, y la de *SoGA3ox2* aumentan con el desarrollo y descienden durante la desecación; (ii) la expresión de *SoACO2* y *SoGA2ox6* incrementan durante todo el período estudiado, mientras que la de *SoGA20ox2* es muy elevada al inicio del desarrollo y desciende fuertemente en la fase final del mismo y durante la desecación. Tomados en conjunto, estos resultados apuntan a que las etapas finales del desarrollo de *S. officinale* no requiere GAs bioactivas, mientras que, y contrariamente a lo que sucede en *B. rapa* (Rodríguez-Gacio *et al.*, 2003), la síntesis de ET parece evidente.

En este trabajo hemos demostrado que las semillas oscuras de *S. officinale* son altamente susceptibles al AR y durante la adquisición del mismo el transcrito *SoGA3ox2* es el único estudiado que aumenta claramente su nivel. La expresión del resto de los genes desciende de forma sustancial con el AR. Es decir, hemos demostrado que las semillas secas son capaces de realizar una actividad transcripcional medible durante el AR. La actividad transcripcional en ambientes tan deshidratados es un hecho sometido a debate. Sin embargo, el descubrimiento en semillas de tabaco sometidas a AR de zonas con altos potenciales hídricos podría explicar esta actividad (Leubner-Metzger, 2005; Manz *et al.*, 2005). Por otra parte, en semillas de *N. plumbaginifolia* y *Hordeum vulgare* se demostró mediante cDNA-AFLP que el AR disminuye fuertemente los transcritos estudiados (Bove *et al.*, 2005; Leymarie *et al.*, 2007); mientras que mediante microarrays se demostró en *Arabidopsis* que la trancripción de al menos 30 genes disminuyen debido al AR (Finch-Savage *et al.*, 2007).

El hecho de que el transcrito *SoGA3ox2* incremente durante el AR, fuertemente sugiere que las GAs bioactivas tienen especial relevancia en el proceso germinativo de *S. officinale* (Iglesias-Fernández y Matilla, 2009). Las GAs bioactivas se requieren para la germinación de *Arabidopsis*, se sintetizan al inicio de la imbibición (Pen *et al.*, 2002; Ogawa *et al.*, 2003; Holdsworth *et al.*, 2008) y las familias génicas *GA3ox* y *GA2ox* desempeñan papeles decisivos en su síntesis e inactivación (Yamauchi *et al.*, 2004; Mitchum *et al.*, 2006). Durante las primeras horas de imbibición de semillas de *S. officinale* tiene lugar un notable aumento en el nivel de los transcritos *SoGA3ox2* y *SoGA20ox2*; mientras que los *SoACS7* y *SoACO2* están fuertemente inhibidos. Este hecho claramente demuestra la notable necesidad de GAs y escasa de ET para la preparación de la radícula para ejecutar su emergencia bajo el estatus de AR. Hecho que no ocurre en semillas no sometidas a AR. Es decir, la participación de ET y GAs parece diferir en semillas con y sin AR (Iglesias-Fernández y Matilla, 2009). Los resultados obtenidos en *S. officinale* usando pulsos de GA_{4+7}, ET e inhibidores de la síntesis y señalización de GAs y ET sugieren que el ET parece menos importante que las GAs para desencadenar la emergencia. Como se demostró en semillas de *Arabidopsis* (Weiss y Ori, 2007; De Grauwe *et al.*, 2008) y de *Fagus silvatica* (Calvo *et al.*, 2004) un claro cross-talk tiene lugar entre el ET y GAs en *S. officinale*. Tomando en conjunto los resultados de la expresión de *SoGA3ox2* y *SoGA20ox2* durante la fase de imbibición podemos concluir que: (i) la biosíntesis de GAs es indispensable para eliminar la dormición en semillas oscuras; (ii) ambos genes son regulados por ET y GAs para realizar la transición dormición-germinación inducida por AR; y (iii) el AR inhibe fuertemente la expresión *SoGA3ox2* en presencia de GA_{4+7}, paclobutrazol (PB) y ET+GA_{4+7} indicando que este gen está sometido a una fuerte regulación *feed-back*, posiblemente para prevenir la acumulación de GAs después de que la señal AR ha sido decidida.

La familia *GA2ox*, implicada en la inactivación de GAs bioactivas, está escasamente estudiada en semillas. Resultados previos a los publicados por nosotros (Iglesias-Fernández y Matilla, 2009) demostraron que *AtGA2ox6* es *down*-regulada por la estratificación en ausencia de luz

(Yamauchi *et al.*, 2004). Por otra parte, Vandenbussche *et al.* (2007) y Dugardeyn *et al.* (2008) demostraron la existencia de *up-* y *down*-regulación por ET de diferentes genes pertenecientes al metabolismo de GAs en plántulas jóvenes de *Arabidopsis*; concluyen que estos hechos están fuertemente relacionados con el cross-talk ET/GAs. Asimismo, los interesantes resultados de Chiwocha con el doble mutante *etr1-2* sugieren que la señalización del ET modula el metabolismo de varias rutas hormonales de semillas (ej. ABA y GAs) (Chiwocha *et al.*, 2005). En esta Memoria demostramos que *SoGA2ox6* se expresa a niveles basales durante los primeros instantes de la imbibición; confirmando la necesidad de la síntesis de GAs bioactivas tanto en presencia como en ausencia de AR. Sin embargo, el AR reguló *aguas arriba SoGA2ox6*, mientras que en semillas sin AR la expresión de este gen está regulada *aguas abajo* (Iglesias-Fernández y Matilla, 2009).

Tomados en su conjunto todos los resultados obtenidos con la expresión de los genes *SoGA3ox2*, *SoGA20ox2* y *SoGA2ox6* en semillas de *S. officinale* podemos hipotetizar que la regulación de la síntesis de GAs bioactivas implicadas en el AR está sometida a un fuerte control. Es decir, si se sobrepasa un nivel umbral de GAs necesario para ejecutar la germinación, la semilla responde con la destrucción de las GAs bioactivas innecesarias; en esta destrucción podría participar *SoGA2ox6*.

La parte final de esta Memoria de Doctorado está dedicada al estudio de la actividad MAN durante la adquisición del AR, la germinación de semillas y el efecto del cross-talk ET/GAs sobre esta actividad enzimática en *S. officinale*. En la actualidad, parece existir un consenso por el que la actividad MAN se requiere para el ablandamiento (*loosening*) del endospermo micropilar; pero no es suficiente para que se complete la germinación (Gong *et al.*, 2005; Gong y Bewley, 2007 y algunas referencias citadas). Se demuestra aquí por primera vez que: (i) el AR induce en semilla seca una actividad MAN mucho más elevada que en semillas sin AR; (ii) esta actividad se mantiene al menos durante 3 h de imbibición; y (iii) la actividad MAN desciende paulatinamente hasta la 12 h (Iglesias-Fernández y Matilla, 2009). La(s) causa(s) de esta gran actividad MAN en semilla seca la desconocemos. Pero aventuramos tres posibilidades: (a) esta proteína forma parte de un *pool* de proteínas inactivas sintetizadas durante el desarrollo como sugiere Ren *et al.* (2008); (b) la MAN desencadena un ablandamiento temprano de los tejidos de la semilla implicados en la emergencia radicular; y (c) la actividad MAN produce azúcares para la nutrición del embrión como propone Wang *et al.* (2004). Las GA_{4+7} incrementa la actividad MAN durante la imbibición y germinación de semillas de *S. officinale* con AR; pero no ocurre este efecto en semillas sin AR. Por otra parte, la actividad MAN es fuertemente estimulada en presencia de PB, lo que apunta a que un descenso en el nivel de GAs bioactivas desencadena una desincronización rápida en la semilla que induce una estimulación no específica de MAN que no afecta a la emergencia radicular. Es conveniente recordar que el PB ejerce una fuerte inhibición de la rotura de la cubierta seminal en *S. officinale*. El ET aumenta la actividad específica MAN y los inhibidores de la síntesis y señalización del mismo no le afectan. Lo que apunta a que el ET es uno de los factores que contribuyen a regular MAN; pero no parece indispensable (Iglesias-Fernández y Matilla, 2009).

Una vez discutidos los resultados de Biol Mol y teniendo en cuenta los datos presentados y publicados de esta Memoria de Doctorado, se propone que la intervención del cross-talk ET/GAs parece probable en semillas de *S. officinale*. Tanto en las que han adquirido el AR como en las que no lo tienen. Sin embargo, si este cross-talk interviene directa o indirectamente sobre el proceso germinativo no está claro actualmente. Es muy probable que el efecto del ET sobre GAs, y viceversa, pueda que sea indirecto, probablemente *vía* ABA. De ahí que esté previsto en nuestro Grupo el estudio del cross-talk ET/ABA.

BIBLIOGRAFÍA

Alboresi A, Gestin C, Leydecker M-T, Bedu M, Meyer C, Truong HN (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. *Plant, Cell & Environ* **28**: 500-12

Ali-Rachedi S, Bouinot D, Wagner MH, Bonnet M, Sotta B, Grappin P, Jullien M (2004) Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana. Planta* **219**: 479-88

Arsovski AJ, Villota MM, Rowland O, Subramaniam R, Western TL (2009) *MUM ENHANCERS* are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana. J Exp Bot* **doi: 10.1093/jxb/erp102**

Bailey CD, Koch MA, Mayer M, Mummenhoff K, *et al.* (2006) Toward a global phylogeny of the *Brassicaceae. Mol Biol Evol* **23**: 2142-60

Baskin JM, Baskin CC (2004) A classification system for seed dormancy. *Seed Sci Res* **14**: 1-16

Beaudoin N, Serizet C, Gosti F, Giraudat J (2000) Interactions between abscisic acid and ethylene signalling cascades. *Plant Cell* **12**: 1103-115

Bethke PC, Libourel IGL, Aoyama N, Chung YY, Still DW, Jones RL (2007) The Arabidopsis aleurone layer responds to nitric oxide, gibberellin, and abscisisc acid and is sufficient and necessary for seed dormancy. *Plant Physiol* **143**: 1173-88

Bove J, Lucas P, Godin B, Oge L, Jullien M, Grappin P (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia. Plant Mol Biol* **57**: 593-612

Calvo AP, Nicolás C, Nicolás G, Rodríguez D (2004) Evidence of a cross-talk regulation of a GA20-oxidase (*FsGA20ox1*) by gibberellins and ethylene during the breaking of dormancy in *Fagus silvatica* seeds. *Physiol Plant* **120**: 623-30

Chiwocha SDS, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, Ross ARS, Kermode A (2005) The *etr1*-2 mutation in *Arabidopsis thaliana* affects the ABA, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. *Plant J* **42**: 35-48

da Silva EAA, Toorop PE, van Aelst AC, Hilhorst HWM (2005) Abscisic acid controls embryo growth potential and endosperm cap weakening during coffee (*Coffea arabica* cv. Rubi) seed germination. *Planta* **220**: 251-61

de Grauwe L, Chaerle L, Dugardeyn J, Decat J, *et al.* (2008) Reduced gibberellin response affects ethylene biosynthesis and responsiveness in the Arabidopsis *gai eto2-1* double mutant. *New Phytol* **177**: 128-41

Dean GH, Zheng H, Tewari J, Huang J, *et al.* (2007) The *Arabidopsis MUM2* gene encodes a β-galactosidase required for the production of seed coat mucilage with correct hydration properties. *Plant Cell* **19**: 4007-21

Debeaujon I, Koornneef M (2000) GA requirements for *A. thaliana* seed germination in determined both by testa characteristics and embryonic ABA. *Plant Physiol* **122**: 415-24

Dinneny JR, Yanofsky MF (2005) Drawing lines and borders: how the dehiscence fruit of *Arabidopsis* is patterned. *BioEssays* **27**: 42-9

Dugardeyn J, Vandenbussche F, van der Straeten D (2008) To grow or not grow: what can we learn on ethylene-gibberellin cross-talk by *in silico* gene expression analysis?. *J Exp Bot* **59**: 1-16

Ellery AJ, Gallagher RS, Dudley SV (2003) Dormancy and germination ecology of annual ryegrass. En: *The biology of seeds: Recent Research Advances* (eds. Nicolás G, Bradford KJ, Côme D, Pritchard HW) Pp 389-96. CABI Pub, Wallingford, Oxon.

Fenner M (1999) Environmental influences of seed size and composition. *Hort Rev* **13**: 183-213

Finch-Savage WE, Cadman CS, Toorop PE, Lynn JR, Hilhorst HW (2007) Seed dormancy release in *Arabidopsis* Cvi by dry after-ripening, low temperature, nitrate and light shows common quantitative patterns of gene expression directly by environment specific sensing. *Plant J* **51**: 60-78

Ghassemian M, Nambara E, Cutler S, Kawaide H, Kamiya Y, Mc Court P (2000) Regulation of ABA signalling by the ethylene response pathway in *Arabidopsis*. *Plant Cell* 12: 1117-26

Gianinetti A., Laarhoven LJJ, Persijn ST, Harren FJM, Petruzzelli L (2007) Ethylene is associated with germination but not seed dormancy in red rice. *Ann Bot* 99: 735-45

Gong X, Bassel G, Wang A, Greewood JS, Bewley JD (2005) The emergence of embryos from hard seeds is related to the structure of the cell walls of the micropylar endosperm, and not to endo-β-mannanase activity. *J Exp Bot* 96: 1165-73

Gong X, Bewley JD (2007) Sorting out the *LeMANs*: endo-β-mannanase genes and their encoded proteins in tomato. *Seed Sci Res* 17: 143-54

Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *New Phytol* 179: 33-54

Iglesias-Fernández I, Matilla AJ, Pulgar I, de la Torre F (2007) Ripe fruits of *Sisymbrium officinale* L. contain heterogeneous endospermic seeds with different germination rates. *Seed Sci Biotech* 1: 18-24

Iglesias-Fernández R, Matilla AJ (2009) After-ripening alters the gene expression pattern of oxidases involved in the ethylene and gibberellin pathways during early imbibition of *Sisymbrium officinale* L. seeds. *J Exp Bot* 60: 1645-61

Imbert E (2002) Ecological consequences and ontogeny of seed heteromorphism. *Perspect Plant Ecology, Evol and System* 5: 13-36

Kucera B, Cohn MA, Leubner-Metzger L (2005) Plant hormone interactions during seed dormancy release and germination. *Seed Sci Res* 15: 281-307

Leubner-Metzger G (2005) Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant J* 1: 133-45

Li CB, Zhou AL, Sang T (2006) Rice domestication by reducing shattering. *Science* 311: 1936-39

Lin Z, Griffith ME, Li X, Zhu Z, *et al.* (2007) Origin of seed shattering in rice (*Oriza sativa* L.). *Planta* 226: 11-20

Liu P-P, Koizuka N, Homrichhausen TM, Hewitt JR, Martin RC, Nonogaki H (2005). Large-scale screening of Arabidopsis enhancer-trap lines for seed germination-associated genes. *Plant J* 41: 936-44

Legesse N, Powell AA (1992) Comparisons of water uptake and imbibition damage in eleven cowpea cultivars. *Seed Sci Tech* 20: 173-80

Leubner-Metzger G, Kucera B, Müller K (2006) Emerging and established model system for endosperm weakening. En: *Seeds: biology, development and ecology.* (eds. Navie S, Adkins S, Ashmore S) Pp. 195-204. CAB International, UK

Lewis MW, Leslie M, Liljegren SJ (2006) Plant separation: 50 ways to leave your mother. *Curr Op Plant Biol* 9: 59-65

Leymarie J, Bruneaux E, Gibot-Leclerc S, Corbineau F (2007) Identification of transcripts potentially involved in barley seed germination: and dormancy using cDNA-AFLP. *J Exp Bot* 58: 425-37

Luzuriaga AL, Escudero A, Pérez-García F (2006) Environmental maternal effects on seed morphology and germination in *Sinapis arvensis* (*Cruciferae*). *Weed Res* 46: 163-74

Manz B, Müller K, Kucera B, Volke F, Leubner-Metzger G (2005) Water uptake and distribution in germinating tobacco seeds investigated in vivo by nuclear magnetic resonance imaging. *Plant Physiol* 138: 1538-51

Matilla AJ (2000) Ethylene in seed formation and germination. *Seed Sci Res* 10: 111-26

Matilla AJ, Gallardo M, Puga-Hermida MI (2005) Structural, physiological and molecular aspects of heterogeneity in seeds: a review. *Seed Sci Res* 15: 63-76

Matilla AJ, Matilla-Vázquez MA (2008) Involvement of ethylene in seed dormancy: Review. *Plant Sci* 175: 87-97

Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, *et al.* (2006) Distinct and overlapping roles of two gibberellin 3-oxidases in Arabidopsis development. *Plant Journal* 45: 804-18

Müller K, Tintelnot S, Leubner-Metzger G (2006). Endosperm-limited *Brassicaceae* seed germination: abscisic acid inhibits embryo-induced endosperm weakening of *Lepidium sativum* (cress) and endosperm rupture of cress and *Arabidopsis thaliana*. *Plant Cell Physiol* 47: 864-77

Nambara E, Marion-Poll A (2003) ABA action and interactions in seeds. *Trends Plant Sci* 8: 213-17

Nguyen H, Brown RC, Lemmon BE (2000) The specialized chalazal endosperm in *A. thaliana* and *Lepidium virginicum* (Brassicaceae). *Protoplasm* **212**: 99-110

Ogawa M, Hanada A, Yamauchi Y, Kuwahara A, Kamiya Y, Yamaguchi S (2003) Gibberellin biosynthesis and response during Arabidopsis seed germination. *Plant Cell* **15**: 1591-604

Pen JR, Harberd NP (2002) The role of GA-mediated signalling in the control of seed germination. *Curr Opin Plant Biol* **5**: 376-81

Penfield S, Meissner RC, Shoue DA, Carpita NC, Bevan MW (2001) *MYB61* is required for mucilage deposition and extrusion in the Arabidopsis seed coat. *Plant Cell* **13**: 2777-91

Petruzzelli L, Müller K, Hermann K, Leubner-Metzger G (2003) Distinct expression patterns of β-1,3-glucanases and chitinases during the germination of solanaceous seed. *Seed Sci Res* **13**: 139-53

Puga-Hermida MI, Gallardo M, Matilla AJ (2003a) The zygotic embryogenesis and ripening of *Brassica rapa* seeds provokes important alterations in the levels of free and conjugated abscisic acid and polyamines. *Physiol Plant* **117**: 279-88

Puga-Hermida MI, Gallardo M, Rodríguez-Gacio MD, Matilla AJ (2003b) The heterogeneity of turnip-tops (*Brassica rapa*) seeds inside the silique affects germination, the activity of the final step of the ethylene pathway, and abscisic acid and polyamine content. *Funct Plant Biol* **30**: 767-75

Ren Y, Bewley JD, Wang X (2008) Protein and gene expression patterns of endo-β-mannanase following germination of rice. *Seed Sci Res* **18**: 139-49

Rodríguez-Gacio MC, Nicolás C, Matilla AJ (2003) The final step of the ethylene biosynthesis pathway in turnip tops (*Brassica rapa* L. cv. Rapa): molecular characterization of the 1-aminocyclopropane-1-carboxylate oxidase *BrACO1* throughout zygotic embryogenesis and germination of heterogeneous seeds. *Physiol Plant* **121**: 132-40

Schopfer P, Plachy C (1984) Control of seed germination by ABA. II. Effect on embryo water uptake in *Brassica napus* L. *Plant Physiol* **76**: 155-60

Vandenbussche F, Vancompernolle B, Rieu I, Ahmad M, Phillips A, Hedden P, Moritz T, van der Straeten D (2007) Ethylene-induced *Arabidopsis* hypocotyl elongation is dependent on but not mediated by gibberellins. *J Exp Bot* **58**: 4269-81

Wang A, Wang X, Ren Y, Gong X, Bewley JD (2005) Endo-β-mannanase and β-mannosidase activities in rice grains during and following germination, and the influence of gibberellin and abscisic acid. *Seed Sci Res* **15**: 219-27

Weiss D, Ori N (2007) Mechanism of cross talk between gibberellin and other hormones. *Plant Physiol* **144**: 1240-46

Western TL, Skinner DJ, Haughn GW (2000) Differentiation of mucilage secretory cells of the Arabidopsis seed coat. *Plant Physiol* **122**: 345-55

Western TL, Burn J, Tan WL, Skinner DJ, Martin-McCaffrey L, Moffatt BA, Haughn GW (2001) Isolation and characterization of mutants defective in seed coat mucilage secretory cell development in *Arabidopsis*. *Plant Physiol* **127**: 998-1011

Western TL, Diana S, Young DS, Dean GH, *et al.* (2004) MUCILAGE-MODIFIED4 encodes a putative pectin biosynthetic enzyme developentally regulated by APETALA2, TRANSPARENT TESTA GLABR1, and GLABRA2 in Arabidopsis seed coat. *Plant Physiol* **134**: 296-306

Williams HA, Bewley JD, Greenwood JS, Bourgault R, Mo B (2001) The cell wall of the endosperm of *Asparagus officinalis* seeds during development and following germination. *Seed Sci Res* **11**: 305-15

Yamaguchi S, Kamiya Y (2002) Gibberellins and light-stimulated seed germination. *J Plant Growth Regul* **20**: 369-76

Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S (2004) Activation of gibberellin biosynthesis and response pathways by low temperatura during imbibition of *Arabidopsis thaliana* seeds *Plant Cell* **16**: 367-78

VII. CONCLUSIONES

VII. – Conclusiones

Se estudia en la crucífera de carácter nitrófilo *Sisymbrium officinale* algunas características morfológicas, fisiológicas y transcriptómicas relacionadas con el desarrollo, dormición, imbibición y emergencia radicular de sus semillas, las cuales son endospérmicas.

Conclusión 1

La silicua madura de *S. officinale* posee semillas heterogéneas con respecto a los siguientes caracteres estudiados: color de la testa (oscuras y claras), densidad, capacidad de producir mucílago (mixospermia), velocidad de imbibición y diferente grado de germinación y sensibilidad a las giberelinas (GA_{4+7}) y etileno. La heterogeneidad no modifica la necesidad del nitrato para germinar; lo que demuestra la notable dependencia de su señalización. En cualquier caso, el hecho de que todas las semillas de esta especie se dispersen al mismo tiempo, abre nuevas expectativas sobre el papel de la heterogeneidad en esta y otras que la posean.

Conclusión 2

La dormición de las semillas oscuras de *S. officinale* se elimina mediante el *after-ripening* (AR), el cual provoca en semilla seca un fuerte incremento en la transcripción del gen *SoGA3ox2*; a diferencia de los otros transcritos estudiados cuyos niveles disminuyen notablemente. Estos resultados nos llevan a concluir que: (i) la rotura de la dormición por AR es dependiente de la síntesis de GAs bioactivas, no evidenciándose la participación del etileno; y (ii) la semilla seca de *S. officinale* es capaz de transcribir, hecho este observado por primera vez en semillas sometidas a AR.

Conclusión 3

El proceso de AR posee cuatro características que afectan a la germinación de semillas oscuras de *S. officinale*: (i) el hecho de que el AR acelere la estimulación de la emergencia radicular en ausencia de nitrato, al cual el AR es incapaz de reemplazar, sugiere la existencia de un *cross-talk* AR/NO_3^-; (ii) el AR amplía el rango de temperturas óptimas para germinar en presencia de nitrato, lo que puede representar un mecanismo para controlar la dormición en ambiente secos; (iii) el AR induce un aumento en la sensibilidad al etileno y GA_{4+7}, hormonas que estimulan la germinación en *S. officinale*; (iv) el AR, a la temperatura óptima de germinación (24 °C), altera positivamente la toma de agua durante la imbibición temprana; esta rápida imbibición, en la que el mucílago no parece implicado, debe de estar muy controlada por la señalización del AR para que el proceso de imbibición se inicie normalmente.

Conclusión 4

Los resultados de expresión de los genes *SoGA3ox2*, *SoGA3ox2*, *SoGA2ox6*, *SoACS7* y *SoACO2* durante el período de imbibición y emergencia radicular de *S. officinale* demuestran que: (i) la participación del etileno y giberelinas difiere en semillas con y sin AR; (ii) la expresión de *SoACO2* en semillas sin AR es inhibida en presencia de GA_{4+7}, (GA_{4+7}+ ET) e inhibidores de la síntesis y señalización de ambas hormonas; esta disminución es revertida por AR; (iii) la expresión de *SoGA3ox2* y *SoGA2ox6* son mucho más susceptibles al AR y ET que la de *SoGA20ox2*; (iv) proponemos que, aunque el *cross-talk* ET/GAs parece intervenir en el proceso del AR, no hemos demostrado si esta intervención es directa o si el ABA colabora en la misma.

Conclusión 5.

Demostramos por vez primera que el AR induce una importante actividad β-mananasa (MAN) in-vitro en semillas secas de *S. officinale*. El estudio de MAN durante el proceso germinativo demuestra que el patrón de actividad y el porcentaje de germinación no se corresponden; lo que nos hace concluir que este enzima puede contribuir al ablandamiento de la zona micropilar y a la emergencia radicular. GA_{4+7} solamente estimulan la actividad MAN en semillas con AR, mientras que el papel del ET es, en este momento, dudoso. Experimentos con mutantes T-DNA en genes *MAN* de *Arabidospis* realizados por nuestro Grupo nos demostraron que, con una expresión *MAN* muy inhibida, el fenotipo de germinación, floración y crecimiento de raíces secundarias está fuertemente alterado.

VIII. ABREVIATURAS

ABA: Ácido Abscísico
aba: ABA deficient
ABRE: ABA Binding Response Element
ACC: Ácido 1-aminiciclopropano-1-carboxílico
ACO: ACC oxidasa
ACS: ACC sintetasa
AR: After-Ripening
ARN: Ácido Ribonucleico
ARNm: Ácido Ribonucleico mensajero
BL: Brasinólido
BRs: Brasinoesteroides
cDNA-AFLP: Complementary Deoxyribonucleic Acid-Amplified Fragment Length Polymorphism
Col: Columbia
Cvi: Cabo Verde Island
DDL: Durmiente Dependiente de Luz
DF: Dormición Fisiológica
DF+DFs: DF+ Dormición Física
DFs: Dormición Física
DFI: Dormición Fisiológica Intermedia
DFP: Dormición Fisiológica Profunda
DFnP: Dormición Fisiológica no Profunda
DM: Dormición Morfológica
DMF: Dormición Morfofisiológica
DOG: Delay of Germination
EBR: Epibrasinólido
ERO: Especies Reactivas de Oxígeno
ET: Etileno
etr: Ethylene insensitive
GA2ox: GA2-oxidasa
GA3ox: GA3-oxidasa
GA20ox: GA20-oxidasa
GAs: Giberelinas
LEA: Late Embryogenesis Abundant
Ler: Landsberg Erecta
LOF: Lost of Function
MAN: Actividad endo-β-mananasa
PAS: Periodic Acid-Schiff
PAs: Proantocianidinas
PME: Pectin-metilestearasa
PP2C: Proteín fosfatasas 2C
QTL: Quantitative Trait Locus
TA: Temperatura Ambiente
TAG: Triacilgliceroles
XTHs: Xiloglucan-endotransglicosilasa /hidrolasas
WT: *Wild Type*

RESUMEN TESIS DOCTORAL

Título: "El cross-talk etileno/giberelinas y la rotura de la dormición de semillas de Sisymbrium officinale L. provocada por el after-ripening"

Resúmen: Se estudia en la crucífera de carácter nitrófilo *Sisymbrium officinale* algunas características morfológicas, fisiológicas y transcriptómicas relacionadas con el desarrollo, dormición, imbibición y emergencia radicular de sus semillas, las cuales son endospérmicas. La silicua madura de *S. officinale* posee semillas heterogéneas con respecto a los siguientes caracteres estudiados: color de la testa (oscuras y claras), densidad, capacidad de producir mucílago (mixospermia), velocidad de imbibición y diferente grado de germinación y sensibilidad a las giberelinas (GA_{4+7}) y etileno (ET). La heterogeneidad no modifica la necesidad del nitrato (NO_3^-) para germinar; lo que demuestra la notable dependencia de su señalización. En cualquier caso, el hecho de que todas las semillas de esta especie se dispersen al mismo tiempo, abre nuevas expectativas sobre el papel de la heterogeneidad en esta y otras que la posean.

La dormición de las semillas oscuras de *S. officinale* se elimina mediante el *after-ripening* (AR), el cual provoca en semilla seca un fuerte incremento en la transcripción del gen *SoGA3ox2*; a diferencia de los otros transcritos estudiados cuyos niveles disminuyen notablemente. Estos resultados nos llevan a concluir que: (i) la rotura de la dormición por AR es dependiente de la síntesis de GAs bioactivas, no evidenciándose la participación del ET; y (ii) la semilla seca de *S. officinale* es capaz de transcribir, hecho este observado por primera vez en semillas sometidas a AR. Asimismo, el proceso de AR posee cuatro características que afectan a la germinación de semillas oscuras de *S. officinale*: (i) el hecho de que el AR acelere la estimulación de la emergencia radicular en ausencia de NO_3^-, al cual el AR es incapaz de reemplazar, sugiere la existencia de un *cross-talk* AR/NO_3^-; (ii) el AR amplía el rango de Tª óptimas para germinar en presencia de NO_3^-, lo que puede representar un mecanismo para controlar la dormición en ambiente secos; (iii) el AR induce un aumento en la sensibilidad al ET y GA_{4+7}, hormonas que estimulan la germinación en *S. officinale*; (iv) el AR, a la Tª óptima de germinación (24ºC), altera positivamente la toma de agua durante la imbibición temprana; esta rápida imbibición, en la que el mucílago no parece implicado, debe de estar muy controlada por la señalización del AR para que el proceso de imbibición se inicie normalmente. Por otra parte, los resultados de expresión de los genes *SoGA3ox2*, *SoGA3ox2*, *SoGA2ox6*, *SoACS7* y *SoACO2* durante el período de imbibición y emergencia radicular de *S. officinale* demuestran que: (i) la participación del etileno y giberelinas difiere en semillas con y sin AR; (ii) la expresión de *SoACO2* en semillas sin AR es inhibida en presencia de GA_{4+7}, (GA_{4+7}+ ET) e inhibidores de la síntesis y señalización de ambas hormonas; esta disminución es revertida por AR; (iii) la expresión de *SoGA3ox2* y *SoGA2ox6* son mucho más susceptibles al AR y ET que la de *SoGA20ox2*; (iv) proponemos que, aunque el *cross-talk* ET/GAs parece intervenir en el proceso del AR, no hemos demostrado si esta intervención es directa o si el ABA colabora en la misma. Por último, demostramos por primera vez que el AR induce una importante actividad β-mananasa (MAN) in-vitro en semillas secas de *S. officinale*. La valoración de MAN durante el proceso germinativo demuestra que el patrón de actividad y el del porcentaje de germinación no se corresponden; lo que nos hace concluir que este enzima puede contribuir al ablandamiento de la zona micropilar y a la emergencia radicular; pero no parece ser la única responsable. GA_{4+7} solamente estimula la actividad MAN en semillas con AR, mientras que el papel del ET es, en este momento, dudoso.