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MIEMBROS DE LA FAMILIA DUF642 COMO POSIBLES REGULADORES DE LA FUNCIÓN DE PROTEÍNAS DE LA PARED CELULAR DURANTE EL DESARROLLO EN ARABIDOPSIS THALIANA

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Resumen

Este trabajo tiene como propósito el estudio de dos miembros de la familia de proteínas DUF642 en *Arabidopsis thaliana* la primera codificada por el gen *At4g32460* denominada *BIIDXI* (que en lengua zapoteca significa semilla) y su gen homólogo *At5g11420*. La pared celular en las células vegetales juega un papel de gran impacto durante el desarrollo y el crecimiento de las plantas, además de ser una barrera que percibe las condiciones bióticas y abióticas del medio. Su composición, a pesar de ser básicamente la misma, varía entre los distintos taxa y hace de su estudio un reto en las diferentes áreas. Una de las preguntas que guía este trabajo tiene como objetivo determinar si la función de estos dos miembros de la familia DUF642 está relacionada con la interacción de ellos y la proteína pectin-metil-esterase 3 de *A. thaliana* (*AtPME3*). La actividad de este tipo de enzimas en procesos relacionados con la morfología y el crecimiento celular es de gran relevancia en la dinámica de la pared celular. El capítulo I aborda esta pregunta mediante los análisis de la expresión de *BDX* en los diferentes tejidos y estadios del desarrollo de *A. thaliana y* mediante la utilización de plantas transgénicas de ganancia de función para *BDX* y *At5g11420* y de pérdida de función para BDX. Los fenotipos correlacionan con los niveles de actividad total de PME en distintos órganos y tejidos proponiéndose que de manera directa o indirecta tanto BDX como At5g11420 podrían estar implicados en la activación de AtPME3.

La regulación de la actividad de PME durante la infección por patógenos también ha sido reportada por lo que el segundo capítulo se encaminó a tratar de vislumbrar la importancia de BDX y otro miembro de la familia DUF642, *At2g41800*, durante etapas tempranas de la infección por el nemátodo *Meloidogyne incognita*. Los resultados de estos experimentos muestran que durante la infección ocurren cambios de expresión y de localización, por lo que la importancia de estos genes durante la interacción planta-patógeno puede ser de gran relevancia.

Finalmente en el capítulo tres se exponen datos no publicados relacionados con la expresión de *At5g11420* y se discute el papel del fotoperíodo durante el transporte de nutrientes y el llenado de la semilla con relación a la expresión de BDX en el haz vascular. También se muestran resultados preliminares al utilizar plantas transgénicas que expresan a la proteína codificada por *At5g11420* bajo la secuencia regulatoria de *BDX*, los cuales permiten sugerir funciones fisiológicas distintas entre ambos genes y nuevamente se enfatiza la regulación de la actividad de PME durante la formación de órganos. Con base en los resultados se discute la importancia de esta familia en la evolución de las plantas con semilla y se propone un modelo general de la función de las proteínas DUF642.

Abstract

The aim of this paper is study two cell wall DUF642 members in *Arabidopsis thaliana.* BIIDXI (which means in zapotec language "seed") encoded by *At4g32460* and its homologous gene *At5g11420*. Plant cell wall plays a main role during plant development and growth, being a barrier that senses biotic and abiotic environmental conditions. Cell wall composition has basically the same structure among different taxa however varies among groups; these differences make its study a challenge. The main question that led this work was to determine whether the function of these two DUF642 members is related to its interaction with *Arabidopsis thaliana* pectin methyl esterase protein 3 (AtPME3). The activity of these enzymes is associated to morphological and cell growth processes and has great importance in the dynamics of the cell wall. Chapter I deals with this question through analysis of *BDX* expression in different tissues and developmental stages of *A. thaliana* and using over expression transgenic plants for *BDX* and *At5g11420* and loss of function for BDX. The phenotypes were correlated with the levels of total PME activity in different organs and tissues proposing that directly or indirectly both BDX as At5g11420 could be involved in the activation of AtPME3.

The regulation of the activity of PME during pathogen infection has also been reported, so the second chapter tries to discern the importance of BDX and another member of the DUF642 family, *At2g41800*, during early stages of infection by the nematode *Meloidogyne incognita*. The results of these experiments showed that during infection changes occurred in expression and localization of both genes. Hence the importance of these genes during plant-pathogen interaction can be of great relevance.

Finally, in chapter three, data related to the expression of *At5g11420* in different tissues are presented and also the role of photoperiod for nutrient transport and filling of the seed in relation to the *BDX* expression in the vascular bundle is discussed. The results using transgenic plants expressing the protein encoded by *At5g11420* downstream BDX regulatory sequence, suggest different physiological functions between these genes and again emphasized PME activity regulation during organ formation. Based on the results the importance of DUF642 family in the evolution of seed plants is discussed and a general model of the biochemical function of the DUF642 proteins is proposed.

Introducción general

Dinámica de la pared celular en plantas

Contrario a lo que intrínsecamente podría ser conceptualizado como una pared, es decir un compartimento rígido que únicamente delimita una zona o espacio, la pared celular en plantas es un compartimento dinámico capaz de experimentar cambios estructurales profundos determinados por el tipo de célula, la etapa y/o el estadío del desarrollo (Palin y Geitmann, 2012).

Bajo ciertas circunstancias y dependiendo del tipo de tejido, las células pueden incrementar su tamaño en varios órdenes de magnitud ligado a un aumento en el volumen. Este aumento implica una ampliación en la superficie celular, que requiere de la síntesis de compuestos para la formación de nuevas membranas y para la construcción de la pared celular. El estudio de la síntesis y recambio de la pared celular durante el crecimiento y elongación celular ha sido un tema de interés desde inicios del siglo pasado. Algunos trabajos de esa época refieren la elongación celular como un proceso llevado a cabo en presencia de la "sustancia de crecimiento" la cual es liberada únicamente en la región de la célula que se elonga sin necesidad de división celular (Bonner, 1934). Actualmente sabemos que la elongación celular es mediada por deposición de compuestos en la pared celular, sintetizados en las cisternas del Golgi y liberados al espacio extracelular a través de vesículas (Geisler *et al.*, 2008, Kim y Brandizzi, 2014). En ocasiones, dicha deposición es polarizada como es el caso del crecimiento del tubo polínico a través del tracto del carpelo y de los pelos radiculares (Gu y Nielsen, *et al.*, 2013; Guan *et al.*, 2013). En otros tipos celulares, la deposición puede ser menos localizada como es en el caso de las células meristemáticas del procambium que, al diferenciarse a células del xilema, pueden llegar a incrementar su tamaño en varios órdenes de magnitud (Cosgrove, 2005). Este proceso también está influenciado por la capacidad de deformación y flexibilidad de la pared existente. El grado de flexibilidad y la dinámica de crecimiento de la pared celular dependen de las propiedades bioquímicas y fisicoquímicas de los polisacáridos que la forman. El estudio de las propiedades de la celulosa ha sido objeto de la mayor parte de los trabajos relacionados con la dinámica de la pared celular de plantas (Sarkar *et al.*, 2014 Somerville, 2006) aunque recientemente también se han enfocado en las propiedades de la matriz péctica en la cual se encuentra embebida (Thimm *et al.,* 2009; Zykwinska *et al.,* 2008, Peaucelle *et al.*, 2012).

El citoesqueleto, específicamente los microtúbulos, juega un papel relevante en la dinámica y remodelación de la pared durante los distintos estadios del desarrollo y durante el ciclo celular, al participar en la organización y deposición de la celulosa dirigiendo los complejos de su síntesis (CSC) a través de la membrana plasmática y guiando su trayectoria y velocidad (Fig. 1). El efecto final es la plasticidad en los patrones de deposición de celulosa.

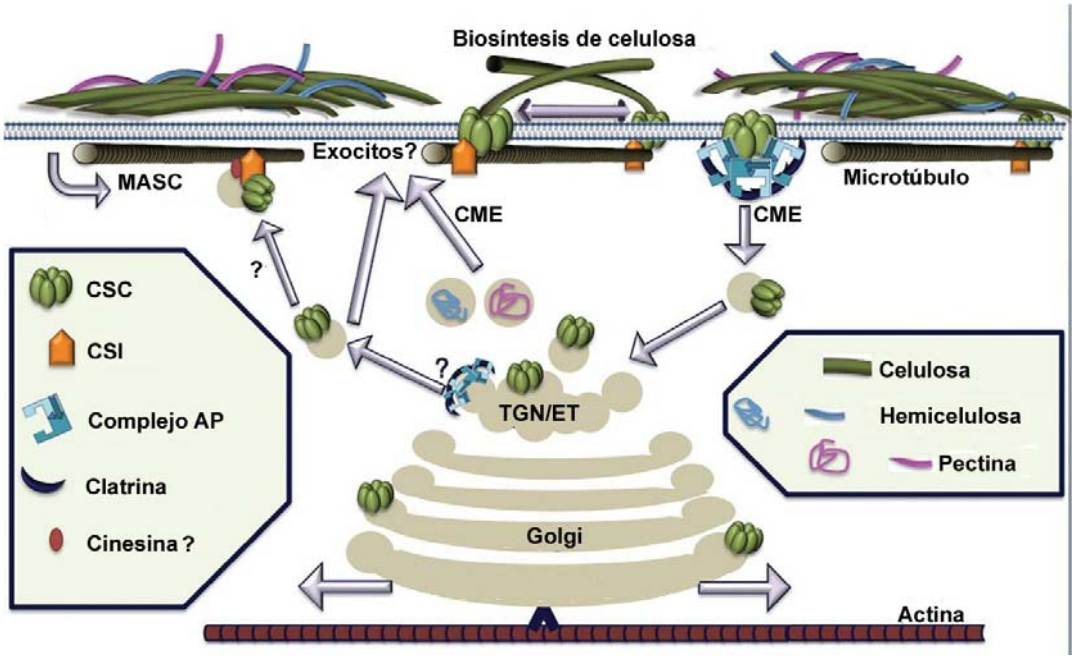


Figura 1: Diagrama que esquematiza las rutas y los mecanismos de tráfico de componentes de la pared celular en plantas. La pectina y la hemicelulosa son sintetizadas y secretadas desde el aparato de Golgi y depositadas en la pared celular a través de vesículas, mientras que la celulosa es sintetizada por los Complejos de Síntesis de Celulosa (CSC) localizados en la membrana plasmática. Los CSC son ensamblados en el aparato de Golgi, el cual es responsable de la distribución de éstos en toda la célula mediante un proceso actina-dependiente. Los CSC son secretados posiblemente desde la Red Trans Golgi/Endosomas Tempranos (TGN/ET). Los mecanismos de fusión de las vesículas que contienen CSC en la membrana plasmática se desconocen pero se llevan a cabo en los microtúbulos corticales. Se ha especulado que complejos exocíticos podrían estar involucrados en los eventos de fusión. Los CSC localizados en la membrana plasmática se mueven bi-direccionalmente a lo largo de los microtúbulos corticales al utilizar la fuerza de polimerización de la celulosa como fuerza de propulsión. La proteína CSI media la interacción entre el CSC y los microtúbulos. La endocitosis de los CSC puede ser mediada por complejos AP2/clatrina y rápidamente ser reciclados a través de la TGN/ET (Modificado de Bashline *et al.*, 2014).

Tipos y composición de la pared celular en plantas

Una característica de las células vegetales es la presencia de pared celular, matriz extracelular y apoplasto. La pared en plantas funciona como una barrera protectora contra el estrés biótico y abiótico siendo el principal compartimento de las células vegetales cuya función está estrechamente relacionada con percibir las señales externas y la transmisión de éstas hacia el interior a través de rutas de señalización.

Existen básicamente tres tipos de paredes o capas celulares en las células vegetales: la lámina media o placa celular que se deposita poco después de la mitosis creando una barrera entre las dos células hijas, la pared primaria la cual se deposita durante todo el proceso de crecimiento y expansión de las células y la pared secundaria que se deposita internamente en la pared primaria una vez que el crecimiento de la célula ha terminado (Popper, 2008; Cosgrove, 2005).

Pared primaria

La composición de la pared celular primaria varía dependiendo del estadío, proceso del desarrollo y/o la especie. Aunque todas las paredes celulares en plantas tienen exactamente la misma composición química básica, ya que el número de monómeros necesarios para la síntesis de polisacáridos es relativamente pequeño, las diferencias en cuanto a estructura, ensamblaje y organización de las macromoléculas hacen que el estudio de este compartimento sea complejo en cada grupo. En dicotiledóneas, como en *Arabidopsis thaliana,* la matriz de polisacáridos en la que se embebe la celulosa consiste principalmente de hemicelulosa xiloglucanos y de pectinas cargadas negativamente (Fig. 2 y 3) (Popper *et al.*, 2011).

Los residuos predominantes de monosacáridos que componen los polisacáridos son la D-glucosa (Glc), D-galactosa (Gal), D-manosa (Man), D-xilosa (Xil), L-arabinosa (Ara) L-fucosa (Fuc), L-ramnosa (Rha) y D-ácido galacturónico (GalA). Las diferentes combinaciones en la composición generan una gran variedad de polisacáridos con diversas características químicas que están relacionadas con la complejidad de la pared celular y la diversificación de las plantas. Esto se ve reflejado en el número de genes involucrados en la síntesis, remodelación y de-construcción de la pared celular. Por ejemplo, en *A. thaliana*

y en *Populus tripocarpa* (álamo) existen respectivamente, entre 1000 y 2500 genes posiblemente relacionados con estos procesos (Sarkar *et al.*, 2009).

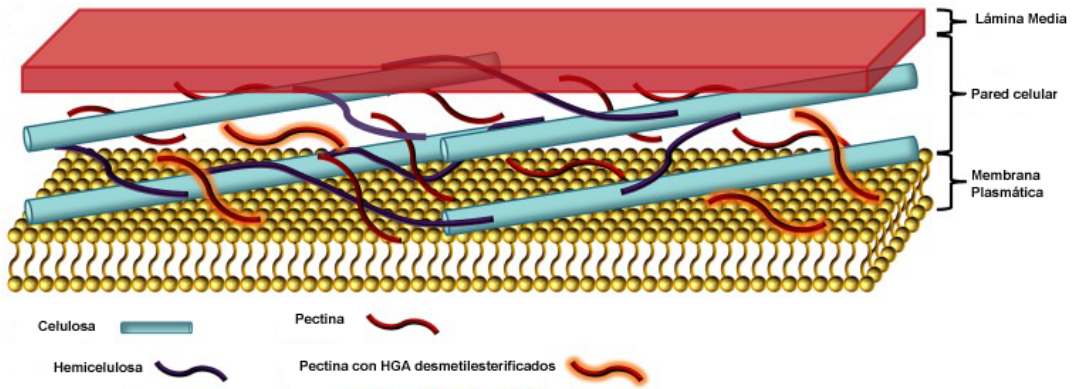


Figura 2: Esquema que representa de manera simplificada la estructura de la pared primaria en plantas (tomado de Malinovsky *et al.*, 2014).

En general, la pared primaria de las plantas es una red de glicanos interconectados por celulosa-hemicelulosa, embebidos en una matriz más soluble de pectinas, glicoproteínas, proteoglicanos, iones y compuestos de bajo peso molecular (Carpita, 1996). En las paredes primarias de las dicotiledóneas (Paredes tipo I), las hemicelulosas más abundantes son los xiloglucanos (XG), los cuales pueden interactuar con la celulosa superficialmente o quedar atrapados entre las microfibrillas durante su síntesis mediante enlaces covalentes a través de reacciones de transglicosilación. Las pectinas son uno de los componentes principales de las paredes primarias de las plantas y también se encuentran abundantemente en la lámina media, entre las paredes primarias, donde su función es regular la adhesión intercelular. Constituyen alrededor del 35% de la pared primaria en dicotiledóneas y en plantas monocotiledóneas no poaceas (Willats *et al.*, 2001). Las pectinas de las plantas superiores forman la familia de polisacáridos estructuralmente más compleja y se ha estimado que durante su síntesis están involucradas al menos 67 actividades enzimáticas distintas que incluyen glicosil transferasas, acetil transferasas y metil transferasas. Con base en su estructura, se han descrito cinco clases de pectinas: homogalacturonanos (HG), xilogalacturonanos (XGA), apigalacturonanos (AP), y ramnogalacturonanos I y II (RG). Los homogalacturonanos (HG) son las pectinas más abundantes, aproximadamente el 65%, y consisten de un polímero lineal de aproximadamente 100 unidades de α-1,4-ácido galacturónico (GalA). Las modificaciones

presentes en los HG son determinantes durante el ciclo de vida de las plantas ya que controlan procesos tales como el desarrollo de órganos, abscisión de las hojas, hidratación de la semilla, maduración del fruto y la filotaxis (Wolf *et al.*, 2009). Estudios *in vitro* e *in situ* muestran que las pectinas son capaces de interactuar con celulosa mediante sus cadenas laterales de arabinanos, galactanos y RG-I. Durante la síntesis de pared celular en bacterias, se ha demostrado que las pectinas inducen alteraciones en la extensibilidad y firmeza de la red de microfibrillas de celulosa, función consistente con los análisis realizados en planta al utilizar cobtorina, un herbicida que perturba las propiedades fisicoquímicas de las pectinas y que también desestabiliza la deposición de las microfibrillas de celulosa (Yoneda *et al.*, 2010, Chanliaud *et al.*, 1999). Las pectinas pueden también formar uniones covalentes con los xiloglucanos.

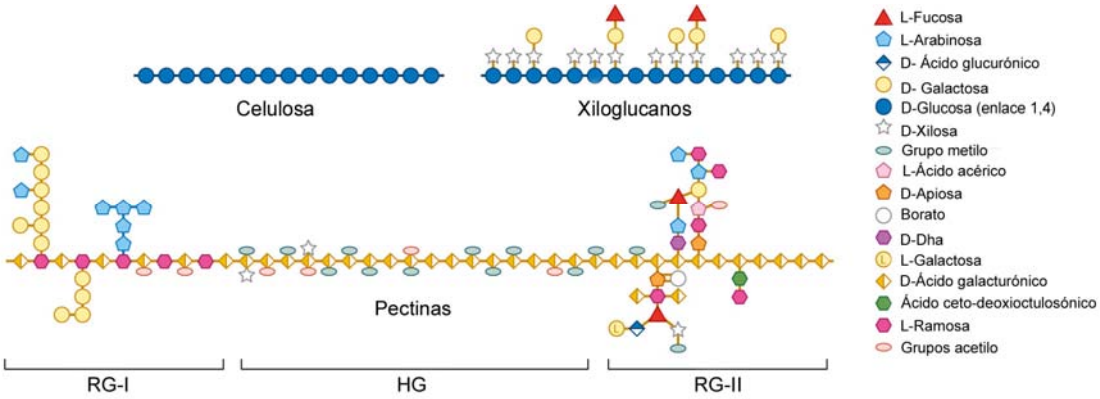


Figura 3: Estructura de algunos componentes de la pared celular. Representación esquemática de la celulosa, xiloglucanos y algunas pectinas (Ramnogalacturonanos tipo I, homogalacturonanos, y Ramnogalacturonanos tipo II. (Modificado de Wolf *et al.,* 2012; Burton *et al.*, 2010).

Otro componente de la pared celular es la calosa, un homopolímero lineal construido con residuos de glucosa ß-1-3 unidos con ramas de tipo ß-1-6. La calosa es sintetizada por las calosas sintasas también denominadas glucan sintasas-"like". La calosa se deposita en regiones determinadas de la pared celular en etapas particulares de diferenciación y crecimiento. Por ejemplo, durante la división celular, la calosa se localiza específicamente en la placa media durante la citocinesis (Chen *et al.*, 2009) y en el cuello de los plasmodesmos permitiendo la regulación de la comunicación intercelular a través de la modificación del límite de exclusión molecular (SEL) (Xie *et al.*, 2011). La deposición de calosa puede también ser inducida por infección por patógenos, heridas, aluminio, ácido absícico y otros tipos de estrés fisiológico (Chen y Kim *et al.*, 2009).

Aproximadamente entre un 10% y un 15% de la pared celular primaria está constituida por proteínas las cuales han sido clasificadas en ocho categorías de acuerdo a sus funciones bioquímicas predichas: (1) proteínas que actúan sobre polisacáridos (glicosil hidrolasas, esterasas, liasas y expansinas) constituyen un 25.9% del total, (2) Oxido-reductasas (peroxidasas y enzimas "berberine bridge") con un porcentaje del 13.5%, (3) proteínas estructurales siendo el 1.5%, (4) proteínas involucradas en señalización (8%), (5) proteasas con un 10% del total, (6) proteínas con dominios de interacción (dominios de lectinas, dominios LRR y enzimas con actividad inhibitoria) también con un 10%, (7) misceláneas (16.5 %) y (8) proteínas de función desconocida (10%) (Jamet *et al.,* 2006). En el anexo I se adjunta un capítulo relacionado con esta clasificación y la importancia de ciertas proteínas durante diferentes eventos en el ciclo de vida de las plantas.

Pared secundaria

La pared secundaria, la cual está restringida a plantas vasculares, está compuesta básicamente de celulosa, xilanos y lignina. La pared secundaria de las gimnospermas también presenta glucomananos. Los xilanos son polisacáridos formados por una cadena de D-xilosa con ramificaciones y sustituciones y son los segundos polisacáridos más abundantes en las dicotiledóneas. La presencia de éstos en las plantas vasculares sugiere una ventaja pre-adaptativa que permitió la ganancia en tamaño y la colonización de ambientes con limitaciones hídricas (Popper 2008).

Los galactoglucomananos son el principal componente de los tejidos maderables con crecimiento secundario tanto en Angiospermas como en Gimnospermas pero también se encuentran en las paredes de los helechos y musgos lo que hace suponer que este polisacárido se originó antes de la diversificación de las plantas vasculares proveyendo de soporte o tensión a las paredes celulares de estos organismos (Popper 2008).

Las ligninas, clasificadas como H-, G- y S- dependiendo del tipo de monolignano del que estén formadas, son un componente esencial en las paredes secundarias de ciertos linajes como son las angiospermas y los licopodios. También se ha descrito la presencia de lignina en las paredes celulares del alga roja *Calliarthron cheilosporioides* aunque no se ha determinado si su presencia es una convergencia evolutiva o se deriva de una relación ancestral entre estos grupos (Popper *et al.,* 2011).

Evolución de la pared celular en plantas.

La pared celular no es exclusiva de las plantas, sin embargo, esta estructura es de vital importancia debido a la condición sésil de estas. Los cambios en la composición de la pared celular han sido determinantes en los distintos grupos, para su diversificación y están relacionados con el establecimiento y la sobrevivencia en ambientes variados. La celulosa, elemento básico para la construcción de una pared rígida, promovió la reducción de la presión osmótica evitando la ruptura de las células sometidas a condiciones osmóticas extremas. Por otro lado, la participación de la pared celular en los mecanismos de señalización y en la percepción de las condiciones externas, tanto ambientales como aquellas relacionadas con el ataque por patógenos y parásitos, también fue clave durante la evolución de las plantas.

Las carofitas o algas verdes corresponden al grupo ancestral viviente más cercano de las plantas terrestres. Las carofitas son algas multicelulares que presentan una pared celular delgada, la cual no puede ser clasificada como una pared primaria o secundaria. La pared de estas algas contiene celulosa y hemicelulosas con alto contenido de manosas, ácido glucurónico, 3-*O*-metil ácido manurónico y ramnosa. Se propone que en las carofitas, la celulosa brindó estabilidad y tensión celular, confiriendo cierta ventaja sobre organismos con paredes celulares con componentes menos estables, como la quitina en los hongos. La formación de una red de microfibrillas de celulosa rígida en las carofitas promovió la colonización de ambientes distintos. Aunque las carofitas son organismos acuáticos, son capaces de soportar temporadas secas debido a la alta cantidad de ácido urónico y 3–*O*-metil ramnosa en su pared celular. La síntesis de estos mismos carbohidratos probablemente esté implicada en la formación del mucílago que las protege durante periodos secos. Por otro lado, la carencia de xiloglucananos, de proteínas ricas en hidroxiprolina, de lignina y de cutina, compuestos comunes en las plantas, les impidió tener un mayor volumen (Popper *et al.*, 2011; Popper, 2008).

Los cambios en la atmósfera y el aumento de competencia por recursos en los océanos promovieron la colonización a ambientes menos hidratados. Las primeras plantas terrestres, las briofitas, presentan un plan corporal particular que incluye el desarrollo de rizoides para la absorción de agua y un sistema de transporte de nutrientes por difusión. Las paredes celulares en los diferentes tejidos son muy delgadas, lo que permite absorber y transportar agua y, al igual que las pareces de las carofitas, no pueden ser clasificadas como primarias o secundarias. Estas paredes celulares presentan altas cantidades de celulosa, hemicelulosa y manosa. También presentan altas cantidades de ácidos urónicos

tales como ácido glucurónico y ácido galacturónico, así como ácidos manurónicos y 3–*O*-metil ramnosa, componentes que tienen un papel de vital importancia al impedir la desecación tal como ocurre en las carofitas. La pared de las briofitas contiene también ramnogalacturonanos, xiloglucananos y proteínas ricas en hidroxiprolina, componentes relacionados con un mayor soporte y fuerza estructural. Las briofitas presentan lignanos como compuestos fenólicos (Popper *et al.*, 2011)).

En las pteridofitas, el cambio de plan corporal de gametofítico (n) a esporofítico (2n), promovió un mayor volumen y altura y la capacidad de sobrevivir alejadas de los ambientes acuícolas. Asimismo, la formación de un sistema de conducción y transporte de agua más eficiente dio lugar a una compartamentalización y al desarrollo de un sistema vascular. Las paredes primarias de las pteridofitas contienen mananos como uno de los componentes principales que interconectan glicanos con la celulosa, proceso importante para la adhesión celular y la elasticidad además de contribuir en la fuerza de tensión de la pared (Silva *et al.,* 2011). De igual manera, la formación de pared secundaria promovió la protección y defensa contra patógenos e insectos. En particular, la lignina fue uno de los componentes diferenciales en la pared celular de las leptoesporangiadas.

La importancia de las proteínas modificadoras de pectinas durante el desarrollo y la evolución en plantas.

La metilesterificación de los HG es de vital importancia en la textura y propiedades mecánicas de la pared celular regulando así el crecimiento y la forma (Wolf *et al.,* 2009). Los HG son sintetizados en la región *cis* del Golgi, metilesterificados en la región media en el carbono 6 (C6), y secretados por la región *trans* con aproximadamente un 80% de grupos metilo. La desmetilesterificación de los HGs se lleva a cabo en la pared celular y resulta en la liberación de metanol y protones. Es catalizada por enzimas con actividad de pectin metilesterasas (PMEs) (Fig. 4). Los HG no están homogéneamente distribuidos en la pared celular, forman microdominios con diferentes patrones de metilesterificación que pueden ser visualizados en las distintas regiones de las paredes celulares mediante anticuerpos específicos. Un mínimo de nueve residuos de GalA continuos no esterificados pueden formar uniones con Ca^{2+}, promoviendo la formación de las denominadas estructuras cajas de huevo ("egg-box") que constituyen regiones más rígidas de la pared celular y son blanco de enzimas degradadoras de pectinas como las poligalacturonasas

(PGs) reguladas por las proteínas inhibidoras (PGIPs) y las pectin liasas (PLs). En procesos de señalización, durante el ataque por patógenos, estas enzimas promueven la acumulación de oligogalacturonoides (OGAs), los cuales son producto del rompimiento de HGs que participan como elicitores promoviendo una respuesta de defensa por parte de la planta (Ferrari *et al.,* 2013) Los OGAs son capaces de inducir cambios en: la expresión de genes, la apertura de estomas, la producción de etileno, el reforzamiento de la pared celular y la producción de ROS.

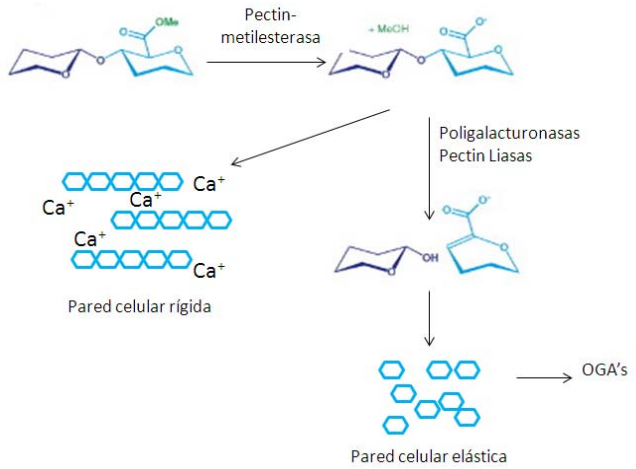


Figura 4: Algunas modificaciones de las pectinas. Las PME catalizan la desmetilesterificación de las pectinas promoviendo la actividad enzimática de otras proteína de pared celular como PGs y PLs, dichas modificaciones influyen en las características de la pared celular haciéndola más o menos flexible, e inducen otras vías de señalización tales como las mediadas por OGAs.

La actividad de las PMEs está regulada por proteínas inhibidoras (PMEI). La regulación del grado de metil esterificación de las pectinas es de vital importancia en procesos tales como la maduración de polen (Francis *et al.*, 2006; Bosch *et al.*, 2005), el crecimiento del tubo polínico (Röckel *et al.*, 2008; Jiang *et al.*, 2005), la liberación del mucílago y la ruptura de la testa durante la germinación en semillas (Rautengarten *et al.*, 2008; Müller *et al.,* 2013, Saez-Aguayo *et al.,* 2013), la regulación de la elongación en hipocotilos (Derbyshire *et al.,* 2007; Pelletier *et al.*, 2010), el control de la filotaxia (Peaucelle *et al.*, 2008, 2011a) y la emergencia de primordios radiculares, vegetativos y florales (Peaucelle *et al.*, 2011b).

La importancia de la esterificación de las pectinas también se ha evidenciado durante la interacción planta-patógeno, por ejemplo, la sobreexpresión de una PMEI puede inhibir la infección por hongos, mientras que durante la infección por nematodos

ocurre la sobreexpresión de PG y otras enzimas relacionadas con las modificaciones estructurales de las pectinas (Raiola *et al.*, 2007, Bellincampi *et al.*, 2014).

De acuerdo con análisis filogenéticos, las PME se pueden clasificar en dos grupos. El primero está conformado por las isoformas de PMEs que presentan un dominio catalítico activo y un dominio transmembranal o un péptido señal en la región amino terminal, también se clasifican en este grupo las isoformas solubles. En *A. thaliana* existen al menos 23 PME con dichas características. En el segundo grupo se encuentran las PMEs que presentan un dominio catalítico y además un dominio inhibitorio en la región N-terminal o región Pro (proPME) (Micheli, 2001). Dicho dominio inhibitorio es similar a las PMEIs. En *A. thaliana* este grupo está conformado por 43 isoformas (Wang *et al.,* 2013).

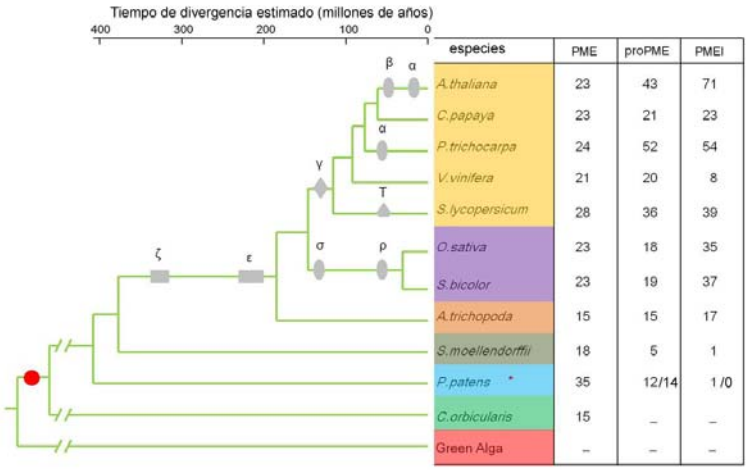
Los análisis filogenéticos realizados por Wang y colaboradores sugieren que el origen de los genes que codifican las PMEs y sus proteínas inhibidoras, está estrechamente relacionado con la aparición de las pectinas. De acuerdo con estos autores la diversidad funcional de las enzimas modificadoras de pectinas refleja lo complejo de su metabolismo, además de que tanto la diversificación de los proPME como de los PMEI está ligada con la síntesis de paredes celulares especializadas en los distintos grupos de plantas terrestres (Wang *et al.,* 2013). Por otro lado McCarthy y colaboradores reportan la ausencia de PMEI y PGIPs en *Physcomitrella patens* (Fig. 5) lo que sugiere que al menos alguna de estas familias de proteínas reguladoras de las modificaciones estructurales de las pectinas, se originaron después de la divergencia de las plantas con semilla (McCarthy *et al.*, 2014).

Aunque no se han reportado proteínas activadoras de las PMEs en plantas, el nematodo *Heterodera schastti* secreta una proteína de unión a celulosa (CBP) cuya sobreexpresión en *A. thaliana* promueve un incremento en la actividad total de PMEs. A pesar de que el mecanismo de acción de esta proteína no se ha demostrado, la interacción *in vitro* con AtPME3 sugiere la participación directa de esta CBP en la activación de las PMEs (Hewezi *et al.*, 2008).

Proteínas de unión a carbohidratos

Algunas de las enzimas responsables de los cambios estructurales de los polisacáridos que integran la pared celular pueden ser secretadas por ciertos parásitos y bacterias mientras otras son sintetizadas por la planta. Glicosil hidrolasas, liasas y carbohidrato-esterasas están clasificadas en familias tomando en cuenta la homología en la secuencia de sus aminoácidos (Carbohydrate Active EnZymes (CAZy)). Frecuentemente

dichas enzimas localizadas en la pared celular son modulares y contienen, además del dominio catalítico, uno o más módulos de unión a carbohidratos (CBMs). Se ha sugerido que la función de los CBMs está relacionada con aumentar la eficiencia de estas enzimas al permitir un mejor contacto entre el dominio catalítico y su substrato (Bae *et* al., Gilbert *et al.*, 2013).



especies	PME	proPME	PMEI
A.thaliana	23	43	71
C.papaya	23	21	23
P.trichocarpa	24	52	54
V.vinifera	21	20	8
S.lycopersicum	28	36	39
O.sativa	23	18	35
S.bicolor	23	19	37
A.trichopoda	15	15	17
S.moellendorffii	18	5	1
P.patens *	35	12/14	1 /0
C.orbicularis	15	–	–
Green Alga	–	–	–

Figura 5: Número de copias de PME, proPME y PMEI en diferentes especies. Árbol ultramétrico modificado de Jiao, Popper y Lee; *et al.* Las cajas grises (ζ, ε) diamantes (γ, T) y elipses (α, ß, σ, ρ) representan eventos de duplicación del genoma completo en las distintas especies, el punto rojo indica la aparición de las pectinas en la pared celular de estos organismos. El * indica que en *Physcomitrella patens* de acuerdo con McCarthy y colaboradores existen en total 14 PMEs y ningún PMEI. (Modificado de Wang *et al.*, 2013).

Los CBMs han sido clasificados en tres tipos; A, B y C, definidos como CBMs que se unen a superficies cristalinas, cadenas de glicanos y secuencias cortas de oligosacáridos, respectivamente (Boraston *et al.*, 2004 en Gilbert *et al.*, 2013). Gilbert y colaboradores proponen definir al tipo B como aquellos CBM que interactúen internamente a cadenas de glicanos (tipo-endo), mientras que los del tipo C se unen a glicanos terminales (tipo-exo). Otro aspecto importante es que los CBMs pertenecientes a distintas familias pueden utilizar el calcio durante el reconocimiento del componente de la pared celular con el que interactúan, posiblemente aumentando la afinidad por su sustrato y contribuyendo a la especificidad. Los CBMs codificados junto al dominio catalítico que modifica carbohidratos, además de dirigir la interacción entre el sustrato y el módulo catalítico, podrían tener la función de mediar el reconocimiento de la enzima con regiones de la pared celular que están siendo activamente degradadas por otras enzimas (Gilbert *et al.*, 2013).

Las proteínas DUF642 y sus múltiples posibles interactores

Los análisis proteómicos de pared celular de plantas han sido útiles en gran medida para determinar las aproximadamente 400 proteínas que se localizan en este compartimiento celular y cuya importancia es primordial durante el desarrollo y defensa de estos organismos (Zúñiga-Sánchez y Gamboa de Buen, 2009, Anexo I). Dentro de las proteínas detectadas se han determinado al menos 8 representantes o familias clasificadas por el proyecto Pfam del Instituto Sanger (http://pfam.sanger.ac.uk/) con un dominio de función desconocida. Dentro de este grupo se encuentran las proteínas DUF642 (DUF por sus siglas en inglés, Domain Unknown Function), las cuales se caracterizan por la presencia de un único dominio de función desconocida.

La familia DUF642 es una familia muy conservada y específica de plantas, representada tanto en Angiospermas como en Gimnospermas (Albert *et al.*, 2005, Vázquez-Lobo *et al.*, 2012). En *A. thaliana,* dicha familia está representada por 10 genes. En otras especies como *Populus tricocarpa* y *Oriza sativa*, se han descrito 11 y 10 miembros respectivamente. Reconstrucciones filogenéticas para esta familia describen la existencia de cuatro clados o ramas principales (Vázquez-Lobo *et al.*, 2012) que agrupan proteínas con características específicas. Los miembros del clado A y B corresponden a los descritos en pared celular (6 de 7 miembros ya que el gen *At2g41810* es específico de flores y no ha sido descrito en ningún proteoma de pared celular hasta la fecha) mientras que los miembros del clado C y D (3 miembros) están anclados a glicosilfosfatidilinositol (GPI) y han sido descritos en proteomas de membrana celular (Borner *et al.*, 2003). Todas las proteínas de DUF642 de *A. thaliana* se caracterizan por la presencia de un péptido señal. Sin embargo, algunos de ellos pueden tener un tipo de edición alternativa o primer exón alternativo (FAE, por sus siglas en inglés) que omite la expresión de la región del péptido señal, lo que sugiere que también podría existir una versión de ciertos miembros cuya localización subcelular es intracelular. Esto ha sido descrito para los genes *BIIDXI* (*BDX*, *At4g32460*) y *At3g08030* y teóricamente puede presentarse en los otros genes de los clados A y B debido a que algunos otros miembros también tienen un ATG en marco de lectura muy cercano al inicio del segundo exón, después de la región que codifica para el péptido señal, (The Arabidopsis Information Resource http://www.arabidopsis.org/) (Fig. 6).

Análisis de interacción *in vitro* han permitido identificar distintos tipos de unión entre miembros de la familia DUF642 tanto con componentes de la pared celular como con

componentes del citoesqueleto. Por ejemplo, se ha demostrado una interacción *in vitro* de BDX y la proteína codificada por *At5g11420* con el dominio catalítico de AtPME3 y FLOR1, una proteína similar a PGIPs (Zúñiga-Sánchez y Gamboa de Buen, 2012, Anexo I). La proteína codificada por *At3g08030,* otro miembro de la familia DUF642, interactúa *in vitro* con celulosa y con NUP43 proteína relacionada con el poro nuclear (Vázquez-Lobo *et al.*, 2012; Tamura *et al.,* 2010). Otra interacción relevante es la que se ha reportado ocurre entre BIIDXI y la proteína codificada por *At1g41800* con microtúbulos bajo condiciones de estrés por frío en *A. thaliana* (Sproule, 2008).

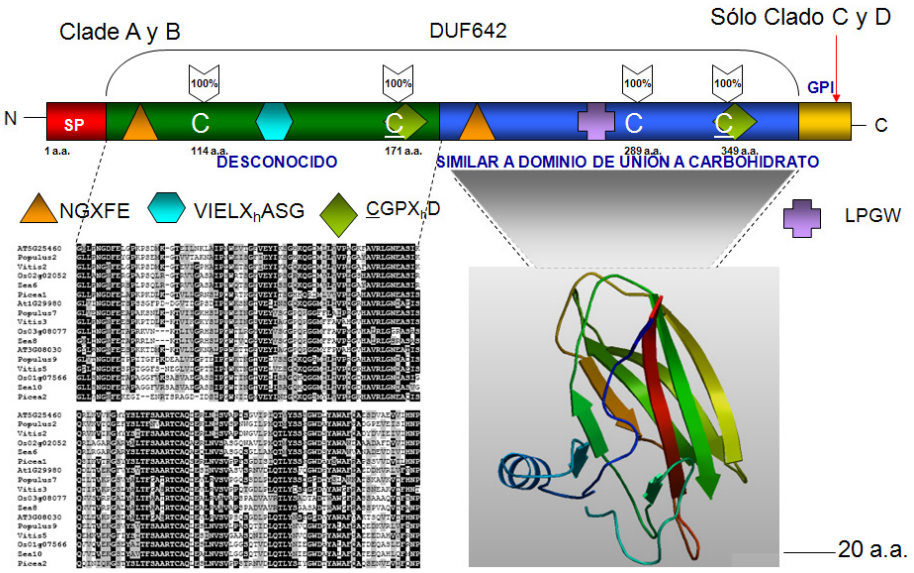


Figura 6: Los módulos de las proteínas clasificadas como DUF642, en rojo el péptido señal, en verde un primer módulo conservado en diferentes taxa con función desconocida, en azul un segundo módulo de interacción a carbohidratos, en amarillo secuencia de anclaje a membrana solo en ciertos miembros de ésta familia. Con flechas se remarca la conservación en todos los taxa de cuatro cisteínas (Vázquez-Lobo *et al.*, 2012). Con distintas figuras se destacan los motivos conservados en los diferentes dominios.

Hipótesis

La función de las proteínas DUF642 codificadas por *BIIDXI* (*At4g32460*) y *At5g11420* está estrechamente relacionada con los cambios en la estructura y remodelación de la pared celular durante el desarrollo y con los procesos de señalización que regulan su reestructuración, promovidos tanto por señales ambientales como internas.

Objetivo general

Estudio de los miembros de la familia DUF642 BIIDXI (32460) y 11420 como posibles reguladores de la función de proteínas de pared celular y su participación durante eventos específicos del desarrollo en *Arabidopsis thaliana*.

Objetivos particulares

1. Determinación de la expresión de los genes DUF642, *BIIDXI* (*BDX*, *At4g32460*) y *At5g11420* durante etapas específicas del desarrollo vegetativo y reproductivo de *A. thaliana*.

2. Obtención de líneas de localización subcelular de la proteína codificadas por *At4g32460* .

3. Obtención y análisis de líneas de plantas que sobre-expresen los genes *At4g32460* y *At5g11420* para llevar a cabo comparaciones fenotípicas y fisiológicas.

    a) Determinación del efecto de la sobreexpresión de los genes *BDX* y *At5g11420* sobre la germinación.

    b) Determinación del efecto de la sobreexpresión de los genes *BDX* y *At5g11420* sobre la actividad de PME.

4. Obtención y análisis de plantas de pérdida de función.

    a) Caracterización fenotípica y fisiológica de líneas antisentido (AS) para el gen *BDX.*

    b) Caracterización fenotípica y fisiológica de líneas mutantes de T-DNA para *BDX.*

5. Determinación del fenotipo de plantas con la expresión de la proteína codificada por *At5g11420* bajo la región regulatoria del gen *BDX.*

Materiales y métodos

Plantas transgénicas para determinar los patrones de expresión de los genes BDX y At5g11420.

Con el objeto de clonar la posible región promotora, se llevó a cabo la amplificación de las regiones río arriba del sitio de inicio de la traducción para *BDX y At5g11420* a partir de DNA genómico de hojas de roseta. La región intergénica para el gen *BDX* es de aproximadamente 3 kb y para el gen *At5g11420* es de aproximadamente 2 kb. En el primer caso, se diseñaron oligos para amplificar 1983 pb mientras que para el segundo gen se amplificó un fragmento de 1386 pb. Estos fragmentos fueron clonados en pGEMT-easy, secuenciados (M. en C. Laura Márquez, Instituto de Biología) y subclonados río arriba de la secuencia codificante para la proteína verde fluorescente (GFP) en el plásmido pBIN-ERGFP. La inserción de los fragmentos en dicho plásmido se confirmó mediante análisis de restricción y PCR.

Los plásmidos purificados se usaron para transformar la cepa de *Agrobacterium tumefaciens* C4. La transformación se confirmó mediante PCR. Cultivos de esta cepa fueron empleados para transformar plantas silvestres de *A. thaliana* ecotipo Columbia (Col.) de acuerdo con el protocolo descrito en el apéndice II. De las semillas obtenidas se llevó a cabo la selección de plantas transformadas en placas de medio MS con kanamicina. (Protocolo, Apendice II). Se seleccionaron varias plantas resistentes para ambas líneas, es decir tanto para la construcción con el promotor de *BDX* como de la construcción con el promotor de *At5g11420*.

En ambas construcciones la generación T1 fue heterocigota (50% de resistencia a kanamicina), la segunda generación o T2 fue homocigota con todas las plantas resistentes a kanamicina.

Tres líneas independientes en cada construcción fueron crecidas en medio MS y en tierra. Observaciones de raíces en diferentes estadios del desarrollo, hojas, flores y embriones se llevaron a cabo en un microscopio confocal Olympus FV100 a longitudes de onda entre los 576-670 nm para el yoduro de propidio, y 485-545 nm para la GFP.

Plantas transgénicas de ganancia de función para los genes BDX y At5g11420

Se extrajo RNA de las hojas de roseta con Trizol de Sigma (No. de catálogo 15596018) y se sintetizó cDNA con Super Script II Transcriptasa Reversa de Invitrogen (No de catálogo 18064). Se diseñaron oligos específicos para la amplificación de los genes *BDX* y *At5g11420* con y sin péptido señal (tabla 1 Apéndice II). Los cuatro productos de PCR se clonaron y secuenciaron en el plámido pGEMT-easy. Una vez secuenciados, los productos se subclonaron en el plásmido pBIN-ERGFP bajo el promotor del virus del mosaico del tabaco (CaMV 35 S promoter). Las secuencias codificantes de los genes *BDX* y *At5g11420* con y sin péptido señal se confirmaron mediante PCR. Dichos plásmidos fueron utilizados para electroporar *A. tumefaciens*. Se transformaron plantas silvestres ecotipo Col. con las construcciones antes mencionadas. Se sembraron y seleccionaron transformantes en medio MS con kanamicina de cada una de las construcciones. Plantas transgénicas de líneas distintas tanto de la construcción con péptido señal como de la construcción sin péptido señal fueron sembradas en tierra y en agar para determinar si existía algún cambio en el fenotipo al sobreexpresar dichos genes.

Plantas transgénicas para la localización subcelular

Se clonaron y amplificaron las secuencias codificantes con y sin péptido señal de *BDX* en el plásmido pGEMT-easy, una vez secuenciados para descartar posibles errores o cambios en el marco de lectura se procedió a subclonarlas en el plásmido pBIN-ER-GFP en marco de lectura con la proteína verde fluorescente (GFP).

Las semillas obtenidas de dicha transformación (T_0) fueron sembradas en medio MS con kanamicina. Las plantas sobrevivientes fueron trasplantadas en macetas con tierra. Al menos tres líneas distintas para cada construcción fueron crecidas en MS y en tierra, plantas en diferentes estadios fueron observadas en el microscopio confocal.

Líneas antisentido para BDX.

Se amplificó la secuencia codificante para *BDX* a partir de cDNA, con oligos diseñados con la finalidad de que la secuencia codificante de este gen pudiera insertarse en la construcción que contenía su región regulatoria y la proteína GFP río abajo. La clonas cuya secuencia fueron idénticas a la región codificante se subclonaron en el

plásmido pBIN-PROBDX:GFP. El producto de dicha construcción fue una construcción antisentido (AS) para *BDX* pBIN-PROBDX::BDX:GFP y al mismo tiempo una construcción del gen completo *BDX* sin intrones bajo su propio promotor y la proteína verde fluorescente como gen reportero.

Se obtuvieron clonas para ambos caso en sentido y en antisentido, los plásmidos obtenidos fueron empleados para electroporar *A. tumefaciens*. Una vez en esta cepa fueron confirmadas las construcciones mediante PCR. Plantas silvestres ecotipo Col. fueron transformadas con dichas construcciones. Las plantas resistentes a kanamicina (T_0) fueron trasplantadas y crecidas hasta obtener la generación T_3. Se realizaron comparaciones fenotípicas entre plantas antisentido y plantas silvestres. Se realizaron observaciones en el microscopio confocal de plantas transgénicas con la construcción sentido para determinar la localización subcelular de la proteína quimérica BDX-GFP.

Líneas de inserción de T-DNA para BDX.

Se analizaron semillas de plantas de inserción de T-DNA SALK_142260 y SALK_054867 para conocer el sitio de inserción del T-DNA. Para ello se extrajo DNA de hojas de roseta y flores de plantas transgénicas T1 y plantas wt. La inserción fue corroborada con el primer LBb1 que se una al borde izquierdo del T-DNA y un primer reverso que se une específicamente al gen *BDX* en ambos alelos. Las bandas amplificadas fueron clonadas y secuenciadas. Las secuencias permitieron corroborar la inserción del T-DNA en la línea SALK_142260 al final del segundo exón mientras que en el alelo SALK_054867 la inserción se localizó al final del tercer exón.

Plantas T2 para ambos alelos fueron sembradas y analizadas fenotípicamente, dichos análisis mostraron una proporción 3:1 de plantas con fenotipo silvestre: contra plantas con fenotipos aberrantes. El fenotipo de estas plantas de inserción de T-DNA es similar al fenotipo observado en las plantas AS.

Líneas transgénicas que expresan la región codificante de At5g11420 bajo la región regulatoria de BDX.

Con la finalidad de conocer si existía redundancia funcional entre las proteínas codificadas por *BDX* y *At5g11420* , dado el alto porcentaje de similitud e identidad entre ellas (Fig. 14), se llevó a cabo una construcción con la región codificante de *At5g11420*

bajo la secuencia regulatoria de *BDX.* Para ello se utilizó el plásmido pBIN-PROBDX:GFP previamente construido al que se le insertó la secuencia codificante de *At5g11420*. Plantas silvestres fueron transformadas con dicha construcción y las semillas obtenidas (T_0) fueron sembradas en medio MS con kanamicina. Las plantas seleccionadas se sembraron en tierra y las semillas obtenidas (T_1) fueron resembradas.

CAPÍTULO I

La importancia de *BIIDXI* durante la germinación y la reproducción en *A. thaliana*.

En este capítulo se describe el posible papel de *BDX* (*At4g32460*) en la regulación de la actividad de PMEs, durante la germinación y la generación de semillas. Pero también se incluyen resultados relacionados con el gen *At5g11420*, el cual está filogenéticamente relacionado con *BDX*, durante la germinación. Como ya se ha mencionado, la interacción de estas dos proteínas con el dominio catalítico de AtPME3 sentó las bases para un estudio más profundo sobre su participación en la regulación de a actividad de PME *in vivo.* La regulación de la actividad de PME es de gran relevancia en diferentes procesos del desarrollo de la planta (Fig. 7). Uno de estos procesos es la germinación que en *A. thaliana* ocurre en dos pasos: el primer paso está relacionado con la ruptura de la testa y el segundo implica la ruptura del endospermo por la radícula emergente (germinación *sensu stricto*). En la ruptura de la testa participan activamente las PMEs. El ácido abscísico (ABA) hormona que inhibe la ruptura del endospermo pero no la ruptura de la testa (Müller *et al.,* 2013) regula negativamente la expresión de algunas enzimas encargadas de la remodelación de la pared celular durante la germinación, como son las xiloglucan-endotransferasas (XET) y las expansinas, mientras que el ácido giberélico (GA) las induce junto con otras enzimas cuya actividad incrementa durante la ruptura del endospermo en diversas especies, como son las β-manasas y β-manosidasas (Fig. 7). Nuestros resultados demuestran que en *A. thaliana* la germinación de semillas de plantas de ganancia de función de BDX y At5g11420 se adelanta con respecto a las semillas de plantas silvestres como consecuencia de un aumento en el tiempo de inicio de la ruptura de la testa. Es posible que esto ocurra debido al aumento en la actividad total de PME registrado en estas semillas. Aunque estas plantas no presentaron fenotipos morfológicos, el aumento de actividad de PMEs se registró en diferentes tejidos. En relación a las plantas de pérdida de función de *BDX* (AS y T-DNA), la actividad total de PME en hojas de roseta fue significativamente menor con respecto a las hojas de las plantas silvestres. La producción de semillas fue afectada por la disminución de los niveles de expresión de *BDX*. Las silicuas de las plantas AS son pequeñas y carecen de semillas mientras que las plantas heterocigotas de T-DNA también presentan silicuas pequeñas con pocas semillas. Trabajos relacionados han mostrado que la disminución del tamaño de las silicuas y de la producción de semillas está estrechamente vinculado con una reducción en la actividad de

PME en plantas transgénicas que sobreexpresan un PMEI (Müller *et al.*, 2013). Estos resultados sugieren que las DUF642 podrían participar en diferentes procesos de desarrollo de la planta a través de la inducción de la actividad de PME.

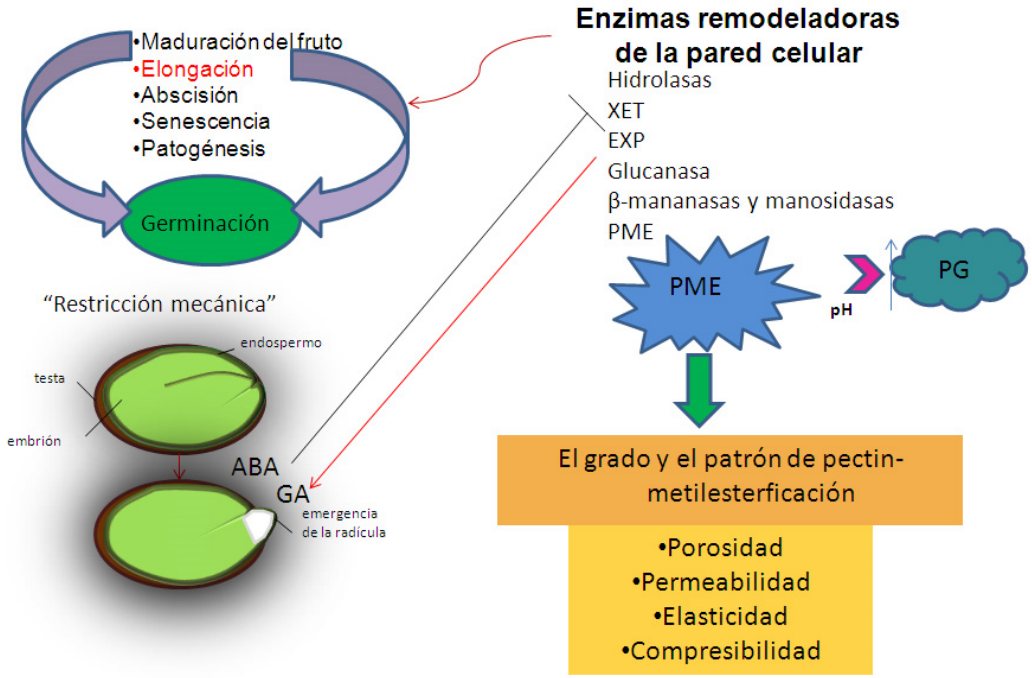


Figura 7: Esquema que muestra algunos procesos relacionados con la actividad de PME, con énfasis en la germinación en *A. thaliana*. Durante la maduración del fruto, la elongación celular, la abscisión de órganos, la senescencia y patogénesis la actividad de PME juega un papel determinante. Específicamente en el proceso de germinación se induce la actividad de diversas enzimas relacionadas con la remodelación de la pared celular lo cual conlleva a la ruptura de la testa y el endospermo. La expresión de algunas de estas enzimas es inducida por ácido giberélico (GA), mientras que el ácido abscísico las reprime (ABA) inhibiendo también la ruptura del endospermo pero no la de la testa. La actividad de PME determina el grado de esterificación de las pectinas en la pared, lo cual determina ciertas características físicas de ésta. Cambios en el pH y un alto grado de pectinas desmetilesterificadas induce la expresión de otras enzimas que rompen pectinas como son las poligalacturonasas (PG).

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BIIDXI, the *At4g32460* DUF642 gene, is involved in pectin methyl esterase regulation during *Arabidopsis thaliana* seed germination and plant development

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Abstract

Background: DUF642 proteins constitute a highly conserved family of proteins that are associated with the cell wall and are specific to spermatophytes. Transcriptome studies have suggested that members of this family are involved in seed development and germination processes. Previous in vitro studies have revealed that *At4g32460*- and *At5g11420*-encoded proteins interact with the catalytic domain of pectin methyl esterase 3 (AtPME3, which is encoded by *At3g14310*). PMEs play an important role in plant development, including seed germination. The aim of this study was to evaluate the function of the DUF642 gene *At4g32460* during seed germination and plant development and to determine its relation to PME activity regulation.

Results: Our results indicated that the DUF642 proteins encoded by *At4g32460* and *At5g11420* could be positive regulators of PME activity during several developmental processes. Transgenic lines overexpressing these proteins showed increased PME activity during seed germination, and improved seed germination performance. In plants expressing *At4g32460* antisense RNA, PME activity was decreased in the leaves, and the siliques were very short and contained no seeds. This phenotype was also present in the SALK_142260 and SALK_054867 lines for *At4g32460*.

Conclusions: Our results suggested that the DUF642 family contributes to the complexity of the methylesterification process by participating in the fine regulation of pectin status during plant development.

Keywords: DUF642 proteins, Pectin methyl esterases, Germination

Background

DUF642 proteins constitute a highly conserved family of cell wall-associated proteins specific to spermatophytes [1]. Although proteins in this family have been detected in cell-wall proteomes from a variety of plants and tissues, only one functional study on this protein family has been published so far. *At5g25460* is highly expressed in seedlings during the early developmental stages, and plants of the *At5g25460*-null mutant have shorter roots and smaller rosettes than those of wild-type plants [2].

Transcriptome analyses have revealed the differential expression of five DUF642 genes during seed barley germination, suggesting a possible function of this protein family during the germination process [3]. In addition, differential spatial expression of DUF642 genes among various seed compartments during germination has been reported in *Arabidopsis thaliana*. A gene expression study showed that *At3g08030* and *At5g11420* transcripts are enriched in the micropylar endosperm before testa rupture, whereas *At4g32460* is expressed in this compartment after testa rupture [4]. In *Brassica oleracea* seeds, the expression of the *At5g25460* gene ortholog increases during germination [5]. *At3g08030* transcript is present in after-ripened seeds, and the transcript levels increased in seeds subjected to controlled imbibitions in

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soil or water (matrix-primed and hydroprimed seeds). Notably, *At3g08030* transcript is absent from aged seeds with low germination performance [6].

DUF642 proteins have been detected in the cell-wall proteomes of multiple tissues [7]. A transcriptome analysis of stigmatic papillae cells revealed high transcript levels of two DUF642 genes, *At4g32460* and *At2g41800*, and genes encoding other cell wall-related proteins, including a pectin methyl esterase. Cell wall remodeling of the stigma is involved in successful pollination, likely via regulating the penetration of the pollen tube through the transmitting tract [8]. In *Lilium longiflorum*, an analysis of proteins in the stigmatic exudate revealed a DUF642 protein [9].

Proteins encoded by *At4g32460* and *At5g11420* interact in vitro with the catalytic domain of pectin methyl esterase 3 (AtPME3, encoded by *At3g14310*) [10]. Protein interactome data have proved to be an useful resource for formulating and testing hypotheses [11]. One potential physiological function of the DUF642 proteins encoded by *At4g32460* and *At5g11420* is related to the regulation of PME activity. Several studies have shown that the degree of pectin methylesterification, a highly regulated process, is critical for fine-tuning the biomechanical properties of the cell wall during various developmental processes [12-14]. The demethylesterification of pectins is mediated by PMEs, and PME catalytic activity is regulated by PME inhibitor (PMEI) proteins [15].

Unesterified pectins, especially homogalacturonans (HGs), are the substrates for polygalacturonases (PGs), enzymes regulated by polygalacturonase inhibitor proteins (PGIPs) that are involved in cell separation processes [16]. Differences in pectin methylesterification have been described during pistil, silique, and seed development. In olive, low methylesterified HGs are detected in the stigma and in the transmitting tissue during pollination [17]. In *A. thaliana*, silique growth is related to a decrease in the degree of methylesterification [18]. In seeds of *A. thaliana*, the cell walls within the embryo have low levels of unesterified pectins, the endosperm cell walls contain abundant unesterified HG, and the testa cell walls are rich in highly methylesterified HG [19]. In *A. thaliana,* the genes encoding pectin-modifying enzymes and their regulators are highly regulated during the first 24 h of seed germination [20]. In yellow cedar seeds, PME activity positively correlates with germination performance [21].

In *A. thaliana* and related endospermic species, germination is a two-step process that requires testa and endosperm rupture for radicle protrusion [22]. During *A. thaliana* germination, PME activity increases until testa rupture is complete, and decreases during endosperm breakdown. Overexpression of PMEI led to accelerate endosperm breakdown and an improved capacity for radicle emergence. Delays in endosperm rupture caused by abscisic acid significantly extend the period of high PME activity [13]. Conversely, PGIP overexpression inhibits germination, a process that is enhanced in *pgip* mutant seeds [23].

The aim of this study was to study the function of the DUF642 gene *At4g32460* during seed germination and plant development. We evaluated the role of the BDX protein in the regulation of PME activity, focusing on the periods of seed germination and plant growth. We demonstrated that the overexpression of either *At4g32460* or its homolog *At5g11420* increased PME activity and promoted germination, primarily by accelerating testa rupture. We also demonstrated that total PME activity was inhibited in *At4g32460* antisense transgenic plants and that the morphological changes in these plants included small siliques with no seeds. This phenotype was also observed in SALK T-DNA mutants. In accordance with these results, we named *At4g32460* as *BIIDXI* (*BDX*), which means 'seed' in the Zapotec language. Our data suggest that DUF642 proteins are involved in the regulation of PME, thereby remodeling the cell wall during various processes in plant development.

Results

BIIDXI is expressed in the embryos of imbibed seeds, roots, leaves, stems, and various floral organs (bar.utoronto.ca). To determine whether the cloned region (Additional file 1: Figure S1C) was sufficient to drive expression in a pattern similar to that described previously for *A. thaliana*, we produced transgenic plant lines containing the cloned fragment fused to the green fluorescent protein (GFP) reporter. Three transgenic lines were produced, and we monitored GFP fluorescence throughout their growth and development. GFP driven by the *BDX* promoter was highly expressed in the vascular tissue of primary and lateral roots, and in leaves, stamens, and petals (Figure 1). GFP fluorescence was detected in the vascular tissue of radicles from seeds that had been germinating for 48 h and 72 h (Figure 1A and B). During the seed imbibition process, GFP fluorescence was detected from 6 h until germination was complete (Additional file 1: Figure S2). In the primary roots of 6-day-old seedlings, GFP fluorescence was detected exclusively in the provascular tissue of the meristematic and transition zone (Figure 1C). In the roots of 8-day-old seedlings, GFP fluorescence was detected in pericycle cells in the differentiation zone (Figure 1D). In the roots of 22-day-old plants, GFP fluorescence was detected in the vascular tissue in specific regions of the mature zone (Figure 1E). GFP fluorescence was also detected in the vascular tissue of fully expanded leaves (Figure 1F). During different stages of flower development, *BDX* promoter-driven GFP expression was detected exclusively in the vascular tissue of stamen filaments and anthers, in petals, and in the stigmatic papilla, as described previously [8] (Figure 1G and H).

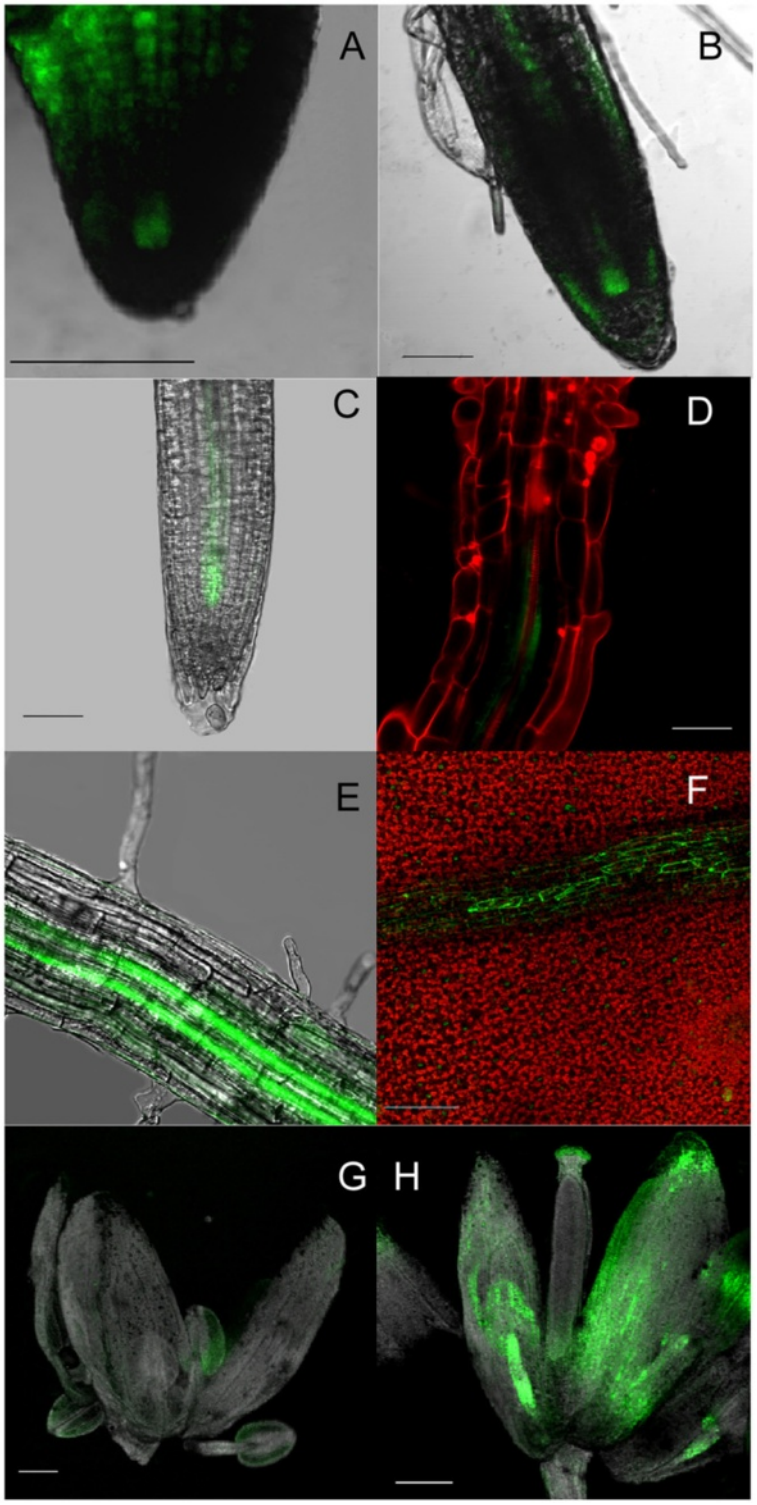

Figure 1 (See legend on next page.)

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Figure 1 Identification of *At4g32460* promoter activity during *Arabidopsis thaliana* development using pBDX::ER-GFP plants (Additional file 1: Figure S1C). A) GFP fluorescence in radicle tissue of a 48-h germinating seed. **B)** GFP fluorescence in radicle tissue of a 72-h germinating seed. **C)** GFP fluorescence in different cell types of meristematic and transition zones of primary roots from 4-day-old seedlings. **D)** GFP fluorescence in vascular tissue of maturation zone of primary roots of 22-day-old plants. **E)** GFP fluorescence in pericycle-differentiated cells that constitute primordium of lateral root emerging from maturation zone of primary roots of 22-day-old plants. **F)** GFP fluorescence in vascular tissue of fully expanded leaves. **G)** GFP fluorescence in anthers and petals of stage-6 flowers. **H)** GFP fluorescence in vascular tissue of stamen filaments, anthers, petals, and stigma of stage-12 flowers. Scale bars =50 μm in A and B, 15 μm in D, 100 μm in E and F, and 300 μm in **C**, **G**, **H**, and **I**. Images **A**, **B**, **E**, **F**, **G**, and **H** are projections of confocal Z-stacks. C and D are longitudinal sections.

Next, we analyzed *BDX* promoter activity in embryos at various stages; heart stage, torpedo stage, and mature embryos (Figure 2). GFP fluorescence was detected in provascular cells from the radicle meristematic region of the mature embryo (Figure 2A) and in embryos at the torpedo (Figure 2C) and heart stages (Figure 2E). The expression pattern of GFP driven by the *BDX* promoter was primarily associated with vascular tissue during different stages of plant development, consistent with previous reports.

Transcriptome analyses have revealed that *BDX* expression is induced by auxin [24] and also by gibberellic acid (GA) during germination [25]. In silico analysis of the putative promoter region (pBDX) revealed at least two auxin response factor motifs [26] and two gibberellic acid response element (GARE) [27]. We performed hormone induction analyses to test whether the pBDX fragment contained information for auxin and GA responses. Auxin and GA treatments, for 2 h or 48 h, altered *BDX* expression in the roots of 7-day-old seedlings. In both treatments, *BDX* expression was detected in vascular tissue, as previously observed in control seedlings, but also in cortical cells (Additional file 1: Figure S3).

To understand the physiological function of *BDX*, we generated overexpression lines (OEBDX; Figure 3) in which the full-length *At4g32460* coding sequence was expressed under the control of the cauliflower mosaic virus 35S promoter (Additional file 1: Figure S1A). Several independent and homozygous transgenic lines were obtained. We examined dry seeds from two lines to determine their PME activity and *BDX* transcript levels.

BDX transcript was not detected in wt dry seeds, but was presented at high levels in the overexpression lines (Figure 3A). Although there were no morphological differences among the different lines at all developmental stages (results not shown), there was increased total PME activity in vegetative-meristem-enriched samples and in seedlings (p <0.001), compared with that in wt. There was no significant difference in PME activity in leaves between the overexpression lines and wt (Figure 3B). In wt *A. thaliana* seeds, PME activity has been reported to increase before testa rupture and to decrease at the beginning of endosperm rupture [13]. Based on this information, we performed a germination analysis of matrix-primed seeds of two OEBDX lines and wt plants. Plants transformed with the empty vector were used as a negative control (Additional file 1: Figure S4). Compared with wt, OEBDX lines showed a shorter initial time of testa rupture (p =0.02, Figure 3C), and their seeds showed a shorter time to endosperm break initial time (p =0.004, Figure 3D).

Phylogenetic studies have demonstrated that *BDX* and *At5g11420* resulted from a recent duplication event, and that their respective encoded proteins interact with AtPME3 in vitro [1,10]. Also, recent studies have shown that *At5g11420* expression in the micropylar endosperm increases prior to testa rupture, suggesting a potential role for *At5g11420* in this process [4]. To evaluate the possible role of *At5g11420* in testa rupture, we generated overexpression lines (OE11420, Figures 1B and 4). There were no morphological differences among the different lines and wt (results not shown). *At5g11420* transcript was detected at high levels in dry OE11420 seeds (Figure 4A). Compared with wt, the OE11420 lines showed a significant increase in PME activity in vegetative-meristem-enriched samples and seedlings (p =0.003), but no significant change in PME activity in the leaves (Figure 4B). Also, OE11420 matrix-primed seeds showed improved germination, compared with that of wt seeds (Figure 4C and D). Specifically, the lag time until the initiation of testa and endosperm rupture was shorter in OE11420 seeds than in wt seeds (p =0.004 and p < 0.001, respectively). There was an increase in the rate of endosperm rupture in OE11420 lines (p <0.001). Germination analysis of OEBDX and OE11420 matrix-primed seeds suggests that the overexpression of these genes in dry seeds improves their germination, possibly through enhancing testa rupture performance (Figures 3 and 4).

To determine the effects of the overexpressed genes on seed germination and PME activity during germination, we tested seeds without a priming pre-treatment. The initial testa rupture and endosperm rupture times were shorter for OE seeds than for wt seeds (p =0.01 and p < 0.01, respectively). The seeds of OEBDX and OE11420 did not show different testa rupture rates, but seeds of both lines showed significantly lower endosperm rupture rates compared with that of wt seeds (p =0.01, Figure 5A and B). For wt seeds, PME activity increased before testa rupture was complete and decreased thereafter, similar to the pattern

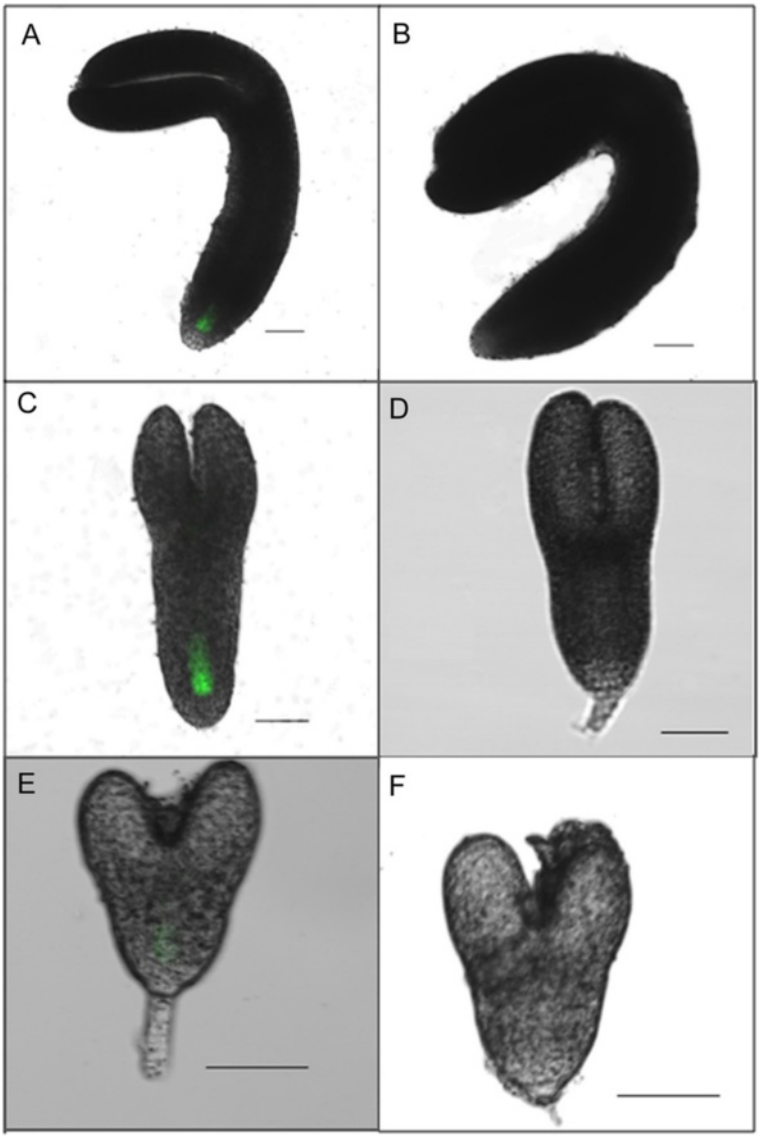

Figure 2 Identification of *At4g32460* promoter activity during *Arabidopsis thaliana* embryo development using pBDX::ER-GFP plants (Additional file 1: Figure S1C). A) GFP fluorescence in provascular cells from radicle meristematic region of mature embryo. **C)** GFP fluorescence in provascular cells from radicle meristematic region of torpedo-stage embryo. **E)** GFP fluorescence in provascular cells from radicle meristematic region of heart stage embryo. **B)**, **D)**, and **F)** Images of wt embryo stages. Scale bars =50 μm. All images are projections of confocal Z-stacks.

reported previously [13]. The same pattern of PME activity was observed in OE11420 seeds. At 20 h of germination, PME activity was significantly higher in OE11420 seeds than in wt seeds. However, the PME activity pattern for OEBDX seeds was different; the PME activity did not decrease at 34 h of germination. In the 1-h and 34-h germinating seeds, PME activity was higher in OEBDX seeds than in wt seeds (p <0.001, Figure 5C).

Pectins are the main component of *A. thaliana* seed mucilage, and can be detected by staining with ruthenium red [28]. In water-imbibed OEBDX seeds, mucilage release was similar to that of wt imbibed seeds (Additional file 1: Figure S5).

Next, we used antisense RNA technology to silence *BDX* expression. An *At4g32460* RNA antisense transgene driven by the cognate promoter of the endogenous *BDX* gene (BDX::BDX antisense RNA transgene; Additional file 1: Figure S1D) was transformed into wild-type *A. thaliana* plants to generate ASBDX plants (Figure 6). Five independent transgenic lines were obtained by kanamycin selection. All of these transgenic lines exhibited phenotypic variation in the T1 progeny, and the segregation analysis revealed a 3:1 ratio of wt phenotypes to defective phenotypes. This ratio was previously observed for an antisense construct with a cognate promoter in rice [29]. Analyses of morphological and developmental defects were conducted

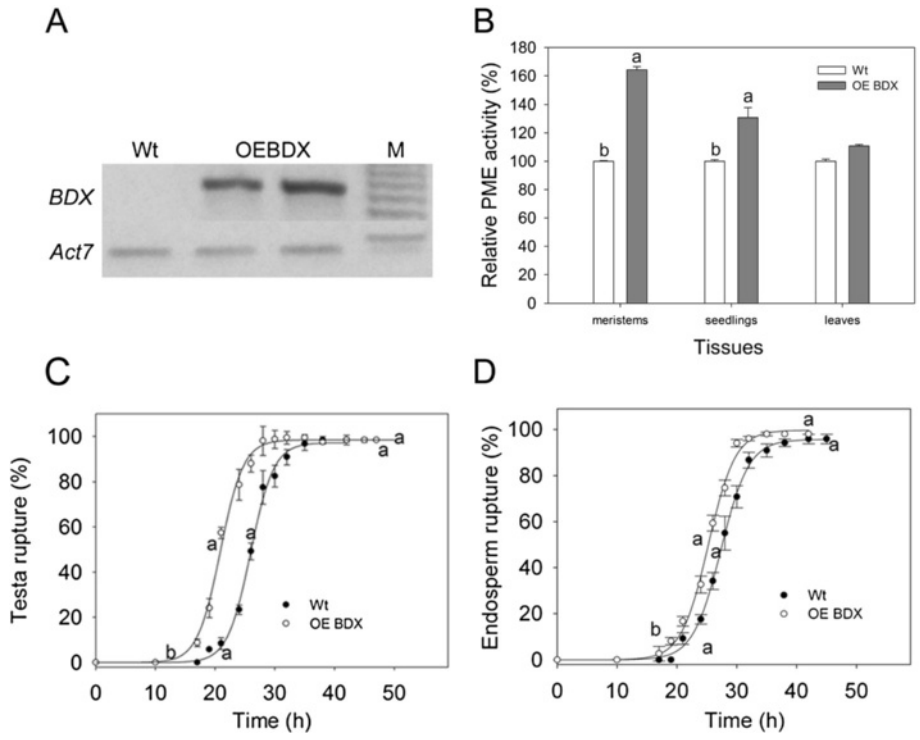

Figure 3 Effects of *BDX* overexpression on *BDX* transcript level, PME activity, and germination of matrix-primed seeds. A) Detection of *BDX* transcripts in dry seeds from wt plants and two independent OEBDX lines. *ACT7* expression was used as an internal control. **B)** PME activities in meristem and leaves of *A. thaliana* plants (18 days after sowing) and seedlings (5 days post-germination). **C)** Cumulative testa rupture curve. **D)** Cumulative endosperm rupture curve. Germination assays were performed in triplicate at 20°C. All experiments were performed at least three times for each line, with different seed lots. Values shown are mean ± standard error; different letters indicate significant differences among lines.

using three independent antisense lines. All three lines showed reduced silique size and did not produce seeds (Figure 6A, B, C and D). Analysis of a transverse section of a stage 13 ASBDX flower bud revealed abnormal carpel morphology. The size of the stigma was decreased, and the septum had engrossed regions. In mature ovules, different tissues could not be differentiated (Figure 6D, close-up image). The mean silique length for ASBDX lines 1, 2, and 3, was 3.3, 3.1, and 4 mm, respectively, corresponding to a size reduction of at least 60% (Figure 6E). Compared with wt, all three ASBDX lines showed significantly lower PME activity in the leaves (p =0.014, p =0.001, and p =0.003 for lines 1, 2, and 3, respectively; Figure 6F). A quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis revealed that all three ASBDX lines showed a 40% reduction in *At4g32460* transcript levels, compared with that in wt inflorescences (p <0.001 for line 1 and 2, and p =0.004 for line 3; Figure 6G).

We conducted a phenotypic analysis of two SALK lines, SALK_142260 and SALK_054867, each of which have a T-DNA insertion in the *BDX* coding sequence. The results provided further evidence that *BDX* plays roles in reproductive development, and possibly in modulating PME (Figure 7). The T-DNA insertion in the SALK_142260 line is at end of the second exon and that in the SALK_054867 line at the end of the third exon of the *At4g32460* locus (Figure 7A). Heterozygous plants of the two SALK lines showed a reduction in silique length, similar to that in ASBDX plants (Figure 7B and D). However, in the SALK lines, the siliques contained a few seeds (average, 4 ± 1 seeds). Some seeds showed an abnormal morphology and were not viable (Figure 7C). Seeds with normal morphology generated either heterozygous or wt plants but not homozygous plants. A qRT-PCR analysis showed a significant decrease in *BDX* transcript levels in T-DNA heterozygous plants (p =003, Figure 7E).

Discussion

DUF642 is a highly conserved family of cell wall-related proteins specific to spermatophytes. This family shows a high level of amino acid identity among different plant species, suggesting that members of the DUF642 play an important function in plant cell wall properties [1]. Our results showed that *BDX* was expressed in embryos, in imbibed seeds, in 48-h seedlings, and throughout the adult plant, where it was primarily localized in vascular tissue, as has been described for *At5g25460* [2] (Figures 1 and 2). *BDX* was detected in the initial cells of the different stages

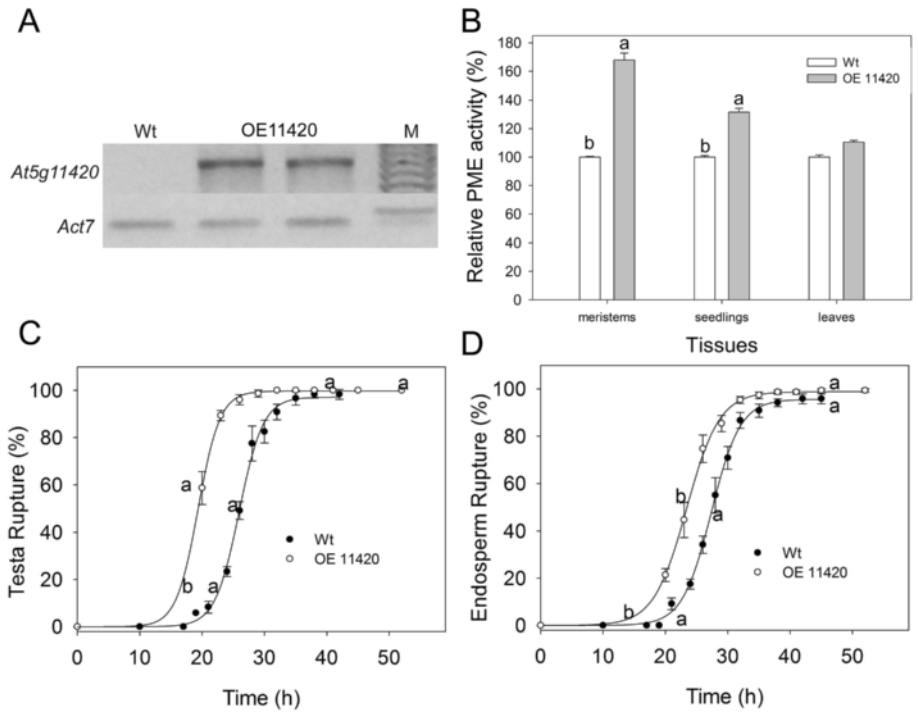

Figure 4 Effects of *At5g11420* overexpression on *At5g11420* transcript level, PME activity, and germination of matrix-primed seeds. **A)** *At5g11420* transcript level in dry seeds of wt and two independent OE11420 lines. *ACT7* expression served as the internal control. **B)** PME activities in meristem and leaves of *A. thaliana* plants (18 days after sowing) and seedlings (5 days post-germination). Values are means ± standard error. **C)** Cumulative testa rupture curve. **D)** Cumulative endosperm rupture curve. Germination assays were performed in triplicate at 20°C. Different letters indicate significant differences among lines. All experiments were performed at least three times for each line, with different seed lots.

of the embryo and primary root, mainly in the pericycle cells before lateral root emergence; therefore, its expression in vascular tissue in other organs could be related to these types of cells.

Auxin signaling promotes the emergence of lateral root primordia [30]. In the present study, auxin induced *BDX* expression in roots (Additional file 1: Figure S2), and *BDX* was highly expressed in stigma tissue of stage 12 flowers (Figure 1H), where auxin-signaling genes are overrepresented [31]. Auxin distribution in the stigma plays an important role in the pollination process. In the ASBDX plants, the siliques were very short and contained no seeds; in the heterozygous T-DNA plants, the siliques were short but they contained a few seeds (Figures 6 and 7). The high transcript level of *BDX* in stigma tissue could be related to the low seed yield of ASBDX and T-DNA plants. It has been suggested that the regulation of cell wall structure, and especially pectin status, in female tissue is fundamental for pollen tube penetration [32]. Previous studies have shown that methylesterification of the transmitting tract decreases before and during pollination and that PME activity increases during silique development [17,18,32]. Whereas *pme3*-null mutants showed no morphological changes in silique development [33], PMEI5 OE plants grew short and wrinkled siliques containing only one or two seeds. A decrease in total PME activity is detected in PMEI5 OE [13] and in ASBDX plants (Figure 6).

BDX was also expressed during embryogenesis (Figure 2). In three generations, homozygous T-DNA plants were not obtained, suggesting that there was an embryo development defect in null mutant plants.

Transgenic plants overexpressing AtPME3 showed an increase in total PME activity, but their only morphological changes are longer roots and taller shoots than those of wt [33]. In the present study, the *A. thaliana* plants overexpressing *BDX* and *At5g11420* had no morphological changes, as compared with wt, during different vegetative stages of the life cycle, although they showed higher total PME activity in the meristem, seedlings, and imbibed seeds (Figures 3 and 4). The increased PME activity could be related to improved germination performance in OEBDX and OE11420 seeds. Compared with wt seeds, OEBDX and OE11420 seeds showed shorter initial times to testa rupture, which were correlated with the increase in PME activity (Figure 5). A previous study showed that the *At5g11420* transcript is enriched in the micropylar endosperm before testa rupture [4]. In the present study, there were high levels of PME activity in the hours before testa rupture in the OE11420 line,

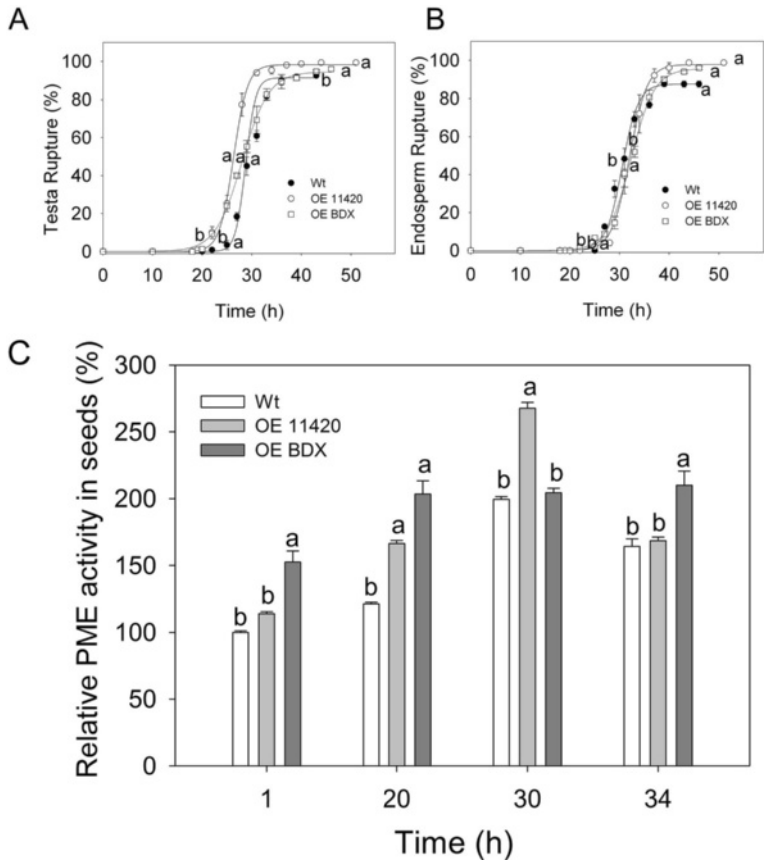

Figure 5 PME activity during germination of OEBDX and OE11420 control (dry) seeds. A) Cumulative testa rupture curve. **B)** Cumulative endosperm rupture curve. **C)** PME activity. PME activity assays were performed in triplicate. All experiments were performed at least twice for each line, with different seed lots. For each time point, letters indicate significant differences. Error bars show SE.

suggesting that *At5g11420* plays a physiological role in germination.

Conclusions

Our data indicated that the DUF642 proteins encoded by *BDX* and *At5g11420* are positive regulators of PME activity in *A. thaliana*. They may affect PME activity directly, or indirectly via altering cell wall properties. The relevance of cell wall modifications during plant development has been studied extensively, and PMEs have been shown to play important roles in modulating methylesterification during fruit development and germination [34]. In this study, the lines overexpressing *BDX* and *At5g11420* showed increased PME activity during seed germination, a two-step process that is highly correlated with changes in PME activity. We speculate that PME activation leads to faster completion of germination by improving the capacity for testa rupture. Previous studies have shown that reduced cell wall pectin methylesterification allows improved access of PG to degrade pectin and promote cell separation in the testa. In addition, the modulation of PG activity by PGIP was shown to inhibit germination [23].

Many morphogenetic events during algal and plant development are related to pectin chemistry [34,35]. In *Chara corallina*, pectin de-esterification promotes cell expansion [36]. Phyllotaxis and organ initiation depend on the regulation of pectin status of the meristem [37]. During pollen tube elongation, a spatial gradient in pectin methyl-esterification is shown to be precisely controlled by PMEs and PMEI proteins [38].

The evolution of the complexity of the methylesterification process is an important process in plant diversification and adaptation to different environments. The expression of pectin and PME in cell walls first occurred in charophytes. The inhibitory domain of PMEs appeared in the PMEI family in land plants during the divergence of mosses from charophytes [39]. The cellulose binding protein (CBP) of the nematode *Heterodera schachtii* increases PME activity in plants through directly interacting with AtPME3; other than this exception, no other positive regulator of PMEs has been described [33]. Our results suggest that DUF642 proteins, a spermatophyte-specific family, contribute to the complexity of the methylesterification process by participating

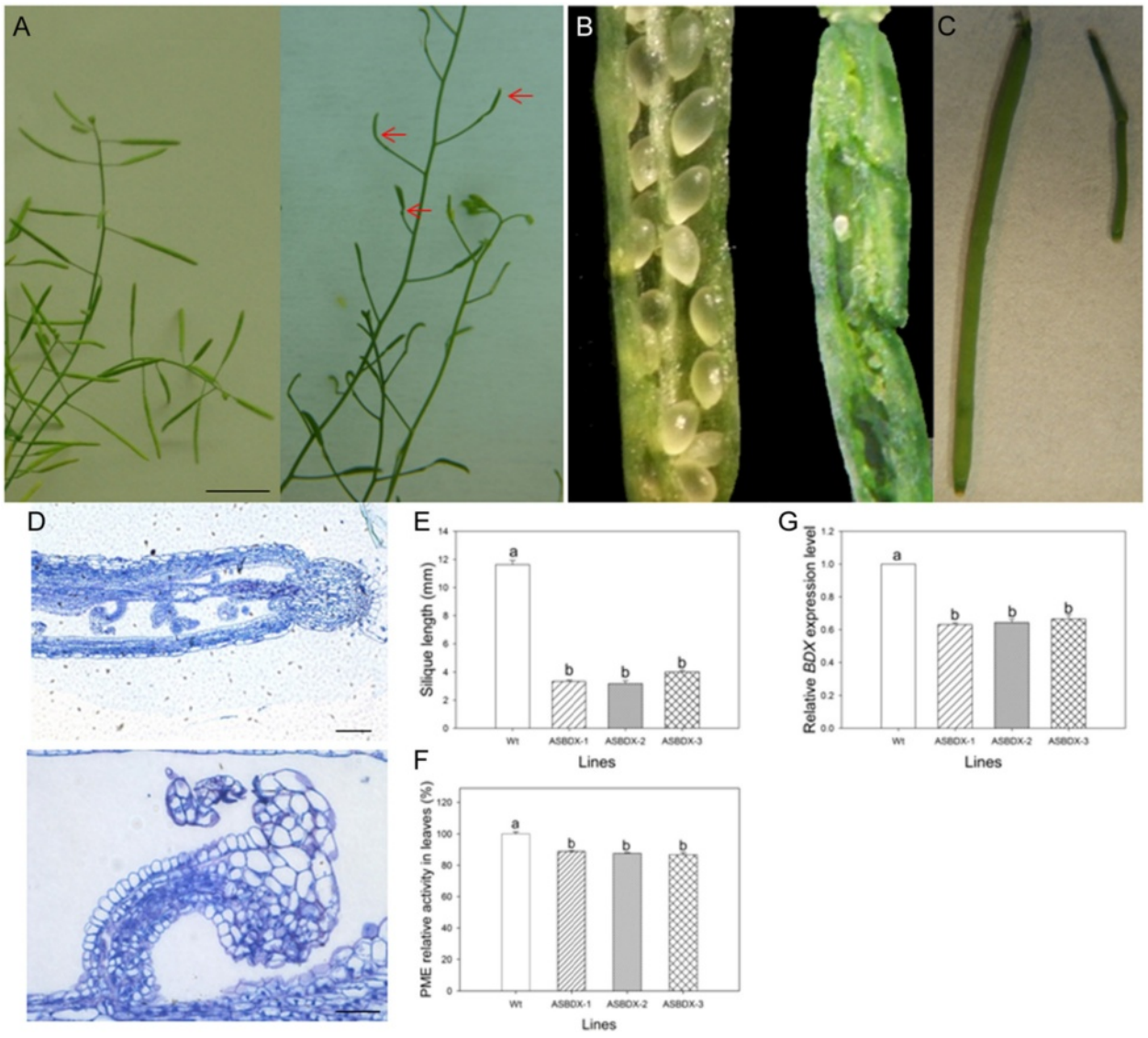

Figure 6 Phenotypic, molecular, and biochemical analyses of pBDX::BDX antisense transgenic lines. A) Representative wt and ASBDX plants, arrows indicate differences in siliques respect to wt. **B)** Representative open siliques from wt and ASBDX plants **C)** Close up from wt and ASBDX siliques. **D)** Longitudinal section of pistil from stage 13 flower from ASBDX. Scale bar =200 μm. A close-up of one ovule is shown. Scale bar =25 μm. **E)** Quantitative analysis of silique length in wt and ASBDX lines 1, 2, and 3. **F)** PME activity in total protein extracts from rosette leaves of wt and ASBDX lines 1, 2 and 3. For all lines, experiments were performed at least three times with different plants. Values shown are mean ± standard error. **G)** Expression of pBDX::BDX antisense transgene significantly decreased endogenous *BDX* transcript level in inflorescences of all three lines. Different letters indicate significant differences.

in the fine regulation of pectin status during plant development.

Methods

Plant materials

Wild-type Columbia (Col) ecotype *A. thaliana* plants (wt) and lines overexpressing (OE) *At4g32460* and *At5g1120* were sown in soil in pots, or on Murashige and Skoog (MS) medium (pH 5.7) in Petri dishes. The plants were grown in a chamber (E-15, Conviron, Manitoba, Canada) at 21°C under a long-day photoperiod (16-h light/8-h dark). Five-day-old seedlings and vegetative meristems (comprising the young organ primordia and the shoot apical meristem proper) and rosette leaves (from 18-day-old plants) were collected, frozen in liquid nitrogen, and stored at −80°C until use in PME activity and RNA analyses.

To determine the expression patterns of *At4g32460* and *At5g11420* during imbibition, 50 mg wt *A. thaliana* seeds were sown on 1% (w/v) agar plates and placed in a

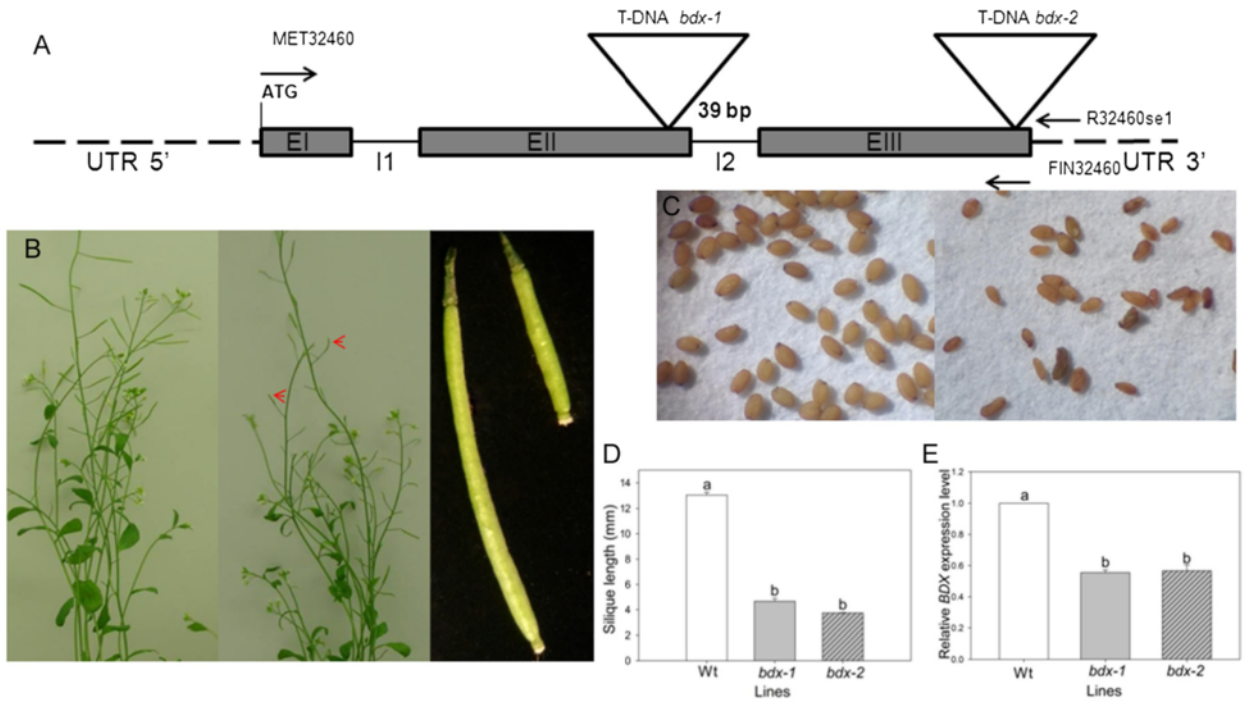

Figure 7 Phenotypic, molecular, and biochemical analyses of T-DNA insertion lines *bdx-1* and *bdx-2*. A) Schematic representation of T-DNA insertion at end of second exon of *BDX* for *bdx-1* and at end of third exon for *bdx-2*. **B)** Phenotypic comparison between heterozygous *bdx-1* plant and wt. All siliques from heterozygous *bdx-1* plants were shorter than those from wt. **C)** Many seeds from *bdx-1* plants were small and wrinkled (right). Similar phenotype was observed for T-DNA *bdx-2* siliques and seeds (Additional file 1: Figure S6). **D)** Quantitative analysis of silique length in wt, *bdx-1*, and *bdx-2*. **E)** *BDX* endogenous gene expression was significantly decreased in inflorescences of heterozygous *bdx-1*and *bdx-2*, compared with that in wt inflorescences. Different letters indicate significant differences.

growth chamber (Lab-Line Instruments Inc. Melrose Park, IL, USA) at 21°C under a 16-h light/8-h dark photoperiod. Seeds were collected at 2, 4, 6, 8, 12, 24, and 48 h post-imbibition, frozen in liquid nitrogen, and then stored at −80°C until RNA extraction.

DNA extraction, RNA extraction, and seed cDNA synthesis

Genomic DNA was extracted from rosette leaves and inflorescences using the phenol chloroform isoamyl alcohol method (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was extracted from rosette leaves and inflorescences using the Trizol method (Invitrogen) according to the manufacturer's instructions.

Total RNA was isolated from 0.05 g *A. thaliana* wt and OE seeds according to the protocol described elsewhere [40]. A Nanodrop spectrophotometer (Nanodrop Lite; Thermo Scientific; www.thermoscientific.com) was used to quantify RNA. For semiquantitative RT-PCR, cDNA was synthesized from 100 ng RNA that had been treated with DNase-I (Qiagen, Valencia, CA, USA) using SuperScript II Reverse Transcriptase (Invitrogen) and oligo (dT) primers. We used the primer pair FAT32460 (forward, 5′-GTGTCCCAAAGCCATTATTC-3′) and RAT 32460 (reverse, 5′-AGCGACGAATCTCAATGAC-3′) to amplify *At4g32460*, and the primer pair 11420LSF (forward, 5′-TCTAGAATGAAAGGAGGCAGCCTCT-3′) and 1142 0LSR2 (reverse, 5′-GGATCCCGGCTTACGAGCACTGA GGAGTT-3′) to amplify *At5g11420. Actin* (*ACT7*) was used as an internal control.

Quantitative RT-PCR

cDNA samples synthesized from 100 ng RNA from antisense, T-DNA, and wt inflorescences were used for amplification with SYBR Green Master Mix using an Applied Biosystems StepOne platform (Applied Biosystems, Foster City, CA, USA). The PCR conditions were as follows: 50°C for 2 min for DNA polymerase activation, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C; finally, samples were subjected to 15 s at 95°C, 1 min at 60°C, and 15 s at 95°C for melting curve analysis. Three independent biological replicates with three technical replicates were analyzed. *ACT7* served as the endogenous control. To analyze gene transcript levels in inflorescences of antisense plants, the forward primer consisted of a sequence from the UTR 5′ region (5′-CTCTCGCTC ACTCTTCTCCAA-3′) and the reverse primer consisted of a sequence from the beginning of the second exon (5′-CGACAAAGCCTGAGAGTTCCC-3′, BDXR). For analyses of the T-DNA lines, we used the *BDX* forward

primer (5′-GCTTCAATGATGGACTACTACC-3′, BDXF) and BDXR. Samples were compared with C_T and slope values and analyzed using the mathematical model established by Pfaffl [41] to obtain the relative expression ratio. Data were subjected to a natural log transformation before the Student's *t*-test.

Plasmid construction

For *At4g32460* (GENE ID 829381) expression analysis, a 1983-bp fragment of the *At4g32460* intergenic region ending at ATG was amplified from leaf-extracted genomic DNA using the following primers: PRO32460F (forward, 5′-AAGCTTGCATGGGAGAATTGACCACT-3′) and PRO32460R (reverse, 5′-GGATCCTTGGAGAAGA GTGAGCGAGAG-3′). This PCR fragment was cloned into pGEM-T Easy and then sequenced. The fragment was then partially digested with HindIII followed by BamHI, and then cloned into the pBIN-m-GFP-ER plasmid (PRO32460: ER-GFP, Figure 1).

For overexpression of *At4g32460* and *At5g11420* (GENE ID 831013), their coding regions were amplified from cDNA synthesized from leaf mRNA using the primers pairs F32460se1 (forward, 5′-GGATCCATGAA AGAGATGGGAGTGATAG-3′) and R32460se1 (reverse, 5′-GAGCTCTCACGGCCTCCGAGCACT-3′); and F1 1420se1 (forward, 5′-GGATCCATGAAAGGAGGCA GCCTCT-3′) and R11420se1 (reverse, 5′-GAGCTCT TACGGCTTACGAGCACTGA-3′), respectively. These PCR fragments were cloned into pGEM-T Easy and then sequenced. After digestion with BamHI and SacI, the fragments were subcloned into the pBIN plasmid and expressed under the control of the CaMV 35S promoter (Figure 1C and D). For gene silencing analysis, we constructed a plasmid with the *At4g32460* antisense transgene under the control of its cognate promoter.

Plant transformation

Wt Col *A. thaliana* plants were transformed using the floral-dip method via *Agrobacterium tumefaciens* C58 [42]. Collected seeds were sown on plates containing MS medium (pH 5.7) with kanamycin for selection, and green seedlings were transplanted into soil in pots. At least 10 independent transgenic lines were selected. The homozygous T3 seed generation was used for germination experiments (two independent lines) or confocal microscopy (three independent lines). For antisense transgenic plants, at least 10 independent lines were selected. For phenotype analysis, five homozygous (T3) lines were used, and three lines were used for PME activity and qRT-PCR analyses.

T-DNA lines

The insertion in the T-DNA SALK_142260 line was verified by PCR, and the region of interest was sequenced using the T-DNA left border primer LBb1 (5′-GCGTGGACCGCTT GCTGCAACT-3′) and a specific primer for *BDX*, R32460se1, located at the end of the second exon. For the SALK_054867 line, the reverse primer consisted of a sequence located at the end of the third exon.

Laser confocal scanning microscopy

To label the cell walls, seedlings were incubated in a propidium iodide solution (1.7 mM) for 30 s before confocal imaging analysis. Propidium iodide and GFP were imaged at 485 and 545 nm, respectively, using an aFV100 Laser Confocal Scanning Microscope (Olympus, Tokyo, Japan). Images from all plant tissues without fluorescence were used as controls. The images were assembled using Photoshop, v. 5.0 (Adobe Systems, San Jose, CA, USA).

Matrix priming treatment (M)

A. thaliana seeds were enclosed in cellophane and buried at 2 cm depth in a pot filled with field-capacity humid soil (−0.027 MPa water potential). The pot was covered with aluminum foil and incubated at 22°C for 24 h. Seeds were exhumed in the dark and air-dried at room temperature (25°C). Subsequently, the seeds were used for germination testing or frozen in liquid nitrogen and stored at −80°C.

Germination testing

For germination testing, dry mature seeds were stored at 20°C for 3 months of after-ripening. Seeds were sown on 1% (w/v) agar plates and transferred to a plant growth chamber (Lab-Line Instruments Inc.) at 20°C under a 6-h light/18-h dark photoperiod. All germination experiments consisted of five replicates with 30 seeds per replicate. Biological replicates were performed with different seed lots of wt and the different overexpression lines for *BDX* and *At5g11420*. Germination was scored using an Olympus dissection microscope. Seeds with visible endosperm were considered to have reached testa rupture. Seeds with a radicle tip emerging through the endosperm were considered to have reached endosperm rupture. The percentages of seeds with testa and endosperm rupture over time were fitted to sigmoid models using Table Curve 2D v.3 software (AISN, Software, Chicago, IL, USA). From these models, we obtained the lag time of germination and the germination rate after an arcsine transformation to meet the assumptions of the test. The results were analyzed with an analysis of variance (ANOVA) and Tukey's post hoc test (Sigma Plot v.11, Systat Software Inc., San José, CA, USA). Germination percentages were also arcsine-transformed to meet the assumptions of the test. To compare the slope between wt and OE lines, the testa and endosperm fits were included.

PME activity assays

PME activity was determined according to a previously described method [28] with the following modifications: 8–20 μg protein in equal volumes (5–20 μl) was loaded into a gel matrix prepared in 50-mm Petri dishes (instead of 6-mm-diameter wells). The gel consisted of 0.1% (w/v) ≥85% esterified citrus fruit pectin (Sigma-Aldrich), 1% (w/v) agarose, 12.5 mM citric acid, and 50 mM Na_2HPO_4 (pH 6.5). The PME activities in different tissues were normalized to the corresponding wild-type average activity (=100). Significant differences were assessed using a Mann–Whitney *U* test and two-way multivariate analysis of variance (MANOVA) for seed imbibition.

Histological analyses

Silique samples from plants harboring the antisense construct were fixed in FAA (by volume, 4% formaldehyde, 2% acetic acid, 50% ethanol). The samples were dehydrated through a graded ethanol series and embedded in paraffin. Sections (1–2 μm) were cut with a rotary microtome, and then stained with toluidine blue. Histological sections were observed and photographed under an Olympus microscope.

Availability of supporting data

The supporting data of this article is included in the additional file.

Additional file

Additional file 1: Figure S1. Schematic presentation of transgene constructs. (**A**, **B**) *At4g32460* and *At5g11420* cDNA fragments were cloned into pBIN to generate CAMV 35S::BDX (OEBDX) and CAMV 35S::*At5g11420* (OE11420) constructs, respectively. **(C)** To construct pBDX::mGFP-ER, a 1983-bp fragment of *At4g32460* intergenic region was cloned into pBIN-m-GFP-ER. **(D)** pBDX::BDX antisense consisted of *At4g32460* RNA antisense transgene driven by the cognate promoter of *At4g32460* (ASBDX). Black arrow, CaMV promoter; striped arrows, cognate promoters; gray arrows (right), sense fragments; gray arrows (left), antisense fragment. **Figure S2.** Early and differential transcription of *BDX* and *At5g11420* during seed imbibition. *ACT7* was analyzed as internal standard. **Figure S3.** Auxin- and gibberellic acid-inducible expression of *At4g32460* in primary roots. Transgenic 5-day-old seedlings were treated for 0 h (**A**, **D**) or 24 h with 2 μM GA (B,E) or for 48 h with 2 μM IAA (**C**,**F**). **A)** GFP fluorescence in provascular tissue of meristematic zone (as in Figure 2). **B)** GFP fluorescence in provascular and vascular tissue of meristematic and transition zone. **C)** GFP fluorescence in provascular and vascular tissue of meristematic and transition zone and was also detected in cortical cells of transition zone. **D)** GFP fluorescence in pericycle cells of vascular tissue of maturation zone (as in Figure 2). **E)** GFP fluorescence in vascular tissue and cortical cells of maturation zone. **F)** GFP fluorescence in vascular tissue and cortical cells of maturation zone. **Figure S4.** Effect of empty plasmid on germination performance. A) Cumulative testa rupture curve. **B)** Cumulative endosperm rupture curve. **Figure S5.** OEBDX and OE11420 seeds did not show defective mucilage release after imbibition in water [43]. **Figure S6.** Phenotypic analyses of *bdx-2*. **A)** Phenotypic comparison between heterozygous *bdx-2* plant and Siliques of heterozygous *bdx-2* plants were shorter than those of wt. **B)** Many seeds from *bdx-2* plants were small and wrinkled (right).

Abbreviations

BDX: *BIIDXI*; DUF642: Domain unknown function 642; PME: Pectin Methyl Esterase; AtPME3: Pectin Methyl Esterase 3 from *Arabidopsis thaliana*; PMEI: PME inhibitor; HGs: Homogalacturanans; PG: Polygalacturonase; PGIP: Polygalacturonase inhibitors proteins; ABA: Abscisic acid; GFP: Green fluorescent protein; GA: Gibberelic acid; GARE: Gibberelic acid response elements; OE: Overexpression; OEBDX: BDX Overexpression; ASBDX: Antisense BDX; CBP: Cellulose Binding Protein; Col: Columbia ecotype; wt: Wild-type; MS: Murashigee and Skoog; IAA: Indole-3-acetic acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EZ and DS carried out the molecular genetic studies and participated in germination analysis. EMB and AOS participated in the design of the study. AGB conceived of the study and participated in its design and coordination. EZ, DS, EMB, AOS and AGB wrote the article. All authors read and approved the final manuscript.

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CAPÍTULO II

El papel de proteínas de la pared celular y las auxinas durante la infección por nematodos en plantas.

Durante la inducción de la formación de estructuras de alimentación por parásitos como los nematodos (NFS Nematode Feeding Site, por sus siglas en inglés), ya sea por nematodos formadores de un sincicio o aquellos conocidos como nematodos agalladores, ocurren importantes cambios relacionados con la reestructuración de la pared de las células que formarán parte del NFS. Se ha descrito una degradación de las paredes de las células que delimitan el sincicio, la deposición de una capa translucida y la formación de crecimientos, de la pared celular, hacia el interior, los cuales permiten un incremento en la tasa de intercambio de solutos y nutrientes desde el apoplasto hacia el simplasto (Bohlmann y Sobczak, (2014); Sobczak *et al.*, 2011). También se han descrito cambios en la expresión de genes que participan en la síntesis de polisacáridos como el UDP- ácido glucurónico el cual es un precursor que contribuye hasta en un 50% con la biomasa de la pared celular. Mutaciones en las enzimas que lo sintetizan inhiben el desarrollo adecuado del sincicio (Siddique *et al.*, 2012).

El incremento en la expresión de genes que codifican para proteínas modificadoras de la pared como son expansinas, endoglucanasas, PME's y extensinas también ha sido reportado (Jammes *et al.,* 2005; Wieczoreck *et al.,* 2006). Hewezi y colaboradores enfatizan la importancia de una de éstas proteínas (AtPME3) durante procesos de infección por un nematodo parásito del betabel, *Heterodera schachtii*, Durante la infección de *A. thaliana* por este nematodo se ha descrito la expresión de ciertos genes del parásito. Entre los genes identificados extraídos de las glándulas subventrales activas se identificó a una proteína con dominio de unión a celulosa CBP (Cellulose Binding Protein) entre otras proteínas. CBP es sintetizada por el nematodo durante las primeras etapas de la infección y se expresa más abundantemente durante la formación del sincicio y no durante las etapas más tardías. Esta proteína se caracteriza por la presencia de un péptido señal, su alta afinidad a la celulosa y carecer de un dominio catalítico. Muy poco se sabe de de los efectos de la CBP de *H. schachtii* sobre la pared celular de las plantas, sin embargo, análisis de interacción proteína-proteína han permitido determinar que CBP interactúa específicamente con AtPME3 (Hewezi *et al*, 2008). Los análisis de localización subcelular para AtPME3 con y sin péptido señal (solo región catalítica) permitieron localizarla en

pared celular y citoplasma en el primer caso y solo en el citoplasma en el segundo lo cual corrobora la interacción *in vivo* entre CBP y AtPME3. Las plantas que sobreexpresan la proteína CBP del nematodo tienen un incremento en la actividad de AtPME3 y al igual que las plantas que sobreexpresan AtPME3, presentan raíces más largas y son más susceptibles a ser infectadas. Las mutantes nulas de AtPME3 además de tener raíces más cortas son menos susceptibles a ser infectadas por este nematodo. De acuerdo con los autores AtPME3 es necesaria para la función de CBP además de estar probablemente involucrada en reducir localmente la esterificación de las pectinas de las paredes de las células pre-sinciciales induciendo su relajamiento durante etapas tempranas de la formación del sincicio (Hewezi *et al*, 2008).

Al igual que durante la formación de las raíces laterales, durante la formación de los sitios de alimentación por nematodos, ya sea por nematodos formadores de sincicio o de nematodos agalladores, las auxinas juegan un papel de vital importancia. Algunas de las primeras observaciones mostraron que las plantas de durazno tratadas con esta hormona e infectadas por nematodos eran mucho más susceptibles a la infección. En *A. thaliana* se ha reportado un incremento en los niveles de expresión del marcador de respuesta a auxinas (DR5) después de la infección por nematodos. El incremento de auxinas en los sitios de alimentación de ambos tipos de nematodos, podía ocurrir, tanto porque el nematodo fuese capaz de secretar la hormona como porque la planta llevase a cabo la liberación de las auxinas hacia estos sitios. Un estudio realizado con nematodos sinciciales y con nematodos formadores de células gigantes, demostró la activación del promotor del transportador *AUX1,* sugiriendo la estimulación del influjo de auxinas en los sitios de alimentación. Otro estudio con los transportadores de eflujo de auxinas *PIN1* y *PIN7* que se expresan normalmente en el haz vascular, mostró que al inicio de la formación del NFS la expresión de estos genes se inhibía disminuyéndose así el transporte de auxinas. Esto sugirió que los nematodos son capaces de regular directa o indirectamente la expresión de dichos transportadores. Este mismo estudio muestra que tanto *PIN3* como *PIN4* se expresan específicamente y de manera muy abundante en el NSF (Grunewald *et al.*, 2009 a y b).

La infección de *Arabidopsis thaliana* por *Meloidogyne incognita* induce la expresión de los genes DUF642 regulados por auxinas *BDX* y *At2g41800* y promueve su localización en la pared celular.

Alexis Salazar Iribe[1], Esther Zúñiga-Sánchez[1*], Emma Zavaleta Mejía[2], Alicia Gamboa-deBuen.

Meloidogyne incognita es un nematodo formador de agallas que infecta diferentes plantas incluyendo *Arabidopsis thaliana*. Los juveniles de estadío 2 (J2) penetran la raíz en la región de elongación cercana a la región meristemática y migran intercelularmente hacia el ápice de la raíz. Posteriormente se lleva a cabo el desarrollo de las células gigantes inducidas por el nematodo a partir de las células del procambium para la formación de la estructura de alimentación del nematodo (Williamson y Hussey, 1996). Durante la migración, el nematodo secreta una gran cantidad de enzimas modificadoras de la pared celular incluyendo enzimas que degradan pectinas, principal componente de la lámina media, para facilitar la separación de las células (Davis *et al.*, 2004). A pesar de que la migración entre las células genera poco daño, desde esta etapa, el nematodo manipula las redes de distribución de las auxinas en la raíz que también responde incrementando la producción de especies reactivas de oxígeno (Grunewald *et al.*, 2009 a y b, Melillo *et al.*, 2006). La activación de proteínas modificadoras de la pared celular de la planta se lleva a cabo principalmente durante el desarrollo de las células gigantes (Jammes *et al.*, 2005). La secreción de una proteína que interactúa con una pectin metil esterasa (AtPME3) que facilita la invasión también ha sido descrita en el caso de *Heterodera schasstii*, un nematodo sincicial (Hewezi *et al.*, 2008).

BIIDXI (*At4g32460*) es una proteína de pared celular que pertenece a la familia DUF642, que interactúa *in vitro* con AtPME3 (Zúñiga-Sánchez y Gamboa de Buen, 2012). La actividad total de pectin metil esterasa es mayor en las plantas que sobreexpresan esta proteína y menor en las plantas mutantes (Zúñiga-Sánchez *et al.*, 2014). La relación de la función de otro gen de esta familia, *At2g41800*, con la activación de pectin metil esterasas también ha sido establecida (Salazar-Iribe *et al.*, en preparación). La familia DUF642 es exclusiva de espermatófitas y está muy conservada. De los 10 genes presentes en *A. thaliana*, se ha detectado una inducción de la expresión de dos de ellos en respuesta a patógenos formadores de agallas o tumores; la inducción de la expresión de *At1g29980* ha sido reportada durante los eventos tempranos (3 dpi) de la formación de la células gigantes inducidas por *M. incognita* (Barcalá *et al.*, 2010) mientras que la expresión de *At3g08030* se incrementa notablemente en las primeras horas de la invasión por *Agrobacterium tumefaciens* (Deeken *et al.*, 2006; Lee *et al.*, 2009).

El objetivo de este estudio es determinar la expresión de *BIIDXI* y *At2g41800,* dos genes cuya expresión se induce por auxinas, y la localización subcelular de ambas proteínas en raíces primarias durante una etapa muy temprana de invasión de *M. incognita* utilizando plántulas de 5 días de *A. thaliana*.

Para los estudios de expresión, se utilizaron plantas transgénicas con la región promotora de *BDX* y *At2g41800* y GFP como gen reportero (PROBDX y PRO41800, respectivamente). Para los estudios de localización subcelular se utilizaron plantas transgénicas con las regiones promotora, codificante y para la proteína GFP en el extremo 3´ para cada gen (Zúñiga-Sánchez *et al.*, 2014; Salazar-Iribe *et al.,* en preparación). Las semillas de las plantas transgénicas y control se sembraron después de ser escarificadas durante 2-3 días a 5 ºC en cajas de petri con 0.5 X de medio basal Murashige y Skoog adicionado con sacarosa 0.5% y fueron colocadas verticalmente en una cámara CONVIRON con fotoperiodo largo a 20-22 ºC.

Los juveniles de estadío 2 (J2) fueron obtenidos a partir de masas de huevos de *M. incognita* presentes en raíces infectadas de *Capsicum annum.* Plántulas de 5 días crecidas según lo descrito se inocularon con 250 J2s por planta. Plántulas transgénicas DR5::GFP fueron usadas como control positivo de la infección, la GFP se detectó en todas las células de la raíz de las plántulas PROBDX infectadas con *M. incognita* (Fig 1B), y como control negativo se utilizó la solución de preservación de los nematodos. Por otro lado, también se realizaron ensayos con *Nacobus aberrans,* un nematodo que no infecta *A. thaliana*, sin embargo, se detectó GFP en las células epidérmicas de raíces de las plántulas transgénicas DR5::GFP (Fig 1C). Los juveniles J2 fueron obtenidos a partir de masas de huevos presentes en raíces infectadas de *Lycopersicon esculentum*. La infección se llevó a cabo de la misma manera descrita para *M. incognita*. Las plantas transgénicas y las plantas control se infectaron con el mismo procedimiento.

La detección de GFP se llevó a cabo a 545 nm con un microscopio Confocal Olympus FV100. Se utilizaron como controles las imágenes de las plantas control.

La infección por *M. incognita* indujo la expresión de los genes *BDX* y *At2g41800* mientras que la infección *N. aberrans* no alteró esta expresión (Fig. 1). Para BDX, la detección de GFP en raíces de plántulas de 7d tratadas con la solución está restringida al haz vascular y a la epidermis tal como ha sido previamente descrita (Fig. 1A; Zúñiga-Sánchez *et al.*, 2014). La GFP fue detectada en todos los tipos celulares de las raíces de las

plántulas infectadas con *M. incognita* mientras que la infección con *N. aberrans* no altera el patrón (Fig 1B y 1C). Resultados similares se observaron utilizando las plántulas transgénicas PRO41800; (Fig. 1G), la GFP se detectó en todos los tipos celulares de la raíz infectada por *M. incognita* incluyendo las células del haz vascular, a diferencia de las raíces control (no infectadas) (Fig.1H recuadro). En las raíces que fueron tratadas con la solución y en las raíces infectadas con *N. aberrrans* sólo se detectó la GFP en las células epidérmicas correspondiente al patrón previamente descrito (Fig 1I).

Las proteínas codificadas por *BDX* y *At2g41800* han sido detectadas en proteomas de pared de diferentes tipos celulares. Sin embargo, la proteína 41800 también ha sido localizada intracelularmente durante la división celular y, posteriormente en la pared durante la formación de la lámina media (Salazar-Iribe *et al.*, en preparación). La infección por *M. incognita* promueve la localización de BDX en la pared celular mientras que la de At2g41800 la induce y promueve su localización en este compartimento (Fig. 2).

Se ha descrito de manera detallada lo que ocurre durante la migración del nematodo antes de su establecimiento pero poco se sabe de los eventos que ocurren durante la infección temprana que son determinantes para el establecimiento y reproducción del nematodo en la planta. Los análisis transcriptómicos de células gigantes (CG) en etapas muy tempranas de infección (3 dpi) han permitido la obtención de un perfil genético durante este período, dicho perfil, es similar para la formación de agallas en la infección por *A. tumefaciens* (Barcalá *et al.*, 2010). Un aumento en la concentración de auxinas y la expresión de genes que promueven la progresión del ciclo celular sin citocinesis en el sitio de alimentación del nematodo formador de agallas son característicos en esta etapa de la interacción. Otra característica es la expansión y engrosamiento de la pared celular sin digestión de ésta (Abad *et al.*, 2003). Durante estas modificaciones están implicadas no solamente aquellas proteínas modificadoras de la pared secretadas por el nematodo sino también las sintetizadas por la planta (Wieczorek *et al.*, 2014). Nuestros resultados demuestran que la infección de *A. thaliana* por *M. incognita* en etapas muy tempranas induce la expresión de dos genes de la familia DUF642 y promueve su localización hacia la pared celular. Los procesos relacionados con la esterificación de las pectinas durante etapas tempranas de infección es muy distinto en nematodos agalladores y nematodos formadores de un sincicio, se ha reportado una gran cantidad de pectinas de-metilesterificadas en las paredes de las células sinciciales a los 5 dpi, mientras que en las paredes de las células gigantes hay un mayor número pectinas metiladas lo cual

correlaciona con una menor actividad de PME. BDX al igual que la CBP interactúa con AtPME3 y promueve su actividad, sin embargo, es necesario realizar más estudios con la finalidad de conocer si al igual que ésta proteína, BDX facilita el parasitismo de nematodos agalladores o tiene otro papel en etapas tempranas de la infección. Por otro lado la expresión de *At2g41800* y su localización en la pared celular en etapas tempranas de la infección podría estar correlacionada con su participación durante el crecimiento y diferenciación de las células gigantes. Es posible que ambos genes estén involucrados tanto en los procesos tempranos de defensa de la planta como en las redes de genes cuya expresión es manipulada por las secreciones del nematodo con el fin de establecer una interacción exitosa. Estos resultados son de gran relevancia ya que proponen que la reestructuración de la pared y las modificaciones específicamente en las pectinas podrían jugar un papel muy importante desde el inicio de la infección, además de sugerir que al menos un gen DUF642 interviene en los eventos tempranos que determinan la formación del NSF.

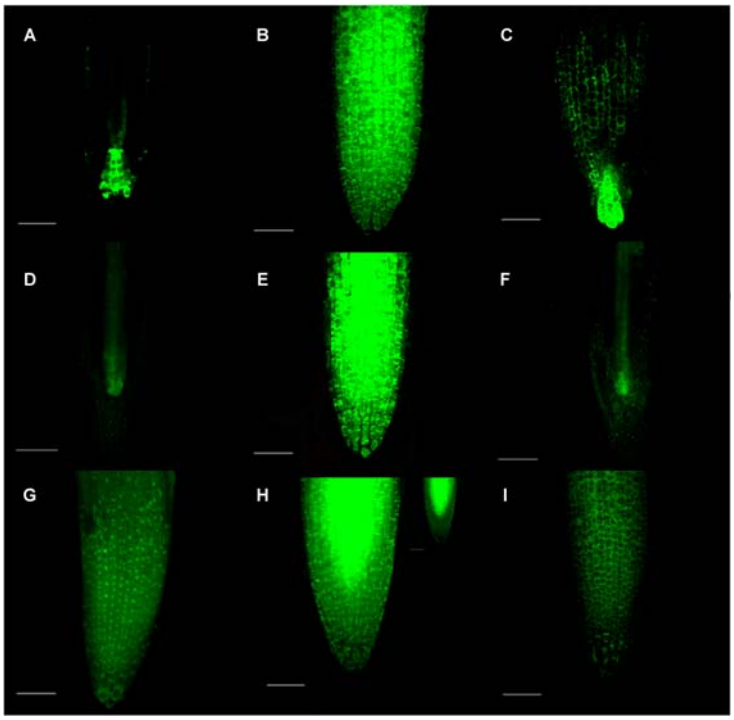

Figura 1: La expresión de *BDX* y *At2g41800* es inducida durante etapas tempranas de la infección por *M. incognita*. Raíces principales de plantas transgénica de 7 días. A) DR5::GFP B) DR5::GFP infectada con 250 nemátodos en J2 de *M. incognita*. C) DR5::GFP infectada con 250 nemátodos en etapa J2 de *N. aberrans*. D) En PROBDX, la señal de GFP se detectó en las células provasculares y epidérmicas de la zona meristemática. E) PROBDX infectada con *M. incognita* J2 (2 dpi), la GFP se detectó en todos los tipos celulares de la zona meristemática y de transición F) En PROBDX infectada con *N. aberrans* en J2 (2 dpi) se detectó en células provasculares y epidérmicas de la región meristemática similar a la raíz control. G) PRO41800 con señal de GFP en las células epidérmicas de la zona meristemática y de transición. H) PRO41800 infectada con *M.*

incognita (2 dpi), la señal de GFP se detectó en todos los tipos celulares de la zona meristemática y de transición incluyendo el tejido vascular (recuadro). I) La señal PRO41800 infectada con *N. aberrans* (2 dpi) se detectó en células epidérmicas de la región meristemática como ocurre en la radícula control. Barra de escala =100 µm. Todas las imágenes confocales son proyecciones en Z excepto la del recuadro en H (corte medio).

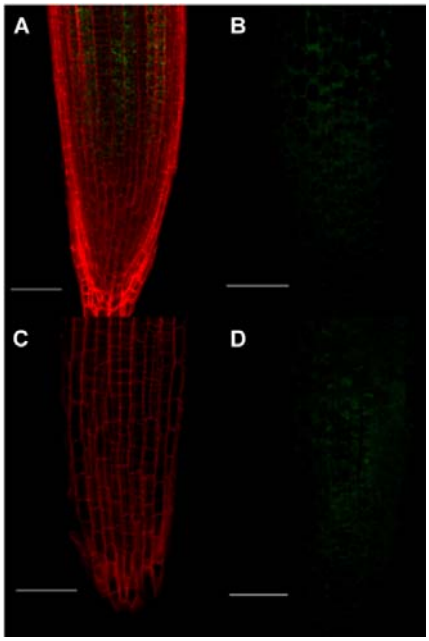

Figure 2: La infección por *M. incognita* promueve la inducción y localización de BDX y At2g41800 en la pared celular. Plantas transgénicas de 7 días fueron infectadas con J2 de *M. incognita* A) Raíz principal teñida con ioduro de propidio, de planta transgénica PROBDX::BDX-GFP con señal de GFP en la zona meristemática detectada en compartimentos citoplásmicos. B) Zona meristemática de raíz principal PROBDX::BDX-GFP infectada con *M. incognita* (2 dpi), la GFP se observa en la pared celular. C) Raíz principal teñida con yoduro de propidio, de planta transgénica GC41800, no se detecta señal de GFP en la zona meristemática. D) Zona meristemática de raíz principal GC41800 infectada con *M. incognita* (2 dpi), la GFP se observa en la pared celular y en otros compartimentos. Barra de escala=50 µm. A y C corresponden a cortes, B y D son proyecciones en Z.

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CAPÍTULO III

Resultados adicionales

Resultados relacionados con el gen *BIIDXI*

La proteína BIIDXI puede localizarse en citoplasma y en pared celular.

La localización de BDX en pared celular ha sido sugerida por su abundante detección en los proteomas de pared celular de hipocotilos etiolados y de tallos maduros (Feiz *et al.*, 2006; Minic *et al.*, 2007; Irshad *et al, 2008*). Previamente se describió que este gen es uno de los dos con posibilidad de presentar un exón alternativo que puede dar lugar a una proteína con o sin péptido señal. El péptido señal de ésta proteína dirige su excreción hacia la pared celular a través de la ruta secretora o posiblemente a través de alguna ruta no descrita relacionada con la excreción de proteínas hacia este compartimento o el apoplasto. (De Caroli, *et al.,* 2011).

En la figura 8 se observa la raíz principal de una planta transgénica de 7 días transformada con una construcción para la localización subcelular de la región codificante de BDX bajo el promotor CAMV 35S. En las distintas líneas observadas al microscopio confocal, la GFP fue detectada con patrón puntuado y difuso en las células epidérmicas de la zona meristemática de la raíz principal, lo que sugiere una localización en vesículas o en el citoplasma.

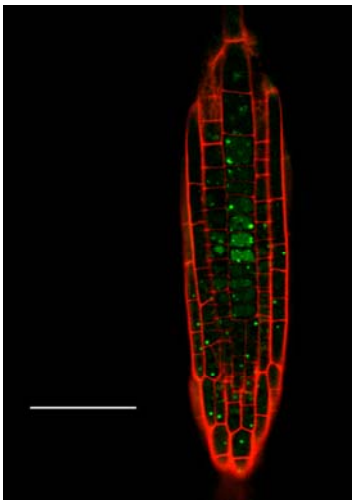

Figura 8: Raíz principal de una planta transgénica de 7 días expresando la proteína codificada por *BDX* bajo el promotor CAMV 35S. La señal de GFP se observa difusa en el citoplasma y en un patrón puntuado. Barra de escala=50 μm.

En las plantas transgénicas transformadas con la construcción del gen BDX bajo su propio promotor, la señal de GFP se localizó en general de manera puntuada en citoplasma en las células epidérmicas y del cortex de la región meristemática de la raíz principal (Fig 9 A y B), mientras que en plantas tratadas con NaCl, BDX se localizó en la pared celular (Fig. C y D). Es necesario llevar a cabo más estudios con el fin de establecer las condiciones necesarias que determinan ésta última localización.

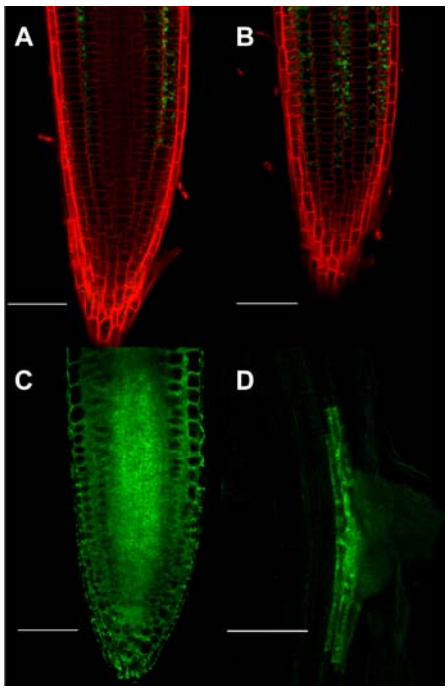

Figura 9: Localización subcelular de BDX en la raíz principal de plantas que expresan ésta proteína bajo su propio promoto crecidas en condiciones controladas de luz y temperatura en medio MS control (A y B) y con NaCl (C y D). A) Raíz principal teñida con yoduro de propidio y señal de GFP intracelular en células del cortex y la endodermis de la región meristemática. B) Raíz principal con señal intracelular de GFP en células epidérmicas de la región meristemática. C) Raíz principal de planta crecida en presencia de 50 mM de NaCl con señal de GFP en pared celular. D) Raíz lateral de planta de 11 días con señal de GFP en la pared de células del haz vascular. Barra de escala=50 μm. A y B son cortes, C y D son proyecciones en Z.

Estudios con diferentes proteínas de pared celular, descritas en los proteomas, demuestran que estas se encuentran generalmente en el interior de la célula, posiblemente dentro de vesículas, y se movilizan hacia la pared celular en respuesta a un estímulo determinado. Por otro lado, también se ha demostrado que la movilización a pared puede ser consecuencia del daño ocasionado por el proceso al que se someten las plantas para su estudio.

La obtención de semillas de plantas AS homocigotas es dependiente del fotoperiodo.

Las plantas AS crecidas en condiciones de fotoperiodo largo (FL, 18 horas luz/ 6 oscuridad) no producen semillas tal como se describió en el artículo del capítulo I (Fig 6), sin embargo, cuando las plantas crecen en condiciones de luz continúa (LC), las silicuas crecen aproximadamente un 20 % más con respecto a las silicuas de plantas crecidas en FL (Fig. 10) y además son capaces de producir un promedio de 55 semillas por planta, siendo toda la progenie homocigota.

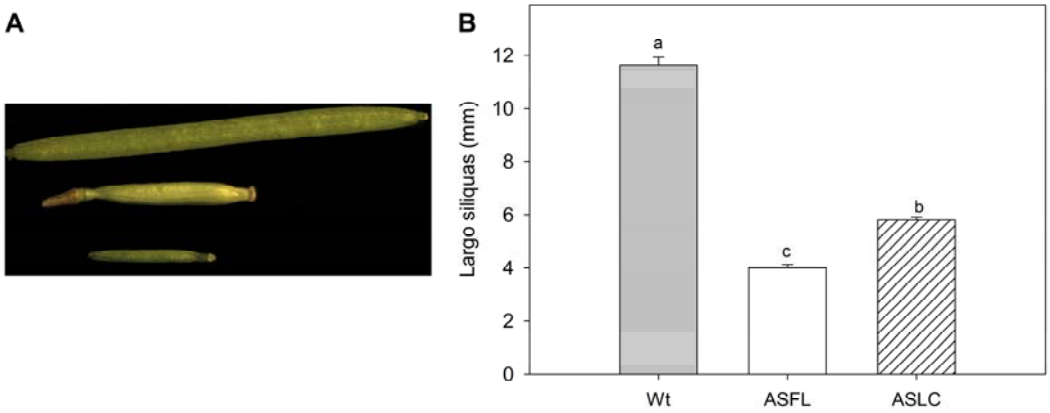

Figura 10: El tamaño de las silicuas y la producción de semillas en las plantas antisentido (AS) para BDX. A) El aumento en el tamaño de las silicuas de las plantas AS para BDX es inducido bajo condiciones de luz continua. A) De arriba para bajo silicuas de planta control, silicuas de planta AS crecida en condiciones de luz continuas (LC) y silicuas de planta AS bajo condiciones de fotoperiodo largo (FL). B) Gráfica que muestra las diferencias en longitud de las silicuas de plantas silvestres crecidas en FL y silicuas de planta AS crecidas en FL y LC. Las letras denotan diferencias significativas.

Se ha descrito que el llenado de grano en *A. thaliana* depende de la fotosíntesis de la silicua durante el periodo de luz y de la movilización de reservas de los órganos fuente durante las horas de oscuridad (Fig. 11) (Andriotis *et al.,* 2012). Las plantas transgénicas AS sólo producen semillas en condiciones de luz continua sugiriendo que la movilización de reservas podría ser una limitante para el llenado.

De manera cualitativa se determinó que la cantidad de almidón en hojas de roseta cortadas al final de la noche y al final del día es mayor en las plantas AS respecto a las plantas silvestres (wt). Aunque los resultados obtenidos son preliminares, éstos sugieren que la movilización del almidón está afectada en estas plantas.

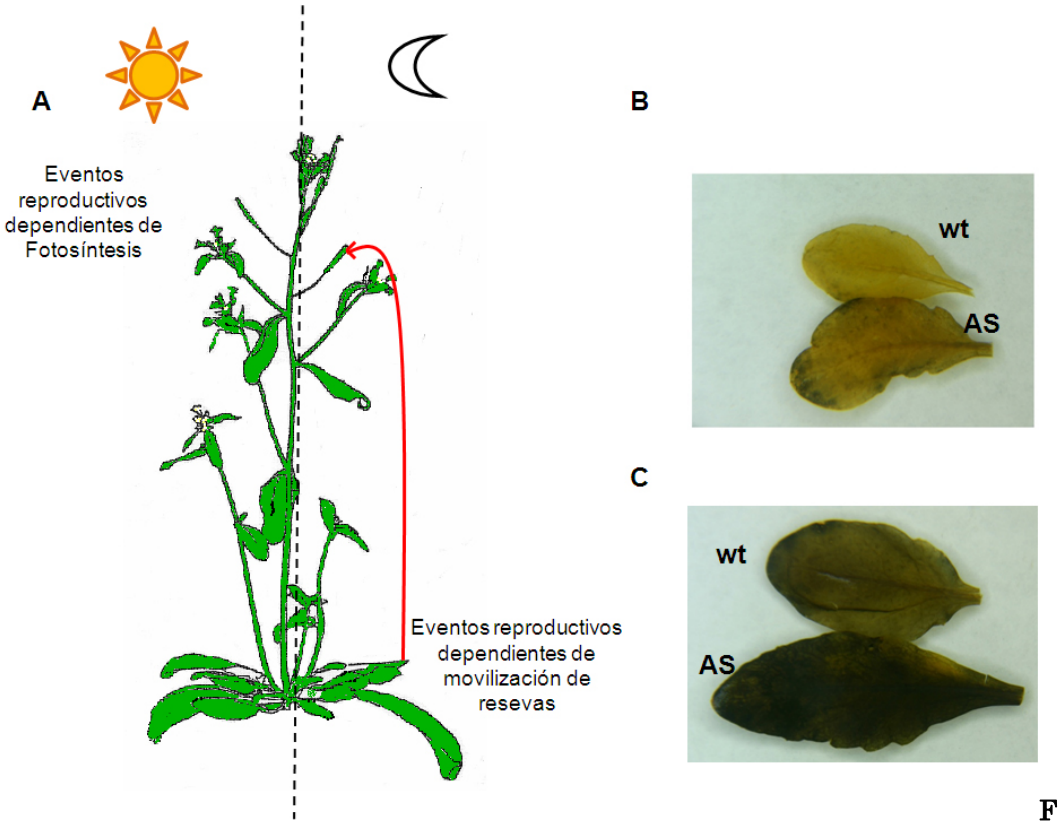

Figura 11: El llenado de la semilla y el transporte de almidón en plantas antisentido (AS) para BDX. A) En *A. thaliana* el desarrollo del embrión es dependiente de la actividad fotosintética durante el día, mientras que durante la noche el transporte de nutrientes se lleva a cabo desde órganos fuente. B) Hojas de roseta teñidas con Lugol de plantas AS para BDX y plantas silvestres (wt) cortadas al termino de un período de oscuridad de 16 horas (C) y al termino de un período de luz de 8 horas.

Resultados relacionados con el gen *At5g11420*

Patrones de expresión del gen *At5g11420* en diferentes órganos y tejidos de *A. thaliana.*

La expresión de *At5g11420* restringe a ciertos tipos celulares en la raíz principal (Fig. 12 A), raíz lateral (Fig. 12 B y C), flor (Fig. 12 D, E y F), embrión (Fig G) y es inducido por auxinas en hipocotilos (Fig. H e I). En la raíz principal, la GFP se detectó en las células de la epidermis de la región meristemática (Fig. 12 A) mientras en la raíz lateral en las células epidérmicas de la zona de emergencia (Fig. 12 B y C). En las flores la expresión de este gen se restringe a la zona de dehiscencia de los verticilos florales y a los pétalos (Fig. 12 D, E y F). En embriones la expresión se detectó en los cotiledones y en células del tejido vascular de la región meristemática de la radícula (Fig.12 G). La expresión de este gen se indujo en presencia de 10 mM de auxinas, en hipocotilos de 10 días (Fig. H e I). Como se puede observar los niveles de expresión son menores y más delimitados a ciertos tipos celulares con respecto a su gen homologo BDX.

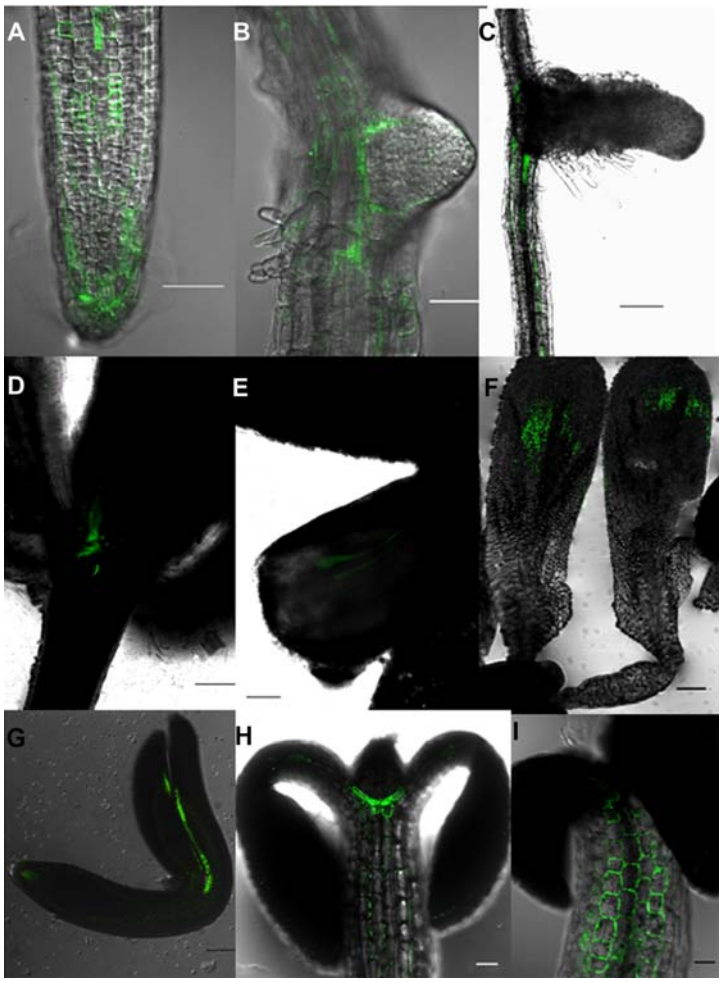

Figura 12: Análisis de expresión de *At5g11420* en plantas PRO11420::GFP en distintos órganos, tejidos y estadios de desarrollo de *A. thaliana*. A) Raíz principal de planta de 10 días con señal de GFP en células epidérmicas de la región meristemática. B y C) Raíces laterales de plantas de 11 días con señal de GFP en células epidérmicas de la región donde se lleva a cabo la emergencia de la raíz lateral. D) Flor de planta transgénica PRO11420::GFP con señal de GFP en la zona de dehiscencia de los verticilos florales. E y F) Pétalos de flores de plantas transgénicas con señal en células del haz vascular y células del parénquima. G) Embrión de planta transgénica con GFP en cotiledones y en la radícula en células vasculares de la región meristemática. H e I) Plántulas de 5 días crecidas en medio MS en presencia de 10 mM de auxinas con señal de GFP en la base del domo del meristemo y en células epidérmicas del hipocotilo.

La expresión ectópica de *At5g11420* induce la desorganización del SAM en *A. thaliana.*

Las plantas que expresan la región codificante de *At5g11420* bajo el promotor de BDX presentaron un fenotipo distinto al de las plantas silvestres en la generación T_2. Dicho fenotipo consistió en plantas cuya progenie tenía un porcentaje bajo de germinación (Fig.13 A y D), algunas de las plántulas tenían al menos dos meristemos apicales (Fig. 13

B y C) y la filotaxis de las plantas adultas era desorganizada con respecto a la de una planta silvestre (Fig. 13 F y G). En la parte subterránea se observó un menor número de raíces laterales y de menor tamaño (Fig. E). La proteína codificada por *At5g11420* comparte con BDX un 79 % de identidad y un 81% de similitud (Fig. 14) esta homología sugiere una redundancia funcional entre ambas proteínas, sin embargo, los resultados obtenidos nos muestran que la expresión diferencial de ambas proteínas puede tener efectos funcionales.

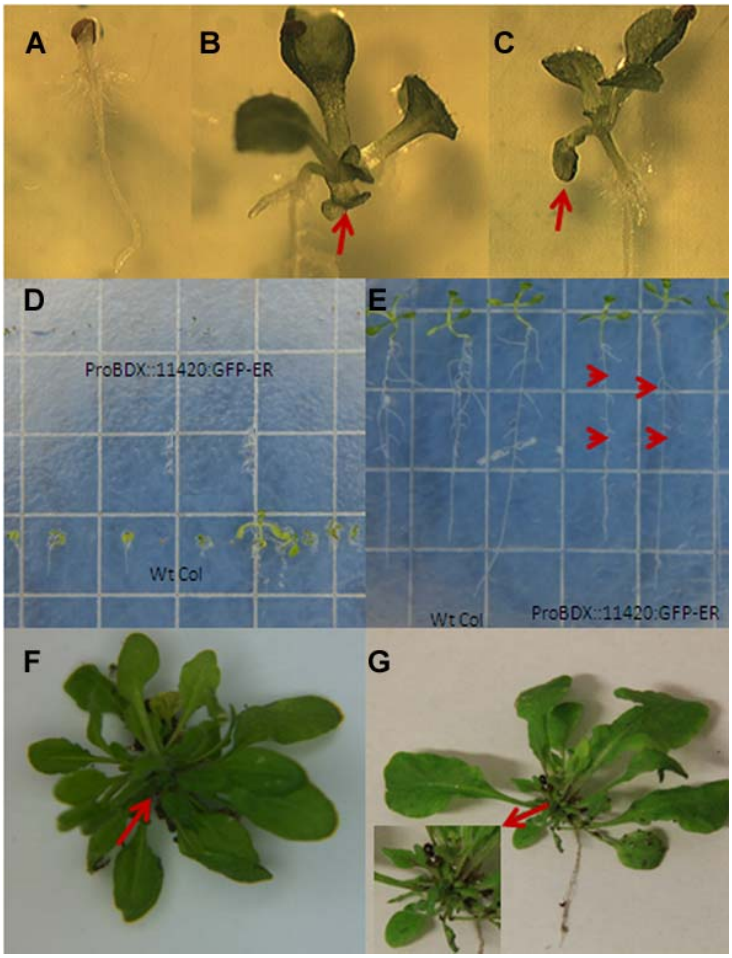

Figura 13: Plantas transgénicas que expresan la región codificante de *At5g11420* bajo la región regulatoria de *BIIDXI*. A) Plántula transgénica cuya emergencia de cotiledones está retrasada. B y C) Plántulas de 10 días, las flechas señalan la presencia de meristemos y cotiledones adicionales. D) La germinación de las semillas se vio afectada en estas plantas. E) Plantas de 12 días con raíces laterales de menor tamaño. F y G) Plantas de 21 días antes de la emergencia del escapo con alteraciones en la filotaxis.

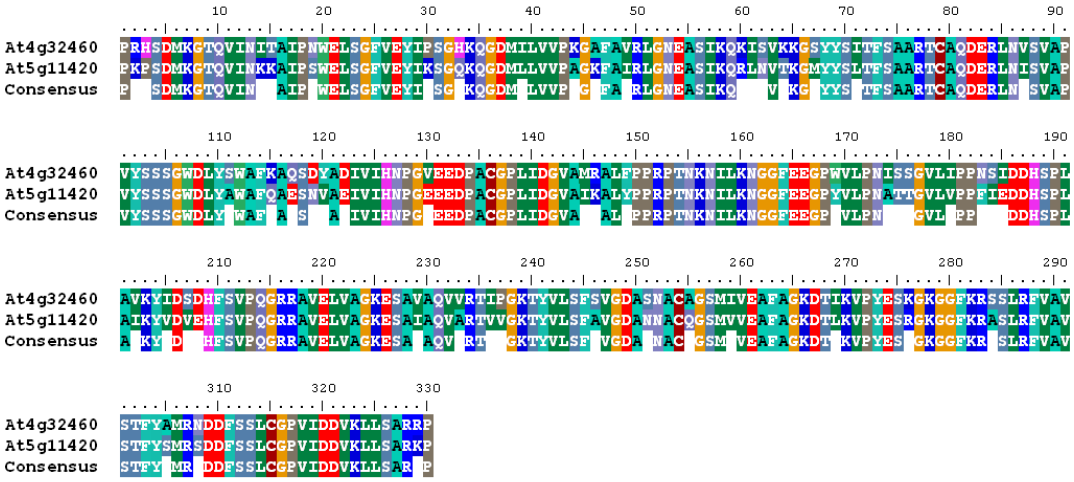

Figura 14: Alineamiento de la secuencia de aminoácidos de las proteínas codificadas por *BIIDXI* y *At5g11420* sin péptido señal y el consenso. BioEdit v. 9

Discusión general

El estudio de la pared celular de plantas ha tenido un gran impacto en los últimos años principalmente debido a que las paredes celulares de este grupo son la fuente más abundante de carbono orgánico sobre la biosfera, por lo que las investigaciones con enfoques biotecnológicos para su consumo en forma de energía han sido primordiales (Pauly y Keegstra 2010; Gilbert *et al.*, 2008; McCann y Carpita 2008). Algunas de estas investigaciones se han encaminado principalmente al análisis de los componentes y la forma en que éstos pueden ser manipulados por enzimas específicas (Qing *et al.*, 2010; Himmel *et al.*, 2007; Yang y Wyman 2006)). Otras tienen como principal objetivo conocer los aspectos evolutivos que llevaron hacia la síntesis de las paredes celulares especializadas en cada uno de los taxa vegetales (McCarthy *et al.*, 2014; Wang *et al.*, 2013; Yin *et al.*, 2010). Nuestro interés por el conocimiento acerca de la función de las proteínas de pared la familia DUF642 abarca distintos aspectos. Uno de ellos se desarrolló en trabajos previos relacionados con la evolución de las proteínas de ésta familia realizados a través de los análisis de su estructura primaria y su ocurrencia en los distintos grupos en plantas (Vázquez-Lobo *et al.*, 2012). De acuerdo con dichos análisis, las proteínas DUF642 se caracterizan por ser exclusivas de espermatofitas. Estudios recientes relacionados con la evolución de algunas proteínas de la pared celular han mostrado la importancia de las familias de proteínas exclusivas de plantas con semilla. Dichos trabajos han enfatizado la complejidad del metabolismo y la síntesis de algunos carbohidratos, además han establecido la relación que existe entre dicha complejidad y la aparición de las proteínas que los modifican, la cual fue posterior a la síntesis del carbohidrato; esto sugiere que algunas de la proteínas modificadoras de carbohidratos resultaron en caracteres evolutivos que promovieron la divergencia de los grandes grupos tales como el de las plantas espermatofitas (McCarthy *et al.*, 2014; Wang *et al.*, 2013). Otros datos relevantes extraídos de los análisis evolutivos de la familia de proteínas DUF642 confirmaron la presencia de un péptido señal, dos módulos ó subdominios con una alta similitud y cada uno de ellos con ciertos motivos o firmas característicos, producto posiblemente de una duplicación genética, así como cuatro cisteínas conservadas en todos los taxa. La presencia de un péptido señal sugiere la forma en la que los miembros de esta familia pueden ser secretados hacia la pared o hacia el apoplasto. Aunque la mayoría de las proteínas de pared celular son secretadas a través de la TGN y la ruta secretora, no se ha descartado la existencia de otras formas de excreción de proteínas principalmente de aquellas que se excretan hacia el apoplasto (De Caroli *et al.*, 2011). Dentro de las proteínas apoplásticas

puede encontrarse la proteína ortóloga de durazno codificada por *At5g11420* que al ser expresada en células de jitomate se localiza en éste compartimento subcelular (Bustamante *et al.*, 2012). Otras proteínas de la pared tales como la PME codificada por *At1g23200* también han sido localizadas en este compartimento.

La edición alterativa contribuye en la complejidad proteínica y puede dar como resultado la transcripción de un gen en distintos tejidos a bajo condiciones específicas. En plantas el primer exón alternativo (FAE) es una forma de edición que utiliza un promotor alternativo para mejorar la traducción o la eficiencia de transporte del transcrito hacia afuera del núcleo. En este caso los exones compartidos río abajo contienen codones de inicio y el mismo marco de lectura (ORF), lo cual produce la síntesis de proteínas idénticas, aunque algunos otros casos pueden también tener codones de inicio alternativos dando como resultado proteínas con N-terminales variantes o proteínas totalmente distintas (Chen *et al.*, 2007). Dentro de la familia DUF642, este tipo de edición ha sido reportada para los genes *BDX, At1g29980* y *At3g08030* pero no se descarta que ocurra en otros miembros. De acuerdo con ciertos reportes en otras especies de plantas, la expresión de modalidades diferentes de un gen puede relacionarse con una expresión diferencial lo cual a su vez correlaciona con el desempeño de funciones fisiológicas distintas.

Dentro de la estructura primaria de las proteínas DUF642 el subdominio ubicado en la región C-terminal presenta homología de acuerdo con ciertas clasificaciones a los dominios de unión a carbohidratos específicamente a galactosa y como ya se mencionó pudo ser producto de una duplicación del primer subdominio, en este sentido se sabe que la mayoría de las proteínas de unión a carbohidratos que han sido descritas en otros organismos como hongos y bacterias necesitan más de un dominio de unión para su función (Jones y Ospina-Giraldo 2011; Hervé *et al.,* 2010; Bae *et al.,* 2007) lo que explicaría la presencia de los dos subdominios en esta proteínas, sin embargo, también existe la posibilidad de que la formación de dímeros entre los miembros de esta familia sean necesarios para que puedan llevar a cabo su función éstas proteínas. En relación también con el subdominio C-terminal Vázquez-Lobo y colaboradores describieron que al menos un miembro de esta familia de *A. thaliana* (*At3g08030*) interactúa con celulosa *in vitro*. Por otro lado, la interacción *in vitro* de BDX con la pectin metilesterasa 3 ha sido de igual modo reportada. En general, las características estructurales de la familia DUF642 podrían estar relacionadas con los estudios *in vitro* que señalan que proteínas con módulos de unión a celulosa (cellulose-binding CBMs) de las familias 2a y 3a (CAZy) también

pueden regular positivamente la actividad de enzimas remodeladoras de pectinas, específicamente de PLs Estos trabajos concluyen que los CBM anexados son capaces de dirigir a la proteína hacia carbohidratos distintos a sus sustratos. Si el carbohidrato con el que interactúa el CBM se encuentra en baja cantidad o está ausente en la pared celular el impacto que puede tener el modulo sobre la actividad de la enzima se reduce considerablemente o simplemente desaparece. El CBM es capaz de proveer una ventaja a la enzima cuando las paredes celulares presentan un empaquetamiento hermético de difícil acceso hacia los polisacáridos. También se ha registrado que la remoción de polisacáridos de la pared como son los HG, incrementa el acceso del CBM a la celulosa (Hervé *et al.,* 2010). Esto puede de igual manera ocurrir a niveles de regulación más finos, por ejemplo, con el grado de esterificación de las pectinas lo que experimentalmente se ha comprobado al utilizar el herbicida cobtorina el cual perturba la orientación paralela entre los microtúbulos corticales y las microfibrillas de celulosa además de causar una distribución aberrante de pectinas metiladas. Las plantas silvestres tratadas con este fármaco presentan una deposición desorganizada de las microfibrillas de celulosa lo cual altera la morfología y promueve una hinchazón de las células. En las plantas de sobreexpresión de enzimas degradadoras de pectinas tales como PGs y PMEs se disminuye el efecto de dicho fármaco sobre la morfología celular. La cobtorina por lo tanto, afecta la deposición de las microfibrillas de celulosa, al perturbar las modificaciones y la distribución de las pectinas (Yoneda *et al*., 2010). La importancia de este tipo de procesos reside en el posible papel que tienen las proteínas de la familia DUF642 al funcionar como proteínas de andamiaje a ciertos carbohidratos como la celulosa, permitiendo que su o sus interactores sean más activos en ciertas regiones específicas de la pared al dirigir la interacción de éstos a zonas con características específicas, tales como pectinas con un mayor o un menor grado de esterificación. En este sentido el trabajo del capítulo I describe la importancia de BDX y At5g11420 como genes reguladores positivos de la actividad de PME durante la germinación y el desarrollo en *A. thaliana*. La participación directa o indirecta que estos genes pueden tener con relación a las modificaciones que sufren las pectinas influye en una gran variedad de procesos ya que la matriz péctica juega un papel de gran relevancia en el desarrollo y la morfología de las plantas. La modulación de la actividad de PME, puede afectar la dinámica de la pared celular ya sea a través de las modificaciones directas de las pectinas o a través de los efectos de esta modulación sobre la deposición de otro tipo de polisacáridos.

Trabajos recientes enfatizan la importancia de la actividad de PME durante el desarrollo embrionario (Levesque-Tremblay *et al.,* 2015) pero como se describe en el capítulo II la regulación de la actividad de PME tiene consecuencias tanto sobre procesos involucrados en el desarrollo como en la interacción planta-patógenos. AtPME3 tiene una función relevante en procesos de resistencia contra patógenos al afectar las propiedades fisicoquímicas de la pared celular de las plantas haciéndola más o menos accesible a enzimas digestivas y también ha sido descrita como una proteína relacionada con señalización intra y extracelular vía plasmodemos durante infecciones virales en distintas especies de plantas (Bruce *et al.*, 2014). Asimismo la actividad de AtPME3 ha sido relacionada recientemente con la proteína cinasa asociada a pared celular 2 (WAK2). Esta proteína pertenece a una familia de WAKs con una región transmembranal y una extracelular que se une a pectina en la matriz extracelular especialmente a pectinas desesterificadas. Esta familia participa en la expansión celular durante el crecimiento y en procesos relacionados con patógenos y estrés abiótico (Bruce *et al.*, 2014).

Al igual que *BDX, AtPME3* se expresa abundantemente en la raíz específicamente en el tejido vascular. Las observaciones *in vivo* de la expresión de *At4g32460* en el haz vascular coinciden con el trabajo de Koziel, en el que se determina mediante microarreglos la expresión de los genes más abundantes en el tallo y el haz vascular de *Cannabis sativa.* En éste se describe una expresión diferencial de genes DUF642 pertenecientes al clado B en el floema con respecto al xilema, siendo más abundante su expresión en el floema, además de ser inducidos por aluminio (Koziel, 2010). *AtPME3* por su parte también está implicada en el estrés por otro metal, el Zn^{2+}. Plantas mutantes que presentan una versión de AtPME3 dañada en su procesamiento son hipersensibles a este metal (Weber *et al.*, 2013). La capacidad de inmovilización de metales iónicos por la pared celular específicamente por las pectinas ha sido descrita. Los grupos carboxílicos de las pectinas generados por la actividad de PME interactúan con iones $Ca^{2+,}$ por lo que la modulación de las pectinas se correlaciona con la función de la pared celular como intercambiador de cationes (Weber *et al.*, 2013).

La disminución de la expresión de *BDX* en el haz vascular podría estar correlacionada con la mayor acumulación de almidón en las hojas de roseta de las plantas AS para BDX (Fig. 11). La disminución en la movilización de recursos hacia los órganos demanda, como son las semillas y los meristemos, puede ser consecuencia de la pérdida de función de este gen desde etapas muy tempranas en el desarrollo del tejido vascular incluyendo el embrión (Fig. 2, capítulo I).

Por otro lado, la expresión de *At5g11420* en tejidos y etapas en las que normalmente se expresa *BDX* resulta en alteraciones en el desarrollo de las plantas. De acuerdo con nuestras observaciones preliminares, las plantas heterócigotas presentan un desarrollo anormal desde las primeras etapas del desarrollo, tales como la presencia de más de un meristemo apical en las plántulas. En las plantas adultas, la cantidad y el largo de las raíces adventicias es menor y el arreglo de las hojas de roseta es distinto al de las plantas silvestres. La mayoría de las semillas homocigotas no germinan (Fig. 13). Estos fenotipos podrían explicarse en función de un cambio en el patrón espacio-temporal de expresión de *At5g11420*. Es posible que en los tejidos y tipos celulares donde normalmente no se expresa no se regule adecuadamente la abundancia de los transcritos y de la proteína. Las alteraciones del meristemo también pueden explicarse en relación a la función potencial de estas proteínas como reguladores positivos de PMEs ya que el grado de esterificación de las pectinas determina la formación de los primordios en el meristemo apical. En las plantas transgénicas que sobreexpresan inhibidores de PMEs (PMEI) la formación de primordios en el meristemo es menor y presentan alteraciones en la filotaxis. Dichas alteraciones están relacionadas con la abundancia de pectinas metilesterificadas en el domo del meristemo. Por otro lado, la sobreexpresión de PME promueve la abundancia de pectinas desmetilesterificadas que a su vez causa la formación de órganos en el domo del meristemo (Peaucelle *et al.,* 2008*,* 2011, Wolf and Greiner, 2012*)*.

Estos resultados podrían sugerir que es primordial tanto una expresión como una localización adecuada de la proteína codificada por *At5g11420* y que por lo tanto no existe una redundancia de tipo fisiológica entre *BDX* y *At5g11420*.

Los resultados de este trabajo resaltan tanto la importancia del estudio de las características estructurales de los carbohidratos y de las modificaciones que éstos pueden tener, como el estudio de las proteínas de pared celular que están relacionadas con la síntesis y la regulación de las pectinas. De acuerdo con investigaciones recientes las modificaciones en las pectinas tienen un papel muy importante en la transición de un ambiente acuático a un ambiente terrestre (MacCarthy *et al.,* 2014). La producción de proteínas novedosas involucradas en la modificación de la estructura de las pectinas y su especialización impactó substancialmente sobre las propiedades fisicoquímicas de la pared celular lo que posibilitó a las plantas la colonización de diversos ambientes

Los resultados obtenidos en este trabajo sugieren que la función general de la familia DUF642 está relacionada con la regulación positiva, directa o indirecta, de la actividad de PMEs, sin embargo, existen otras evidencias que recientemente han permitido dejar más claro el papel que llevan a cabo las proteínas de la familia DUF642 dentro y fuera de la pared celular. Uno de ellos es la participación de estas en etapas específicas del ciclo celular, además la interacción de miembros de esta familia con componentes del citoesqueleto y proteínas asociadas al poro del núcleo sugiriendo una función especializada de cada uno de los miembros en distintos eventos en los que la construcción, remodelación o reciclaje de la pared celular estén implicados. De acuerdo con nuestro modelo funcional (Fig. 15) se propone que las proteínas de esta familia regulan positivamente la actividad de PME al interactuar físicamente con ella promoviendo la desmetilesterificación de las pectinas en microdominios específicos de la pared celular. Considerando que la deposición adecuada de las microfibrillas de celulosa está relacionada con el bajo grado de esterificación de las pectinas, la interacción de las proteínas DUF642 con celulosa podría influenciar la capacidad de la PME para interaccionar con las pectinas metilesterificadas, promoviendo la actividad de este tipo de enzimas en regiones donde es necesario que ocurra la deposición. Por otro lado, la interacción de proteínas DUF642 con tubulina sugiere que la función principal de esta familia de proteínas está relacionada con una capacidad de andamiaje o "docking". Los distintos tipos de interacciones establecidas por los miembros de esta familia podrían ser determinantes durante la formación o la deposición de pared celular *de novo* ó durante su remodelación sin ser degradada por otras enzimas tales como PGs. La interacción de dos miembros de la familia DUF642 con FLOR1 (de la familia de PGIPs) podría estar involucrada con la inhibición de PGs. lo que aumenta la complejidad de las redes de interacción influyendo en los distintos procesos que son capaces de mediar cada uno de los componentes implicados.

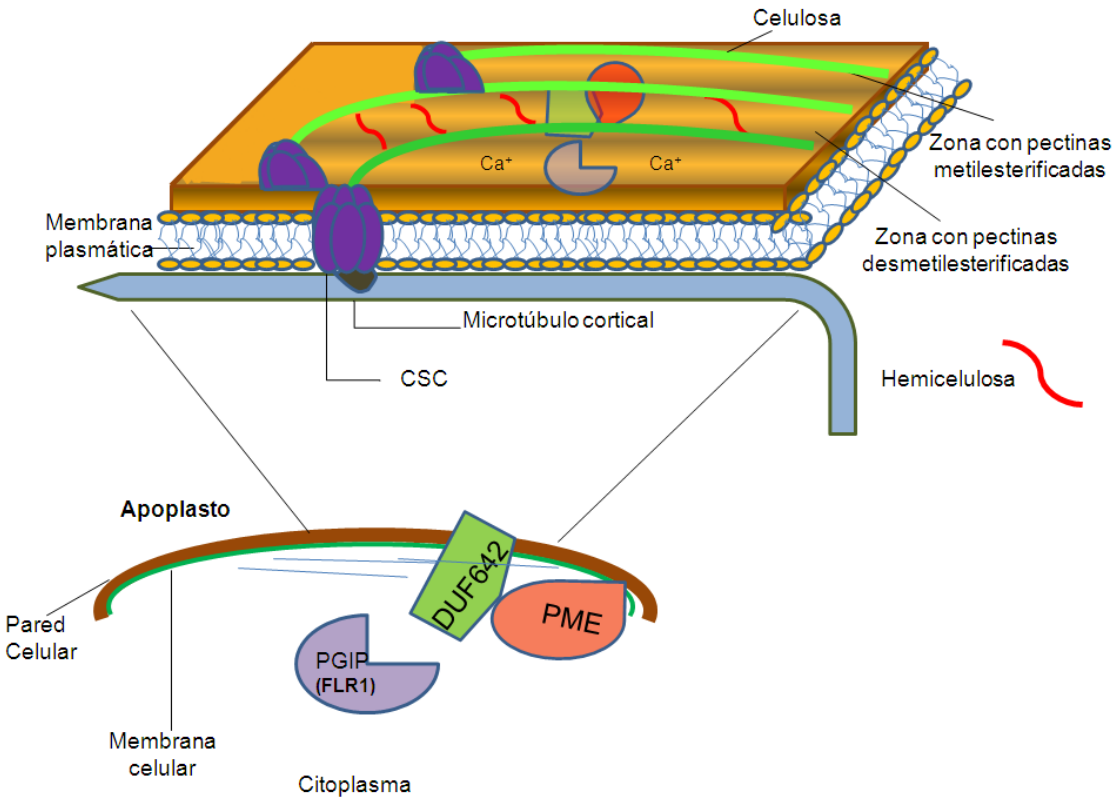

Figura 15: Modelo que explica la función general de miembros de la familia DUF642 en la pared celular de *A. thaliana*. De acuerdo con este modelo la regulación positiva de AtPME3 se lleva a cabo por miembros de esta familia en las zonas de deposición de microfibrillas de celulosa La interacción de proteínas DUF642 con otras proteínas relacionadas con la inhibición de la degradación de la pared y con microtúbulos también sugiere su participación en eventos de gran relevancia tales como la deposición *de novo*.

Conclusiones

- ✓ El papel de las proteínas de pared celular BDX y la codificada por At5g11420 está íntimamente relacionado con la regulación de la actividad de PME. Nuestros resultados sugieren que estos genes pueden ser capaces de modular positivamente la actividad de AtPME3 lo que hasta el momento no se ha descrito para ninguna otra familia de proteínas de plantas.

- ✓ La expresión de BDX en el tejido vascular en diferentes órganos y tejidos sugiere que este gen podría tener un papel relevante durante la diferenciación y el desarrollo de este tejido.

- ✓ La infección por nematodos agalladores incrementa los niveles de expresión y localiza a dos miembros de la familia DUF642 en la pared celular durante etapas tempranas de la infección por lo que el estudio de su intervención durante interacciones planta-patógeno puede ser de gran relevancia para entender las modificaciones que ocurren en la pared celular durante este tipo de eventos.

- ✓ Las diferencias en los niveles de expresión entre *BDX* y *At5g11420*, la presencia de los transcritos en distintos tipos celulares y tejidos así como los fenotipos de las plantas PROBDX::11420::GFP sugieren que no existe redundancia funcional entre ambos genes.

- ✓ La participación de las proteínas DUF642 en las modificaciones de las pectinas sugiere que esta familia es de gran relevancia en los eventos relacionados con el aumento de la complejidad estructural de la pared celular, correlacionado con la divergencia de los plantas.

- ✓ El modelo propuesto sobre la función general de las proteínas DUF642 permite hacer predicciones sobre los procesos de la pared celular en los que estas proteínas podrían estar involucradas.

Referencias

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Apendice I

Additional file

1. **Schematic presentation of the constructed transgenes.**

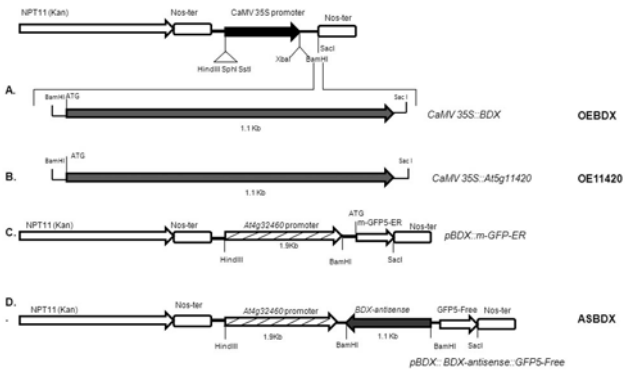

Figure 1. Schematic presentation of transgene constructs. (A, B) Independently amplified *At4g32460* and *At5g11420* cDNA fragments were cloned into pBIN to generate CAMV 35S::BDX (OEBDX) and CAMV 35S::*At5g11420* (OE11420) constructs, respectively. (C) To construct pBDX::mGFP-ER, a 1983-bp fragment of *At4g32460* intergenic region was cloned into pBIN-m-GFP-ER. (D) pBDX::BDX antisense consisted of *At4g32460* RNA antisense transgene driven by the cognate promoter of the endogenous *At4g32460* (ASBDX). Black arrow, CaMV promoter; striped arrows, cognate promoters; gray arrows (right), sense fragments; gray arrows (left), antisense fragment.

2. **Early and differential gene expression of *At4g32460* and *At5g11420* during seed imbibition.**

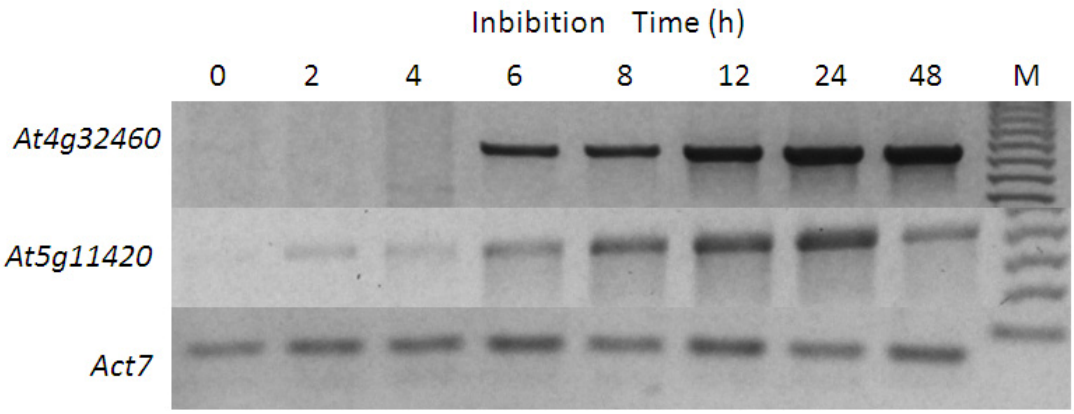

Figure 2. Early and differential transcription of *BDX* and *At5g11420* during seed imbibition. Transcript level of *ACT7* was analyzed simultaneously as an internal standard.

3. Auxin and Giberellic acid promoted *At4g32460* expression in roots.

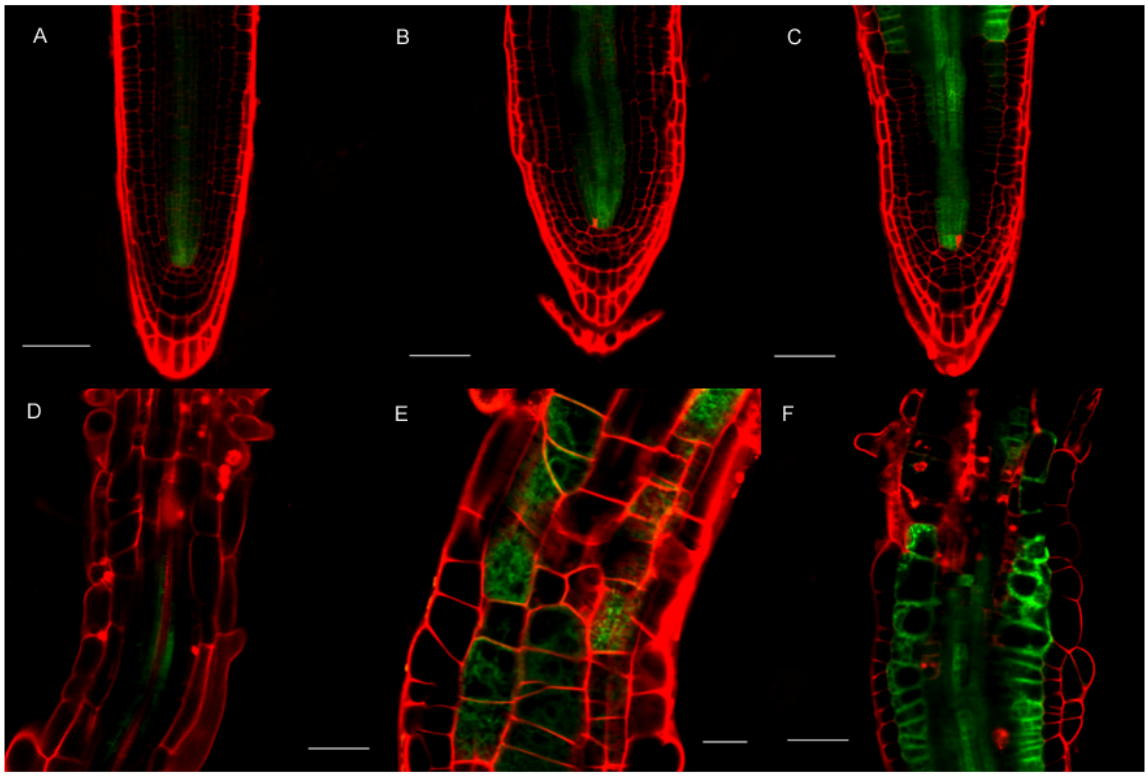

Figure 3. Auxin- and gibberellic acid-inducible expression of *At4g32460.* Transgenic 5-day-old seedlings were treated for 0 h (A, D) or 24 h with 2 μM GA (B,E) or for 48 h with 2 μM indole-3-acetic acid (IAA) (C,F). A) GFP fluorescence in provascular tissue of meristematic zone in primary roots (as in Figure 2). B) GFP fluorescence in provascular and vascular tissue of meristematic and transition zone in primary roots. C) GFP fluorescence in provascular and vascular tissue of meristematic and transition zone in primary roots. GFP fluorescence was also detected in cortical cells of transition zone. D) GFP fluorescence in pericycle cells of vascular tissue of maturation zone in primary roots (as in Figure 2). E) GFP fluorescence in vascular tissue and cortical cells of maturation

zone in primary roots. F) GFP fluorescence in vascular tissue and cortical cells of maturation zone in primary roots.

4. Negative control for germination assays.

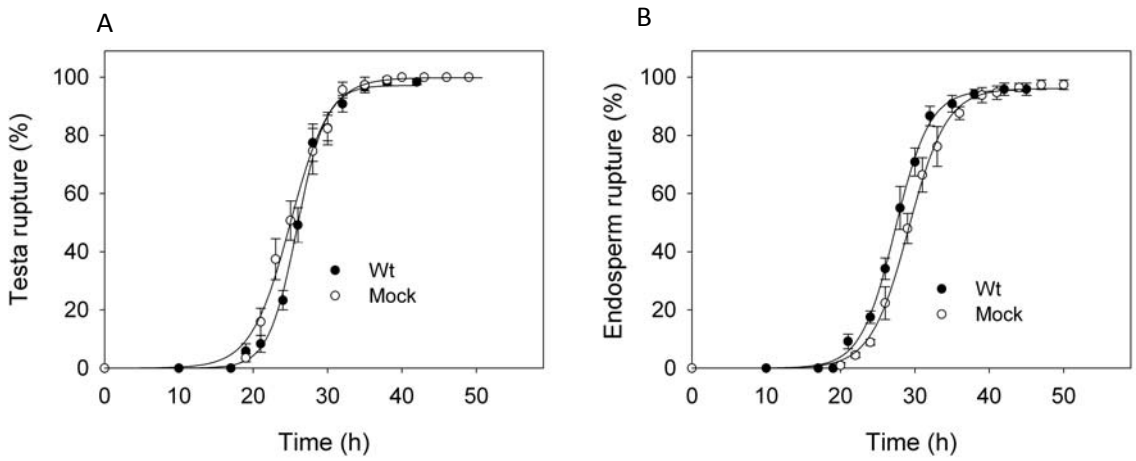

Figure 4. Effect of empty plasmid on germination performance. A) Cumulative testa rupture curve. B) Cumulative endosperm rupture curve. Germination assays were carried out in triplicate at 20 °C.

5. Mucilage release in water imbibed OE lines

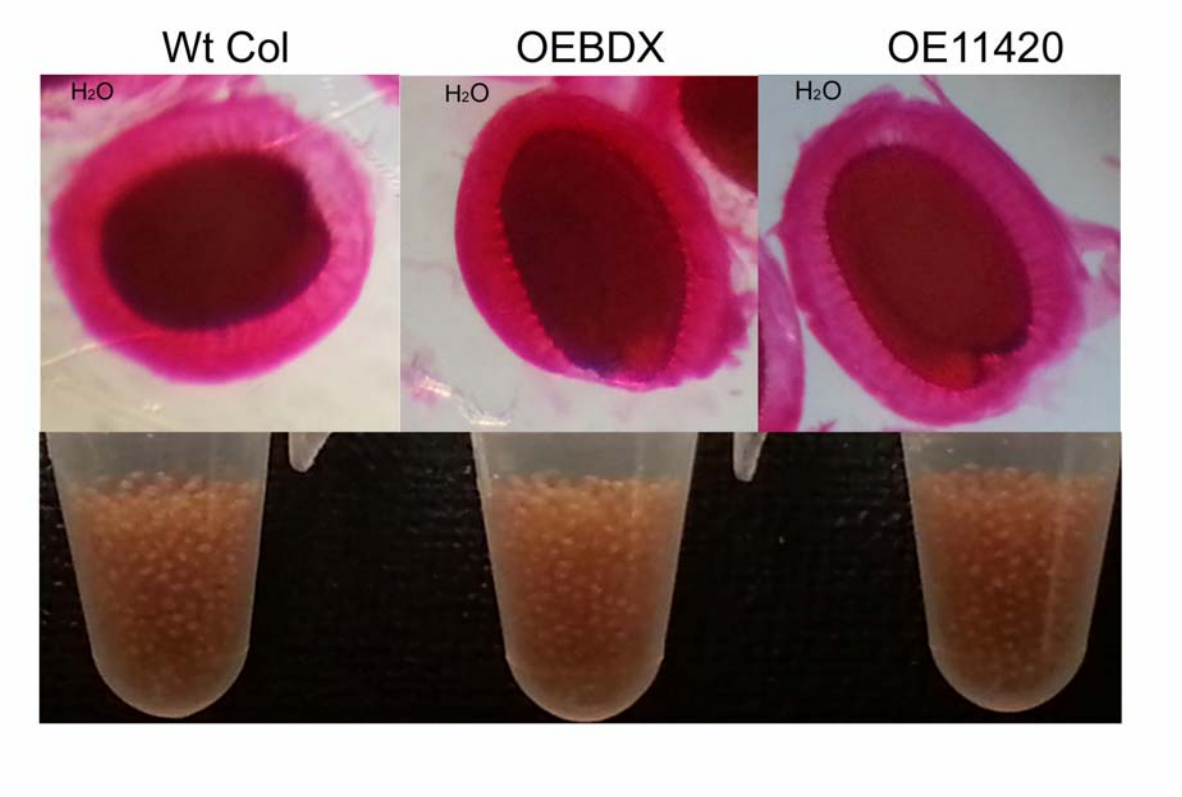

Figure 5. OEBDX and OE11420 seeds did not show defective mucilage release after imbibition in water. Dry seeds (10 mg) were hydrated (43) and stained with ruthenium red to detect pectin (upper panel).

6. Phenotypic analyses of T-DNA *bdx-2*

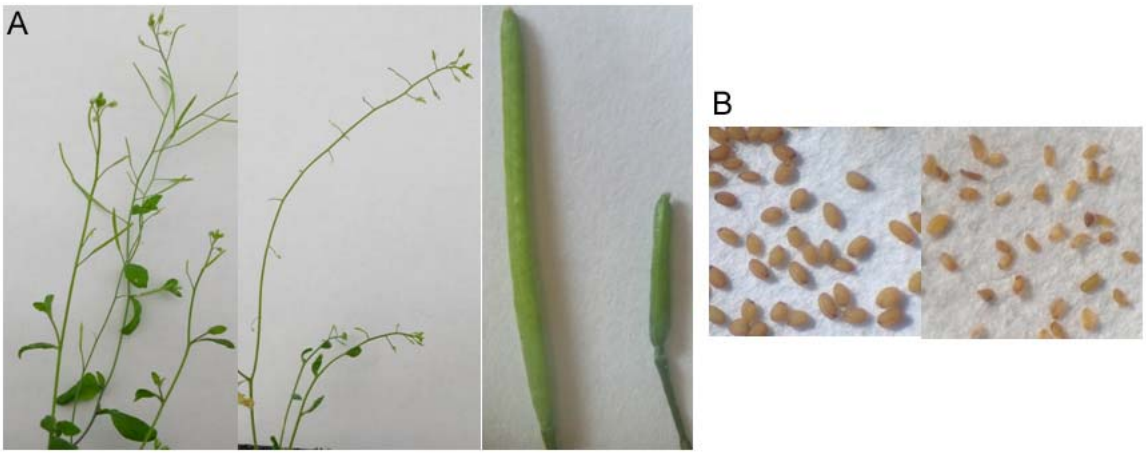

Figure 6. Phenotypic analyses of T-DNA *bdx-2*. A) Phenotypic comparison between heterozygous *bdx-2* plant and wt (control). Siliques of heterozygous *bdx-2* plants were shorter than those of wt. B) Many seeds from *bdx-2* plants were small and wrinkled (right).

7

The Two DUF642 *At5g11420* and *At4g32460*-Encoded Proteins Interact *In Vitro* with the AtPME3 Catalytic Domain

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1. Introduction

The plant cell wall provides structural integrity to plant tissues and regulates cellular growth and form. The cell wall is a dynamic compartment that varies in composition and structure during plant development and in response to different environmental signals. During cell division, the cell plate is rapidly generated. The biogenesis of this new cell wall requires the delivery of vesicles containing newly synthesised material. Cell surface material that includes plasma membrane proteins and cell wall components can be also rapidly delivered to the forming cell plate (Dhonukshe et al., 2006). The three different layers that can compose the cell wall are the middle lamella, primary cell wall and secondary cell wall. The middle lamella, which is a pectinaceous interface, is deposited soon after mitosis to create a boundary between the two daughter nuclei and is important for the adhesion of neighbouring cells. The primary cell wall is deposited throughout cell growth and expansion. These two processes require a continuous synthesis and exportation of cell wall components that have to be reorganised in the cell wall network. The secondary cell wall is deposited when cell growth has ceased and is not present in all cell types.

1.1 Polysaccharide composition of the cell wall

The primary cell wall is composed of diverse polysaccharides (85-95%) and cell wall proteins with different functions (5-15%, CWP). Cellulose, hemicelluloses (e.g., xyloglucans) and pectins (e.g., homogalacturonans) are the main types of polysaccharides present in cell wall. Cellulose microfibrils confer rigidity to the cell wall and interact with hemicelluloses to provide structure to the network. These polysaccharide interactions could restrict access of enzymes to their substrates; however, this network can be modified during plant development by different proteins that interact with the network components or by enzymes that modify the polysaccharides (Harpster et al., 2002). The polysaccharides are not the only contributor to cell wall integrity during plant development. Recently, it was demonstrated that the presence of cellulose is essential to maintain the polar distribution of proteins at the plasma membrane. The polar distribution of PIN transporters for the phytohormone auxin is disrupted by a pharmacological interference with cellulose or by

mechanical interference with the cell wall (Feraru et al., 2011). Pectins, which are a major component of primary cell wall, are a large group of complex polysaccharides that are synthesised in the Golgi and transported to the cell wall by secretory vesicles (Sterling et al., 2001). Methylesterification of homogalacturonan (HG) occurs in the plant Golgi apparatus, possibly by a S-adenosylmethionine (SAM) methyltransferase named Cotton Golgi-Related-3 (CGR3) (Held et al., 2011). HG is delivered to the cell wall in a highly methylesterified state, and the modulation of this state is a very important process in plant development. Highly esterified pectins are present in the proliferating zone of different tissues, whereas the cell walls of differentiating cells present abundant non-esterified pectins (Barany et al., 2010).

1.2 Protein composition of the cell wall

The cell wall composition is continuously modified by enzyme action during growth and development and in response to environmental conditions (Cassab, 1998). Proteins with enzyme activity and modulatory activity are present with different abundances in different cell types. Approximately 400 cell wall proteins that have been detected in cell wall proteomes have been classified into eight categories on the basis of predicted biochemical functions (Jamet et al., 2006). Members of seven of the eight groups have been previously defined as cell wall proteins involved in different aspects of cell wall dynamics. Many proteins have been detected in cell wall proteomes isolated from apoplastic fluids obtained from seedlings and rosette leaves (Charmont et al., 2005; Boudart et al., 2005), vegetative tissue that included etiolated hypocotyls and stem (Ishrad et al., 2008; Minic et al., 2007) and cell suspension cultures (Chivasa et al., 2002; Bayer et al., 2006, Bordereis et al., 2002). These proteins present a domain with an unknown function and are grouped together. The study of the function of the different families of this group of proteins will provide information about the dynamic processes of the cell wall.

1.2.1 Proteins acting on polysaccharides

Xyloglucan endotransglycosylase/hydrolase (XTH) is a family of glycosyl hydrolases that transglycosylate xyloglucan to allow expansive cell growth. These hydrolases are involved in cell growth, fruit ripening, and reserve mobilisation following germination in xyloglucan-storing seeds. In *Arabidopsis*, 33 genes have been identified that code for these hydrolases. Different temporal and spatial expression patterns for these *XTH* genes suggest that this family is involved in the change of cell wall properties related to every developmental stage. For example, *XHT5* is expressed in hypocotyls, root tips, and anther filaments, whereas *XHT24* is localised in vasculature tissue from the cotyledons, leaves, and petals. However, there is also an overlapping of the *XTH* gene expression pattern that suggests a combinatorial action of this enzyme group (Becnel et al., 2006).

Pectin modification is catalysed by a large family of pectin methylesterases (PMEs). In *Arabidopsis*, 66 genes have been suggested to potentially encode PMEs and are expressed differentially during organ and tissue development. A pro-domain is present in approximately 70% of the *Arabidopsis* PME family members (Micheli, 2001). It has been suggested that this domain has an inhibitory function during transportation to the cell wall by vesicles. The carboxylic fragment with the catalytic domain has been detected in cell wall

proteomes, but the complete protein is required for secretion (Wolf et al., 2009). The interaction of PME with proteins that inhibit its activity, which are called pectin methyesterase inhibitors (PMEIs), contributes to the modulation of the degree of the methylesterified state of the pectin in the cell wall during different developmental processes (Pelloux et al., 2007). During pollen germination, the pollen tube wall presents highly methylesterified pectins in the tip region and weakly methylesterified pectins along the tube. It has been suggested that the activity of PMEs during pollen tube growth is highly regulated by PMEIs (Dardelle et al., 2010). Local relaxation of the transmitting tract cell wall also results from changes in the methylesterification of pectins that possibly facilitate the growth of the pollen tubes in the extracellular matrix of this female tissue (Lehner et al., 2010). An important role of pectin modifications in the regulation of cell wall mechanics in the apical meristem tissue has also been suggested (Peaucelle et al., 2011). The demethylesterification of pectin by PME activity results in random and contiguous patterns of free carboxylic residues. These contiguous patterns promote Ca^{++} binding, which generates a rigid cell wall. PMEs might also be involved in maintaining apoplastic Ca^{++} homeostasis. PME activity has been suggested to maintain apoplastic Ca^{++} homeostasis during heat shock. The resulting cell wall remodelling maintains the plasma membrane integrity to confer thermotolerance to the soybean (Wu et al., 2010). The random release of protons promotes pectin degradation by polygalacturonases, which are enzymes that also affect the pectin network. Polygalacturonases (PGs) promote pectin disassembly and might be responsible for various cell separation processes. PG activities are associated with seed germination, organ abscission, anther dehiscence, pollen grain maturation, fruit softening and decay, and pollen tube growth. In *Arabidopsis*, 69 genes encode PGs with different spatial and temporal patterns. For example, *At1g80170* is specifically expressed in the anther and pollen (González-Carranza et al., 2007).

Expansins are cell wall proteins that modify the mechanical properties of the cells to enable turgor-driven cell enlargement. Expansin genes are highly conserved in higher plants, and there are four different expansin families in plants. Multiple expansin genes are often expressed in association with developmental events such as root hair initiation or fruit growth. They are also involved in processes such as fruit ripening and abscission, although cell wall modification occurs without expansion. Expansins may also be involved in embryo growth and endosperm weakening during germination (Sampedro and Cosgrave, 2005). The localised expression of expansins is associated with the meristems and growth zones of the root and stems (Reinhardt et al., 1998).

1.2.2 Oxido-reductases

Peroxidases are implicated in many physiological phenomena that include cross-linking of cell wall components, defence against pathogens, and cell elongation. These enzymes have a great variety of substrates and can regulate growth by controlling the availability of elongation-promoting H_2O_2 in the cell wall (Passardi et al., 2004). In *Arabidopsis*, 73 genes have been reported to code for putative peroxidases (Valério et al., 2004), and AtPrx33 and AtPrx34 function is specifically related to root elongation (Passardi et al., 2006).

Germins are oligomeric enzymes with oxalate oxidase activity that are associated with the extracellular matrix. In *Arabidopsis,* this family contains 12 members that are expressed in

almost every organ and developmental stage. *AtGer1* has been implicated in germination, whereas *AtGer2* is involved in seed maturation (Membré et al., 2000).

1.2.3 Proteases

Proteases cleave peptide bonds and are classified into four catalytic classes: Cys proteases, Ser carboxypeptidases, metalloproteases and Asp proteases. The *Arabidopsis* genome encodes 826 proteases that are classified into 60 families with high functional diversity. Plant proteases are key regulators of different biochemical processes that are related to meiosis, gametophyte survival, embryogenesis, seed coat formation, cuticle deposition, epidermal cell fate, stomata development, chloroplast biogenesis, and local and systemic defence responses (van der Hoorn, 2008). Some proteases have been detected in cell wall proteomes, especially in cell suspension cultures.

1.2.4 Proteins that have interacting domains with no enzymatic activity

LRR proteins are frequently implicated in protein-protein interactions and are localised in the different subcellular compartments (Kajava, 1998). The LRR superfamily includes polygalacturonase-inhibiting proteins (PGIPs) that are present in the cell wall and are involved in disease resistance as well as growth and development (Di et al., 2006). FLOR 1, a putative PGIP protein, has been detected in cell wall proteomes but is also localised intracellularly, as more than 70% of the PGIP in *Pisum sativum* was reported to be distributed in the cytoplasm (Acevedo et al., 2004; Hoffman & Turner, 1984).

Pectin methyl esterases inhibitors (PMEIs) are a diverse group of proteins that belong to the family of invertase inhibitors (INHs). PMEIs share with INHs a domain that is characterised by four conserved cysteine residues that can form two disulfide bonds (Juge, 2006). In *Arabidopsis*, there is an spatial patterning of cell wall PMEI at the pollen tip (Röckel et al., 2008).

Lectins are a diverse group of carbohydrate specific binding proteins that are involved in signal transduction (Lannoo et al., 2007). This group of proteins has interacting domains but does not show catalytic activity. The group presents with varying cellular localisation, which suggests a role in signal transduction between the different cellular compartments (Van Damme et al., 2004).

1.2.5 Proteins involved in signalling

In plants, there is a large subclass of receptor-like kinases that have extracellular LRRs in the receptor domain and are involved in signal transduction during development or defence (Clark et al., 1997). Arabinogalactan proteins (AGPs) are hydroxyproline-rich glycoproteins that are also involved in signalling. This family contributes to defensive, adhesive, nutrient and guidance function during pollen-pistil interactions (Cassab, 1998).

1.2.6 Proteins related to lipid metabolism

Lipases (LTPs) are hydrolytic enzymes with multifunctional properties. GDSL lipases are mainly involved in the regulation of plant development, morphogenesis, synthesis of secondary metabolites and defence responses (Ruppert et al., 2005).

1.2.7 Structural proteins

LRR-extensins were the only group of structural proteins detected in cell wall proteomes. This family may be involved in the local regulation of cell wall expansion. Eleven genes have been described in *Arabidopsis*; four of them are pollen specific (Baumberger et al., 2003).

1.2.8 Unknown proteins

Approximately 5 to 30% of the total proteins from different cell wall proteomes have been classified as hypothetical, expressed, putative, unknown or with a domain of unknown function (DUF), especially in cell suspension culture. A domain is considered to be a discrete portion of a protein that folds independently of the rest of the protein and possesses its own function. Eight DUF protein families (DUF26, DUF231, DUF246, DUF248, DUF288, DUF642, DUF1005, DUF1680) are represented by one (or more) member(s) of the cell wall proteomes.

DUF26 is a plant-specific protein family composed of 40 members in *Arabidopsis.* Some members include DUF26 receptor-like kinases (RLKs), which are also known as cysteine-rich RLK (CRKs). These proteins are involved in pathogen resistance and are transcriptionally induced by oxidative stress and pathogen attack (Wraczeck et al., 2010). *At5g43980* encodes a protein present in the apoplastic fluid from rosette leaves that has been described as a plasmodesmal protein (PDLP1) involved in cell-to-cell communication processes (Thomas et al., 2008). The other DUF26 protein, which was detected in the cell wall proteome from cell suspension cultures, has not yet been assigned a function.

DUF231 is present in the proteins of the *TRICHOME BIREFRINGENCE/TRICHOME BIREFRINGENCE-LIKE* (TBR/TBL) plant family with 46 members in *Arabidopsis.* The role of this family in cellulose biosynthesis has been recently described; *tbr* mutants presented decreased levels of crystalline secondary wall cellulose in trichomes and stems (Bischoff et al., 2010a). Loss of TBR also results in increased PME activity and reduced pectin esterification, which suggests that TBL/DUF231 proteins are "bridging" proteins that crosslink different cell wall networks (Bischoff et al., 2010b). *At5g06230* (TBL9) was found in a cell wall proteomic analysis of etiolated hypocotyls (Ishrad et al., 2008).

The domain unknown function 246 is considered to be a GDP-fucose o-fucosyltransferase domain in animals. This protein family has 16 members in *Arabidopsis,* and one of them, *At1g51630*, was detected in the proteome of cell suspension cultures.

DUF248 is a putative methyltransferase-related family of proteins with an ankyrin-like protein domain that is related to dehydration-responsive proteins. There are 29 proteins of this family in *Arabidopsis*, but only one, *At5g14430*, has been described in the cell wall proteome of cell suspension cultures (Bayer et al., 2006).

DUF288 is not a plant-specific family; this domain is also found in *Caenorhabditis elegans* proteins. In *Arabidopsis,* there are two members: *At2g41770* and *At3g57420*. *At3g57420* encode protein was purified from the apoplastic fluid of the cell wall proteome of rosette leaves (Boudart et al., 2005).

The DUF1005 domain has five integrants in *Arabidopsis* with two members that are similar to IMP dehydrogenase/GMP reductase from *Medicago trunculata.* The integrant isolated from

the cell wall proteome of mature stems (*At4g29310*) does not have the other domain (Minic et al., 2007).

Two loci are described in *Arabidopsis* for the DUF1680 family, and one of them was purified from the cell wall proteome of mature stems.

The most important family of unknown proteins detected in cell wall proteomes is DUF642, which is a highly conserved plant-specific family that is present in angiosperms and gymnosperms (Albert et al., 2005, Vázquez-Lobo, personal communication). *Arabidopsis* has ten members. The *At3g08030*-encoded protein is present in all cell wall proteomes and is the only unknown protein that was also detected in a seed proteome from the *Arabidopsis* accession Cape Verde Island (Cvi) that has deeper seed dormancy (Chibani et al., 2006). *At2g41800* and *At1g80240*-encoded proteins were only found in cell suspension cultures (Bayer et al., 2006), whereas *At5g25460*-encoded protein was found in vegetative and cell wall suspension cultures. *At4g32460* and *At5g11420*-encoded proteins were both detected in apoplastic and vegetative tissues. The consistent presence of 6 members of this family in all cell wall proteomes suggest that the biochemical function of the DUF642 family is related to the regulation of the activity of cell-wall-modifying enzymes at different stages of plant development.

1.3 DUF642 family

The DUF642 protein family is highly conserved, is widespread in plants, and might be involved in important basic developmental processes. Members of this family have been observed in basal angiosperms such as *Amborella*, in both monocots and dicots and also in gymnosperm species. The relevance of the DUF642 family to plant evolution was discussed by Albert and collaborators (2005). The proteins encoded by the DUF642 gene family have a unique, highly conserved domain with no assigned function that shares similarity with the galactose-binding domain. The ten members of this family identified in *Arabidopsis* contain a signal peptide of 20 to 30 amino acids in the N-terminus region that could promote their localisation in the endomembrane system or in the cell wall. Three of the ten *Arabidopsis* genes (*At1g29980*, *At2g34510* and *At5g14150*) encode proteins have been described as glycosil-phosphatidyl-inositol anchored proteins (Figure 1) (Borner et al., 2003, Dunkley et al., 2006). The *At2g41800*-encoded protein has been detected in the *Arabidopsis* cell wall proteome. The proteins encoded by *At5g11420* and *At2g34510* contain a ATP/GTP binding site motif that has been described in many proteins involved in signal transduction processes.

Although a function has not yet been assigned for this family, it has been suggested that some members could be involved in different developmental processes. Organ-specific expression has been described for the flowers of two DUF642 members, *At3g08030* and *At5g11420* (Wellmer et al., 2004), and for the stems for a DUF642 *Medicago sativa* gene (Abrahams et al., 1995). *At4g32460*, *At5g14150* and *At2g41800* have been described as papillar cell-specific genes in flowers (Tung et al., 2005). Changes in DUF642 gene expression have been also detected under specific environmental conditions. Saline stress promotes the expression of *At2g41810* (Kreps et al., 2002), and an RNA increase in the three DUF642 *Arabidopsis* homologs (*At3g08030*, *At5g25460* and *At4g32460*) was described during the priming and germination of *Brassica oleracea* seeds (Soeda et al., 2005).

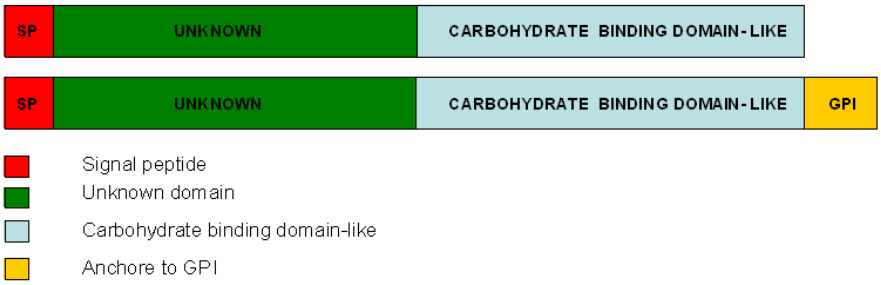

Fig. 1. DUF642 proteins have a basic structure divided into two subdomains and a signal peptide. N-terminus subdomain has not function or putative function assigned while C-terminus subdomain has homology with a carbohydrate binding domain. Some DUF642 proteins present in their C-terminus a GPI anchored motive.

We characterised the plant-specific DUF642 protein family using different approaches. We determined mRNA expression in different plant tissues, characterised sequence features and detected the potential interaction of proteins with two members of this family in *Arabidopsis* (*At5g11420* and *At4g32460*-encoded proteins). The proteins identified by LC/MS/MS analysis were the leucine-rich repeat protein FLOR1 (FLR1), a vegetative storage protein (VSP1), and a ubiquitous pectin methylesterase isoform (PME3) isolated from *Arabidopsis* flowers and leaves. Based on the structural characteristics of the DUF642 family of proteins and the associated affinity chromatography analyses, we propose that these proteins could interact specifically with other cellular components via their DUF642 domain and are therefore potentially involved in developmental plant processes. Our results provide a starting point for defining the function of the DUF642 family in plant development.

2. Materials and methods

2.1 Plant material and sample collection

Arabidopsis thaliana from the Columbia (Col) ecotype plants were grown on MS plates (1X Murashige and Skoog basal salt mixture, 0.05% MES, 1% sucrose as carbon source and 0.8% agar) in a REVCO growth chamber under a long photoperiod (16-h light 8-h darkness) at 20ºC. Fifteen-day-old seedlings were transferred to pots containing Metro-Mix 200 (Scotts Company) soil and grown under the same controlled conditions.

2.2 Reverse transcriptase–polymerase chain reaction (RT-PCR)

Arabidopsis samples from different tissues were collected from 15-day-old seedlings and flowering plants, immediately frozen in liquid nitrogen and stored at -80ºC until analysis. Total RNA from different tissues was isolated using TRIZOL according to the supplier's instructions (INVITROGEN™). cDNA templates for the amplification by PCR were prepared using SuperScript II reverse transcriptase (INVITROGEN™) according to the manufacturer's

instructions. Based on the sequence of each gene member of the DUF642 family of *Arabidopsis*, the following primers were synthesised:

At2g41800: F 5′tcctcctcctatctctctgc 3′ and R 5′aaacggttctcttcctgc 3′;
At2g41810: F 5′atgggccaaaaaaacac 3′ and R 5′atgtctctcgttctctctc 3′;
At3g08030: F 5′ggttcccaaagccattattc 3′ and R 5′acaatctcgtcaatgacagg3′;
At5g25460: F 5′cttccttcttttcatcgcc 3′ and R 5′acgagaaatcatcgctcc 3′;
At5g11420: F 5′ccatgggcttcagtgacgggatg 3′ and R 5′agatctgagtgtcttttcccgc 3′;
At4g32460: F 5′gtgatagtgcttcttctccttcac 3′ and R 5′ agcgacgaatctcaatgac 3′;
At1g80240: F 5′aaaagcagcactcctcttag 3′ and R 5′ atcattggtccctcacaac 3′;
At1g29980: F 5′ccgagcaacaatagatgc 3′ and R 5′actgtagaacgcaactctgg 3′;
At2g34510: F 5′ttggtctctccattgtggc 3′ and R 5′ccttaacgtcatcaatcacagg 3′;
At5g14150: F 5´ttgcgcctcttcagattttt3′and R 5′cttctcaccagagccagtcc 3′.

Polymerase chain reaction (PCR) was performed under the following conditions: 94ºC 5 min; 35 cycles of 94ºC 30 sec, 60-62ºC 30 sec, 72ºC 1 min 30 sec, 72ºC 5 min.

2.3 Sequence analysis and database search

The 10 DUF642 protein sequences of *Arabidopsis* were obtained from GenBank (NP_973938: *At1g29980*; NP_178141: *At1g80240*; AAC02768: *At2g41800*; AAC02767, NP_181712: *At2g41810*; AAC26689: *At2g34510*; AAO00904: *At3g08030*; ABF19001: *At4g32460*; NP_196919: *At5g14150*; AAN31807: *At5g11420* and AAP37805: *At5g25460*). A multiple sequence alignment, using only the DUF642 protein domain, was performed using ClustalW from the Bio Edit Sequence Alignment Editor. The possible secondary structure of the proteins coded for by *At5g11420* and *At4g32460* was compared on-line using the Draw an HCA (Hydrophobic Cluster Analysis) program (http://ca.expasy.org/tools/) as described in Gaboriaud et al. (1987).

2.4 Recombinant 5xHis-tagged DUF642 proteins and the resin-bound DUF642 protein affinity column

The entire open reading frame of the DUF642 genes *At5g11420* and *At4g32460*, without the signal-peptide-coding region, was amplified using PCR. The primers used for the *At5g11420* were MET11420 (5′ccatgggcttcagtgacgggatg3′), which includes an in-frame ATG, and primer 11420FIN2 (5′agatctagtgtcttttcccgca3′). For the amplification of the carboxyl-terminus truncated protein, the *At5g11420* (Δ11420) forward primer MET11420 and the reverse primer 11420FIN3 (5´agatctcggcttacgagcactgag3´) were used. *At4g32460* was amplified using the following primers: MET32460 (5′ccatgggcttcaatgatggactactacc3′) and 32460FIN2 (5′agatctgcgtaaaacgtactgtaga3′). The amplified regions of these genes were cloned into the pQE60 vector using the NcoI and BglII restriction sites. A negative control was performed using the empty pQE60 vector. Protein expression and purification were performed following the supplier's instructions, and the recombinant proteins with the histidine tail were detected using western blot analysis with a Ni-NTA conjugate (QIAGEN). The three recombinant proteins were eluted as a single band and were identified to have the histidine tail. No protein was detected when the empty vector was used. The elution process was the only step omitted when the column was prepared for each recombinant protein.

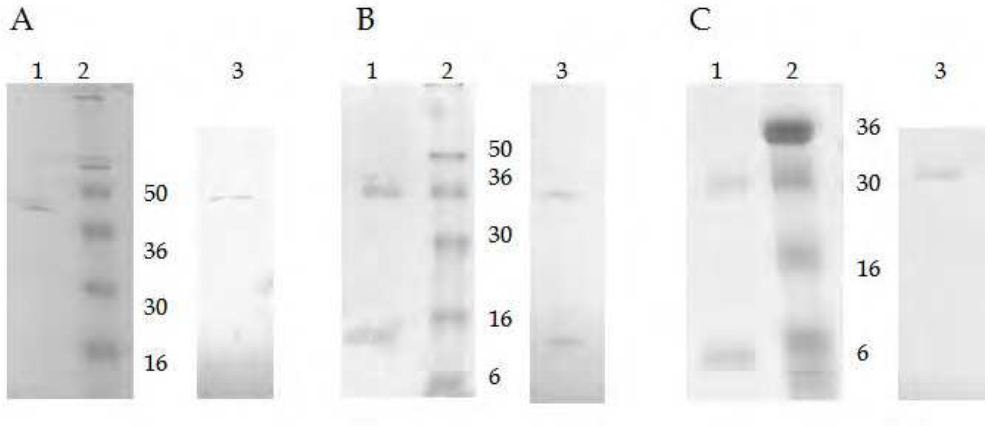

Fig. 2. Recombinant 5xHis-tagged DUF642 proteins.
A) Purification of the 32460 recombinant protein. 12% PAGE Gels were stained with Coomassie Blue. The column was eluted with 250 mM Imidazole (Lane 1). Western Blot of the eluted fraction (NiNta beads with phosphatase alkaline secondary antibody). The band of approximately 40 kDa corresponds to the calculated molecular weight for this protein (Lane 3).
B) Purification of the 11420 recombinant protein. 12% PAGE Gels were stained with Coomassie Blue. The column was eluted with 250 mM Imidazole (Lane 1). Western Blot of the eluted fraction (NiNta beads with phosphatase alkaline second antibody). The band of approximately 40 kDa corresponds to the calculated molecular weight for this protein (Lane 3).
C) Purification of the Δ11420 recombinant protein. 12% PAGE Gels were stained with Coomassie Blue. The column was eluted with 250 mM Imidazole (Lane 1). Western Blot of the eluted fraction (NiNta beads with phosphatase alkaline second antibody). The band of approximately 32 kDa corresponds to the calculated molecular weight for this protein (Lane 3).

2.5 Affinity chromatography of flower or leaf protein extracts

Frozen flowers or leaves from *Arabidopsis* plants (10-20 g) were ground with a mortar and pestle and placed in two 40 ml tubes with 14 ml of extraction buffer (50 mM Tris-HCl pH 7.5, 3 mM $MgCl_2$, 1 mM PMSF). The crude homogenate was centrifuged at 15,000 x*g* for 30 min, and in the case of DUF642 affinity columns, the supernatant was loaded onto a previously equilibrated DEAE-Sephacel column (2x 10 cm) with extraction buffer at 4ºC. The resulting fraction was then used for affinity chromatography. The affinity column was prepared beforehand as described above and equilibrated with extraction buffer. The protein extracts from the different tissues were mixed for 1 h with the prepared resin at 25ºC using gentle agitation in a ratio of 10 ml of extract/0.2 ml of agarose. The column was washed with 50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$ (50 vol) buffer to remove unbound proteins. Bound proteins were eluted with the same buffer containing different NaCl concentrations (100 to 1000 mM). These fractions were precipitated with cold acetone. Agarose and the empty vector column were used as negatives controls, and no bound proteins were detected (Gamboa et al., 2001).

The fractions obtained in the affinity chromatography assays were analysed on denaturing 12% SDS-PAGE gels and stained with silver. Bands of interest were extracted from the gels and sent to the Proteomics Platform of the Eastern Genomics Center, Quebec, Canada, where the in-gel digest and mass spectrometry experiments were performed. Tryptic digestion was performed according to Shevchenko et al. (1996) and Havlis et al. (2003). Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nano/LC) and analysed by electrospray mass spectrometry (ES/MS/MS).

Database searching. All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.2.0)

Criteria for protein identification. Scaffold (version Scaffold-01_07_00, proteome Software Inc. Pórtland Oregon, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm.

Only one protein was identified for the protein bands derived from the two chromatography steps, DEAE-Sephacel and affinity chromatography (11420 and 32460 column affinity protocols).

3. Results and discussion

3.1 Gene structure of the DUF642 family in *Arabidopsis thaliana*

The DUF642 domain was only present in the ten *Arabidopsis* members described before, and all members had the same gene structure, which consisted of three exons and two introns (Figure 3). The first intron encoded the signal peptide, and an alternative usage of the first exon was detected for *At1g29980* and *At3g08030*. The first intron was also included in the mRNA sequence for the *At3g08030* gene. The expression of two different mRNAs has been found in different tissues, which suggests a possibly different protein subcellular localisation.

3.2 DUF642 members are widely expressed in all *Arabidopsis thaliana* plant tissues

The RT-PCR expression analysis of the ten DUF642 genes in different tissues including seedlings, stems, cauline leaves, rosette leaves, flowers, inflorescences and roots is shown in Figure 4. The genes with broad expression patterns are *At1g80240*, *At5g11420*, *At5g25460* and *At2g41800*, whereas *At1g29980* and *At4g32460* were not detected in cauline leaves. *At2g41810* expression was restricted to inflorescence tissue. The *At2g41810*-encoded protein exhibits 81% identity and 89% similarity to the *At2g41800*-encoded protein. In the inflorescence tissue, the *At2g41800* transcript contained an additional region of 100 bp corresponding to the first intron, which suggests an alternative use of the first exon described for *At3g08030* and *At1g29980*. The gene with the most divergent sequence in the family, *At5g14150*, was also detected in the stem, flower, inflorescence, and root tissues and was detected at low levels in cauline leaves.

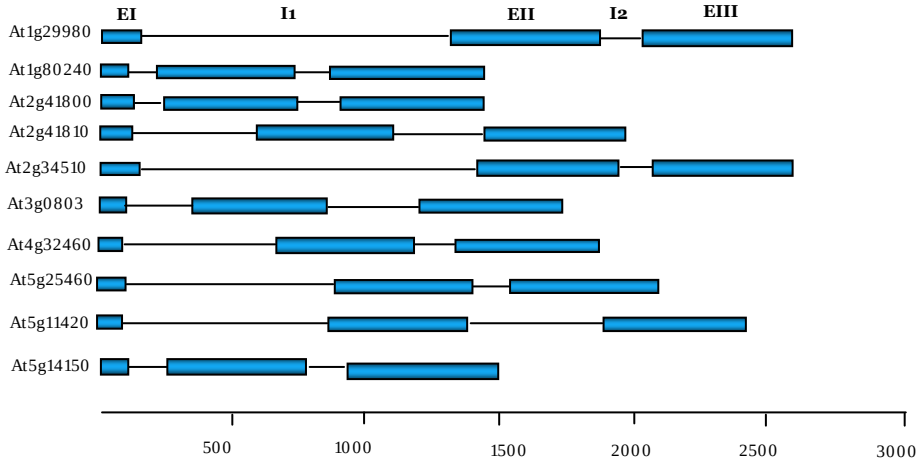

Fig. 3. Gene structure of DUF642 family in *Arabidopsis thaliana*.
EI: Exon 1, **I1**: Intron 1, **EII**: Exon 2, **I2**: Intron 2, **EIII**: Exon3

Our results are consistent with the microarray data described in the Gene Investigator Atlas (http://www.genevestigator.ethz.ch/), except for the *At2g41810* gene. We did not find *At2g41810* expression in the roots, but the Atlas indicated high expression. However, Kreps and collaborators (2002) demonstrated that the expression of this gene in the roots is induced by NaCl stress. These discrepancies in the results obtained in different studies could therefore be related to the different growth conditions used. Spatio-temporal expression analyses of this family will provide important information about its function. Cell-type-specific expression in the roots of the auxin-inducible DUF642 genes *At2g41800* and *At4g32460* was recently reported (Goda et al., 2004; Salazar-Iribe & Gamboa-deBuen, unpublished data).

Transcriptomic analyses suggest that the expression of this family of genes is also affected by different environmental conditions. The expression of genes that encode DUF642 proteins could be inhibited or stimulated by different pathogens. Indeed, invasion by necrotrophic pathogens or insect attack has been shown to significantly reduce the expression of *At5g11420*, *At5g25460*, *At4g32460* and *At1g29980* in plant tissues (Hu et al., 2008; Ehlting et al., 2008). Conversely, an increase of DUF642 gene expression in response to biotrophic organisms has been reported in *Arabidopsis* transcriptomic analyses of sink-heterologous structures, such as galls. Furthermore, the *At3g08030* and *At1g29980* genes have been found to be up-regulated in response to *Agrobacterium tumefaciens* and *Rhodococcus fascians* invasion (Depuydt et al., 2009, Lee et al., 2009). *At1g29980* has also been shown to be highly expressed in the giant cells induced by the root-knot nematode, *Meloidogyne incognita* (Barcalá et al., 2010), and the development of such sink structures is related to an increase in auxin (Grunewald et al., 2009). The study of the effect of nematode invasion on the gene expression of the DUF642 family will provide important functional insights.

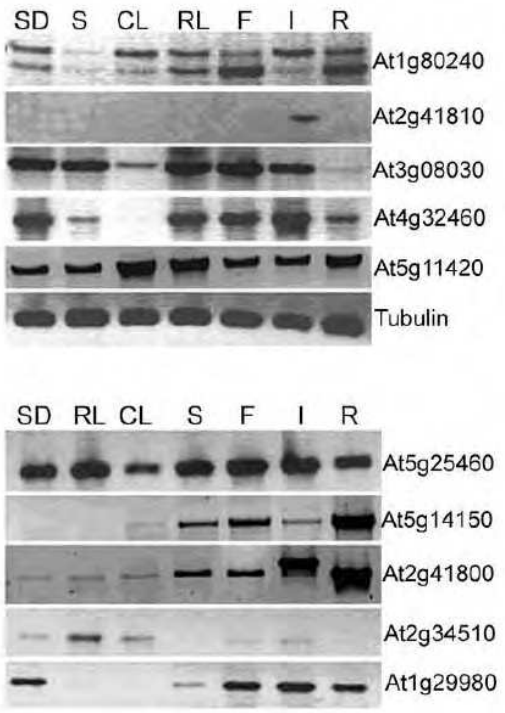

Fig. 4. RT-PCR expression of *Arabidopsis thaliana* DUF642 genes in various tissues. Seedlings (SD), rosette leaves (RL), cauline leaves (CL), stems (S), flowers (F), inflorescences (I), and roots (R). The expression of tubulin was analyzed simultaneously as an internal standard.

3.3 Comparison of the primary sequence of the ten *Arabidopsis thaliana* DUF642 family members

The DUF642 gene family encodes proteins with an estimated molecular mass ranging from 39 to 44 kDa. These proteins contain the DUF642 amino acid domain, preceded by a 20-30 amino acid signaling peptide on the amino terminus. This signaling peptide could be involved in the cell wall localisation of DUF642 proteins in several plant organs. Alignment analysis of the ten *Arabidopsis* members shows an extensive conservation of the DUF642 domain; the percentage of identical and similar amino acids varies from 30% to 85% and 43% to 92%, respectively (Figure 5A). About 30% of the amino acids distributed throughout the sequence of the DUF642 domain are hydrophobic. These residues are not identical, but they are similar among the different proteins. The comparison of the hypothetical secondary structure of *At5g11420* and *At4g32460*-encoded proteins shows that the hydrophobic clusters present are similar (Figure 5B). Four conserved cysteine residues are present in all of the sequences as previously described for the pectin methyl esterase inhibitors localised in the cell wall (Juge, 2006). Because no catalytic activity has yet been assigned to the DUF642 domain, this family could be involved in specific carbohydrate or protein interactions.

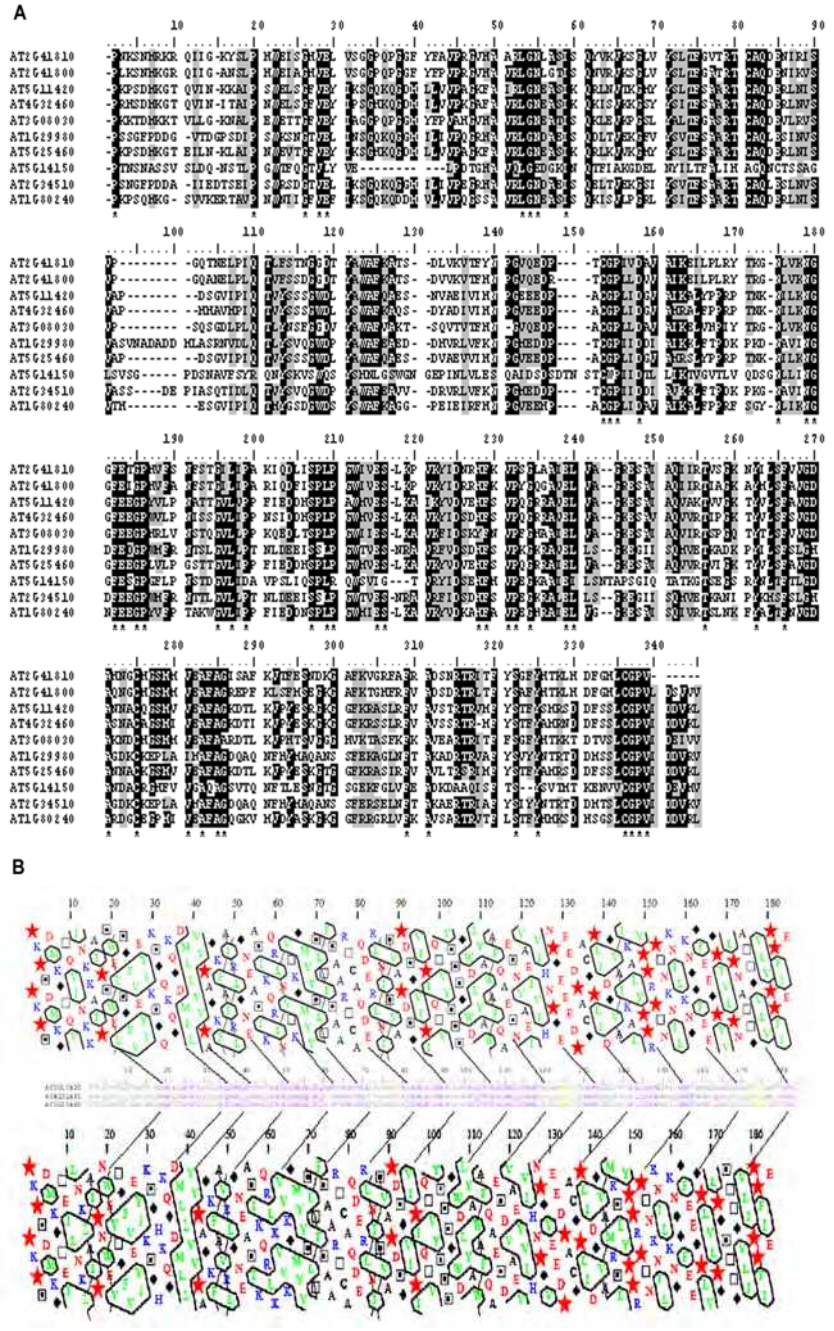

Fig. 5. DUF642 amino acid sequence and features.
(A) Clustal W alignment (BioEdit) of the DUF642 domain of the 10 *Arabidopsis* proteins is

shown. The N-terminal region (comprising the signal peptide) was eliminated for the alignment. Shading indicates conserved amino acid and dark shading indicates identities. (B). Secondary structure comparison of 11420, top, and 32460, bottom. The initial 180 amino acid sequences of the DUF642 domain of both proteins are compared using "Draw an HCA" online program (http://ca.expasy.org/tools/) (Gaboriaud et al., 1987). Amino acids forming putative hydrophobic clusters are grouped together. Compare similar patterns in both sequences. Star: P; dotted square: S; rhomb: G, and empty square: Y residues; other amino acids in standard abbreviation.

Most of the members of the DUF642 family have a broad expression pattern in different plant tissues. A putative redundancy of function in this family should be considered because of the high conservation of the DUF642 domain; however, it is important to describe the organ, cell type and specific stress-related expression patterns for each gene to determine the individual gene function (Wellmer et al., 2004).

3.4 DUF642 proteins have specific interactors in the flowers and leaves of *Arabidopsis thaliana*

Recombinant 32460 protein interacts *in vitro* with the LRR protein FLR1 (Q9LH52, *At3g12145*), with VSP1 (Q93VJ6, *At5g24780*) and with PME (Q9LUL7, *At3g14310*) in flowers, whereas in leaves, it interacts with the same PME (*At3g14310*) (Figure 6). The recombinant 32460 protein interacts *in vitro* with three proteins with sizes of 38 kDa, 37 kDa and 29 kDa from the flowers (Figure 6A). These proteins were identified as FLR1, PME, and VSP1, respectively (Figures 8A, B and C). It is important to note that FLR1 was not eluted by 500 mM NaCl, and VSP1 is only present in this fraction as determined in the interaction assay using the *At5g11420*-encoded protein. A 37 kDa band was purified in the three salt fractions from leaf extracts and was identified as the same PME isoform described for the flowers. A 29 kDa band was also eluted, and this protein was identified as a possible auxin-binding protein (Figure 6B). For all protein bands analysed, only a significant hit was assigned, as described in the material and methods.

The recombinant DUF642 11420-protein interacts *in vitro* with FLOR1 and VSP1 in flowers, but in leaves, it only interacts with PME (Figure 7). A high-purity protein fraction with two bands was obtained from the 11420-affinity column after the floral crude protein extracts were purified over several steps (Figure 7B). Different ionic strengths were used during elution; one 38 kDa band was eluted at 100 and 200 mM NaCl, whereas a 29 kDa band was obtained at 200 and 500 mM NaCl (see arrows in Figure 7B). The 38 kDa protein was identified as FLR1 (12% coverage) and the 29 kDa band as VSP1 (11% coverage), as described in the methods (Figures 8A and B). A Δ11420 protein without the carboxylic terminus that included the most divergent amino acid sequence was also used as a ligand. FLR1 was the only purified protein, which suggests that the carboxylic region is important for interaction with VSP1 (Figure 7C). *At5g11420* is expressed in all *Arabidopsis* tissues, and therefore, we were interested in the determination of the proteins in the leaves that interact with the *At5g11420*-encoded protein. The same procedure, using the affinity column with a leaf extract protein fraction, was used. In the first two fractions, two bands of 45 kDa and 32 kDa were detected. In the 500 mM NaCl fraction, three major bands of the following sizes were detected: 45 kDa, 32 kDa and 14 kDa (Figure 7D). The identified 32 and 14 kDa bands

correspond to a PME (40% coverage, Q9LUL7, *At3g14310*). The PME 14 kDa band was also identified when the Δ11420 protein was used as the ligand (Figure 7E). The two lower-molecular-weight bands contained the carboxyl region that includes the catalytic domain of the PME, and therefore, it is possible that the differences in their electrophoretic mobility are the result of post-translational modifications.

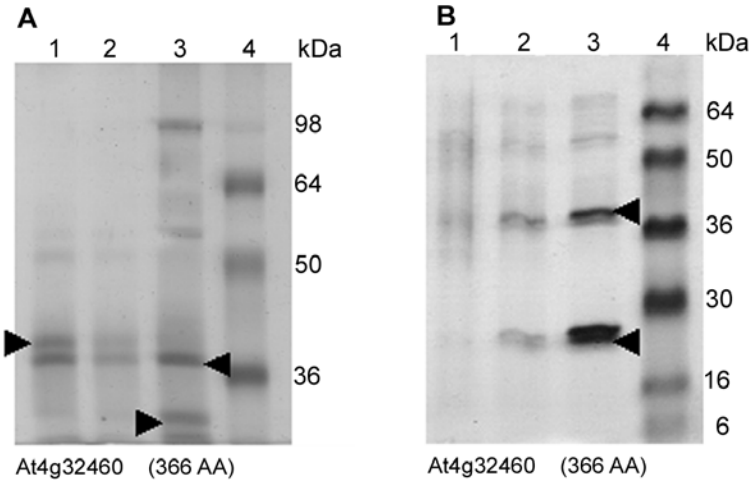

Fig. 6. 32460-protein *in vitro* interactors.
(A) Recombinant 32460 amino acid sequence. (B C) affinity chromatography assays of 32460 interactors from DEAE-Sephacel flow-through protein fraction from *Arabidopsis thaliana* flowers (B) and leaves(C). Silver staining of 12% SDS-PAGE gel showing: (1) NaCl 100 mM, (2) NaCl 200 mM, (3) NaCl 500 mM elution fractions, and (4) molecular weight reference. (B) Flower interactors of the 32460 recombinant protein. In (1) and (2) two main protein bands are seen; with molecular masses of 38 and 37, corresponding to FLR1 (arrow in (1)) and PME (upper arrow in (3)) respectively. In (3) the two bands with molecular masses of 37 and 29 were identified as PME and VSP1 respectively (see arrows in (3)).
(C) Leaf interactors of the 32460 protein. Fraction (3) was highly enriched with two bands with molecular masses of approximately 37 and 29. The 37 kDa band was identified as the catalytic domain of a PME, while the 29 kDa band was identified as a possible auxin-binding protein.

The proteins that interacted *in vitro* with the DUF642 11420 and 32460 proteins, i.e., FLOR1 and AtPME3, were detected in the cell wall proteomes (Figures 7A, B and C). Similar expression patterns reflect a possible *in vivo* interaction. FLOR1 is an LRR protein related to polygalacturonase inhibitors (PGIPs) that are highly expressed in vascular and meristem tissues. An intracellular localisation of FLOR1 has been also reported (Acevedo et al., 2004). AtPME3 (*At3g14310*) is expressed in the vascular tissue of seedlings, leaves, stems and roots and is involved in adventitious root formation (Guénin et al., 2011). Recently, we demonstrated that *At4g32460* is also expressed in the meristems and in vascular tissue (Zúñiga-Sánchez & Gamboa-deBuen, unpublished data).

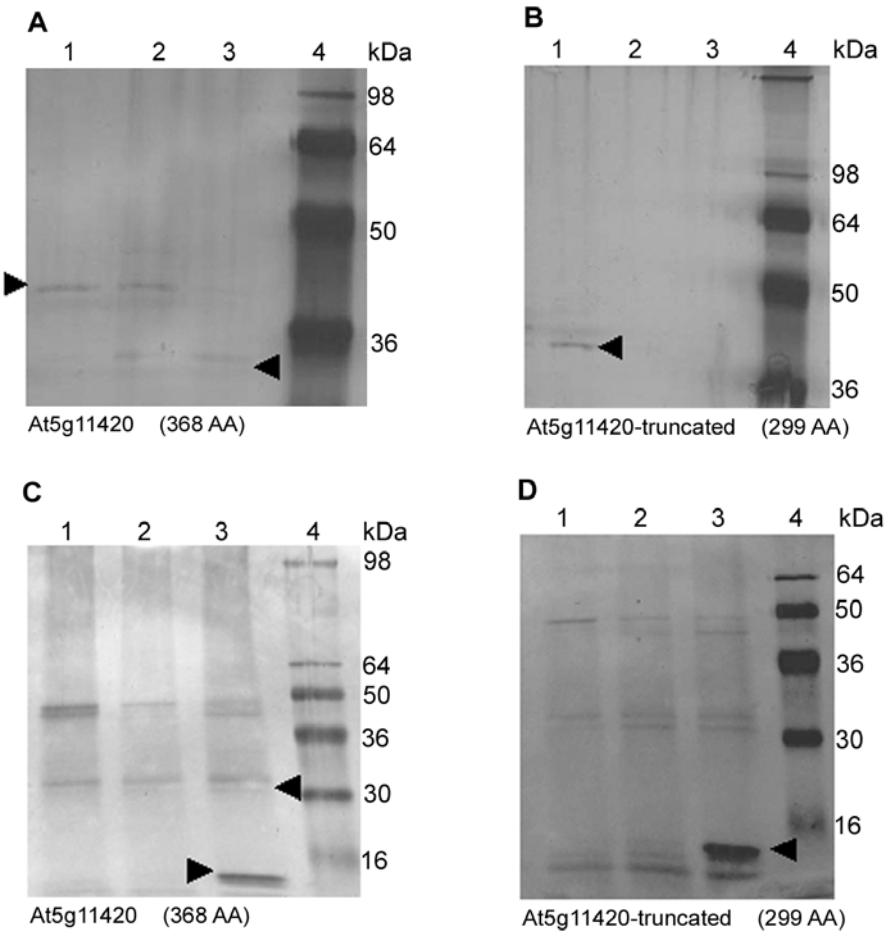

Fig. 7. 11420-protein *in vitro* interactors.
(ABCD) Affinity chromatography assays of 11420 interactors from DEAE-Sephacel flow-through protein fraction of *Arabidopsis thaliana* flowers (B,C) and leaves (D,E); Silver staining of 12% SDS-PAGE gels showing: (1) NaCl 100 mM, (2) NaCl 200 mM, (3) NaCl 500 mM elution fractions, and (4) molecular mass references.
(A) Flower interactors of the 11420 recombinant protein: Two protein bands are seen with molecular masses of 38 and 29 corresponding to FLR1 and VSP1 respectively (see arrows).
(B) Flower interactors of the 11420-truncated protein (Δ11420): In (1) only a 38kDa protein is detected, corresponding to FLOR1 (see arrow).
(C) Leaf interactors of the 11420 recombinant protein. In (1) and (2) two main protein bands are seen, corresponding to molecular masses of 37 and 32. In (3) Three proteins are detected with molecular masses of approximately 45, 32 and 14. The 32 kDa and 14 kDa bands were identified as the catalytic domain of a PME (see arrows).
(D) Leaf interactors of the 11420-truncated protein. The 14 kDa band shown in (3) (arrow) was identified as the same PME as in (C).

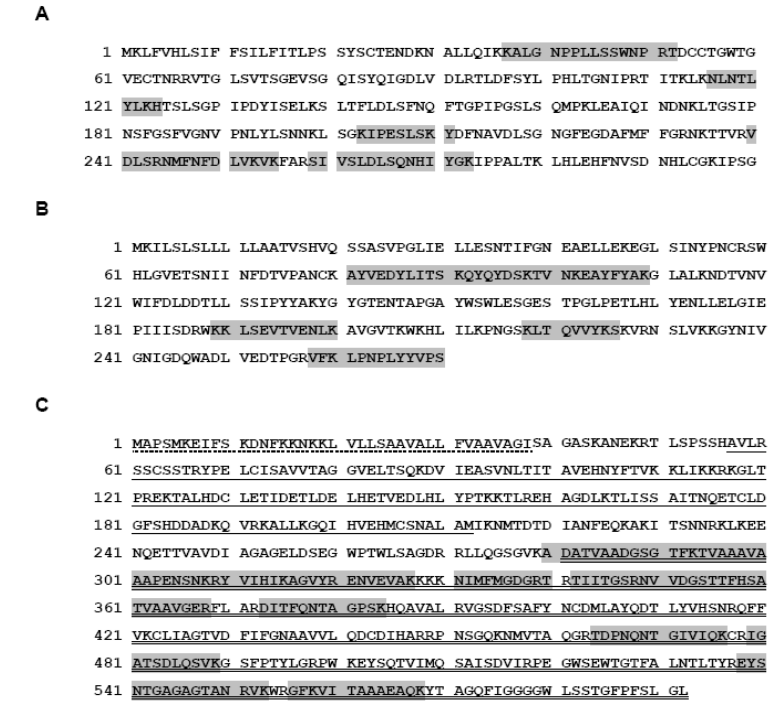

Fig. 8. Sequences of protein bands identified by LC-MS/MS from the pull down essays using DUF642 proteins. Bands were excised from the gels and sent to the Proteomics Platform of the Eastern Genomics Center, Quebec, Canada for their identification. One protein with high hit was identified for each band sent. Peptides identified are shaded.
(A) FLR1 (Q9LH52, *At3g12145*) amino acid sequence showing all the peptides identified in different protein fractions.
(B) VSP1 (Q93VJ6, *At5g24780*) amino acid sequence showing all the peptides identified in different protein fractions.
(C) PME (Q9LUL7 *At3g14310*) amino acid sequence showing all the peptides identified in different protein fractions. Underlines show signal peptide (_ _ _), inhibitory domain (____) and catalytic region (___). Note that all the peptides identified for this protein match the catalytic domain.

Subcellular localisation is also an important criterion for putative *in vivo* protein interactions. Three bands of 37, 32 and 14 kDa were identified as fragments of the catalytic domain sequence from AtPME3 in leaf protein extracts. This electrophoretic pattern has been previously described in a purified citrus PME fraction. The enzymatic activity of the citrus PME fraction was not affected (Savary et al., 2002). However, this modification could be related to the subcellular localisation of AtPME3. The carboxylic 14 kDa fragment, which interacts with the *At5g11420*-encoded protein, was previously detected in the apoplastic fluid of rosette leaves (Boudart el al., 2005), whereas the complete AtPME3 catalytic domain that specifically interacts with 32460 protein was identified in the cell wall proteomes of different plant tissues (Feiz et al., 2006). FLOR1 was also detected in cell wall proteomes from different tissues.

The *in vitro* interactions of AtPME3 with the tested DUF642 proteins appear to be specific because no other PME was isolated with the affinity column. In particular, AtPME2 (*At1g53830*) shares a 90% sequence similarity to AtPME3, which is also present in the leaves. This result and the high similarity of the primary and secondary structures of both DUF642 proteins suggest that DUF642 proteins can interact with the same protein but with different isoforms that result from posttranslational modifications (Figures 6 and 7). A specific protein interaction of AtPME3 has been previously described. The cellulose-binding protein (CBP) secreted by the nematode *Heterodera schachtii* and that is involved in the infection process specifically interacts with AtPME3, and no interaction was detected with AtPME2 (Hewezi et al., 2008).

The interaction of PMEs with proteins is highly involved in cell wall remodelling. The interaction of PME with proteins that inhibit its activity contributes to the modulation of the methylesterified state of the pectin in the cell wall during different developmental processes (Pelloux et al., 2007). An important role of pectin modifications in the regulation of cell wall mechanics in the apical meristem tissue has been suggested (Peaucelle et al., 2011). In root tips, highly esterified pectins were found in the proliferating zone, and non-esterified pectins were abundant in the cell walls of differentiating cells (Barany et al., 2010). During pollen germination, the pollen tube wall presents highly methylesterified pectins in the tip region and weakly methylesterified pectins along the tube (Dardelle et al., 2010). It has been suggested that a local relaxation of the transmitting tract cell wall resulting from changes in the methylesterification of pectins could facilitate the growth of the pollen tubes in the extracellular matrix of this female tissue (Lehner et al., 2010).

4. Conclusions

The DUF642 domain contains a carbohydrate-binding module (CBM) that could be involved in cell wall polysaccharides. The presence of these modules has been described in enzymes from bacteria that hydrolyse hemicelluloses and pectins to degrade the plant cell wall (Kellet et al., 1990; Mc Kie et al., 2001). The function of these modules appears to be related to a precise targeting to polymers in specific regions of plant cell walls during developmental processes. Plant cell wall proteins can act as bridging proteins that target specific cell wall regions and crosslink different networks (Hervé et al., 2010). Additionally, 32460 and 11420 proteins interact *in vitro* with a PME and a LRR protein that are closely related to PGIPs. These two DUF642 proteins could be scaffold proteins that promote the complexation of PME and LRR proteins to prevent the targeting of non-esterified pectins by pectin-degrading enzymes such as polygalacturonases.

Our results suggest that FLOR1 and AtPME3 interact with the 11420 and 32460 DUF642 proteins, but the precise biochemical and biological functions remain to be determined.

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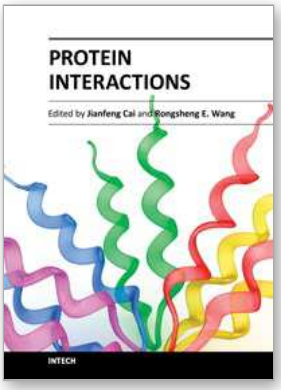

Protein Interactions

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Protein interactions, which include interactions between proteins and other biomolecules, are essential to all aspects of biological processes, such as cell growth, differentiation, and apoptosis. Therefore, investigation and modulation of protein interactions are of significance as it not only reveals the mechanism governing cellular activity, but also leads to potential agents for the treatment of various diseases. The objective of this book is to highlight some of the latest approaches in the study of protein interactions, including modulation of protein interactions, development of analytical techniques, etc. Collectively they demonstrate the importance and the possibility for the further investigation and modulation of protein interactions as technology is evolving.

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6. Proteins involved in cell wall dynamic during different plant reproduction processes

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Abstract. Plant cell walls are dynamic compartments whose composition and structure vary during development or in response to environmental signals. Structural modification of cell wall is involved in stamen filament, silique, petal and sepal growth, pollen maturation, anther dehiscence, pollen tube growth and its adhesion to the transmitting tract epidermis of the style and seed germination. In this chapter, a comparison between different cell wall proteomes that included vegetative tissues, cell suspension cultures, and apoplastic fluids was done. The cell wall proteins identified in pollen proteome was also used in this analysis. Sixty four proteins were found in at least two different proteomes indicating that these proteins are cell wall proteins. An analysis of the eight groups classified by Jamet *et al.* 2006 [1] was also done. Proteins acting on polysaccharides are a common group in cell wall from vegetative and apoplastic fluids proteome. Approximately a 20% of the genes that codified for acting on polysaccharide proteins were represented in cell wall proteomes. Proteases group was highly represented in cell suspension cultures. Proteins having interacting domains, proteins involved in signalling and proteins

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related to lipid metabolism were also detected in different subcellular proteomes. Eight Domain Unknown Protein families (DUF26, DUF231, DUF246, DUF248, DUF288, DUF642, DUF1005, DUF1680) are also represented in cell wall proteomes. DUF642 was considered an important family because is present in all cell wall proteomes described until now. Changes in cell wall structure during reproductive were discussed based on transcriptomic and proteomic analysis. Special emphasis was done to the possible role of DUF642 family on germination.

Introduction

Plant cell walls are dynamic compartments whose composition and structure vary during development or in response to environmental signals. The two principal processes related with changes in cell wall structure are cell wall elongation, which implies a newly delivered cell wall matrix material, and cell wall deposition thickness or restructuration. The expansion of cells during elongation requires a synthesis and exportation of cell wall components that are reorganized in the cell wall network. Cell wall thickness can be reduced, increased or unchanged in growing cells compared with no growing cells, but it is related with an important reorganization of the different elements [2]. Cell wall dynamic is involved in the success of reproduction events. Growth by cell elongation has been described in stamen filaments, siliques, petals and sepals. Pollen tube growth and radicle growth during germination also involves an important increase in cell volume that occurs together with a relaxation of the cell wall. Cell wall remodelation has been described in anther dehiscence and pollen maturation, siliques dehiscence and seed germination. Structural modification of cell wall is also involved in adhesion, an essential event in cell-cell communication in plants. For example, adhesion of pollen tubes to the transmitting tract epidermis of the style is essential for the proper delivery of the tube cell to the ovary [3].

Cell wall composition

Cell walls are composed of three types of layers: the middle lamella, the primary cell wall and the secondary cell wall. The middle lamella is deposited soon after mitosis creating a boundary between the two daughter nuclei. The primary cell wall deposition continues throughout cell growth and expansion meanwhile the secondary cell wall is deposited once cell growth has ceased. Secondary cell walls are not present in all cell types; parenchyma and collenchyma cells frequently have only a primary cell wall.

The primary cell wall is composed of 95% of polysaccharides and 5-10% of cell wall proteins (CPWs). Polysacharides are represent by three types of polymers; cellulose, hemicellulose and pectin. Cellulose microfibrils and hemicelluloses constitute a network with structural proteins that are embedded in a gel-like matrix of pectins. Pectins are especially abundant in the middle lamella and are very important for maintaining cell-to-cell cohesion [4]. Localization of different classes of pectins within the cell wall appears to depend on species, organ, tissue, and cell type, with the pectin network being temporally and spatially regulated [5].

Cell wall proteins

Cell wall composition is continuously modified by enzyme action during growth and development and in response to environmental conditions [6]. Proteins with enzyme activity and its activity modulators are present with different abundances in different cell types. Proteomic analysis of different plant species has been done including *Zea mays* [7], *Medicago sativa* [8], *Cicer arientinum* [9] and *Arabidopsis thaliana* [10]. In *Arabidopsis* cell suspension cultures [11–15], etiolated hypocotyls [16, 17], etiolated seedlings [18], stems [19] and rosettes [20] have been used for cell wall proteomic analysis. The approximately 400 proteins identified in the cell wall are classified in eight categories on the basis of predicted biochemical functions but there are important differences between them that reveal that cell wall structure and composition are strongly regulated during development [1]. Most of the unknown function proteins have been detected in cell suspension culture proteomes. In order to compare pollen proteome [21–23] with cell wall proteomes, we established three categories: apoplastic fluids from seedlings and rosette leaves [18, 20], vegetative tissue that include etiolated hypocotyls and stem [17, 19] and cell suspension culture [12, 13, 24] (Fig. 1). Fourteen proteins were detected in proteomes from the three cell wall proteomes meanwhile five cell wall proteins from pollen proteome, were detected in cell suspension cultures and only one was detected in apoplastic fluids. Only five cell wall proteins are present in seed proteome (http://seedproteome).

Although the cell wall proteomes analysis have been done with different protein extraction and separation methods, this comparison reveals that at least twenty percent of the protein described is involved in cell wall processes and provides information to detect putative important groups of proteins with no function assigned (Domain Unknown Function families, DUF). One of the most consistent DUF families in cell wall proteomes is the DUF642.

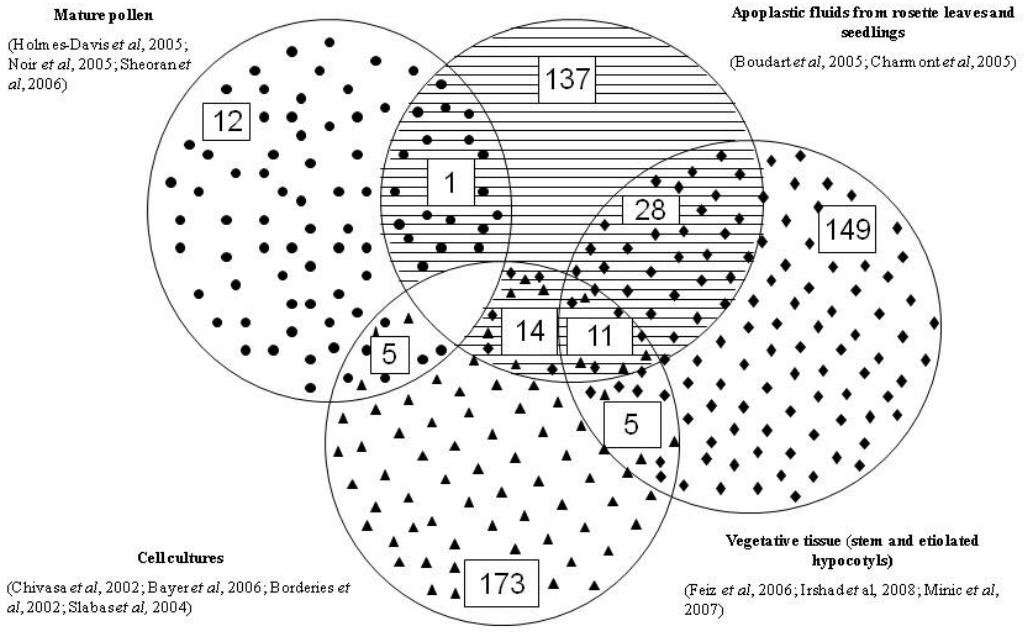

Figure 1. Venn diagram of proteins identified in four classes of cell wall proteome analysis of *Arabidopsis:* Mature pollen, vegetative tissue (stem and hypocotyls), cell culture, apoplastic fluids from rosette leaves and seedlings. Protein number found in specific or share in different classes are shown in square.

Cell wall proteins classification

1) Proteins acting on polysaccharides: 22-35% of total proteins, includes glycosil hydrolases, glycosil transferases, carbohydrate esterases, carbohydrate lyases, and expansins.

Xyloglucan endotransglycosylase/hydrolases (XTHs) are glycosil hydrolases that transglycosylate xyloglucan allowing expansive cell growth. They are involved in cell growth, fruit ripening, and reserve mobilization following germination in xyloglucan-storing seeds. It has been described 33 genes in *Arabidopsis*. Differential temporal and spatial expression patterns for these XTHs genes suggest that this family is involved in the change of cell wall properties related to every developmental stage. There is an overlapping of the XTHs genes expression patterns suggesting a combinatorial action of this enzyme group [25]. Proteomic analysis has detected seven XTHs from stems and hypocotyls and only one was purified from cell suspension cultures. A broad expression pattern was described for XHT5 and XHT24, detected in hypocotyls. However, there is a localized expression of GUS reporter fusion genes in the different tissues; XHT5 is expressed in hypocotyls, root tip, and anther filaments meanwhile XHT24 is

localized in the vasculature tissue from cotyledons, leaves, and petals [25]. This specific localization in a determinate tissue suggests that, although there is a general expression pattern among tissues, these proteins are involved in local cell wall restructuration processes that could be related either to wall thickness or elongation.

Polygalacturonases (PGs) promote pectin disassembly and could be responsible for various cell separation processes. PGs activity are associated with seed germination, organ abscission, anther dehiscence, pollen grain maturation, fruit softening and decay, and pollen tube growth. In *Arabidopsis*, there are 69 genes that codified for PGs with different spatial and temporal patterns [26]. Nine PGs has been detected in proteomic studies from stems, leaves and etiolated hypocotyls. The other one was detected in cell suspension culture (At3g15720; [15]). No expression was reported for the protein from cell suspension cultures meanwhile the other eight are expressed in almost all organs tested (Flower, silique, stamen, leaf and root, [27]). For At1g80170, anther and pollen specific expression in flowers was observed with GUS fusion expression [26].

Pectin Methyl Esterases (PMEs) regulated the degree of pectin esterification. Demethyl esterification of pectin exposed its carboxyl groups and increase cell wall firmness by forming complexes with calcium ions [28]. PMEs contribute both to the firming and softening of the cell wall in several physiological processes; PME activity was detected in the micropylar endosperm and embryo of germinating tomato seeds and PMEs expression profile in siliques suggests an important role of this family in fruit development [29]. In *Arabidopsis*, 66 genes have been suggested to potentially encode PMEs and are expressed differentially during organ and tissue development. A pro-domain that inhibits enzyme activity is present in some family members. This region is not present in the enzymes localized in the cell wall [30]. From eight PMEs detected in hypocotyls and leaves, six are expressed almost in all tissues except pollen and stamens, three are mainly expressed in pollen and stamens and the last one is expressed in petals, sepals, and leaves.

Expansins are wall proteins that modify the mechanical properties enabling turgor-driven cell enlargement. Expansins genes are highly conserved in higher plants and there are four expansin families in the different plant families [31]. Multiple expansins genes often are expressed in association with developmental events such as root hair initiation or fruit growth. Localized expression of expansins is associated with the meristems and growth zones of the root and stems [32]. They are also involved in processes such as fruit ripening and abscission although cell wall modification occurs without expansion. Expansins may also be

involved in both embryo growth and endosperm weakening during germination [31]. In *Arabidopsis*, it has been reported 26 expansin (alfa) genes, three of them detected exclusively in cell suspension culture proteomic analysis. In proteomic analysis from etiolated hypocotyls, five expansins were detected [31].

It is important to point out that almost all the family proteins included in the interacting carbohydrate group were found in etiolated hypocotyls. Almost all the proteins detected in cell suspension cultures were only exclusively from this preparation.

2) Oxido-reductases: 7 to 16 % of the total proteins, includes peroxidases, multicopper oxidases, berberin-bridge enzyme (S)-reticulin:oxygen oxido-reductases, and germins.

Peroxidases are implicated in many physiological processes that include cross-linking of cell wall components, defence against pathogens, and cell elongation. These enzymes have a great variety of substrates and can regulate growth by controlling the availability of H_2O_2 in the cell wall that promotes elongation [33]. Seventy three genes coding for putative peroxidases have been described in *Arabidopsis* [34]. Hypocotyls and stems cell wall proteomes presented exclusively these 11 peroxidases. Two peroxidases were detected both in stems and hypocotyls and cell suspension cultures and two were exclusive from cell suspension culture. No peroxidases were detected in proteomic analysis of leaves [20]. A role in cell elongation has been described for two of the hypocotyls peroxidases, AtPrx33 and AtPrx34, specifically in root elongation [35].

Multicopper oxidases constitute an enzyme superfamily present in plants, yeast and animals. In plants, some of these enzymes are involved in lignin biosynthesis in various cell types at different stages of plant development. Laccase-like multicopper oxidase (LMCO) gene family in *Arabidopsis* is composed by seventeen genes. The members of this family seems to be also involved in other physiological process than lignification since some of them have a constitutive expression as well as tissue-specific expression in minimally lignified tissues [36]. Only one protein related to this family has been detected in proteomic analysis (At2g30210, [17]).

SKS (Skewed5-Similar) family is included in the multicopper oxidase like protein superfamily and is composed by 19 members in *Arabidopsis*. Functional analysis of SKS5 and SKS6 were done suggesting their role in plant growth processes through cell wall remodelling. SKS5a is a cell wall and membrane protein involved in root growth meanwhile SKS6 participates in the vascular development of cotyledons [37, 38]. Five SKS proteins have been described in the proteomic analysis from leaves and stems. SKS5a was also detected in cell suspension cultures.

Germins are oligomeric enzymes with oxalate oxidase activity associated with the extracellular matrix. Germin like-proteins mRNAs can be found in almost every organ and developmental stage. AtGER1 has been implicated in germination meanwhile AtGER2 is involved in seed maturation [39]. This family contains 12 members in *Arabidopsis*; six of them were detected in leaves and hypocotyls proteomic analysis. This group of proteins was also mainly detected in vegetative tissues.

3) Proteases 7 to 24% of the total proteins, Cysteine proteases, Serine carboxypeptidases, and Aspartic proteases.

Proteases cleave peptide bonds and are classified in five catalytic classes: Cysteine proteases, Serine proteases or subtilases, carboxypeptidases, metalloproteases and Asp proteases. The *Arabidopsis* genome encode for 826 proteases that almost 50% belongs to ser-proteases. They are classified in 60 families with a high functional diversity. Plant proteases are key regulators of different biochemical processes that are related to meiosis, gametophyte survival, embryogenesis, seed coat formation, cuticle deposition, epidermal cell fate, stomata development, chloroplast biogenesis, and local and systemic defence responses [40].

Cysteine-proteases. These proteases play a role in programmed cell death (PCD), in response to both developmental cues and pathogens and are also involved in regulate epidermal cell fate, flowering time, inflorescence architecture, and pollen or embryo development. Four cys-proteases were reported in cell wall hypocotyls and one of them was also detected in proteomes from vacuole [41].

Serine-proteases. The *Arabidopsis* genome encodes approximately 70 subtilases and most of them have been implicated in intercellular signalling through activation/inactivation of their substrates [42]. Five members have been described in stems, two in hypocotyls and three in leaves. At2g05920 encoded protein was also detected in cell suspension cultures and it was described its expression in castor endosperm [43]. Subtilisin–like proteases had been involved in epidermal surface formation [44].

Serine carboxipeptidases. There are nearly 60 serine carboxipeptidases in the *Arabidopsis* genome. They are active only at acidic pH, often accumulate in vacuoles and are also involved in intercellular signalling [40]. Three of this group of proteins has been detected in stems, three in cell suspension cultures and two in hypocotyls.

Aspartyl-proteases. These proteases are very abundant in plants but there is no much information about them. Some aspartyl-proteases exhibits both proteolytic and chloroplast DNA-binding activities *in vitro* [45]. The aspartyl-proteases detected in proteomic analysis are pepsin like; six were detected in stems, hypocotyls and leaves meanwhile the other five were exclusively from suspension cell culture proteomics.

The protease group is highly represented in cell culture proteomics.

4) Proteins having interacting domains: 7 to 23 % of the total proteins, included Leucine Rich-Repeat (LRR)-proteins, Polygalacturonase inhibitor proteins (PGIPs), lectins, and protease inhibitors.

LRR-proteins are frequently implicated in protein-protein interactions [46].

LRR superfamily includes LRR proteins involved in defence mechanisms. Six LRR proteins were detected in proteomic analysis from leaves. At1g33590, At1g33600, At3g20280 encoded proteins were also detected in chloroplast, membrane or both cellular compartments proteomic analysis respectively [47, 48] and one was detected from cell suspension culture.

PGIPs are glycoproteins which reduce the hydrolytic activity of polygalacturonases, mainly fungal enzymes. PGIPs are LRR proteins that are involved in disease resistance but also are related to growth and development [49, 50]. In *Arabidopsis*, two LRR proteins have been described as PGIPs [51] and both are present in the proteomic analysis from hypocotyls and in cell cultures (PGIP1, PGIP2). Two PGIP homologs have been also detected in leaves and hypocotyls. At3g12145/At3g12148 (FLOR1) encoded protein was previously localized in the cytoplasm and interacts *in vitro* with the MADS transcription factor AGAMOUS [52, 53]. Hoffman 1984 [54] reported that 70%-90% PGIP from *Pisum sativum* is distributed in the cytoplasm meanwhile the rest of the protein is localized in the cell wall.

Lectins are a diverse group of carbohydrate interacting proteins involved in signal transduction with a diverse biological function that are localized in different cellular compartments [55, 56].

Nine lectins were detected from leaves, stems and hypocotyls proteomic analysis. At1g78850 encoded protein was also detected in vacuole proteomic analysis [57], At1g78830 protein was detected in membrane [58], At3g15356 protein was detected in chloroplast [47], and At3g16530 protein was detected in nucleus [59].

The intracellular localization of many proteins that have been detected in cell wall proteomic analysis suggests that this group could be involved in signal transduction mechanisms between different cellular compartments.

5) Proteins involved in signalling 0 to 3% of the total proteins, included LRR-receptor protein kinases.

In plants, there is a large subclass of receptor-like kinases that have extracellular LRRs in the receptor domain and are involved in signal transduction during development or defence [60].

Arabinogalactan proteins (AGPs) are hydroxyproline-rich glycoproteins also involved in signalling. This family plays diverse roles like contributing to defence, adhesive, nutrient and guidance function during pollen-pistil interactions [6].

6) Proteins related to lipid metabolism: 0 to 10% of the total proteins, included lipases.

Lipases are hydrolytic enzymes with multifunctional properties. GDSL lipases that have been characterized are mainly involved in the regulation of plant development, morphogenesis, synthesis of secondary metabolites and defence response. [61, 62]. In *Arabidopsis* many sequences of GDSL lipases have been deposited in the databases and six extra cellular lipases EXL1-6 has been reported in pollen coat proteome [63]. The GLIP1 protein has both lipase and anti-microbial activities and is able to disrupt fungal spore integrity and Ara-1 lipase is expressed only in ethylated shoots. Seven lipases with GDSL motif have been purified from different *Arabidopsis* cell wall proteomes including were found hypocotyls, stems and apoplastic fluids from rosette leaves. Several of theses proteins have been also describe in vacuoles.

7) Structural proteins: 0 to 1.8% of total proteins. LRR-extensins were the only group of structural proteins detected in cell wall proteomes. One possibly function of this family is related to locally regulate cell wall expansion. Eleven genes are described in *Arabidopsis*; four of them are pollen specific [64]. Four proteins encoded by “vegetative” genes were detected in cell suspension cultures and hypocotyls; two proteins were described in both proteomes, one was specific of cell suspension culture and the other was specific for hypocotyls.

8) Unknown proteins: 5 to 30% of the total proteins from cell wall different proteomes have been classified like hypothetical, expressed, putative, unknown or with a domain of unknown function (DUF). A domain is considered a discrete portion of a protein that fold and function independently. Eight DUF proteins families (DUF26, DUF231, DUF246, DUF248, DUF288, DUF642, DUF1005, DUF1680) are represented by one o more member in cell wall proteomes.

DUF26 family is composed by 36 members in *Arabidopsis*, but also members of this family are found in *Oriza* sativa and *Vitis vinifera*. Some members share functional domains with the extracellular region of receptor-like kinase (RLKs) or serine threonine kinases domains which are implicated in signal transduction. Two members of this family, At5g43980 and At3g22060, have been described in cell wall proteomes of apoplastic fluid from rosette leaves and cell suspension culture respectively.

There are 16 DUF231 members in *Arabidopsis* and one member of this family At5g06230 is found in cell wall proteomic analysis of etiolated hypocotyls.

The DUF 246 has 16 members in *Arabidopsis* one of them, At1g51630, was detected in cell suspension culture proteome, and any function has been assigned to this unknown domain until now.

DUF248 is a putative methyltransferase related family and have also similitude with ankyrin-like protein domain. This family is grouped by 29

genes but just one of them has been described in cell suspension culture cell wall proteome, At5g14430 [15].

DUF288 is not a plant specific family; this domain is also found in Caenorhabditis elegans proteins. In *Arabidopsis* there are two members At2g41770 and At3g57420, last protein has been purified from apoplastic fluids of rosette leaves cell wall proteome [20].

DUF1005 domain has 5 integrants in *Arabidopsis* with two similar members to IMP dehydrogenase/GMP reductase from *Medicago trunculata* in other domain. The one described in proteomic cell wall from mature stems (At4g29310) has not the other domain [19].

Two loci are described in *Arabidopsis* for DUF1680 family and one of them purified from mature stems cell wall proteome.

The most important family of unknown proteins detected in cell wall proteomes is DUF642, a highly conserved plant specific family that is present either in angiosperms and gimnosperms [65]. *Arabidopsis* has ten members grouped in 4 clades; A, B, C and D. Members of clade A and B were detected in all cell wall proteomes. Proteins from clade C and D were described as GPI membrane associated proteins (Fig. 2, [14]). At3g08030 encoded protein is present in all cell wall proteomes and is the only unknown protein that was also detected in a seed proteome from the *Arabidopsis* accession Cape Verde Island (Cvi) that has deeper seed dormancy [66]. At2g41800 and At1g80240 were only found in cell suspension cultures meanwhile At5g25460 was found in vegetative and cell wall suspension culture proteomes. At4g32460 and At5g11420 proteins, both detected in apoplastic and vegetative tissues, interact *in vitro* with the phosphatase acid Vegetative Storage Protein 1 (VSP1), LRR protein FLOR1, and PME AtPME3 [67]. The last two proteins were also detected in apoplastic and vegetative cell wall proteomes. The consistent presence of 6 members of this family in all cell wall proteomes and their interaction with a pectin methyl esterase suggest that the biochemical function of DUF642 family is related with the regulation of the activity of cell wall modifying enzymes at different stages of plant development.

Plant reproduction process

Changes in cell wall structure during reproductive have been extensively report. Flowers are the reproductive organ of angiosperms; a perfect flower has four different organs that form the whorls: sepals, petals, the male stamens, and the female carpel [68–70]. In *Arabidopsis*, a successful fecundation depends on a coupling process that involves a coordinate stamen

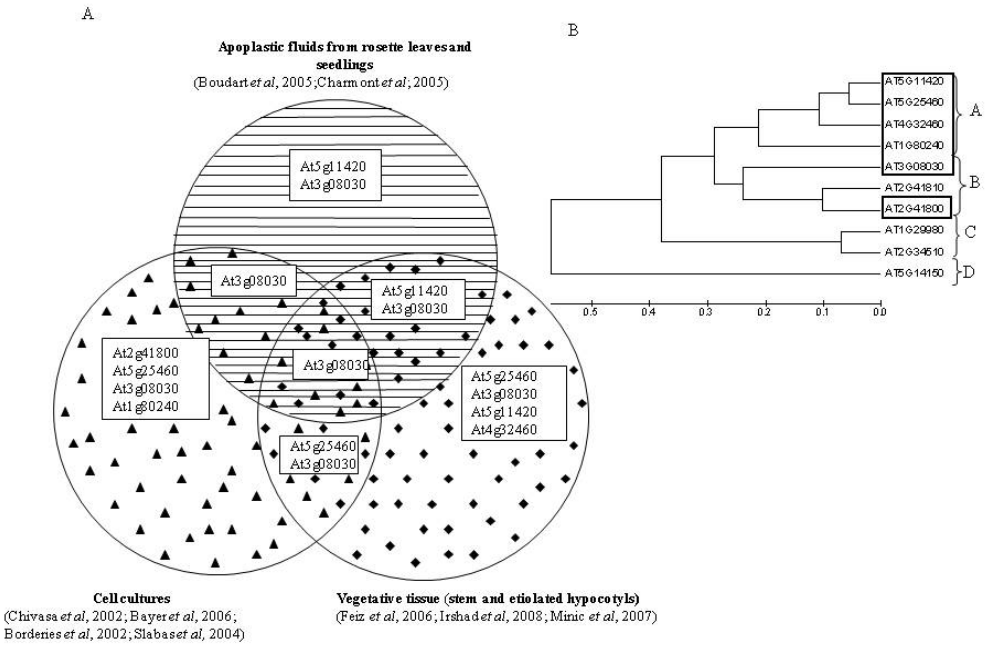

Figure 2. A) Venn diagram of DUF642 proteins found in three classes of cell wall proteome analysis of *Arabidopsis*. B) Phylogenetic reconstruction for DUF642 family members in *Arabidopsis*. Four clades A, B, C and D are shown. Proteins found in cell wall proteomics belong to A and B clades.

filament and pistil growth with pollen maturation and it release through anther dehiscence. In summary, sepals, petals, stamens and pistil elongation, anther dehiscence, pollen maturation, pollen recognition by stigma epidermis, pollen tube growth, post pollination abscission of stamens, sepals and petals, and siliques dehiscence are developmental processes related to plant reproduction in plants where witch the cell wall reorganization is involved (Fig. 3). All these processes involve cell wall modification either by constructing or degrading its different components in localized regions. This spatial and temporal restructuration of the cell wall could be a result of specific signalling pathways that induce the local changes in order to coordinate the different events related to a successful fecundation [71].

Pollen maturation

Male gametogenesis begins with the division of a diploid sporophytic cell. The daughter cells of this division form the tapetum; the tissue that supplies nutrients to the developing pollen mother cell. Pollen mother cell undergoes meiosis to yield a tetrad of haploid cells called microspores. Pollen wall deposition also begins and a prime (exine) layer develops around each microspore, visible at tetrad stage of pollen maturation. In most species,

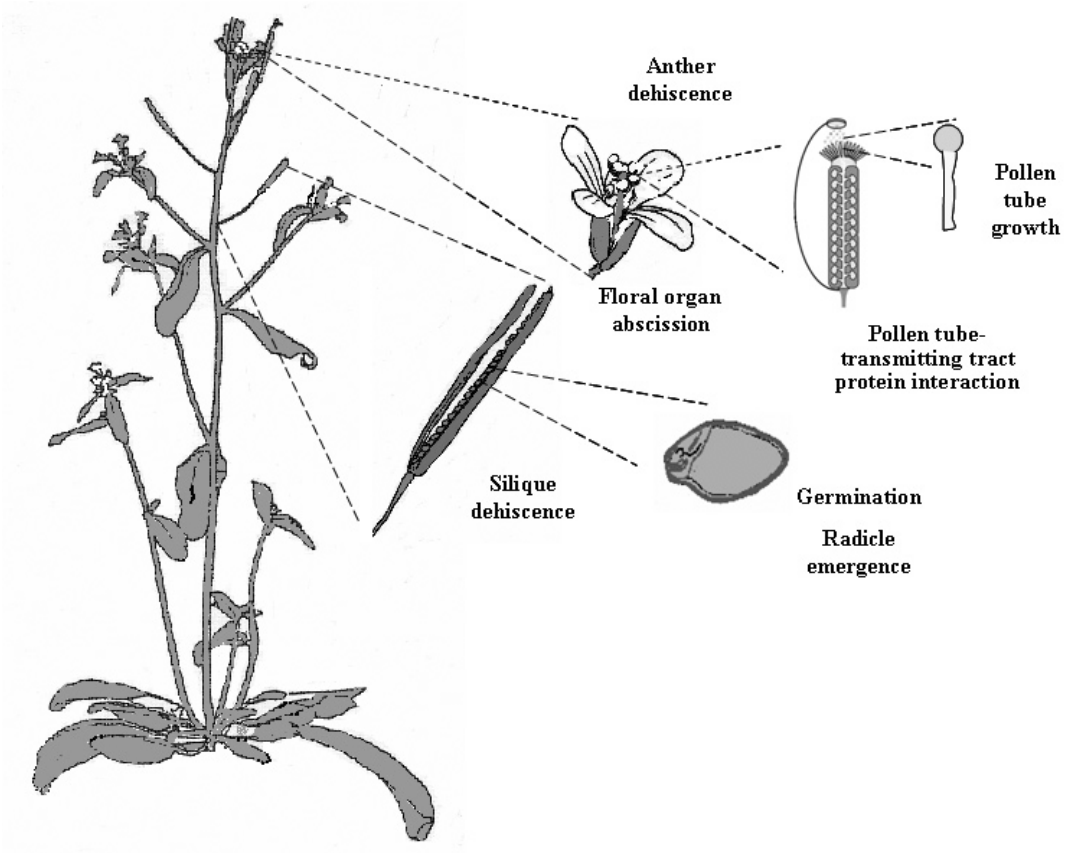

Figure 3. Reproductive process and cell wall dynamic in *Arabidopsis*. During development different modification in cell wall either reestructuration or elongation occur, specifically during reproductive process such as sepals, petals, stamens and pistil elongation, anther dehiscence, pollen maturation, pollen recognition by stigma epidermis, pollen tube growth, abscission of stamens, sepals and petals, siliques dehiscence and germination.

dissolution of the callose wall by callases releases the haploid microspores from the tetrad. Once freed from the callose wall, this uninucleate microspore undergoes an asymmetric mitotic division to form bicellular pollen grain.

Some of the processes involved in changes in cell wall of pollen mother cell during pollen maturation and development has been described. In *Arabidopsis*, the gene *glucan synthase-like5* (*AtGs15*) that encodes a plasma membrane-localized protein is highly expressed in flowers and possibly is required for deposition of callose in pollen [70–72].

In pollen transcriptome, the first category of five principal functional categories of genes was assigned for cell wall related proteins and five of the six most abundant expressed transcripts in pollen are related to cell wall. These genes codified for polygalacturonases and pectinesterases and four of them are pollen specific [73]. Cell wall biosynthesis and regulation genes are

the 24 % of the 150 genes that have been classified as pollen specific genes. It has been proposed that part of the 52.5% unknown function genes could also be related with cell wall [74]. Only one PME detected in hypocotyl proteome was found in pollen specific transcriptome; this study discriminates the genes that were present in both pollen and vegetative tissues. Pollen specific genes with a high expression are a putative cellulose synthase (At4g38190) and a callose synthase (At2g13675). The polygalacturonase At3g07820, that is also high expressed in pollen, could be involve on pollen tube wall modification and on the penetration in the stigmatic tissue.

Proteomic analysis of pollen cell wall proteins confirms transcriptome results. From 135 proteins identified, 20% are predicted to function in metabolism, 17% in energy generation, and 12% in cell wall structure [21].

Male sterility is also related to defects on pollen development that involved cell wall reestructuration. Pollen development is arrested just after microspore release from tetrads in *ms1* mutants. *MALE STERILE1* (MS1) encoded a possible transcription regulator related PHD-finger family that regulates later pollen wall formation genes [75]. *Arabidopsis quartet* mutants present an abnormal separation of microspores that result in male sterility. QUARTET3 is a specific pollen PG that seems to be involved in the degradation of the surrounding pollen mother cell wall [76]. Also, several mutations affecting pollen development that exhibit abnormal callose deposition have been described (*ms37*, *ms32*, *ms31*, *7219* and *7593*).

Anther dehiscence

Anther dehiscence is a process necessary for pollen release and, therefore, has an important role in crop production. Anther is comprised by four cell layers; epidermis, endothecium, middle layer, and tapetum [77]. Before anther dehiscence, endothecium secondary cell walls become thickened and there is an enzymatic breakdown of the stomium, a longitudinal line of weakness in the epidermis. Finally, to release pollen, there is a retraction of the anther wall and full opening of the stomium caused by the dessication of the endothecium [78]. Defects associated with the failure of secondary thickening are related to loss of anther dehiscence and male sterility [75]. In *delayed-dehiscence1*, *2*, *3*, *4 and 5*, anther dehiscence and pollen release are delayed preventing successful pollination, but their pollen morphology and viability is indistinguishable from wild type [79]. In *non-dehiscence1* mutant plant, anthers contain wild-type pollen but do not dehisce [79].

MALE STERILE35 (*MS35*/*MYB26*) is expressed during early anther development and is involved in endothecium formation and thickening, and

in anther dehiscence [75, 80]. MYB26 transcription factor may be a regulator of *NAC SECONDARY WALL-PROMOTING FACTOR1* (*NST1*) and *NST2*, promoters of secondary wall thickening [75]. Although an important number of anther-specific mRNAs that encoded for PGs and proline rich cell wall proteins have been isolated [81] there are not yet evidence that the expression of these genes is regulated by the transcription factors involved in anther dehiscence.

Jasmonate and auxins regulate anther dehiscence in order to coordinate pollen release with stigma maturation. Mutants in genes related to jasmonic acid (JA) biosynthesis are defective in anther dehiscence; *DEFECTIVE IN ANTHER DEHISCENCE1* (*DAD* [82]), *CORONATINE INSENSITIVE1* (*COI1* [83]) and *DELAYED DEHISCENCE1* mutants (*DDE1* [79]). Anthers defective in dehiscence are present in double mutants of the *ARF6* and *ARF8* auxin transcription factors [84]. Exogenous application of JA to these mutants promoted normal anther dehiscence, suggesting that auxin effect on anther dehiscence is related to JA perception [85]. Auxin regulation of the expression of genes involved in cellulose remodelling has been described in *Arabidopsis* [86] thus it is possible that the effect of auxin and jasmonate on anther dehiscence involves cell wall proteins that regulate enzyme activity necessary for cell wall remodelling.

Pollen cell wall exhibits gradients in composition, as well as in extensibility [87]. Once that the pollen grain is in contact with the stigma of the carpel, the pollen grains are hydrated and cell expands by polar growth forming the pollen tube. Polarized growth of pollen tube involves biosynthesis and precise organization of the various wall components. The pollen tube must generate significant amounts of cell wall material, which must be rapidly organized at the pollen tube tip to allow the penetration of the migrating tube to the extracellular matrix of the transmitting tissue [88]. The primary pollen tube wall consists primarily of pectin, together with hemicellulose and cellulose, while the secondary wall is composed of callose. However, the callose lining is absent from the tip and is thus apparently not part of the extension process [89]. In addition to polysaccharides, the pollen tube cell wall contains proteins that have an important impact on the mechanical properties of pollen cell wall. Pectin methylesterases (PME) play an important role in tube growth; esterified pectins are secreted largely at the extreme apex and become de-esterified showing a gradient from esterified to de-esterified pectins that perfectly correlated with the degree of cell wall rigidity [87, 90]. The homogalacturonan (HGA) is a major pectin component of the cell wall. Spatial control of HGA esterification status is essential for pollen tube tip growth. This control could involve different PME and PME inhibitors

(PMEI) isoforms that are highly expressed in pollen and pollen tubes [91]. Specific pollen PME VANGUARD1 (VGD1) is localized in the plasma membrane and the cell wall either in the pollen grain and the tube [92]. A second PME pollen-specific characterized to date is AtPPME1, a protein localized around the cell periphery and in the cytoplasm and the ER/Golgi endomembrane system [93]. On the other hand, the inhibition of the pollen tube growth from *Lilium fotmosonum* and tobacco was detected after application of exogenous PME to pollen grains [94]. The overexpression of PME caused a similar effect on inhibition of the tube growth.

A whole range of cell wall hydrolytic and cell wall loosening enzymes such as arabinogalactan proteins, polygalacturonases, pectate lyases, glycosyl hydrolases, expansins are expressed in pollen grains [95]. Extensin and extensin-like proteins genes are highly expressed in pollen grains [74]. Extensin-like proteins present a differential localization pattern along of pollen tube [96]. PEX-1 (for pollen extension-like protein) has been implicated in pollen development [97]. Arabinogalactan proteins (AGPs) also participate in the control of cell wall formation and specifically during pollen tube growth [98]. Microarray studies have demonstrated elevated and specific expression of *AGP* genes in stamens and pollen [99, 100].

Interaction between pollen and stigmatic cells results in pollen hydratation and pollen tube growth. The pollen tube is conducted along the female style and enters the ovule where the sperm cells are released into the embryo sac [101, 102] This focused process implies that the pollen tube cell wall is adhered to the different cells of the transmitting tract. Pollen tube is guided to the ovary by an epidermis extracellular matrix (TTE). Stigma/style cysteine-rich adhesion (SCA) protein and low esterified pectin polysaccharide play a role during the interaction [103–105]. The level of the SCA mRNA is high in stigma/style tissue, petals and young leaf but is absent in pollen tubes [105]. The mechanism of action of both SCA and pectin is unknown.

The identification of genes expressed in *Arabidopsis* pistils specifically in the path of pollen tube growth was done comparing the whole-genome transcriptional profiling of ablated and wild-type tissues from stage 13 floral buds. It was identified 115 stigma-specific genes and 34 transmitting tract-specific genes. Predicted secreted proteins, including potential signalling components and proteins that could contribute to reinforcing, modifying, or remodelling the structure of the extracellular matrix during pollination were highly present in the stigma and transmitting tract [106]. From 115 genes stigma-specific 9, corresponding to 7.8%, and from 34 genes transmitting-tract specific 2 corresponding to 5.8% are describe in cell wall proteomic analysis.

Petal, sepal, and anther abscission

Abscission is a critical and highly coordinated process in the life cycle of a plant. Abscission of external whorls; stamens, petals and sepals, occurs after pollination. This process is restricted to a predetermined small region named abscission zones (AZ) located at site of organ detachment. Lost of adhesion between cells, that results in abscission, is consequence of the activation of multiple pectin and hemi cellulose-modifying proteins. Expansins also contribute to the cell separation process [106]. Ethylene-induced leaf and flower abscission promotes PG expression [107] meanwhile two PG At2g41850 (*PGAZAT*) and At3g57570 (*PGDZAT*) are expressed specifically in AZ cells during the shedding of floral organs and within the dehiscence zone cells of anthers, pod and seed abscission in oilseed rape. The gene At1g80170, that codifies for a PG reported in cell wall proteome [19], was detected in young, mature and dehiscing anthers, in pollen, in sepal:petal junction, at the base of the flower until the floral organs were shed and on the stigmatic surface [26]. This localization pattern suggests a common mechanism for abscission.

Seed germination

Germination is considered as the period from the start of imbibition of a dry seed until the radicle first emerges from any tissues enclosing it [108]. Cell walls play an important role in all the process. Initially, the rates of water uptake into dry seeds are controlled by the permeability of the testa. A rapid water uptake is observed during phase I meanwhile a slowly increase in seed water content is observed during phase II. Cell walls of the living tissues of the seed control further water uptake and swelling. Both embryo cell wall extensibility and the resistance of enclosing tissues to expansion can be involved in determining the water content achieved during phase II of imbibition [109].

Seeds of many plant species contain living endosperm tissues that completely enclose the embryo. In these seeds, the endosperm possesses two biological functions: first, to provide reserves for seed germination and second to control the timing of germination. Endosperm weakening, an enzyme-mediated degradation process, is a prerequisite for radicle protrusion. The regulatory role of the endosperm in germination is realized mainly through the micropylar endosperm that is composed by small cells with thin cell walls. Inner surface of the endosperm cell walls is increasingly degraded following imbibition. No changes occur in lateral endosperm after radicle emergence [110].

A number of cell wall proteins and hydrolases and the genes encoding them have been identified that are likely to have a mechanistic role in endosperm weakening.

Expansins may be involved in both embryo growth and endosperm weakening during germination. A tomato seed expansin, LeEXPA4 is specifically expressed in the micropylar endosperm cap region within 12 h of imbibition, a time when endosperm weakening had just begun [111]. The role of XTHs in germination is suggested by the differential upregulation of *Arabidopsis* seeds XTH genes; AtXTH5, AtXH9, and AtXTH31 are upregulated within 6h and two (AtXTH3 and AtXTH33) after 12h of imbibition [112]. In germinating tomato seeds, PME activity was detected in the micropylar endosperm and in the embryo [113].

The PG gene is detected in developing and germination seeds, specifically in the region immediately adjacent to the site of penetration of the radicle [26]. The presence of multiple cell wall hydrolases and expansins in the endosperm during seed germination suggests their concerted action to cause endosperm cell wall disassembly.

Embryo growth is essential for germination of all seeds. The expansion of the embryo occurs during Phase II and transition to Phase III of imbibition mainly by cell elongation [109]. In *Arabidopsis*, the AtXTH5 gene is expressed exclusively in the embryonic axis, suggesting that this enzyme is potentially involved in cell wall loosening associated with embryo growth [112]. PMEs are also expressed in the embryo during tomato seed germination, primarily in the radicle tip and in a discrete band just distal to the elongation zone after germination [113].

Proteomic analysis of seeds have been done in *Arabidopsis* during germination and priming [114], during germination of gibberellin-deficient seeds [115] and during seed aging [116]. Seed germination proteomic was also done in rice [117] and barley [72]. The major changes in protein abundance are reported for seed storage proteins. Recently, it has been suggested that there is a poor quality separation of cell wall proteins by two dimensional electrophoresis (2D-E) [17]; method always used for seed proteome. However, very important changes in expression of genes that codified for cell wall proteins have been detected in seed germination transcriptomic analysis suggesting their role in germination vigour.

Transcriptomic analysis have corroborated that seeds are continuously sensing environment conditions and responding to them. An important developmental phase, after-ripening, is related to the increase in sensitivity to the environmental conditions that promote germination [118]. This process was studied by comparing the expression profile of mutant related to

dormancy and germination and wild type plants [119]. In this study, seed after-ripening (AR) is considered a discrete developmental pathway with nineteen specific genes associated. These genes are suggested as the AR signature; five of them encoded for cell wall proteins meanwhile four membrane protein genes are also present. A germination signature was also obtained with fifteen genes; cell wall and membrane proteins are represented with 4 and 5 genes respectively [119].

Cell wall protein gene expression is strongly regulated by gibberellic acid (GA) during germination. GA regulated genes were obtained by adding GA to promote germination of ga1-3 mutant seeds. Expression of three genes that encoded for alpha expansins was increased after 3 h of treatment. After 6 hours of treatment, ten genes that encoded for PMEs, XTHs and one DUF642 protein were abundant expressed meanwhile after 12 h, eight genes for expansins, XTHs, glucanases and one PME and one DUF642 protein were highly detected. Almost all the codified proteins of these genes were detected in cell wall proteomes suggesting that these proteins are involved in the elongation and cell wall remodelation processes present in the different tissues. GA differential regulation of genes encoding enzymes and other factors that degrade cell wall of endosperm and seed coat suggests that GA stimulates germination by promoting embryo growth, elongation of the embryo axis and breakage of seed coat [112, 120]. The regulation of cell wall dynamics by GA in germination was also described in the DELLA dependent transcriptome [121]. DELLA proteins are identified as plant growth repressors and are putative transcription regulators involved in the negatively regulation of the expression of some GA-response genes. GA triggers the degradation of DELLA proteins [122]. Eleven of the genes that were described as GA regulated were also DELLA down-regulated genes [112, 121]. In *Arabidopsis*, GA regulates the expression of the two DUF642 genes, At4g32460 and At2g41800 during germination meanwhile a differential expression of DUF642 genes was observed in barley germination. The role of GA in triggering cell wall modifying transcripts during germination was also described in barley seeds [123]. All these data suggested that the GA promotion of germination in different plant species is strongly related with the reduction of the physical constraint of the embryo-surrounding seed coat and endosperm to induce radicle protrusion.

Conclusions

The importance of cell wall dynamic during plant reproduction was established by comparing both proteomic and transcriptomic analysis. This

comparison indicates that there are many specific cell wall proteins involved in pollen maturation and pollen tube growth meanwhile common proteins are involved in filament, petal, sepal and pistil growth and abscission, pollen tube adhesion to the transmitting track and germination.

Cell wall proteomics could be an important tool to determine the cell wall dynamics during a specific developmental stage. Recently, an excellent system in which it has been possible to do a distinction between elongations or deposition process was performed using *Arabidopsis* etiolated hypocotyls [17].

Proteomics is also a suitable method to identify CWP codified by genes with no function assigned. Proteins having interacting domains, proteins involved in signalling and unknown proteins, some also located in subcellular compartments, could be involved in signalling between cell types or cellular compartments. Recently, it has been suggested that there is a sequestering mechanism in cell wall and sequestered proteins are release to function in the cytoplasm [124]. We consider that the consistent isolation of DUF642 in different cell wall proteomes and the gene expression regulation during germination indicates that the function of this family could be related to cell wall modifying process during plant development.

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Apéndice II

Oligos diseñados para líneas de sobreexpresión de las proteínas BDX y 11420 con y sin péptido señal.

Primer sentido	Primer antisentido	Producto de PCR (pb)
F11420se1 ggatccATGAAAGGAGGCAGCCTCT	R11420se1 gagctcTTACGGCTTACGAGCACTGA	1101
SP11420SEF ggatccATGTTACCAAACGGCGACT	R11420se1 gagctcTTACGGCTTACGAGCACTGA	1029
F32460se1 ggatccATGAAAGAGATGGGAGTGATAG	R32460se1 gagctcTCACGGCCTCCGAGCACT	1104
SP32460SEF ggatccATGAAAGGAACACAAGTTATCAAC	R32460se1 gagctcTCACGGCCTCCGAGCACT	984

Oligos diseñados para la obtención de líneas de expresión tejido específica BDX y *At5g11420*.

Primer sentido	Primer antisentido	Producto de PCR (pb)
PRO11420F GCGAGAAGCCGTGAATAATG	PRO11420R ggatccTGTGGACGACCAAAGTAAAGAG	1386
PRO32460F GCATGGGAGAATTACCACT	PRO32460R ggatccTTGGAGAAGAGTGAGCGAGAG	1983

Oligos diseñados para la obtención de líneas de localización subcelular para las proteínas BDX 11420 y con y sin péptido señal.

Primer sentido	Primer antisentido	Producto de PCR (pb)
11420LSF ATGAAAGGAGGCAGCCTCT	11420LSR CGGCTTACGAGCACTGAGGACTT	1104
SP11420LSF ATGTTACCAAACGGCGACT	11420LSR CGGCTTACGAGCACTGAGGACTT	1025
32460LSF ATGAAAGAGATGGGAGTGATAG	32460LSR CCTCCGAGCACTGAGAAGCTTAAC	1098
SP32460LSF ATGAAAGGAACACAAGTTATCAAC	32460LSR CCTCCGAGCACTGAGAAGCTTAAC	981

Protocolo de transformación para *A. thaliana* (floral deep) (Clough y Bent 1998)

1. Inocular 5 ml de LB conteniendo 50 µg/ml de antibiótico rifampicina y ampicilina y 25 µg/ml de kanamicina (para la cepa C58). Incubar 2-3 días en agitación a 28º C.

2. Escalar a 250 ml de LB (50 µg/ml rifampicina y ampicilina y 25 µg/ml de kanamicina). Incubar toda la noche a 28 ºC en agitación.

3. Verter en frascos para centrífuga de 250 ml, los frascos deben estar fríos y en todo el procedimiento mantener las células sobre hielo.

4. Centrifugar 15 min. A 7500 rpm, a 4 ºC

5. Preparar medio de infiltración como se indica, la cantidad a utilizar es opcional.

6. Disolver la pastilla de células en una pequeña cantidad de medio de infiltración, una vez disuelta ajustar al volumen final deseado. Utilizar una charolita pequeña que permita introducir los botones florales de la planta.

7. Se prepara una charola para plantas con sanitas mojadas para ambientar una cámara húmeda.

8. Las plantas se sumergen en el medio de infiltración por 15-20 segundos y se colocan sobre las sanitas húmedas en posición horizontal.

9. La charola se cubre con plástico se sella bien y se cubre con papel aluminio para mantener las plantas en ambiente húmedo y oscuro. Mantener este tratamiento hasta el día siguiente.

10. Sacar las plantas, colocarlas en posición vertical en el cuarto de crecimiento.

Medio de infiltración.
202 g de MS
50 g de Sacarosa
500 Ml de Silwet 77
dH_2O estéril, ajustar a 1 L.

Medio MS sólido, cantidades para preparar 1 Litro:
5 g de Sacarosa
0.88 g de Medio Murashige and Skoog
0.5 g de MES
6 g de agar
Para selección de plantas transgénicas: kanamicina 50µg/ml