UNIVERSIDAD DE SALAMANCA
Departamento de Fisiología Vegetal
Facultad de Biología

Identificación de proteínas implicadas en la interacción *Arabidopsis-Pseudomonas* mediante tecnología **"phage-display".** Caracterización funcional de AtERF1 y ERF1 en respuesta a estrés

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Salamanca, Julio 2012

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Identification of proteins involved in *Arabidopsis-Pseudomonas* **interaction by "phage-display"** technology. Functional characterization of AtERF1 and ERF1 in stress responses

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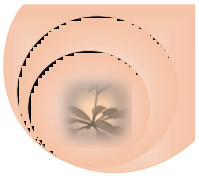


1. RESUMEN

Las plantas están en continua interacción con el ambiente y debido a su naturaleza sésil, han evolucionado y desarrollado complejos mecanismos de respuesta al ataque de patógenos (estrés biótico) o a las condiciones adversas (estrés abiótico). En esta Tesis se ha profundizado en los mecanismos moleculares de las respuestas a estrés, tanto biótico como abiótico en *Arabidopsis thaliana*. En el primer capítulo de este trabajo se ha estudiado la interacción *Arabidopsis-Pseudomonas* desarrollando una nueva estrategia para la búsqueda de proteínas vegetales implicadas en la percepción del patógeno. Empleando la tecnología *phage-display* se ha expresado el transcriptoma de Arabidopsis en respuesta a tres cepas bacterianas (*P.aeruginosa* PA14, el aislado virulento *P.syringae* DC3000 y su variante avirulenta *P.syringae* DC3000+*avrRpt2*) representando así, diferentes grados de especificidad en la interacción planta-microorganismo. Las genotecas "phage display", construidas a partir de cDNA de plantas infectadas, representan aproximadamente $2x10^7$ transcritos vegetales diferentes que se expresan de forma funcional en la superficie del fago T7. Mediante una técnica complementaria denominada *biopanning*, se han seleccionado e identificado aquellos fagos individuales que expresen una proteína heteróloga capaz de unirse físicamente a células bacterianas, entre ellos ATERF-1 (Factor de Transcripción de Respuesta al Etileno 1). El papel de ATERF-1 en respuesta al patógeno ha sido también demostrado *in vivo*. Se ha estudiado la localización de ATERF-1 en plantas de Tabaco y Arabidopsis y se ha visto que la construcción *GFP-ATERF-1* se transloca desde el núcleo al citoplasma tras el contacto con la bacteria. Adicionalmente, el mutante de pérdida de función *aterf-1* es más susceptible a la infección que el genotipo silvestre Col-0, demostrando así, el papel de la proteína en la respuesta inmune. La selección de clones con afinidad física por células vivas de *Pseudomonas* ha sido monitorizada mediante la hibridación de microarrays con el

objeto de comparar la colección de clones inicial *vs.* seleccionados. Esto ha permitido por una parte cuantificar el enriquecimiento de clones específicos, y por otra identificar un total de 418 genes de Arabidopsis que posiblemente participen en la percepción de *Pseudomonas*, o que puedan tener afinidad por alguna de las partes del microorganismo. De entre todos estos genes, algunos ya estaban anotados como genes de defensa y otros podrían ser anotados a partir de este estudio.

En el segundo capítulo de esta tesis, se ha estudiado el papel de ERF1 en la respuesta a estrés abiótico. Esta proteína pertenece a la misma familia que ATERF-1, identificada en el cribado de la primera parte de esta tesis, y es el miembro mejor estudiado de la subfamilia AP2/ERF (Apetala 2/Factores de transcripción de Respuesta al Etileno) en relación con la respuesta a patógenos necrótrofos y a la integración de las señales de Etileno (ET) y Jasmónico (JA). En esta Tesis se ha demostrado que la sobreexpresión de *ERF1* en plantas adultas confiere tolerancia a la sequía y desreprime a los factores de transcripción AtERF4 y DEAR5, propuestos como dianas directas de la regulación transcripcional por ERF1. Esta respuesta mediada por ERF1 es también dependiente de EIN2, un componente clave de la ruta de señalización por ET. Por otro lado, plantas transgénicas que sobreexpresan ERF1 presentan un retraso en el desarrollo temprano de plántulas cuando son sometidas a otros estreses abióticos (salino y osmótico), o cuando son tratadas con la fitohormona Ácido Abscísico (ABA), que regula el cierre de los estomas en las hojas como respuesta a la sequía. El retraso en la germinación de semillas está relacionado con un aumento en los niveles de ABI5, una proteína clave en el proceso post-germinativo que se acumula en semillas transgénicas que sobreexpresan *ERF1.* Por último, en este trabajo se ha demostrado que ERF1 es epistático en la ruta de señalización del ABA y que actúa debajo de HAB2, una proteín-fosfatasa de tipo PP2C implicada en el complejo receptor del ABA y que actúa como regulador negativo, dando evidencias de una interacción hormonal ET-ABA durante el establecimiento de la plántula en Arabidopsis.

2. INTRODUCCIÓN

2.1 Concepto de estrés

La interacción de las plantas con el medio que las rodea implica que en ocasiones estén expuestas a condiciones desfavorables que afectan a algunos procesos del crecimiento y desarrollo, al rendimiento o incluso a la supervivencia, y es lo que se conoce como estrés ambiental. Debido a su naturaleza sésil, las plantas han desarrollado mecanismos específicos que les permiten detectar cambios precisos en el ambiente y responder frente a complejas condiciones de estrés para minimizar el daño en la planta, mientras conservan recursos valiosos para seguir creciendo y reproducirse. Las respuestas de las plantas a organismos patógenos (estrés biótico) y a condiciones ambientales adversas (estrés abiótico) son objeto de elevadas investigaciones, debido a las pérdidas económicas que provocan en los cultivos agronómicos. El estudio, a nivel molecular, de estas interacciones nos permite conocer en profundidad los mecanismos de respuesta que poseen las plantas frente a los diferentes estreses y desarrollar aplicaciones biotecnológicas en agricultura para combatirlas.

2.2 Estrés biótico

En la naturaleza las plantas están constantemente amenazadas por un amplio espectro de patógenos y pestes perjudiciales entre los que se incluyen virus, bacterias, hongos, oomicetos, nematodos e insectos herbívoros. El ataque por cualquiera de estos organismos puede producir síntomas de enfermedad en plantas susceptibles y es lo que se conoce como estrés biótico (Agrios, 1997) (Figura 2.1). Los patógenos generalmente se dividen en biótrofos o necrótrofos según su ciclo de vida y modo de nutrición (Glazebrook, 2005). Los necrótrofos primero destruyen las células a través de de la producción de fitotoxinas y luego se alimentan de su contenido. Los biótrofos se alimentan de células vivas, normalmente a través de estructuras especiales, por ejemplo los haustorios que invaginan la célula huésped

sin destruirla. Muchos patógenos desarrollan ambos estilos de vida y nutrición dependiendo del estadio en el que se encuentren y se denominan hemibiótrofos (Pieterse et al., 2009). En esta introducción se hará especial énfasis en el grupo de bacterias patógenas de plantas.

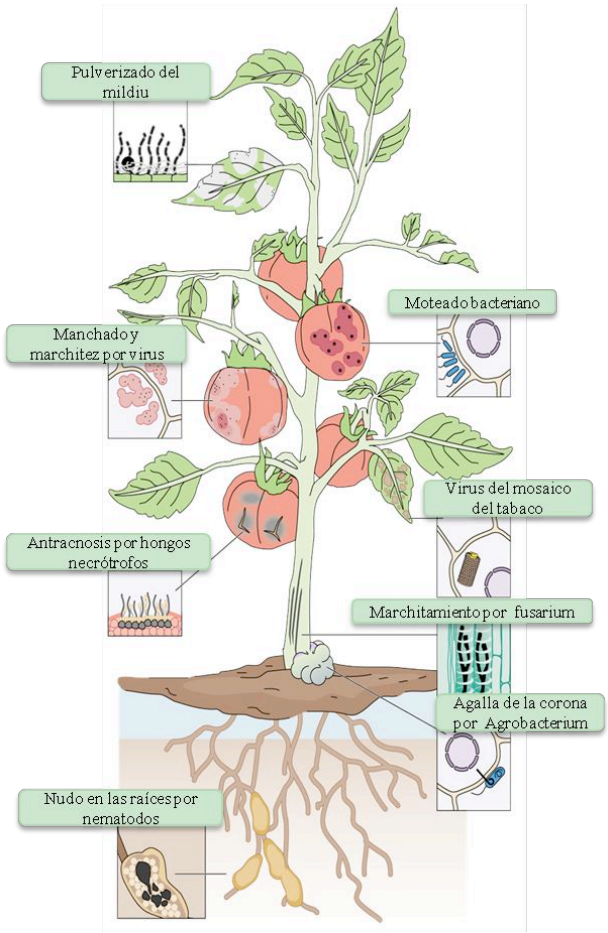


Figura 2.1 Síntomas de enfermedades causadas por diferentes patógenos a lo largo del desarrollo de la planta. Adaptada de Buchanan et al., 2000.

2.2.1 Bacterias fitopatógenas y mecanismos de virulencia

Las plantas son un reservorio de agua y nutrientes para los microorganismos, y muchos patógenos bacterianos, Proteobacterias y Actinobacterias, pueden infectarlas. Las bacterias mejor estudiadas pertenecen al grupo de las

Proteobacterias, ya que infectan un amplio rango de hospedadores, causando un gran impacto económico, y pueden ser fácilmente manipuladas en condiciones experimentales. Este grupo incluye una gran variedad de géneros, tales como *Agrobacterium*, *Erwinia*, *Pseudomonas*, *Ralstonia* y *Xanthomonas*. Todos estos patógenos son bacterias Gram negativas y la mayoría se mueven utilizando flagelos (algunas lo pueden hacer por deslizamiento bacterial -mixobacterias-). Las bacterias pueden dispersarse mediante diferentes mecanismos (viento, lluvia, insectos o prácticas de agricultura extensiva) y penetran en la planta a través de heridas o de aperturas naturales (estomas e hidatodos). Una vez dentro del tejido vegetal, se instalan en los espacios intracelulares (apoplasto) o en el xilema (Abramovitch et al., 2006).

Las bacterias patógenas de plantas causan diferentes síntomas de enfermedad como motas, manchas, clorosis, tizón, marchitamiento, agallas o cancros y pueden causar muerte celular programada en las raíces, hojas, flores, frutos, estambres y tubérculos del hospedador (Agrios, 1997). También han desarrollado complejas estrategias de virulencia, algunas especializadas en tejidos vegetales y otras altamente conservadas entre patógenos de plantas y de animales. La virulencia bacteriana se manifiesta como un incremento en la velocidad de crecimiento o en el tamaño final de la población, así como un aumento en los síntomas de enfermedad que producen en el huésped, que dará lugar a la dispersión del patógeno a través de la planta o a través del ambiente. Los patógenos bacterianos contienen un arsenal de factores de virulencia que facilitan tanto su crecimiento, como su capacidad de causar enfermedad en tejidos vegetales. Entre los factores de virulencia se incluyen la secreción de proteínas específicas o la producción de pequeñas moléculas (Abramovitch et al., 2006).

Existen tres sistemas diferentes de secreción de proteínas en bacterias fitopatógenas (Figura 2.2): i) Sistema de Secreción de Tipo II (T2SS), esencial en microbios que causan podredumbres, característico del género *Erwinia* (Jha et al.,

2005; Toth y Birch, 2005). El T2SS exporta al apoplasto enzimas implicadas en la degradación de la pared celular, incluidas pectinasas, endoglucanasas y celulasas. Estas y otras enzimas que producen son las responsables de causar los fenotipos de pobredumbre y maceración asociados a estas bacterias. ii) Sistema de Secreción de Tipo III (T3SS), estrategia de virulencia más efectiva y está relacionado con el flagelo bacteriano, que forma un *pilus* a través del cual inyecta proteínas de virulencia denominadas *efectores* dentro de la célula vegetal. Una vez dentro de la célula, esos efectores modulan la fisiología de la planta en beneficio del patógeno. Varias bacterias con diferentes estilos de vida, incluidos biótrofos como *Pseudomonas*, bacterias necrótrofas que causan podredumbre e incluso algunas bacterias simbiontes dependen del T3SS para infectar a su hospedador con éxito. Además, los efectores inyectados por el T3SS promueven la virulencia tanto en animales como en plantas (Mudgett, 2005; Alfano y Collmer, 2004; Toth y Birch, 2005; Nomura et al., 2005). iii) Sistema de Secreción de tipo IV (T4SS), tiene un papel crucial en la patogénesis de *Agrobacterium tumefaciens* y es capaz de formar agallas o tumores en las plantas. El T4SS conduce proteínas y su DNA a las células vegetales y se integra dentro del genoma del huésped dando lugar a la producción de hormonas vegetales que inducen los síntomas característicos de la agalla de la corona (Christie et al., 2005). También induce la biosíntesis de nutrientes ricos en opinas que pueden ser catabolizados tan solo por *A.tumefaciens* y se transportan proteínas a través del T4SS que aumentan la eficiencia en la integración del DNA bacteriano (Christie et al., 2005; Tzfira et al., 2004; Gelvin, 2000). Es importante destacar que muchos patógenos dependen de varios de estos mecanismos (Preston et al., 2005). Por ejemplo, muchas especies de *Erwinia* requieren el T2SS y el T3SS para causar enfermedad (Toth y Birch, 2005) y varias cepas de *Xanthomonas* emplean el T2SS, T3SS y T4SS (da Silva et al., 2002).

Las bacterias también utilizan pequeñas moléculas como toxinas, hormonas de plantas, autoinductores y exopolisacáridos (EPS) para causar enfermedad en las

plantas. Toxinas bacterianas como la coronatina, siringomicina, siringopeptina, tabxolina y faseolotoxina tienen especial importancia en la virulencia y síntomas de enfermedad causados en sus hospedadores (Bender et al., 1999). Estas toxinas pueden producir síntomas de clorosis o necrosis empleando diferentes mecanismos de acción que incluyen la biosíntesis o imitación de hormonas vegetales, la formación de poros en las membranas de las plantas o la inhibición de enzimas esenciales del metabolismo del hospedador. En relación con la síntesis de hormonas, muchas cepas de *Pseudomonas* y *Xanthomonas* son capaces de producir auxinas (ácido indolacético, IAA) (Glickmann et al., 1998; Spaepen y Vanderleyden, 2011) y cepas de *Pseudomonas,* y *Ralstonia* pueden producir etileno (ET) (Weingart et al., 1999, 2001; Valls et al., 2006). La producción de hormonas por patógenos está muchas veces correlacionada con la enfermedad. Sin embargo, el papel que juegan las hormonas en este proceso es aún desconocido. Dado que la resistencia a patógenos se ve aumentada cuando los niveles de auxinas disminuyen (Navarro et al., 2006), estas bacterias podrían contrarrestar el nivel de auxinas para suprimir la respuesta de defensa en las plantas (Abramovitch et al., 2006). Cabe destacar que algunos patógenos pueden imitar las hormonas vegetales. Por ejemplo, muchas cepas de *P. syringae* pueden producir coronatina, un análogo estructural de la molécula activa del ácido jasmónico (*JA-isoleucine*; JA) y que en teoría funciona igual que la hormona activa (Fonseca et al., 2009). Las bacterias también producen autoinductores para detectar la densidad de población de una especie o cepa particular (Von Bodman et al., 2003). Este proceso se denomina *quorum sensing* (QS) y se cree que, entre otras cosas, permite a las bacterias regular la expresión génica de factores de virulencia cuando han alcanzado determinados niveles de densidad y de esta forma parasitar la planta. Muchas bacterias de los géneros *Ralstonia* y *Xanthomonas* secretan grandes cantidades de EPS, que son moléculas de azúcar de alto peso molecular, las cuales obstruyen el xilema de la planta y causan los síntomas característicos del marchitamiento (Leigh y Coplin, 1992) (Figura 2.2).

Los EPS aumentan la virulencia del patógeno protegiendo a las bacterias de compuestos antimicrobianos existentes en las plantas, como por ejemplo los factores antimicrobianos presentes en el xilema de la planta. En muchos casos, los mecanismos mencionados anteriormente se solapan para promover la patogénesis. Por ejemplo, se ha observado que el QS puede regular la expresión génica de la producción de EPS en el caso específico de *Pantocea stewarti* (Koutsoudis et al., 2006), indicando que el control de la expresión temporal de EPS es importante para la patogenicidad en bacterias. Todos estos mecanismos de virulencia que han desarrollado las bacterias a lo largo del tiempo le han servido como una estrategia para burlar el sistema de defensa de las plantas. Sin embargo, las plantas a su vez han ido creando mecanismos complementarios para poder defenderse.

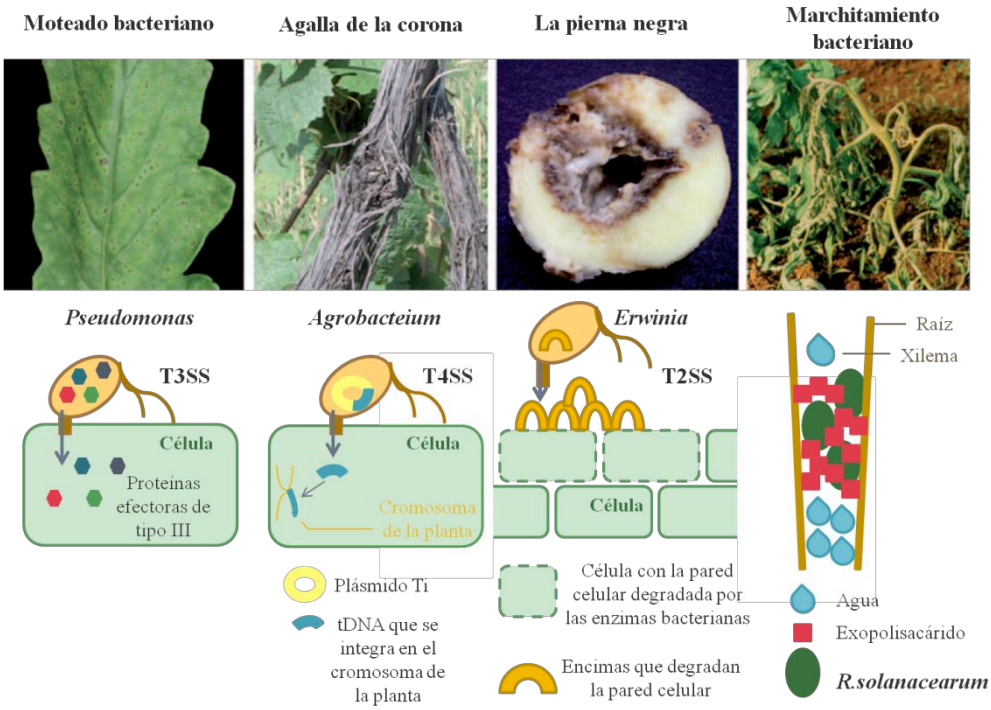


Figura 2.2 Síntomas de enfermedad causados por diferentes tipos de bacterias fitopatógenas en plantas y representación de los mecanismos de virulencia empleados por las diferentes bacterias. Adaptado de (Abramovitch et al., 2006).

2.2.2 El sistema inmune de Arabidopsis frente al ataque de bacterias.

Las plantas, debido a su constante exposición al ataque de patógenos, poseen barreras estructurales para impedir la entrada de microorganismos como la pared celular, lignina, cutícula y ceras, entre otras. Además de estas barreras físicas, las plantas acumulan metabolitos secundarios, que en muchos casos poseen propiedades antimicrobianas, y actúan en los lugares de penetración de los microbios, como estomas e hidatodos. Estas barreras físicas normalmente son suficientes para evitar la entrada del patógeno, sin embargo, en ocasiones el patógeno logra penetrar en la planta, y dependerá de la efectividad de la respuesta de defensa vegetal que la infección se detenga o se propague. Debido a la coevolucion y constante lucha entre plantas y microorganismos los organismos vegetales han desarrollado un sofisticado sistema de defensa que al igual que el sistema inmune de animales, reconoce moléculas no propias o señales de células dañadas y responde a ello mediante la activación de una efectiva respuesta inmune. La inmunidad innata en las interacciones planta-patógeno puede ser fácilmente explicada por el modelo denominado "zigzag" descrito por Jones y Dangl (2006). En este modelo se propone que las plantas detectan a los patógenos mediante un sistema immune que se divide en dos ramas: defensa basal o inmunidad asociada a PAMPs (PTI) e inmunidad asociada a efectores (ETI).

La respuesta PTI ocurre cuando proteínas receptoras de membrana conocidas como Receptores asociados al Reconocimiento (PRRs) detectan moléculas elicitoras altamente conservadas en microorganismos denominadas Patrones Moleculares Asociados a Patógenos/ Patrones Moleculares Asociados a Microorganismos (PAMPs/MAMPs). Con la detección de PAMPs se inicia en la planta una serie de cambios fisiológicos, como por ejemplo la producción de compuestos antimicrobianos y el reforzamiento de la pared celular a través de la deposición de callosa, que dificultan o impiden la entrada de más patógenos (Figura 2.3).

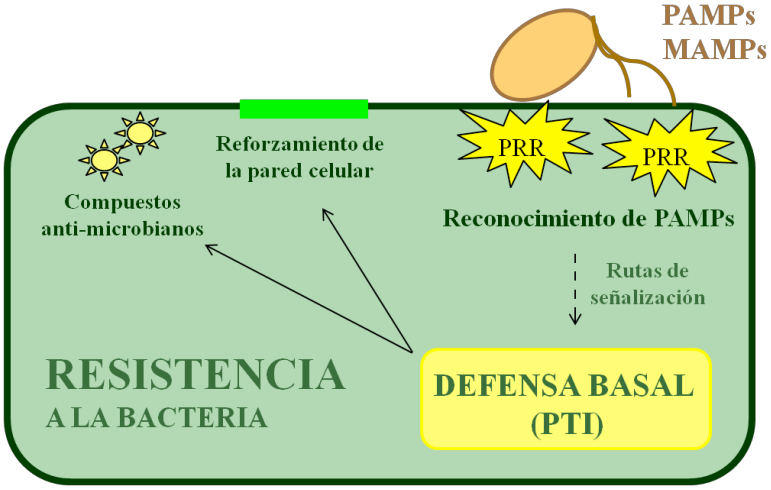


Figura 2.3 Esquema que representa la defensa basal o PTI en las plantas.

Las plantas pueden reconocer un elevado número de PAMPs bacterianos entre ellos la flagelina, el factor de elongación Tu (EF-Tu), proteínas de choque térmico, peptidoglicano (PGN), el lipopolisacárido (LPS) y la superóxido dismutasa (Boller y He, 2009). Aunque la percepción de PAMPs a través de las especies no es ubicua, parece que la habilidad del huésped para reconocer determinados PAMPs viene determinada por los organismos patógenos a los que están expuestos los hospedadores. Además, muchas moléculas que actúan como PAMPs en plantas también actúan como elicitores en vertebrados o no vertebrados, lo que sugiere una presión selectiva común para reconocer las mismas moléculas en mamíferos, insectos y plantas (Gimenez-Ibanez y Rathjen, 2010). La percepción de PAMPs viene dada por los PRRs, que son proteínas que normalmente se localizan en la membrana plasmática y que contienen un dominio extracelular de unión a ligando denominado repeticiones ricas en leucina (*Leucin Rich Repeat*, LRR). Los PRRs pueden ser de tipo Receptor kinasa (RKs), con al menos 610 miembros en Arabidopsis, y Receptores similares a proteín-kinasas (RLPs), con 56 miembros en Arabidopsis descritos hasta el momento. Curiosamente, en los genomas de

mamíferos solo hay una docena de RKs y RLPs, mientras que los genomas de plantas codifican cientos de estas proteínas, y en algunas ya se ha demostrado *in vivo* su participación en la percepción de PAMPs (Gómez-Gómez y Boller, 2000; Shiu y Bleecker, 2003; Zipfel et al., 2006). La expansión de esta familia podría representar una adaptación clave a los cambios ambientales. De momento, y al contrario que en animales, no se han encontrado PRRs intracelulares en plantas. Sin embargo, aún se conoce muy poco de este tipo de receptores y es posible que receptores intracelulares estén esperando a ser descubiertos (Gimenez-Ibanez y Rathjen, 2010).

A pesar de que la defensa basal es muy efectiva, uno de los rasgos característicos de la patogenicidad bacteriana es la capacidad que poseen algunas bacterias de suprimir la defensa basal o PTI mediante la translocación de efectores (o proteínas de virulencia) al interior de la célula vegetal con el T3SS. Una vez dentro, la planta no es capaz de reconocer estos efectores como virulentos y queda rendida a la infección bacteriana resultando en síntomas de enfermedad (Figura 2.4).

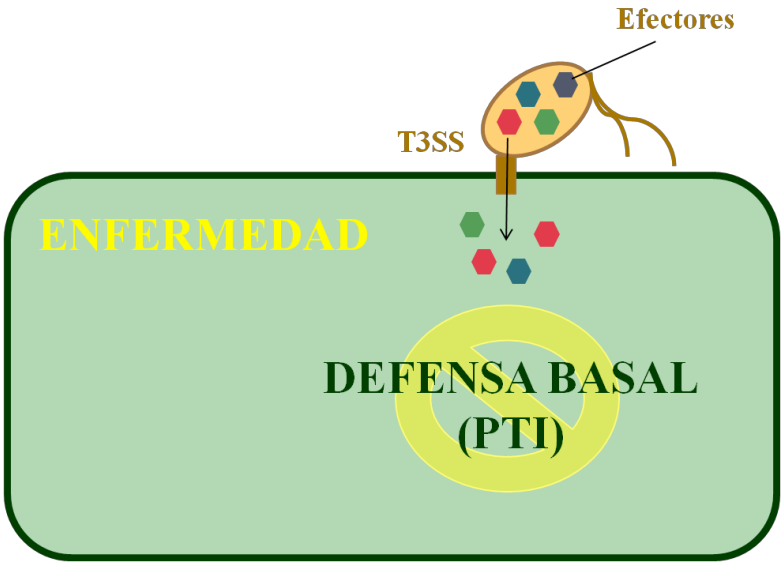


Figura 2.4 Esquema de la supresión de la respuesta PTI mediante la translocación de efectores al citoplasma de la célula por el T3SS

Por su parte, la respuesta inmune denominada ETI, ocurre cuando complejos receptores basados en la unión a nucleótidos y repeticiones ricas en leucina (NB-LRR), conocidos como proteínas de resistencia (R), reconocen a los efectores inyectados en el citoplasma por el T3SS como elicitores bacterianos. Entonces, la planta actúa dando lugar a la Respuesta Hipersensible (HR) que consiste en la muerte celular programada del tejido infectado para evitar así la expansión del patógeno por toda la planta (Figura 2.5). Los efectores bacterianos se caracterizan por ser altamente diversos y la oportunidad de reconocerlos en el citoplasma representa una estrategia del hospedador para identificar moléculas variables del patógeno, al contrario que los PAMPs, que constituyen una diana de reconocimiento estática.

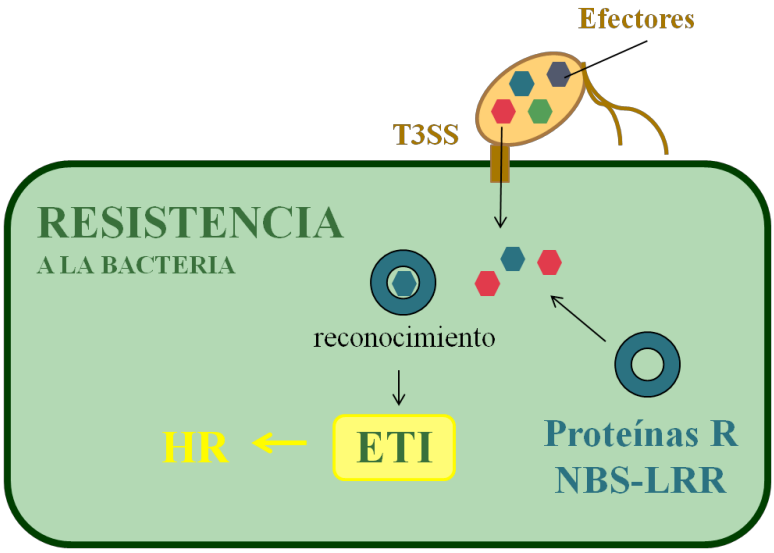


Figura 2.5 Esquema que representa la Respuesta Inmune asociada a Efectores (ETI)

Tanto la respuesta PTI como la ETI contribuyen a la inmunidad de la planta y, en general, sostienen la resistencia de tipo no-hospedador y la específica de hospedador, respectivamente. Sin embargo, esta relación no es exclusiva. La ETI

normalmente se considera una respuesta rápida y amplificada de la respuesta PTI que da como resultado la resistencia a la enfermedad por medio de la HR (Jones y Dangl, 2006). Sin embargo, y a pesar de que la HR has sido siempre considerada un marcador específico de ETI, la PTI también puede dar lugar a una HR (Taguchi et al., 2003), por lo que el concepto no debería generalizarse.

Aunque en el sistema inmune de las plantas existan dos ramas controladas por diferentes tipos de receptores, la mayoría de eventos asociados aguas abajo de la percepción PTI y ETI es la misma. Así, una vez desencadenada la señal de respuesta, nos encontramos con mecanismos específicos de respuesta temprana como los flujos de iones (principalmente Ca^{2+}) que actúan como segundos mensajeros tras la percepción del patógeno, la explosión oxidativa (que está muy ligada a la HR), la activación de proteín-kinasas activadas por mitógeno (MAPKs), la expresión diferencial de genes a través de factores transcripcionales y el cierre estomático que bloquea la entrada de patógenos al apoplasto. También desarrollan mecanismos más complejos de silenciamiento génico, modulación de componentes que afectan la función de NB-LRR para ampliar el espectro de efectores reconocidos y por último, la resistencia sistémica adquirida (SAR) que es un mecanismo de defensa inducida que confiere resistencia frente a un amplio espectro de microorganismos. Aunque la SAR se localiza en el sitio de infección su acción se expande a tejidos distales. Todos estos procesos están regulados por la señalización hormonal, que en bacterias, está gobernada principalmente por las fitohormonas ácido salicílico (SA), JA y ET, aunque también se conoce la participación de otras como ABA, auxinas y giberelinas (GAs). Las rutas de señalización de estas hormonas frente a los diferentes estímulos están muy entrecruzadas entre sí y aunque muchos estudios están enfocados al entendimiento de estos mecanismos de regulación, aún queda mucho por descubrir. En el apartado 2.2.3.2 de esta introducción profundizaremos en la señalización hormonal frente al ataque de patógenos, concretamente en la respuesta de Arabidopsis frente a *P. syringae*.

2.2.3 Arabidopsis-*Pseudomonas*

2.2.3.1 La interacción específica Arabidopsis-*Pseudomonas syringae*

Pseudomonas syringae es una bacteria Gram negativa, baciliforme, aeróbica hemibiótrofa y con flagelos polares que le confieren movilidad. A pesar de infectar a gran variedad de plantas, las interacciones que se dan entre las diferentes cepas de *P. syringae* y las plantas son diversas y específicas del hospedador (Hirano y Upper, 2000). En los años 1980s, se demostró que *P. syringae* era capaz de infectar Arabidopsis, bajo condiciones de laboratorio, y causar en ella síntomas de enfermedad. Desde entonces, el sistema Arabidopsis-*P. syringae* ha contribuido a esclarecer tanto los mecanismos del reconocimiento planta-patógeno y las rutas de señalización que controlan las respuesta de defensa en las plantas, como la susceptiblidad del hospedador y los factores de virulencia y avirulencia del patógeno (Katagiri et al., 2002). Uno de los grandes hitos descubiertos en el sistema Arabidopsis-*P. syringae* fue la demostración de que este sistema de patogénesis se ajusta a la hipótesis gen-a-gen en la que se sostienen la mayoría de interacciones planta-patógeno en la naturaleza. La hipótesis gen-a-gen fue descrita en 1971 y se basa en que cuando un patógeno tiene un gen de avirulencia (avr) y la planta tiene un gen de resistencia (R), la planta es resistente al patógeno. Está definido por un simple gen R en la planta para un único gen en el patógeno (avr). Cuando la planta es resistente, el patógeno se denomina avirulento y la interacción que se da es incompatible. Cuando la planta es susceptible, el patógeno se denomina virulento y la interacción que se da es compatible (Tabla 2.1).

Tabla 2.1 Resistencia gen-a-gen. Adaptada de (Katagiri et al., 2002)

Interacción	Patógeno	Hospedador	Efectos
Compatible	Virulento	Susceptible	ENFERMEDAD
Incompatible	Avirulento (avr)	Resistente (R)	RESISTENCIA

La hipótesis gen-a-gen en Arabidopsis-*P. syringae* se confirmó con el gen de avirulencia *avrRpt2,* aislado de *P. syringae pv. tomato* JL1065, capaz de convertir las cepas virulentas DC3000 y ES4326 en avirulentas (Dong, Mindrinos, Davis, et al., 1991; Whalen et al., 1991), y su correspondiente gen de Resistencia en la planta, RPS2 (Kunkel et al., 1993; Yu et al., 1993), que fue el primer gen de resistencia en clonarse (Bent et al., 1994; Mindrinos et al., 1994). Esta teoría se basa en que la planta detecta al patógeno usando receptores que directamente se unen a moléculas elicitoras producidas por patógeno (Gabriel y Rolfe, 1990). Originalmente se creía que la resistencia gen-a –gen se confería por una interacción directa entre proteínas R y avr (Keen, 1990). Sin embargo, más tarde se descubrieron otros mecanismos indirectos para el reconocimiento del patógeno y la activación de la respuesta defensiva de la planta que se denominó *The guard hypothesis* (Van der Biezen y Jones, 1998). Este modelo se sustenta en que las moléculas del patógeno tienen funciones intrínsecas para promover la virulencia mediante la modulación de componentes en el hospedador que se convertirán en las dianas de virulencia. Se determinó que más que la presencia de moléculas patogénicas, es la manipulación de sus dianas en el hospedador lo que reconocen las proteínas de resistencia R (Chisholm et al., 2006; Jones y Dangl, 2006). Así, las moléculas del patógeno que originalmente fueron referidas como factores de avirulencia son ahora factores de virulencia y actualmente, se utiliza el término "efector" para este tipo de moléculas (Bent y Mackey, 2007; Boller y Felix, 2009).

En paralelo a las investigaciones de la resistencia gen-a-gen, la existencia de inductores de la respuesta de defensa en la planta que no eran específicas de hospedador se hizo evidente. Estos inductores no específicos de la defensa se denominaron elicitores y albergan un amplio rango de tipos de moléculas (Boller, 1995). Durante un largo tiempo se debatió si la defensa inducida por la respuesta a elicitores era fisiológicamente relevante para la inmunidad en plantas y sobre la relación existente entre la resistencia gen-a-gen y la defensa inducida por elicitores.

Con la identificación del primer receptor de un elicitor (FLS2) (Gómez-Gómez y Boller, 2000), la prueba de su papel en la inmunidad de la planta (Zipfel et al., 2004) y la identificación de efectores bacterianos que suprimen este tipo de inmunidad (Hauck et al., 2003), se demostró que ambos tipos de defensa contribuyen a la inmunidad bacteriana. Todos estos experimentos llevados a cabo en *P. syringae* han ayudado a explicar el sistema inmune de una forma sencilla a través del modelo en zig-zag propuesto por (Jones y Dangl, 2006) y explicado en el apartado anterior (2.2.2)

Investigaciones relevantes realizadas con las diferentes cepas de *P. syringae*, sobre todo con la patovariedad (pv.) *tomato* DC3000 han contribuido significativamente a descubrir los paradigmas de la patogénesis mediada por los efectores (Abramovitch et al., 2006; Alfano y Collmer, 2004; Boller y Felix, 2009; Nomura et al., 2005). Gracias al sistema Arabidopsis-*P. syringae* se han descubierto diferentes efectores bacterianos (AvrRps2, AvrRps4, AvrPto, AvrPtoB, AvrRpm1, AvrPphB) y sus respectivas proteínas de resistencia en la planta (RPS2, RPS4, RPS5, RIN4, RPM1, RRS1). También su estudio ha servido para entender los paradigmas del reconocimento de PAMPs. Por ejemplo, han sido ampliamente estudiados y caracterizados la percepción de flagelina mediante el PRR FLS2 y la percepción del EF-Tu mediante el PRR EFR. Además se ha encontrado una proteína (BAK1, BRI1-associated kinase 1) que actúa como un regulador central en la señalización de defensa, actuando como un adaptador para múltiples PRRs en la percepción de PAMPs (Gimenez-Ibanez y Rathjen, 2010). En otros estudios se han analizado a gran escala los genes de Arabidopsis que responden a *P. syringae*, un ejemplo es la reciente publicación del interactoma, que recoge todas las proteínas de Arabidopsis que son capaces de interaccionar con la cepa virulenta DC3000 y la cepa avirulenta DC3000+*avrRpt2* (Mukhtar et al., 2011).Todos estos hallazgos han contribuido al mejor entendimiento de la respuesta inmune, empleando un modelo

sencillo y cómodo para experimentar en condiciones de laboratorio, que después podrán ser extrapolados a las diferentes plantas de cultivo.

2.2.3.2 Señalización hormonal en la defensa contra *P. syringae*

La defensa en las plantas está gobernado por un complejo entramado de rutas de señalización que está gobernado principalmente por SA, ET y JA (Figura 2.7). Generalmente, los mecanismos de defensa dependientes de SA ocurren en respuesta a patógenos (hemi)biótrofos mientras que los de ET y JA se activan por necrótrofos (Grant y Jones, 2009). Existen muchas conexiones entre las tres rutas y por ejemplo, la activación de la ruta del SA mediada por *P. syringae* suprime la señalización de JA y las hojas que habían sido infectadas resultan mucho más susceptibles al hongo necrótrofo *Alternaria brassicicola* (Spoel et al., 2007). La producción de SA está comúnmente asociada con la HR y la resistencia a *P. syringae* (Loake y Grant, 2007) y la señalización de SA se requiere para la inmunidad mediada por NB-LRRs en la mayoría de los casos, pero no para todos. Por ejemplo, la resistencia mediada por RPS2 es dependiente de SA pero la de RPM1 no lo es (Katagiri, 2004). La producción de JA también se activa durante la ETI, sin embargo, no se conoce mucho sobre su papel biológico (Spoel et al., 2003). Se ha demostrado que algunos factores de virulencia suprimen la respuesta de defensa basal de la planta a través de la manipulación de las hormonas vegetales. Por ejemplo, el efector AvrRpt2 promueve la virulencia bacteriana en Arabidopsis mediante la alteración del metabolismo de auxinas, resultando en el aumento de la enfermedad (Chen et al., 2007). Además, otros efectores de tipo III son capaces de activar la ruta de señalización del ABA como principal estrategia de virulencia, resultando en la supresión de la inmunidad del hospedador y en el aumento de la enfermedad (de Torres-Zabala et al., 2007). Como medida de contraataque, las plantas activan las defensas dependientes de SA, muy efectivas para este patógeno, y son capaces de neutralizar algunos efectos de supresión de la respuesta inmune,

como la respuesta a auxinas inducida por efectores (Wang et al., 2007). Sin embargo, la bacteria es capaz de suprimir la defensa mediada por SA. Por ejemplo, HopI es capaz de suprimir la acumulación de SA en el cloroplasto (Jelenska et al., 2007). Además, *P.syringae* produce coronatina, que funciona como un imitador del JA, suprimiendo las defensas mediadas por SA y promoviendo así la susceptibilidad de la planta a este patógeno (Brooks et al., 2005; Uppalapati et al., 2007). Curiosamente, el grado de interacción entre las defensas dependientes de JA y SA difieren entre los ecotipos de Arabidopsis (Koornneef et al., 2008; Traw et al., 2003), lo cual puede deberse a una variabilidad intraespecípica de la comunicación cruzada entre las diferentes rutas. En un contexto ecológico, esta variación natural de las conexiones entre rutas de transducción podría ser un instrumento para evitar las estrategias de engaño que utilizan los patógenos (Pieterse et al., 2009).

Con respecto a la respuesta PTI se ha estudiado ampliamente el papel del SA y se ha demostrado que actúa como un regulador positivo de la PTI (Tsuda et al., 2008). También se ha demostrado que el tratamiento con PAMPs induce la producción de ET y JA (Liu y Zhang, 2004; de Torres Zabala et al., 2009) (Figura 2.7). Ensayos más concretos para profundizar en el papel del ET sugieren que regula de forma negativa la resistencia al patógeno reprimiendo la señal transcripcional iniciada con el reconocimiento de PAMPs. Específicamente, plantas que sobreexpresan el factor de transcripción de la ruta de señalización del ET, EIN3, suprimen la biosíntesis de SA inhibiendo a su vez la PTI (Chen et al., 2009). La acumulación de ET inducida por PAMPs sugiere entonces un bucle de retroalimentación para la regulación PTI. Aunque en principio parezca contradictorio, estos mecanismos de regulación negativa son muy frecuentes en plantas. Recientemente, se ha cuantificado la aportación respectiva de la PTI y ETI midiendo el crecimiento de la cepa virulenta DC3000 en un cuádruple mutante de Arabidopsis (*dde3;ein2;pad4;sid2*) afectado en la señalización de ET, JA y SA (Tsuda et al., 2009). El resultado es que la misma red definida para esos cuatro genes

representó el 80% de ambas respuestas PTI y ETI indicando que la maquinaria de señalización es muy similar para ambos sistemas.

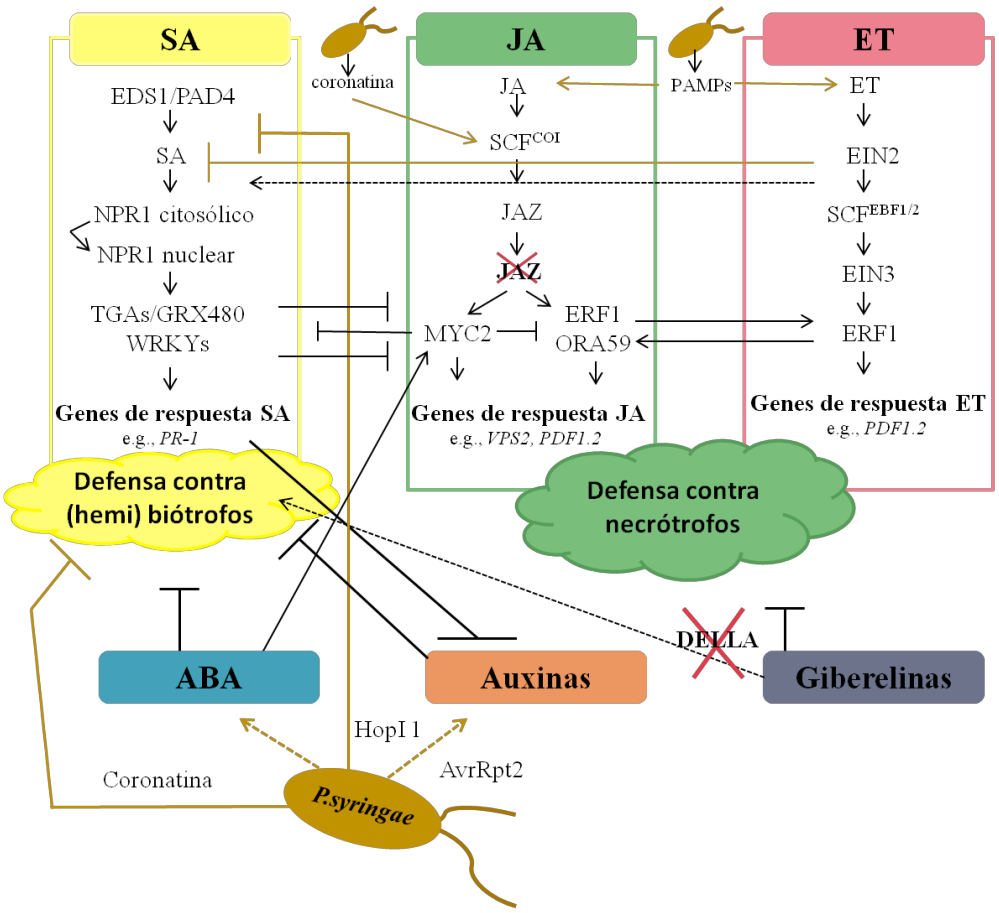


Figura 2.5 Representación de las interacciones hormonales durante la respuesta inmune de las plantas. Las interferencias entre las diferentes rutas de transducción permiten a las plantas adaptar la respuesta de defensa a los diferentes tipos de patógenos. *P.syringae* produce efectores (*e.g.* coronatina, HopI1 y avrRpt2) que pueden manipular la red de señalización para controlar la respuesta inmune y promover la virulencia (interacciones marcadas en marrón ⊣). Las rutas de señalización del SA, JA y ET representan el esqueleto de la red hormonal de señalización de la defensa, junto con otras rutas de señalización hormonal que también interaccionan con el esqueleto. Adaptada de (Pieterse et al., 2009)

Recientes estudios indican que otras hormonas como auxinas, ácido abscísico (ABA) y giberelinas (GAs) están también implicadas en la defensa contra *P. syringae*. La señalización de auxinas aumenta la susceptibilidad a la enfermedad frente a la cepa DC3000 en Arabidopsis (Navarro et al., 2004, 2006; Chen et al., 2007), y el proceso se ve contrarrestado por SA (Wang et al., 2007). También la aplicación de ABA aumenta la susceptibilidad de la planta a la misma cepa (de Torres-Zabala et al., 2007). Por el contrario, las GAs aumentan la resistencia de Arabidopsis a la cepa virulenta de *P. syringae* mediante la degradación de proteínas DELLA. Estas proteínas parecen controlar la respuesta inmune de las plantas mediante la modulación de las respuesta de defensa dependientes de JA y SA (Navarro et al., 2008). A pesar de las evidencias encontradas en la regulación hormonal de la respuesta defensiva contra *Pseudomonas*, todavía quedan por descubrir numerosas interacciones entre las diferentes rutas y probablemente nuevos papeles de las hormonas implicadas hasta la fecha o de otras.

2.2.3.3 La interacción específica Arabidopsis- *Pseudomonas aeruginosa*

Pseudomonas aeruginosa es una bacteria que se encuentra comúnmente en el agua y en el suelo y se caracteriza por su versatilidad, tanto nutricional como ecológica. Es un saprófito que raramente infecta a tejidos sanos pero puede actuar como patógeno oportunista en humanos causando enfermedades nosocomiales (Govan y Deretic, 1996). La efectividad de este organismo para causar infección se debe a su capacidad de producir una gran variedad de factores de virulencia y a la formación de biofilms, que le confieren resistencia contra antibióticos. Los biofilms son ecosistemas microbianos altamente especializados, que se caracterizan por su adhesión a una superficie y la secreción de una matriz extracelular protectora formada por polisacáridos. Normalmente, su formación está regulada por un sistema de QS (Smith y Iglewski, 2003; Costerton et al., 1999). *P. aeruginosa* es también capaz de causar serias infecciones en hospedadores no-mamíferos como insectos

(Jander et al., 2000), nematodos (Mahajan-Miklos et al., 1999; McEwan et al., 2012) y plantas (Rahme et al., 1995; Walker et al., 2004). El modelo Arabidopsis-*P. aeruginosa* fue el primero utilizado para estudiar la patogénesis humana en plantas y dio la primera evidencia de que los factores de virulencia están conservados en la patogénesis de plantas y animales. Además, con este modelo se demostró que *P. aeruginosa* no solo producía síntomas de enfermedad similares a los de bacterias fitopatógenas, sino que también era capaz de multiplicarse rápidamente en el apoplasto y su densidad estaba correlacionada con la severidad de los síntomas de enfermedad producidos en la planta (Rahme et al., 1995, 1997). El modo de infección de *P. aeruginosa* ha sido extensamente estudiado tanto en el tejido foliar (Plotnikova et al., 2000), como en las raíces (Walker et al., 2004). En raíces, se ha visto que la bacteria es capaz de formar biofilms sobre ellas y que compuestos antimicrobianos exudados por las plantas pueden afectar a la formación de biofilms, posiblemente a través de la regulación por el QS (Walker et al., 2004) (Figura 2.6). Debido a que compuestos derivados de plantas han servido para el tratamiento de infecciones microbianas durante siglos, la búsqueda de componentes antimicrobianos en plantas, que atenúen la patogenicidad bacteriana, puede facilitar el descubrimiento de nuevos antibacterianos.

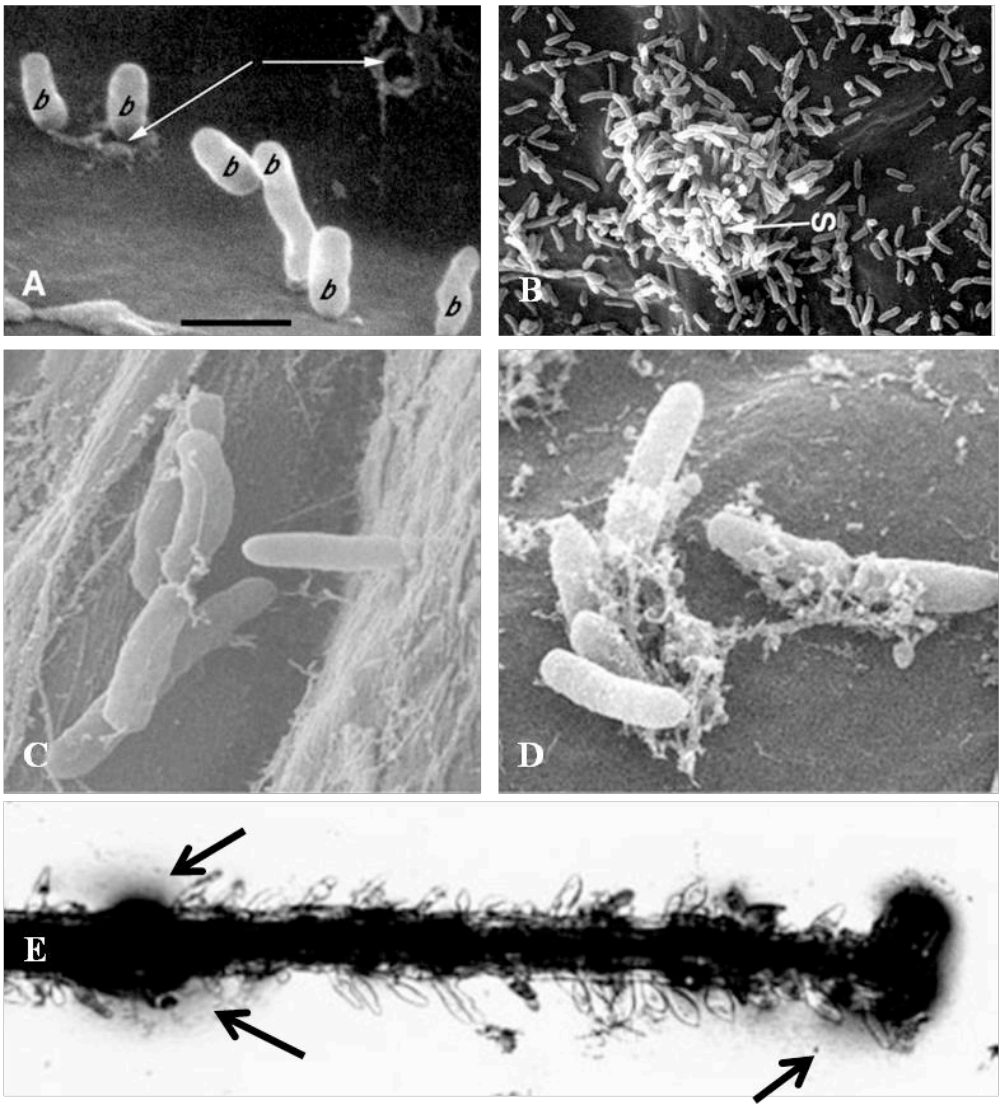


Figura 2.6 Microfotografías de *P. aeruginosa* infectando a Arabidopsis. A. Células de la cepa PA14 atacando la superficie de una hoja. Las flechas indican la digestión de la hoja (b=bacteria; Barra = 1 μm) B. Concentración de células de PA14 alrededor de una apertura estomatal (S=estoma). C. Colonización de raíces de Arabidopisis por células de PA01 D. Células de *P. aeruginosa* embebidas en una matriz extracelular de polisacárido = biofilm. E. Biofilms formados en la superficie de raíces de la planta (las flechas marcan los biofilms). Figura adaptada de (Walker et al., 2004; Prithiviraj et al., 2005).

2.3 Estrés abiótico

Las plantas frecuentemente se encuentran sometidas a condiciones ambientales desfavorables que afectan a su crecimiento y es lo que se conoce como estrés abiótic (Taiz y ZEIGER, 2010). Los factores climáticos como las temperaturas adversas (calor, frío, congelación), sequía (déficit de precipitación o vientos secos) y la contaminación de tierra por altas concentraciones de sal son los principales causantes del estrés abiótico, que limita el crecimiento y desarrollo de las plantas. Este estrés afecta a los cultivos agronómicos y además, juega un papel importante en la determinación de la distribución geográfica de las especies vegetales (Krasensky y Jonak, 2012).

El estrés abiótico puede desorganizar estructuras celulares e impedir funciones fisiológicas clave. La sequía, la salinidad y el estrés por bajas temperaturas imponen en la planta un estrés osmótico que da lugar a una pérdida de turgencia. Durante este proceso las membranas comienzan a desorganizarse, muchas proteínas pierden su actividad o se desnaturalizan y se incrementan los niveles de especies reactivas de oxígeno (ROS) que darán lugar al daño oxidativo. Como consecuencia, en la planta se dan una serie de cambios fisiológicos clave entre los que destacan la inhibición de la fotosíntesis, la disfunción metabólica y daño en las estructuras celulares que contribuyen a la perturbación del crecimiento, la reducción de la fertilidad y la alteración de la senescencia (Krasensky y Jonak, 2012).

Las diferentes especies de plantas son altamente variables con respecto a su ambiente óptimo y una condición adversa que es muy dañina para unas especies, puede no serlo para otras (Munns y Tester, 2008). Este hecho se refleja en la multitud de mecanismos diferentes que utilizan las plantas en respuesta a estrés. Podemos distinguir dos estrategias fundamentales que incluyen evitar el estrés o tolerarlo (Krasensky y Jonak, 2012). La anulación o escape al estrés incluye gran variedad de mecanismos de protección que retrasan o previenen a la planta del impacto a los factores de estrés. Por ejemplo los cactus, que tienen adaptada su

morfología, fisiología y metabolismo a climas áridos. En este caso, la adaptación es estable y hereditaria. Por otro lado, la tolerancia al estrés es la capacidad que tiene la planta para aclimatarse a condiciones de estrés. La aclimatación es un fenómeno plástico y reversible mediante el cual las plantas pueden aumentar su resistencia a varios estreses incluido el salino, la sequía y las altas temperaturas en respuesta a un periodo de exposición gradual a esas condiciones. Por ejemplo, la exposición continua a bajas temperaturas induce el endurecimiento y la aclimatación de las plantas para sobrevivir durante el invierno a temperaturas muy por debajo de cero. Las modificaciones fisiológicas inducidas durante la aclimatación son muy diversas y generalmente se pierden si las condiciones adversas no persisten (Krasensky y Jonak, 2012).

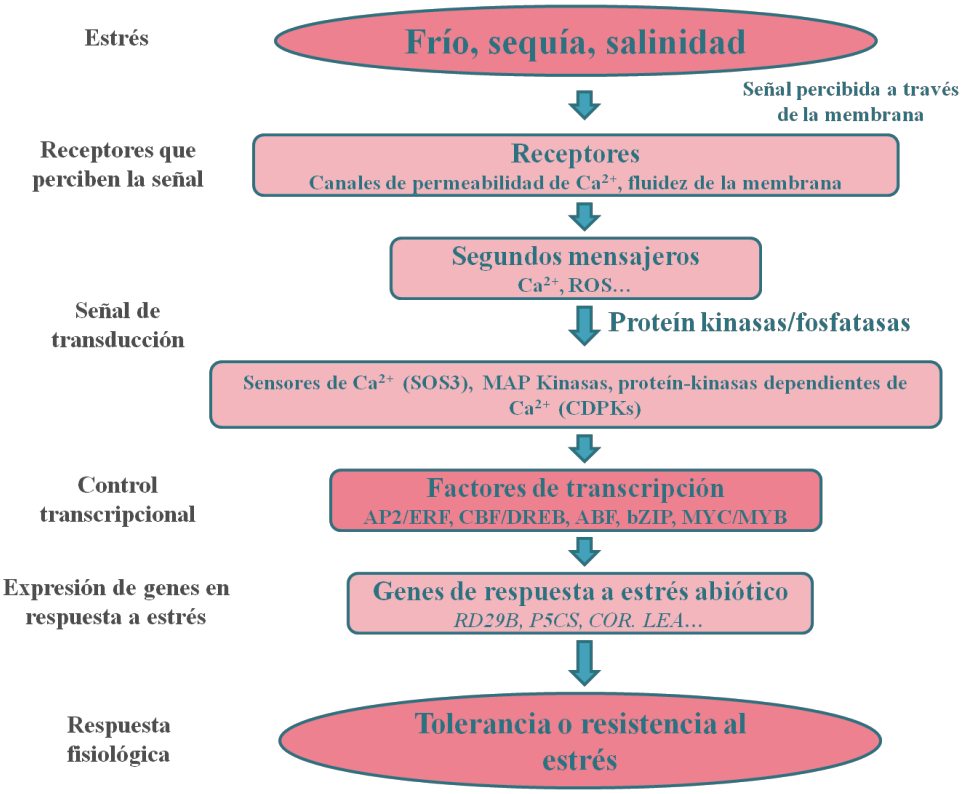
Las respuestas al estrés abiótico ocurren a todos los niveles de organización. Las respuestas celulares al estrés incluyen ajustes en el sistema de membranas, modificaciones en la arquitectura de la pared celular y cambios tanto en la división como en el ciclo celular. Además, las plantas alteran el metabolismo de varias formas, incluyendo la producción de solutos compatibles (*e.g.,*., prolina, rafinosa y glicina betaína). Estos compuestos pueden estabilizar proteínas y estructuras celulares para mantener la turgencia celular mediante ajustes osmóticos, y ajustar el metabolismo redox para eliminar el exceso de ROS y restablecer su balance en la célula (Bartels y Sunkar, 2005; Valliyodan y Nguyen, 2006; Munns y Tester, 2008; Janská et al., 2010; Krasensky y Jonak, 2012). A nivel molecular, la expresión génica está modificada bajo condiciones de estrés (Chinnusamy et al., 2007; Yamaguchi-Shinozaki, 2007) y la regulación epigenética juega un importante papel en la regulación de la expresión génica en respuesta al estrés abiótico (Hauser et al., 2011; Khraiwesh et al., 2012). Los genes inducidos por estrés incluyen genes que participan en la protección directa del estrés como la síntesis de osmoprotectores, encimas detoxificantes y transportadoras, así como genes que codifican proteínas

reguladoras como factores de transcripción (TFs), proteín-kinasas y fosfatasas (Krasensky y Jonak, 2012).

2.3.1 La respuesta general al estrés abiótico

Las plantas hacen frente a los cambios en el ambiente activando rutas de señalización que controlan y coordinan las respuestas fisiológicas y bioquímicas necesarias para la adaptación. Diferentes estreses abióticos dan como resultado efectos generales y específicos en el crecimiento y el desarrollo de la planta. Por ejemplo, la sequía limita el crecimiento de la planta debido a la disminución de la fotosíntesis, al estrés osmótico que le supone y a la disminución de nutrientes disponibles en el suelo durante el déficit hídrico. La salinidad interfiere con el crecimiento de la planta debido a que conduce al estado fisiológico de la sequía y a la intoxicación de iones. El frío, además del efecto que tiene en el metabolismo, también puede causar estrés osmótico. Por tanto, el estrés osmótico y el estrés oxidativo asociado a él parecen ser una consecuencia común de la exposición a la sequía, salinidad y frío. La prevención del estrés osmótico causado por sequía depende de la minimización de la pérdida de agua por los estomas y la cutícula y de maximizar la toma de agua a través del crecimiento de las raíces y el ajuste osmótico. Durante el estrés por sal, el ajuste osmótico parece tener como principal función el mantenimiento de la homeostasis, mientras que la supervivencia durante el estrés osmótico inducido por congelación parece depender de la formación o retraso de núcleos de hielo en las células. Como parte de las respuestas a estrés, la regulación de la expresión génica implica cambios transcripcionales universales o cambios específicos para ciertos genes de la planta (Shinozaki y Yamaguchi-Shinozaki, 2000). Basándonos en la presencia de estos mecanismos de tolerancia general y específicos es lógico esperar que las plantas perciban múltiples estreses y activen diferentes rutas de señalización dándose interconexiones entre ellas a varios niveles (Chinnusamy et al., 2007; Huang et al., 2012).

Existe una ruta de transducción común que señaliza la respuesta a los diferentes estreses osmóticos y que comienza con la percepción del estrés (Figura 2.7). La membrana plasmática juega un importante papel en la percepción y transmisión de señales externas en respuesta a los diferentes estreses. Directa o indirectamente, percibe el estímulo y comienza la ruta de transducción de la señal. Al parecer, los estreses abióticos pueden ser detectados a través de las propiedades físicas de las membranas. Por ejemplo, por su composición de lípidos o ácidos grasos (López-Pérez et al., 2009; Huang et al., 2012). La señal es transmitida por segundos mensajeros como el calcio (Ca^{2+}), especies reactivas de oxígeno (ROS) e inositol-tri-fosfato (IP_3), que modulan los niveles intracelulares de Ca^{2+}. La perturbación de los niveles de Ca^{2+} citosólico es detectada por proteínas de unión a calcio conocidas como detectores o sensores de Ca^{2+} (*e.g.* calmodulina, CaM; proteína altamente sensible a sal 3, *salt overlay sensitive*, SOS3). Estos detectores aparentemente carecen de actividad enzimática pero se sabe que cambian su conformación de una forma dependiente de Ca^{2+} e interactúan en pareja con otras proteínas. Muchas veces esta interacción inicia una cascada de fosforilación, que a su vez modula la mayoría de genes de respuesta a estrés o a los factores de transcripción que controlan esos genes. Por último, los productos de los genes de respuesta a estrés permiten la adaptación o tolerancia de la planta al estrés y le ayudan a sobrevivir o soportar las condiciones desfavorables. De esta forma, las plantas responden al estrés como células individuales y de forma sinérgica como un organismo entero (Figura 2.7). Los cambios en la expresión génica inducidos con el estrés pueden dar lugar a la biosíntesis de hormonas como el ABA y el ET, entre otras. Estas moléculas pueden amplificar la señal inicial e iniciar una segunda ronda de señalización que puede seguir la misma ruta o complementarse con otros componentes de rutas de señalización diferentes (Mahajan y Tuteja, 2005; Xiong et al., 2002; Shao et al., 2007; Huang et al., 2012).



2.7 Ruta general de la respuesta al estrés abiótico en las plantas. La señal de estrés se percibe por los receptores en la membrana y luego se activa una amplia y compleja cascada de señalización intracelular. La cascada de señalización da como resultado la expresión de múltiples genes de respuesta a estrés que codifican proteínas que darán lugar a la tolerancia del estrés directa o indirectamente. Figura adaptada de (Huang et al., 2012)

2.3.2 El papel del ABA en la tolerancia a estreses abióticos

El ABA juega un papel crucial en la respuesta a varias señales de estrés. La sequía, el estrés salino y, en cierta medida, el estrés por frío causan un aumento en la síntesis y acumulación de ABA, que rápidamente es utilizado para ayudar a la planta a enfrentarse al estrés (Xiong et al., 2002). La aplicación de ABA exógeno en las plantas mimetiza el efecto de las condiciones de estrés. Al igual que otros estreses osmóticos, resulta en la desecación celular y la pérdida del balance osmótico. Existe

una superposición de los patrones de expresión de genes marcadores del estrés por frío, sequía, elevada salinidad y tratamientos con ABA. Esto sugiere que varias señales de estrés y el ABA comparten elementos comunes en sus rutas de señalización y que esos mecanismos interaccionan unos con otros para mantener la homeostasis celular (Finkelstein et al., 2002).

La principal función del ABA frente al estrés osmótico producido por sequía o salinidad parece ser la regulación del balance hídrico y osmótico para tolerar el estrés. Así, en tejidos vegetativos el estrés produce un aumento de ABA y da lugar a una variedad de respuestas adaptativas mediadas por ABA como el cierre estomático y la expresión diferencial de genes (Finkelstein et al., 2002; Nambara y Marion-Poll, 2005). El cierre de estomas está regulado por la señalización del ABA en células guarda y ocurre a través de cambios rápidos de flujos iónicos y osmorregulación (Schroeder et al., 2001; Hetherington y Woodward, 2003). Las células guarda se encuentran alrededor de los poros estomáticos en la epidermis y son esenciales para la entrada de CO_2 en las hojas durante la fotosíntesis. En este intercambio de gases con el medio, la planta pierde aproximadamente el 95% de su agua a través de la transpiración. La regulación de las células guarda integra estímulos hormonales, señales de luz, estado de la disponibilidad de agua y CO_2, temperatura y otras condiciones ambientales para modular las aperturas estomatales y así regular el intercambio de gases y la supervivencia de la planta frente a condiciones adversas (Schroeder et al., 2001). Básicamente, lo que ocurre a nivel molecular es que el aumento de los niveles de ABA tras el déficit hídrico provoca un incremento en la concentración de Ca^{2+} citosólico que da lugar a la activación de canales iónicos de membrana (Hamilton et al., 2000; Köhler y Blatt, 2002). Esto conduce a una despolarización de las células guarda, salida de potasio (K^+), pérdida de turgencia y volumen de las células guarda, y finalmente, el cierre de los estomas (Blatt, 2000). Adicionalmente, el ABA también genera un aumento en la producción de H_2O_2 y NO, que actúan como intermediarios promoviendo el cierre estomático (Pei et al.,

2000; Zhang et al., 2001; Garcia-Mata y Lamattina, 2007; Ribiero et al., 2009). La regulación del flujo de transpiración mediada por ABA a través de los estomas es una respuesta crucial de la planta al déficit hídrico. Así, mutantes insensibles a ABA (*abi1* o *abi2*) o deficientes en ABA (*aba1* o *aba2*) presentan un fenotipo de marchitamiento en respuesta a la sequía y cuando el estrés se prolonga, mueren (Zeevaart y Creelman, 1988). Adicionalmente, la ruta de señalización dependiente de ABA regula la expresión génica inducida por estrés dando lugar a la remodelación coordinada de la expresión génica que afecta a más de 1000 genes del transcriptoma de la planta (Hoth et al., 2002; Seki et al., 2002; Takahashi et al., 2004).

2.3.3 Regulación del estrés abiótico a través de factores transcripcionales

Las plantas adquieren resistencia al estrés ambiental mediante la reprogramación del metabolismo y la expresión génica con el objetivo de conseguir un nuevo equilibrio entre el crecimiento, el desarrollo y la supervivencia. En las últimas dos décadas, se han conseguido importantes avances para entender los cambios transcripcionales inducidos por las limitaciones del medioambiente y la identificación de proteínas de señalización y de factores transcripcionales que regulen la expresión génica regulada por estrés. Los resultados muestran un complejo proceso constituido por varias rutas que comienzan en la percepción del estrés y terminan en cambios transcripcionales específicos (Yamaguchi-Shinozaki y Shinozaki, 2006).

Los TFs desempeñan un papel central en la expresión diferencial de genes regulando, como elementos *trans*, la expresión génica aguas abajo a través de la unión específica a elementos *cis* en los promotores de los genes diana. Analizando los promotores de genes de respuesta a estrés, se han identificado muchos elementos *cis* y *trans* implicados en estas respuestas transcripcionales (Yamaguchi-Shinozaki y Shinozaki, 2006) (Figura 2.7).

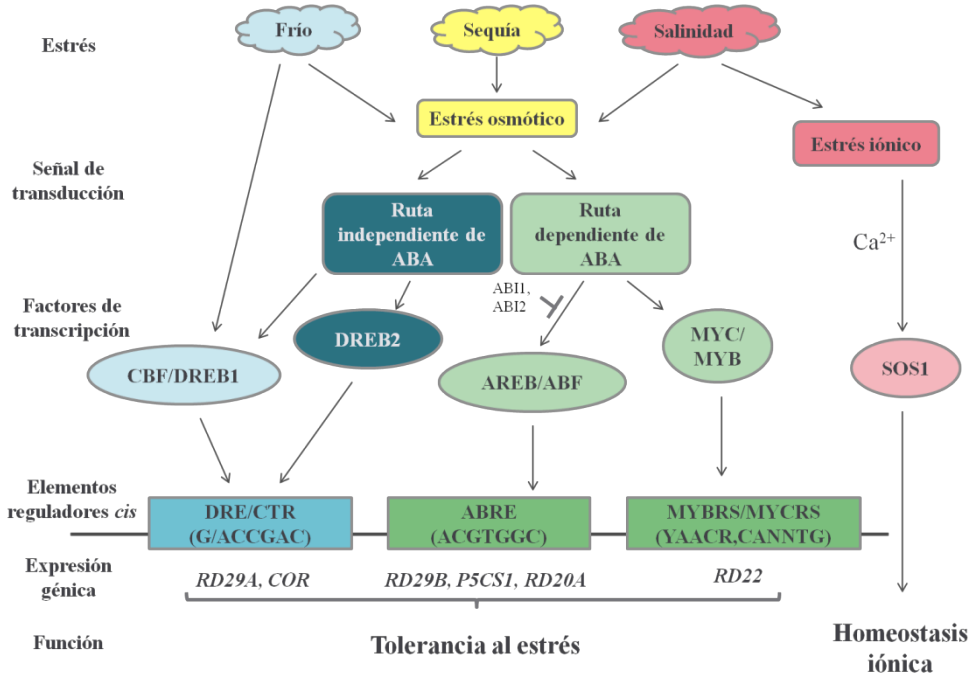


Figura 2.8 Entramado de regulación transcripcional en respuesta al componente osmótico del estrés abiótico con los elementos *cis* y los factores de transcripción independientes y dependientes del ABA. Adaptado de (Huang et al., 2012).

La sequía induce la acumulación endógena de ABA, que desempeña un papel muy importante en la tolerancia al estrés osmótico (sequía y elevada salinidad). Tanto el aumento en los niveles de ABA, como la aplicación exógena de ABA induce genes que responden a la deshidratación. Sin embargo, también se han descrito genes que se inducen por deshidratación pero que no responden al tratamiento exógeno de ABA. Esto sugiere la existencia de cascadas de de transducción de la señal dependientes e independientes de ABA entre el estrés inicial y la expresión de genes específicos (Huang et al., 2012). Existen al menos cuatro rutas independientes de regulación génica en respuesta al estrés por déficit hídrico, dos de ellas son ABA-independientes y las otros ABA-dependientes (Figura 2.7).

Los TFs se agrupan en distintas familias y en plantas se han encontrado hasta la fecha más de 50 (Yamaguchi-Shinozaki y Shinozaki, 2006). Entre estas familias

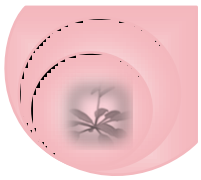
una de las más relevantes es la AP2/EREBP implicada en la señalización en respuesta al estrés osmótico independiente de ABA. Varios de estos TFs pertenecen a la subfamilia DREB/CBF que juega un importante papel en tolerancia al estrés mediante la regulación de genes inducibles por estrés (RD29A, COR78 y LTI78, entre otros) a través de su unión a la secuencia promotora *cis-acting dehydration-responsive element/C-repeat* (DRE/CRT) (Yamaguchi-Shinozaki y Shinozaki, 2006). También algunos miembros que pertenecen a la subfamilia ERF (*Ethylene Responsive Factor*) están implicados en la respuesta a sequía y salinidad (Fujimoto et al., 2000; Park et al., 2001; Seo et al., 2010) y confieren tolerancia a estos estreses cuando se sobreexpresan en plantas transgénicas (Park et al., 2001; Seo et al., 2010). Algunos de ellos, como AtERF1 o AtERF4 pueden unirse al elemento DRE/CRT (Yang et al., 2009). Estudios a escala genómica sobre el análisis de genes AP2/EREBP en álamo (Zhuang et al., 2008), soja (Zhang et al., 2008), tomate (Sharma et al., 2010) y arroz (Sharoni et al., 2011) han identificado muchos genes de la subfamilia ERF que se inducen por altas o bajas temperaturas, deshidratación o elevada salinidad. A pesar de que la función de los TFs ERF en respuesta a estrés abiótico es prácticamente desconocida, se espera que estén implicados en la regulación bajo condiciones adversas de forma tanto dependiente como independiente del ET (Mizoi et al., 2012). Recientemente, se ha descubierto que el estrés osmótico provoca la detención del ciclo celular y el ET es muy importante en este proceso. Cuando las plantas de Arabidopsis se exponen a niveles osmóticos medios (no letales), la producción de ET da lugar a la activación de genes implicados en su señalización, incluidos ATERF-1 (subgrupo B-1) ERF1, ERF2, ERF5 y ERF6 (subgrupo B-3). Aunque en este proceso los genes aguas abajo de estos ERFs sean desconocidos, esta observación sugiere un importante papel de los ERFs en el contexto de adaptación de las plantas a condiciones de estrés abiótico (Mizoi et al., 2012).

En la señalización dependiente de ABA se activan genes como *RD29A, RD29B, P5CS, RD19* o *RD22* a través del elemento *cis* denominado ABRE. Los factores de transcripción de tipo *bZIP ABRE-binding protein/ABRE-binding factor* (AREB/EBF) se unen a la caja ABRE y activan la expresión génica dependiente de ABA (Choi et al., 2000; Uno et al., 2000). Recientemente, se ha demostrado que los TFs MYC/MYB también actúan como reguladores transcripcionales activando sistemas dependientes de ABA (Abe et al., 2003; Valliyodan y Nguyen, 2006).

El llamado *crosstalk* entre las rutas dependientes e independientes de ABA se ha analizado genética y molecularmente y se ha visto que la interacción ocurre a través de los diferentes elementos *cis* (Figura 2.7). Así, muchos genes inducidos por sequía contienen en su promotor tanto el motivo DRE/CTR como el ABRE y se cree que funcionan independientemente (Huang et al., 2012). Sin embargo, al analizar a fondo esos elementos *cis* en la expresión génica de *RD29A* se ha visto que DRE/CTR funciona en cooperación con ABRE como un elemento de anclaje en la expresión génica de respuesta a sequía y dependiente de ABA (Narusaka et al., 2003). Esto indica que las interacciones entre las diferentes maquinarias de trasncripción funcionan para proporcionar interconexiones entre las diferentes rutas de señalización del estrés que le permiten a la planta responder y adaptarse a las condiciones adversas de una forma más eficiente (Huang et al., 2012).

2.3.4 El estrés abiótico en la germinación de semillas

En condiciones ambientales normales, las semillas tienen que superar estreses abióticos para asegurarse la supervivencia en la siguiente generación. La capacidad de tolerar y resistir al estrés osmótico, salino, desecación y frío durante la germinación es muy importante para la supervivencia de las plantas. La toma de agua es esencial durante la germinación, especialmente en la imbibición de las semillas, donde la absorción de agua es clave para el establecimiento de la radícula. Por tanto, es obvio que la germinación *sensu stricto* de las semillas no puede ocurrir



en ausencia de agua. Cuando el estrés abiótico ocurre durante la fase post-germinativa (periodo entre la emergencia radicular y el establecimiento de cotiledones verdes), el proceso de germinación se bloquea. Este proceso está regulado por una red de TFs, entre ellos ABI3 y ABI5 (Finkelstein y Lynch, 2000; Lopez-Molina et al., 2001; Finch-Savage y Leubner-Metzger, 2006; Hilhorst et al., 2007). El mantenimiento o el cese del periodo de bloqueo post-germinativo para completar la germinación está regulado por diferentes rutas metabólicas que incluyen la biosíntesis de hormonas (principalmente ABA), modificaciones de cromatina y regulaciones post-transcripcionales de microRNAs (Daszkowska-Golec, 2011).

Con la finalidad de enfrentarse a la deshidratación y al estrés osmótico, las plantas sintetizan y acumulan metabolitos osmoprotectores que le ayudan a resistir la presión osmótica y mantener la turgencia y el gradiente para la toma del agua. En respuesta al estrés se sintetizan *de novo* dehidrinas, osmotinas y proteínas abundantes durante la embriogénesis tardía (LEA). Las osmotinas y dehidrinas estabilizan la integridad de membranas y proteínas, mientas que las proteínas LEA son capaces de secuestrar iones y agua para proteger los componentes celulares frente al estrés (Hundertmark et al., 2011; Kosova et al., 2007; Hanin et al., 2011).

2.4 Tecnología *phage-display*

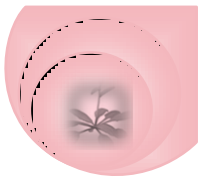
2.4.1 Virus bacteriófagos

Los bacteriófagos, también denominados fagos, son un diverso grupo de virus que infectan células procariotas. Estos virus tienen una estructura muy sencilla en la que básicamente, su material genético (DNA o RNA) está rodeado por una cubierta proteica denominada cápsida (Clark y March, 2006). La mayoría de fagos, exceptuando los filamentosos, tienen una cápsida poliédrica que está conectada a una cola con fibras que le sirven para acoplarse a los receptores específicos de la superficie bacteriana (Ackermann, 1998). Los fagos infectan bacterias y pueden

propagarse mediante un ciclo de vida lítico (por ejemplo T7), en el que los fagos se multiplican vegetativamente y matan a su hospedador, o mediante un ciclo de vida lisogénico (por ejemplo el fago filamentoso M13), en el que los fagos denominados temperados pueden crecer vegetativamente e integrar su genoma en el DNA cromosómico del hospedador y así replicarse durante generaciones (Inal, 2003). Los fagos lisogénicos permanecen en estado de latencia hasta que las condiciones del medio se vuelven adversas, entonces se activan y comienzan la fase lítica para escapar de la bacteria mediante lisis celular (Inal, 2003). Dada la simplicidad de los virus bacteriófagos, tanto estructural como genética, y a su capacidad de replicación en hospedadores bacterianos bajo condiciones de laboratorio, han sido ampliamente utilizados como vectores de clonación en biología molecular (Haq et al., 2012; Bratkovič, 2009).

2.4.2 Bases de la tecnología *phage-display*

Phage-display es un término que describe la expresión heteróloga de péptidos, proteínas o fragmentos de anticuerpos en la superficie de partículas víricas o fagos (Smith, 1985; Winter et al., 1994; Kay et al., 2000). Se describió por primera vez en 1985 cuando se demostró que pequeños péptidos podían ser expresados en la superficie del fago filamentoso M13, como proteínas heterólogas fusionadas a su cápsida, y que partículas víricas individuales podían ser seleccionadas en base a su afinidad de unión por un sustrato determinado (Smith, 1985). El principal foco de interés de esta técnica ha sido la búsqueda y mejora de anticuerpos (McCafferty et al., 1990; Sawyer, 1997; Charlton et al., 2001), sin embargo, el principio se puede aplicar a cualquier proteína o fragmento proteico que pueda ser expresado funcionalmente en *Escherichia coli* (Sternberg y Hoess, 1995). Básicamente, consiste en la incorporación del gen codificante de la proteína o péptido de interés dentro del genoma del fago como una fusión a un gen que codifica una proteína de la cápsida del bacteriófago. Esta fusión asegura que cuando las partículas víricas se



ensamblan, la proteína recombinante que será expresada esté en la superficie del fago maduro y que además la secuencia codificante este contenida en la misma partícula vírica. La proteína de la cápsida sirve únicamente para anclar el péptido expresado heterólogamente y, en teoría, no debería interferir con su estructura. Los elementos clave de esta técnica molecular son: i) la unión física entre genotipo y fenotipo de la proteína expresada, ii) tener un péptido amarrado a una partícula vírica capaz de replicarse a través de una unión peptídica, reteniendo así su estructura/función como si estuviera en una solución. A través de la tecnología del DNA recombinante colecciones de péptidos, variantes proteicas, fragmentos de genes o incluso colecciones de cDNAs pueden clonarse en *phage-display*.

2.4.3 Vectores *phage-display* y sistemas alternativos

Los sistemas *phage-display* se pueden clasificar en no lítico y lítico, según el ciclo de vida del vector elegido. Los sistemas no líticos usan vectores derivados de fagos filamentosos (M13, fl o fd) (Sidhu, 2001; Paschke, 2006). La estrategia más común en este sistema es fusionar la colección de proteínas a expresar al extremo N-terminal del gene *III* de la cápsida (pIII). Sin embargo, fusiones a otras proteínas de la cápsida del fago M13 como pVI, pVII, pVIII y pIX también se han descrito (Kehoe y Kay, 2005). La mayoría de sistemas basados en fagos filamentosos usan fagémidos que son plásmidos que expresan la proteína de fusión de la cápsida con una señal de empaquetamiento y requieren un fago auxiliar que suministre las proteínas silvestres del fago para poder ensamblar el fagémido como una partícula vírica expresando la proteína heteróloga (Kehoe y Kay, 2005; Paschke, 2006). Aunque el genoma de M13 (ap. 5 Kb) es de pequeño tamaño y muy adecuado para la clonación en *phage-display*, existe una limitación en el tamaño de los insertos que puede admitir, ya que el tamaño de la cápsida del fago aumenta proporcionalmente al tamaño del genoma. Para solventar este problema pueden emplearse sistemas de *phage-display* líticos que utilizan otras especies de fagos, tales como Lambda y T7

(Danner y Belasco, 2001; Santini et al., 1998; Zhang, Davis, et al., 2005). En los sistemas líticos, la colección de péptidos o cDNAs se insertan directamente en el genoma de Lamda o T7 y se expresan como una fusión de la proteína de la cápsida. La principal característica de la expresión de proteínas en fagos líticos es que las proteínas heterólogas expresadas en su superficie no necesitan ser secretadas por la membrana del hospedador bacteriano (Krüger y Schroeder, 1981), al contrario que los fagos filamentosos que requieren de este paso para su ensamblaje (Russel, 1991).

Se han desarrollado varios vectores para la expresión de péptidos y proteínas en la superficie del fago T7 (Rosenberg et al., 1996). El genoma de T7 se compone de 39.937 pb en una doble hebra lineal de DNA, contenido dentro de una cápsida icosahédrica formada por el ensamblaje de 60 subunidades repetidas de la proteína 10. Es en esta proteína en la que se realizan las fusiones para la expresión por *phage-display*. Cuando T7 infecta la célula huésped inserta las primeras 850 pb de su genoma en la célula, éstas se transcriben a expensas de la RNA polimerasa del huésped y, como consecuencia, se produce la internalización del genoma completo. Una vez dentro de la célula, los genes para la replicación del DNA y el ensamblaje y maduración de viriones empiezan a expresarse y, en aproximadamente 30 minutos postinfección se forman viriones en el citoplasma celular. Cuando el número de viriones alcanza unos 100, la célula se lisa y los viriones se liberan al exterior para producir una nueva infección. Al contrario que el fago filamentoso M13, el fago T7 es un fago lítico y no necesita atravesar la vía secretora de la bacteria para su extrusión por la membrana. Esto se traduce en la producción de fagos recombinantes con mucha más rapidez que M13. Las partículas víricas son muy robustas y resultan estables en condiciones que inactivan rápidamente otros fagos.

Para el *display* de proteínas se han empleado, además de distintas especies de fagos, fusiones a componentes no fagémicos introducidos en el genoma del fago, por ejemplo, a dominios de unión al DNA de un factor de transcripción humano (Charlton et al., 2001; McGregor y Robins, 2001). También existen sistemas

alternativos como la expresión de péptidos en subunidades ribosomales *in vitro* denominada *ribosome display* (Plückthun, 2012), la expresión de péptidos en la superficie de células eucariotas (Pepper et al., 2008; Gera et al., 2012; Ho y Pastan, 2009), o la expresión de péptidos sobre la superficie de virus eucariotas (Mäkelä y Oker-Blom, 2008; Michelfelder et al., 2007). El poder de la tecnología *phage-display* radica en que conecta de manera funcional fenotipo y genotipo recombinante, permitiendo el coaislamiento simultáneo de una proteína junto a su secuencia codificante.

2.4.4 Selección de péptidos de interés

Mediante *phage-display*, genotecas de cDNAs que representan millones de secuencias nucleotídicas, o incluso un genoma completo (Jacobsson et al., 2003; Faix et al., 2004), pueden convertirse en una población de variantes proteicas expresadas en la superficie de un bacteriófago. Si lo combinamos con la técnica del *biopanning*, en la que fagos individuales pueden seleccionarse por su afinidad de unión a un sustrato/diana determinada, la tecnología se convierte en una poderosa herramienta para la exploración genómica funcional. A lo largo de sucesivas rondas de *biopanning*, lavados, eluciones y amplificaciones la variada genoteca original se va enriqueciendo con los clones capaces de unirse al ligando elegido (Figura 2.9). Dado que tanto el genotipo como el fenotipo de cada proteína está incluido en cada partícula vírica individual, una vez aisladas las proteínas de interés, la secuencia codificante puede ser rápidamente determinada por secuenciación e incluso alterada con la finalidad de refinar las propiedades de unión entre proteína-ligando. Las moléculas utilizadas como ligando pueden ser de naturaleza proteica, como por ejemplo anticuerpos (Zhang, Davis, et al., 2005), o no proteica como ácidos grasos (Gargir et al., 2002), fosfolípidos (Nakai et al., 2005), polisacáridos (Deng et al., 1994), RNAs (Danner y Belasco, 2001) o DNAs (Cicchini et al., 2002). El ligando también puede ser de naturaleza multi-molecular como virus (Lim et al., 2008),

células (Kehoe y Kay, 2005; Zhang et al., 2007), tejidos u órganos (Valadon et al., 2006). Además, la selección por la afinidad del fago a un sustrato puede hacerse tanto *in vitro* como *in vivo* (Li et al., 2006; Valadon et al., 2006). Dada la diversidad de sustratos que se pueden emplear. la tecnología *phage-display* ha tenido un gran impacto en inmunología, biología celular, el descubrimiento de drogas y farmacología y además está ganando importancia en biología de plantas (Chen et al., 2010).

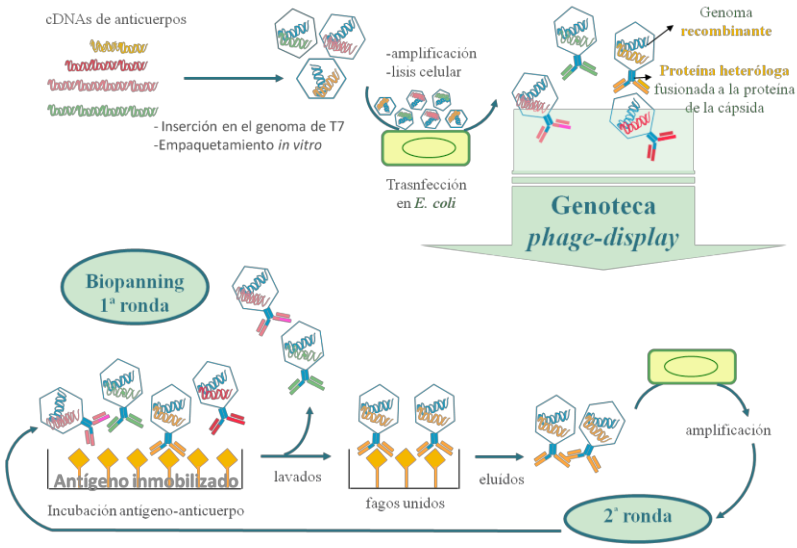


Figura2.9 Construcción de una genoteca *phage-display* y selección por *biopanning*. En este esquema se representa la clonación de una colección de cDNAs de anticuerpos dentro del genoma del fago T7, el empaquetamiento *in vitro* y la amplificación de las partículas víricas mediante la transfección en *E.coli*. Como resultado se obtiene una genoteca *phage-display* de anticuerpos que se incubará contra un antígeno de interés inmovilizado en una placa. Aquellos fagos con afinidad de unión por el antígeno serán recuperados e identificados mediante secuenciación, mientras que el resto se perderán con los lavados.

2.5 Antecedentes y objetivos

Las plantas, debido a su naturaleza sésil, interaccionan continuamente con el medio que las rodea. Esta coevolución con el medio les ha permitido desarrollar diversos y complejos mecanismos para sobrevivir a factores adversos como el ataque de patógenos o las condiciones ambientales desfavorables. El estudio, a nivel molecular, de estas interacciones en organismos modelo como Arabidopsis nos permite conocer en profundidad los mecanismos de respuesta que poseen las plantas frente al estrés tanto biótico como abiótico. Una vez conocidas las estrategias moleculares en especies modelo de investigación, el conocimiento generado puede extrapolarse y aplicarse a otros cultivos de importancia agronómica y mejorar así su producción y rendimiento.

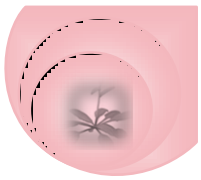
En la respuesta de las plantas frente a los diversos estreses intervienen varias fitohormas, principalmente SA, ET, JA y ABA, que regulan diferentes cascadas de señalización (Pieterse et al., 2009). Sin embargo, se conoce muy poco sobre el *crosstalk* existente entre las diferentes rutas. El estudio del comportamiento de plantas transgénicas y mutantes frente a diversos estreses (tanto bióticos como abióticos) puede ayudarnos a conocer las complejas redes existentes en la señalización hormonal e identificar puntos clave en el entrecruzamiento hormonal.

El sistema Arabidopsis-*Pseudomonas* ha sido ampliamente estudiado desde finales de los años 80, cuando se demostró que *P. syringae* es capaz de infectar Arabidopsis y causar síntomas de enfermedad en condiciones experimentales (Dong, Mindrinos, D, et al., 1991; Whalen et al., 1991). El estudio de esta interacción ha contribuido a entender los mecanismos de reconocimiento de los patógenos por parte de la planta, las rutas de señalización que controlan las respuestas de defensa, la susceptibilidad del hospedador y los factores determinantes de virulencia y avirulencia. Por su parte, la tecnología *phage-display* (Smith, 1985), puede utilizarse como herramienta genética para la expresión de péptidos que representen un genoma

entero (Jacobsson et al., 2003; Faix et al., 2004) y después seleccionar esos péptidos por su afinidad física de unión a un sustrato determinado, incluyendo células enteras (Kehoe y Kay, 2005; Zhang et al., 2007). La expresión del genoma entero de Arabidopsis mediante una estrategia *phage-display* y posterior selección de las proteínas recombinantes capaces de unirse a *Pseudomonas*, podría utilizarse como una herramienta para el cribado de proteínas de Arabidopsis implicadas en el reconocimiento de micoorganismos.

Del mismo modo, la tolerancia a la sequía ha sido ampliamente estudiada debido a que afecta gravemente a la distribución de las plantas y a la producción de cultivos agronómicos. La expresión de genes implicados en la tolerancia o resistencia al estrés abiótico se regula mediante factores de transcripción, que muchas veces participan en diferentes señales de transducción y que por su parte, pueden responder a diferentes estímulos, dando lugar a la interferencia entre las diferentes rutas de señalización. El desarrollo de plantas transgénicas que sobreexpresen genes reguladores de la respuesta a estrés abiótico puede ayudarnos a determinar elementos clave en la tolerancia a un determinado estímulo y profundizar en su regulación usando Arabidopsis como modelo (Kasuga et al., 1999) para después extrapolarlo a diferentes cultivos como tomate (Hsieh et al., 2002) o arroz (Ito et al., 2006).

Con estos antecedentes, el propósito general de este trabajo ha sido profundizar en los mecanismos de respuesta de las plantas frente a diferentes estreses, tanto bióticos como abióticos, estudiando la implicación de diversos factores transcripcionales en la regulación de la expresión génica durante estos procesos. Para ello, se pretenden emplear diversas aproximaciones genéticas y moleculares que nos permitan determinar el papel de los genes y las proteínas seleccionadas en las respuestas a estrés. A lo largo de la experimentación se ha utilizado *Arabidopsis thaliana* como sistema modelo, debido a que las características de esta planta (genoma de pequeño tamaño completamente



secuenciado, amplias colecciones de mutantes y herramientas de genética directa y reversa) facilitan la consecución de los objetivos planteados en este trabajo:

1. Desarrollar una estrategia genética de alto rendimiento para la expresión heteróloga de proteínas de *A thaliana* y la selección de polipéptidos con dominios de unión a microorganismos basada en la tecnología del *phage-display*.

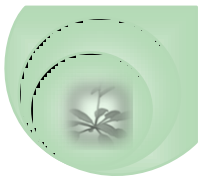
2. Aplicar dicha estrategia para la identificación sistemática de dianas putativas con dominios de unión a microorganismos durante la interacción con especies del género *Pseudomonas*.

3. Confirmar la implicación de alguna de las dianas seleccionadas en la respuesta a estrés biótico *in vivo* como método de validación de la estrategia

4. Estudiar desde un punto de vista fenotípico y molecular plantas transgénicas que sobreexpresan el factor de transcripción ERF1 en respuesta a diferentes estreses abióticos.

5. Analizar funcionalmente el papel de ERF1 en la casacada de señalización en respuesta a estrés mediante ensayos de ganancia de función y profundizar en su interacción con otros factores de transcripción y con el ABA.

La memoria que se expone a continuación recoge en dos capítulos el material, instrumentación y metodología empleados, así como los resultados obtenidos y la discusión de los mismos.



3. CHAPTER 2: Wide Screening of Phage-Displayed Libraries Identifies Immune Targets in Planta

3.1 Introduction

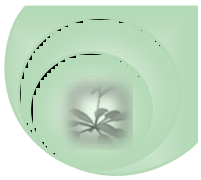
The interactions between plants and micro-organisms in nature are complex and diverse. Microbes can be potential pathogens or beneficial partners, and plants have developed sophisticated mechanism to detect and neutralize them or to make use of their metabolism. Conversely microbes have evolved mechanisms to evade plant immune systems and to use plants as nutritional reservoirs. This co-evolution of plants with micro-organisms has lead to the occurrence of different families of molecules involved in microbial recognition.

Microbe-associated molecular patterns (MAMPs) are structural components of the microbes that can be recognized by the plant and induce pattern-triggered immunity (PTI), the first immune barrier (Chisholm et al., 2006; Jones and Dangl, 2006; Boller and Felix, 2009). MAMPs are conserved within specific microbial families and include diverse molecules such as flagellin, lipopolysaccharide (LPS), fungal chitin or the bacterial EF-Tu elongation factor (Felix et al., 1999; Zeidler et al., 2004; Kunze et al., 2004). Their recognition is also essential for the establishment of beneficial interactions, a process that is coupled to the suppression of PTI (Radutoiu et al., 2003; Van Wees et al., 2008). Plants perceive the different types of MAMPs through specific pattern-recognition receptors (PRRs), the best known of which include LRR (Leucin-Rich Repeat) receptor-like proteins (RLPs) and receptor-like kinases (RLKs) (He et al., 2007). These families of receptors are also found in mammals. In contrast to mammals, plant genomes contain hundreds of genes encoding for RLK and RLP proteins (Shiu and Bleecker, 2003; Eitas and Dangl, 2010). In addition, there are many “orphan” MAMPs that are known to elicit immune response in plants but for which the specific receptors have not yet been discovered. Successful pathogens have also evolved virulence effectors to interfere

with PTI and render the plant susceptible to infection. *Pseudomonas syringae* for example, produces more than 30 different effectors that are secreted upon contact with host plants and target PTI components (Collmer et al., 2000; Chang et al., 2005). As a counterpart, plants have developed corresponding resistance (R) proteins to recognize these effectors and their modified targets, which results in effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI also involves specific families of plant proteins, notably nucleotide-binding-LRR (NB-LRR) proteins (Caplan et al., 2008), which are believed to integrate effector perception and activation of immune-inducible genes through interactions between their modular domains (Takken and Tameling, 2009).

Although PTI and ETI responses trigger different defence mechanisms in plants, the distinction between both types of immunity is not always clear (Tsuda et al., 2009; Thomma et al., 2011). The diversity of MAMPs or virulence effectors that microorganism can display and the multiplicity of the LRR-type receptors that are encoded in plant genomes suggest that a large number of plant proteins could participate in the recognition of bacterial molecules. In this regard, high-throughput protein-interaction screenings are suitable to determine which plant proteins can function as immune receptors for microbial ligands (Thomma et al., 2011; Mukhtar et al., 2011). As an example, by using a yeast two hybrid-based pipeline an interaction network with different pathogen effectors has been created that includes more than 8,000 Arabidopsis proteins (Mukhtar et al., 2011).

Phage display has been used for more than twenty five years as a powerful tool to discover protein-ligand interactions (Smith, 1985; Smothers et al., 2002; Dias-Neto et al., 2009). With this technique, peptides or proteins are functionally displayed on a viral surface as fusions with viral coat proteins, and ligands of interest are used to select for interacting partners. Since the displayed protein and its encoding gene are physically linked in the same viral particle, the identification of selected proteins only requires nucleic acid sequencing. Another key feature of this



technology is that allows for the display of large numbers (up to ~10^{11}) of peptide variants. Individual phage clones are selected from billions of different phage particles on the basis of the binding affinity of their displayed protein for the ligand of choice; selected clones are then amplified and the process iterated to enrich the initial phage population in affinity-binding clones. This so-called bio-panning selection can be manipulated to result in a fine tuning of protein-ligand interaction in the presence of competitive partners.

The possibility of selecting strong protein-ligand interactions between competing partners made phage display a widely-used technology to discover high-affinity antibodies (O'Brien et al., 1999). In addition, the versatility of phage libraries and bio-panning techniques makes the technology suitable for the isolation of a variety of naturally occurring proteins which interact with their physiological ligands. cDNA libraries displayed in phage particles have been used to identify natural protein complexes in a similar way to two-hybrid screening or to discover *in vivo* interactions by injection into living animals and recovery of targeted organs (Pasqualini and Ruoslahti, 1996).

In this study two phage-display libraries were constructed from the cDNA of microbe-challenged Arabidopsis. Recombinant phage displaying plant proteins capable of interacting with different species of *Pseudomonas* were selected by bio-panning using microbial cells as selection ligands. Selected phage were identified by two approaches *i)* sequencing of the dominant clones isolated after bio-panning and *ii)* hybridization of total *vs.* selected cDNAs to Arabidopsis microarrays. The latter was used to compare microbe-binding properties of selected clones on a genome-wide scale. We identified plant proteins involved in defence response and confirmed *in vitro* its capacity to bind microbial cells. The use of different strains of *Pseudomonas* allowed us to discern between common bacterial receptors and specific targets of virulent or avirulent strains.

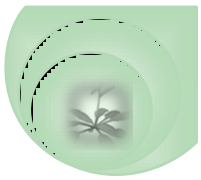
3.2 Materials and Methods

3.2.1 Microbial strains and growth conditions

Pseudomonas aeuriginosa PA14 strain is a hyper-virulent isolate that was kindly provided by Prof. F. Ausubel (Massachusetts General Hospital, Boston, USA). *Pseudomonas syringae pv. tomato* DC3000 strain (*Pst*; wild-type, Rif^r) and the *avrRpt2* strain containing the pV288 plasmid (Pst(avrRpt2)) (Chen et al., 2000) were generous gifts from Dr. Jens Boch (Martin Luther Universitat, Halle, Germany). *Escherichia coli* DH5α (F^- recA Δ*lacU169*(π*80 lacZ*Δ*M15*) *endA hsdR gyrA*) was obtained from Dr. F. Fierro (Universidad de Leon, Leon, Spain) and BLT5103 and BL21 (F^- *ompT gal [dcm][lon] hsdS*$_B$) strains from Novagen. *Agrobacterium tumefaciens* C58C1 strains containing the pGV2260 or the pCH32 plasmid are described in (Deblaere et al., 1985). Unless otherwise noted, bacteria were grown in LB medium supplemented with ampicillin (100 μg/ml), kanamycin, rifampicin or gentamicin (50 μg/ml) when appropriate, at 250rpm and 28ºC (*P. syringae*) or 37ºC (*P. aeruginosa* and *E.coli* strains).

3.2.2 Plant materials

Arabidopsis thaliana accession Columbia-0 (Col-0) is the genetic background used in this work. Arabidopsis seeds were surface-sterilized in 20% bleach and 0.05% Tween-20 for 90 s and washed five times in sterile water before sowing. Seeds were stratified for 3 days at 4°C and then sown on Petri dishes containing Murashige and Skoog medium (MS basal salts, 2-3% (w/v) glucose, 0.6% (w/v) agar pH5.7). Plates were sealed and incubated in a controlled environment growth chamber, under 16-h light/8-h dark cycle. Seven- to ten-day-old seedlings were transferred to individual test-tubes containing 5 ml of liquid MS and incubated in a growth chamber at 90rpm, or sown on pots containing a sterile mixture 3:1 soil-vermiculite and grown in the greenhouse with 16-h light/8-h dark photoperiod.



Transgenic *35S:GFP-ATERF1* seedlings used for microscopic observation were directly sown on 12-well microtiter plates containing liquid MS medium and incubated in a growth chamber at 22ºC and 90 rpm during 10 days. Plants used for the bioassay with *P. syringae* were sown in autoclaved sand and grown in a controlled-environment chamber at 21ºC, 70% relative humidity and 200 µM x m^2/s of cool white fluorescence illumination (10-h light/14-h dark). After 10 days, germinated seedlings were transferred individually to 60-ml pots containing sterile soil-sand mixture (12:5 v/v) (Pieterse et al., 1996). *Nicotiana benthamiana* plants were grown in the greenhouse at 22ºC and 16-h light/8-h dark cycle.

3.2.3 Plant infections with bacterial strains

For infection with *P. aeruginosa*, 25-day-old plants grown in liquid MS were inoculated with OD_{600}=0.02 as previously described (Walker et al., 2004). Infected plants were incubated into a growth chamber at 30ºC and 90 rpm, under long-day light conditions. For infection with *P. syringae* 4-week-old plants growing in pots were infected with OD_{600}=0.002 of bacteria by using the vacuum infiltration procedure (Katagiri et al., 2002). For *aterf-1* bioassays, eight 5-week-old plants were inoculated with OD_{600}=0.0005 from an overnight culture (resuspended in 10mM $MgSO_4$). Bacteria were introduced in 4-5 leaves per plant by pressure infiltration (Katagiri et al., 2002). At 3 days post inoculation (dpi), disease symptom severity was scored and inoculated leaves were collected to determine bacterial titre by homogenizing 2 leaf discs (6 mm diameter) per plant in 400µl of 10mM $MgSO_4$. Serial dilutions of this homogenate were plated on selective KB medium (2% Protease peptone, 0.15% MgSO4-7H_2O, 0.2% KH_2PO_4, 1% Glycerol, 1.2% Agar and 25µg/µl Rifampicin) and incubated for 48 h at room temperature before bacterial colonies were counted.

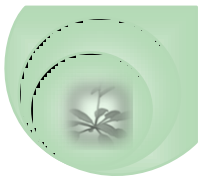
Transgenic *N. benthamiana* plants expressing the histone *H2B* fused to Red Protein Fluorescent (*RFP:H2B)* and the *35S:GFP-ATERF-1* transient-expression

construct were infected with OD_{600}=0.02 of *Pseudomonas* bacteria 3-4 days post-agroinfiltration. Bacterial cultures were centrifuged, resuspended in 10mM $MgSO_4$ and injected with a syringe into the leaves. Boiled bacteria were heated 10 min at 100ºC. After the infection plants were incubated 3 hours into a growth chamber and observed with a confocal microscope.

Transgenic *35S:GFP-ATERF-1* seedlings of *A. thaliana*were grown in liquid medium to avoid any wounding damage prior microscopy and infected with *Pst* using three different concentrations (from 10^2 to 10^4 cfu/ml) of bacteria. Once infected, seedlings were incubated in a growth chamber at 22ºC without shaking. Transgenic roots were observed with a confocal microscope from 1 to 24h after infection.

3.2.4 Construction of T7 -phage-displayed libraries from Arabidopsis cDNA

Plants were infected as described above and frozen in liquid nitrogen at different times post-inoculation (1 h, 3 h, 24 h, 48 h and 3-4 dpi to construct T7LAtPa library and 24 h, 48 h, 3 and 7 dpi for T7LAtPs libraries). For each time point, highly purified, total RNA was isolated from 5 g of frozen plants after homogenization with a micro-dismembrator (Braunn) as described previously (García-Sánchez et al., 2005). mRNA was isolated from 400 µg of pooled RNAs to represent the transcriptional response of the plant during a time-course infection. The cDNA was synthesized from 4 µg of mRNA using OrientExpress cDNA Synthesis Kit (Novagen) and 2 µg of $T_{18}V_3N$ to prime synthesis. A mix 2:1 of MMLV RT (Novagen) and SuperScript III (Invitrogen) was used to synthesize first strand cDNA. Second strand synthesis and end modification were performed as recommended in the manual. End-modified cDNAs were fractionated by gel filtration using the Mini Column Fractionation Kit (Novagen) and higher molecular weight fractions were used for ligation intoT7Select10-3b vector *Eco*RI/*Hin*dIII arms. Different ligations were performed and independently packaged to achieve



optimal vector:insert ratios. Each packaging reaction yielded a different sub-library that was analyzed in order to determine the percentage and the size of the cloned inserts. Final libraries were generated by combining the most representative sub-libraries and scaling the packaging process up.

To calculate the number of primary recombinants, dilutions of the packaging reactions were mixed with *E. coli* BLT5403 and plated on LB + ampicillin plates as described in the T7-Select System Manual. After incubation at 37ºC lysis plaques were counted to calculate phage titres, defined as pfu per unit volume. To determine the percentage of cDNA inserts cloned into the T7Select10-3b vector lysis plaques were transferred to a PCR mix and amplified with PIAG01 (5' AGATTATCGCTAAGTACGC 3') and T7ID (5' GCAAGC$(T)_{18}$ 3') primers; a minimum of 271 (T7LAtPa) or 358 (T7LAtPs) pfu were analysed by this method during the construction of the sub-libraries. Amplification, storage of the libraries and related procedures were performed as recommended by Novagen.

3.2.5 Bio-panning selection

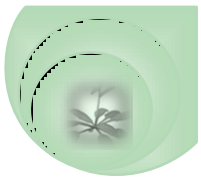
Pseudomonas bacteria were grown to saturation in liquid LB and inoculated (OD_{600}=0.16) into test tubes containing 10-days-old plants grown in 5ml of liquid MS. Plants and bacteria were incubated together for 90 min without shaking at 30 ºC for *P. aeruginosa* or 25ºC for *P. syringae*. Bacteria from 5 plants were recovered by gently rubbing the roots with a 1-ml tip containing MS. MS was then filtered through sterile Whatman paper to remove plant tissues. Bacteria were centrifuged and resuspended in 0.5 ml of the filtered MS before mixing them with 0.5 ml of LB containing $6.3x10^9$ pfu of recombinant phage. Bio-panning was performed for 30 min in 1.5-ml microtubes within a hybridization oven at 25-30ºC and 70rpm. After this time, bacteria were centrifuged 1 min at 13.200 rpm and rinsed by vortexing 1 min in 1 ml TBST (Tris–buffered saline, Tween 0.005%) a total of 5 times. For elution, the bacterial pellet was resuspended by pipetting 3-5 times in 200 µl of

elution buffer (10mM Tris pH7, SDS 1%). 100 µl of this solution were amplified immediately for the next bio-panning round in 50-ml cultures of *E.coli* BLT5403 and 100 µl were preserved in 1 ml of saline mixture (0.5M NaCl) for titrating and further analyzes.

For competitive bio-panning different input mixtures of T7-ATERF-1 or T7-ATUBA1 clones and T7-C1 control phage were prepared to a final concentration of $6.3x10^9$ pfu/ml and panned under the same conditions described above. Input and eluted mixtures were simultaneously titred and replica-analyzed by PCR of 24 to 96 clones from each titration series. The inserts contained in the clones were amplified using PIAG01 and PIAG02 (5' ATAGTTCCTCCTTTCAGC 3') primers to yield a 200-bp band for T7-C1 and a 600-bp or 550-bp band for T7-ATERF-1 or T7-ATUBA1 clones, respectively. 2-3 independent pans were performed from each input mixture to provide the final error estimations. Non-specific binding controls were performed by replacing bacteria with an agarose solution at similar OD_{600}. For the LPS-binding assay, purified LPS from *P. aeruginosa* serotype 10^{22} (SIGMA) was coupled to agarose using ABH (p-Azidodenzoyl hydrazide, Thermo Scientific) as a cross-linker. LPS was cross-linked to a final concentration of 2.5mM, which simulates a living cell. Agarose-coupled LPS was used as the substrate for competitive bio-panning experiments with the T7-ATERF1:T7-C1 input mixtures instead of *Pseudomonas* living cells.

3.2.6 PCR analysis, sequencing and clone rescue

To monitor the enrichment in particular clones during bio-panning a specific PCR-procedure was used. Phage eluted after each round of selection were amplified to $>10^9$ pfu/ml and used to prepare high quality phage DNA by precipitation with 50% PEG 8000 (T7 Select-system Manual) and successive phenol extractions to remove phage capsid proteins. The cDNA amplicons contained in this clone mixture were amplified by PCR [94ºC 2 min, 30 x (94ºC 30 s, 48ºC 30 s, 68ºC 3 min) 68ºC 5



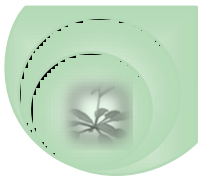
min] with PIAG01 and T7ID primers and Expand high fidelity polymerase (Roche). For individual-pfu analysis phage plaques were directly tipped into the PCR mix and analyzed with Taq (Invitrogen). For sequencing the PCR products were sub-cloned into the pCR2.1 vector (Invitrogen) and submitted to SISTEMAS GENOMICOS as DNA or colony plates when a large number of sequences were required. T7-ATERF-1 and T7-ATUBA1 clones rescued from the plates were amplified from eluates of *Pa* 5B round or *Pst* 3B round. Lysis plaques were picked with a sterile tip and kept in 100µl of Phage Extraction Buffer (PEB, 20 mM Tris-HCl pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$). Aliquots of 1.5 µl were used for PCR amplification with PIAG01 as the common 5' primer and T7-ATERF-1Rvs (5' TCAACAACCTCGCACTTCAC 3') or T7-ATUBA1Rvs (5' AACGTAGGGCAGATGCAGAG 3') as specific 3' primers. Positive clones were amplified from PEB in 2 ml of BLT5403 (OD_{600}=1) until cell lysis was observed and centrifuged at 8000 g for 10 minutes. The supernatant was re-amplified in 20 ml of bacterial culture under similar conditions. Lysates were filtered through a cellulose acetate filter (0.45µm) and kept in 0.5M NaCl.

3.2.7 Labelling of cDNA inserts for microarray hybridization.

To generate microarray probes 100 ng of highly-purified phage DNA was amplified by PCR with the PIAG01 and T7ID primers. PCR was performed in 100 µl with 1.5 units of Expand High Fidelity Plus PCR (Roche) under the following cycles: 94ºC 2 min, 30 x (94ºC 30 s, 48ºC 30 s, 68ºC 3 min) 68ºC 5 min. The product of 5 PCR reactions was purified before labelling with QUIAquick PCR purification columns (Quiagen). Labelling was performed with Alexa 555 or 647 fluorochromes using 4 µg of PCR-amplified DNA and the BioPrime® Plus Array CGH Indirect Genomic Labelling System (Invitrogen).

3.2.8 Microarray hybridization and analysis.

Arabidopsis Genome Oligo Set (AROS) Version 3 microarrays were provided by The University of Arizona. Microarrays were re-hydrated according to the manufacturer's instructions (http://ag.arizona.edu/microarray/methods.html) prior to hybridization. Microarrays were pre-hybridized and washed as previously described (Adie et al., 2007), and hybridized with 60 pmoles of each DNA-incorporated dye after denaturing in 90 ml of hybridization solution (50% formamide, 3X SSC, 1% SDS, 5% Denhard's reagent, 5% dextransulfate). Hybridization was carried out overnight at 42ºC in a Corning hybridization chamber immersed in a water bath. Labelled samples were co-hybridized on the same microarray as follows: T7LAtPa library (L) *versus* 5th bio-panning (5B) round with *Pa* (5BPa*vs*L), T7LAtPs versus 3rd, 2nd or 1^{rst} bio-panning round (3B, 2B or 1B) with *Pst* (3BPs*vs*L, 2BPs*vs*L or 1BPs*vs*L), T7LAPs versus 3rd, 2nd or 1^{rst} bio-panning round with *Pst*(*avrRpt2*) (3BAv*vs*L, 2BAv*vs*L or 1BAv*vs*L). For each B*vs*L comparison 4 replicates microarrays were hybridized swapping the dies of L and B labelled DNAs. Spot signals were captured using a confocal GeneChip scanner (BIO-RAD) and the Vers-Array software. Captured data were lowess-normalised, averaged and statistically analyzed following the workflow for two-colour experiments implemented in the GeneSpring GX Software (Agilent), with L signals used as the control channel for normalization. For significance analysis the GeneSpring *t*-test was used to determinate if the expression values (log B/L centred around 0) for each gene were significantly different from 0. The p-value from the *t*-statistics was computed asymptotically with n=100 permutations. Filtering with volcano plots was performed on p-values ($p<0.1$) and absolute fold change ($>|\pm 1.45|$) of 5B*vs*L for *Pa* bio-panning or 1BvsL, 2BvsL and 3BvsL for *Pst*(*avrRpt2*) and *Pst* bio-pannigs. Corrected p-values were calculated with the Benjamini and Hochberg FDR correction on the minimum set of genes selected after each comparison, ie. 276-gene set for *Pa*; 31, 122 or 9-gene sets (1B, 2B and 3B respectively) for *Pst*(*avrRpt2*) ;



and 171, 142 or 17-gene sets (1B, 2B and 3B respectively) for *Pst*. Supplementary data are shown as exported from GeneSpring GX gene-lists under the corresponding experiment interpretation. Functional categorization was performed with the Gene Ontology tool at TAIR (http://www.arabidopsis.org/tools/bulk/go). Comparisons with the PPIN-1 were performed by importing into GeneSpring a list of 841 genes from *A. thaliana* that produced immune interactions as described in (Mukhtar et al., 2011). Supplemental data is contained in the virtual version.

3.2.9 GFP fusions and *Agrobacterium* infiltration analysis

ATERF-1 (*At4g17500*) full length cDNA was obtained from ABRC (U16643) and translationally fused to the C-terminal region of the GFP gene in the pMDC43 vector (Curtis and Grossniklaus, 2003) by using the GATEWAY technology (Invitrogen). The resulting construct (*35S:GFP-ATERF-1*) was transformed into *A. tumefaciens* C58C1 strain carrying the pGV2260 plasmid, which delivered it into the leaf cells of *RFP:H2B* transgenic *Nicotiana benthamiana*. This transgenic line constitutively expresses red fluorescent protein (RFP) that is targeted to histone 2B in nuclei (Martin et al., 2009). Simultaneous agro-infiltration with the pGV2260 and the pCH32-carrying strains was performed as described (Voinnet et al., 2003) to avoid gene silencing. Basically, *Agrobacterium* cells were resuspended in 10 mM $MgCl_2$, 10 mM MES pH 5.6 and 200 μM acetosyringone and infiltrated in tobacco leaves by using a 10 ml-syringe. After infiltration plants were maintained for 3-4 days in a growth chamber before exposure to *Pseudomonas* strains.

3.2.10 Generation of transgenic Arabidopsis plants

Arabidopsis Col-0 plants were transformed with OD_{600}=0.8 of *Agrobacterium tumefaciens* carrying the construct *35S:GPF-ATERF1* by the floral dip method. Basically, 200ml of the bacterial culture were grown at 28ºC and 250rpm, centrifuged10 minutes at 7000rpm and resuspended into 200ml of transformation

solution (MS medium, 5% sucrose, 4.4 µl BAP (500 µg/µl), 60 µl Silwet L-77). Flowers were dipped into the transformation solution and vacuum-infiltrated twice during 30 sec. Twenty-nine seeds (T_1), from transformed plants (T_0), were selected as transgenic lines on Hygromicin selective MS medium (1% Sucrose, 0.3% Phytagel and 40 µg/ml of Hygromicin) to identify T1 transgenic plants. Approximately 100 of T2 seeds were plated in selective medium to test the segregation ratio. The number of insertions was determined using *chi-square test* with Yates's correction [$\chi^2_{corr}=\Sigma(\Theta\text{-}E\text{-}1/2)^2 / E$] and fixing the limit to accept the hypothesis in $\alpha \leq 0.05$. GFP levels in seedlings were checked as well in the confocal microscope (Table 3.1). Finally, T3 homozygous line number 19, which contained a single insertion and exhibited the stronger and more stable GFP levels in roots, was selected for further studies. Gain-of-function was checked by Real Time-Q-PCR using *ACTIN8* as endogenous control for expression levels and the *ATERF-1* specific primers (qATERF-1-F, 5'-GGAGCTCCGAAGAAGAGGAG-3' and qATERF-1-R, 5'-CACTTCACCGTCAATCCCTTA-3') for the amplification.

Table 3.1 Segregation of transgenic *35S:GFP-ATERF-1* lines from F2, growing in selective medium (Hygromicin). R=resistant, S=sensitive, χ^2=*Chi*^{2}*-test*, alpha≤0.05 (1insertion=1 degree of freedom, 2insertions=2 degree of freedom, 3insertions=3 degre of freedom) and GFP=fluorescence observed.

Line n°	Total	R	S	1 insertion R3:S1 $\chi^2 \leq 3,841$	2 insertions R15:S1 $\chi^2 \leq 5,991$	3 insertions R63:S1 $\chi^2 \leq 7,815$	GFP
1	78	60	18	0,068	34,875	220,953	
2	99	74	25	0,003	57,811	345,993	
4	77	58	19	0,004	41,525	252,618	YES
5	87	68	19	0,310	33,472	219,560	
6	131	112	19	7,148	13,855	134,352	YES
7	132	95	37	0,495	103,184	584,129	YES
8	155	154	1	47,744	7,381	0,356	YES
9	132	122	10	20,455	0,202	27,246	
10	108	101	7	18,778	0,010	13,942	
11	150	141	9	27,876	0,002	16,427	
13	139	108	31	0,405	58,418	375,353	
14	175	168	7	40,048	1,152	5,268	
15	112	92	20	2,679	23,810	182,893	
17	118	115	3	30,554	2,172	0,237	YES
18	150	142	8	29,902	0,087	11,524	YES
19	101	75	26	0,003	62,211	368,374	YES
20	114	93	21	2,292	26,781	199,833	YES
21	102	80	22	0,471	38,277	252,580	YES
22	134	126	8	24,876	0,002	14,181	YES
23	161	153	8	33,393	0,259	10,033	
24	75	66	9	6,084	3,308	46,553	
25	134	118	16	11,502	6,466	87,203	
26	120	98	22	2,500	27,876	208,669	
27	106	85	21	1,258	30,996	217,795	
28	118	92	26	0,407	47,514	308,340	
29	122	98	24	1,574	35,255	248,494	
30	129	106	23	3,165	27,577	211,483	
31	108	88	20	2,086	25,689	191,005	
32	110	90	20	2,376	24,730	186,875	

3.2.11 Fluorescence microscopy

The fluorescence photographs were taken with a Leica SP5 confocal microscope and Bio-Rad Radiance 2100 laser scanning confocal imaging system (LaserSharp v.5 Image software). For GFP and RFP detection, the excitation source was an argon ion laser at 488nm and detection filters between 426-481 nm and 498-554 nm, respectively.

3.2.12 Isolation of the *aterf-1* insertional mutant

Seeds of the *aterf-1* mutant (SALK_036267) were obtained from NASC. Plants homozygous for the T-DNA insertion were confirmed by PCR amplification using a T-DNA specific left border primer (T-DNA SALK Lba_1, 5' TGGTTCACGTAGTGGGCCATCG 3') and a forward *ATERF-1*-specific primer (*ATERF-1*-LPb, 5' CGTCCATCTCATCCGAAAAT 3'). The amplicon confirmed the presence of the T-DNA insertion. The wild type *ATERF-1* locus was identified by PCR amplification using *ATERF-1* specific primers (*ATERF-1*-LPb and *ATERF-1*-RPb, 5' CGTCGGAAGACGAAGAAGAC 3'). Loss-of-function was checked by Real Time-Q-PCR using ACTIN8 as endogenous control for expression levels and the *ATERF1* specific primers (qATERF-1-F and qATERF-1-R) for the amplification.

3.2.13 Subcellular fractionation and Western Blot analysis

Rosette leaves from 4-week-old, *35S:GFP-ATERF1* plants were infiltrated with 0.02 ODs of *Pst* strain, harvested after 3-4 h and frozen in liquid nitrogen. Next, 10 grams of plant material were ground to fine powder by using a mortar and pestle and resuspended in 30 ml of NIB buffer (CelLytic^{TM}PN, SIGMA), 1mM DTT. The suspension was filtered and centrifuged at 1260 *g* for 10 min. Pellets were completely resuspended in 1 ml of NIBA (1x NIB, 1 mM DTT and 1% Protease Inhibitor Cocktail), 0.3% TRITON X-100, and centrifuged at 12000 *g* for 10 min.

The supernatants were collected as the cytosolic fractions whereas pellets were washed in 1 ml of NIBA for crude nuclei preparation. Protein concentration for each fraction was determined by the Bradford Protein Assay (Bio-Rad). Sixty µg of protein were loaded per well onto a 10% SDS-PAGE gel and transferred to an Inmobilon™-P PVDF membrane (Millipore). Membranes were blocked in PBS-T containing 5% ECL Advance Blocking Agent (Amersham) and probed with anti-GFP monoclonal (JL-8) antibodies (Living Colors® A.v., Clontech). To monitor for the cleanness of fractions control blots were performed with antibodies against nuclear H3 histone (Abcam) or cytoplasmic RuBisCo-Large subunit (Agrisera). Membranes were incubated with the corresponding secondary antibodies (ECL-peroxidase, Amersham) and chemi-luminescence signals produced with the ECL Advance Western Blotting Detection Kit (Amersham). Signals were detected with the Intelligent Dark-Box II, LAS-1000 scanning system (Fujifilm).

3.3 RESULTS

3.3.1 Construction of Arabidopsis cDNA libraries for phage-display

Two species of *Pseudomonas* were used to elicit immune response in Arabidopsis. This genus includes very ubiquitous bacteria able to parasite a wide range of hosts. *P. aeruginosa* is typically an opportunistic pathogen of humans which also infects other vertebrates, insects and plants (Rahme et al., 2000), whereas *P. syringae* is a natural pathogen of plants with different host-specific pathovars. Both species share common MAMPs and effectors (Rahme et al., 2000; Prithiviraj et al., 2005) but differ in their adaptation to specific host biology and defence mechanisms (Collmer et al., 2000; Mithani et al., 2011). The library T7LAtPa was constructed with cDNA obtained from plants infected with the PA14 strain of *P. aeruginosa* (*Pa*) (Rahme et al., 1995; Walker et al., 2004). In this infection model, plants were grown in liquid medium and bacteria were inoculated as described by these authors; mRNA was purified at different times post-infection and pooled for cDNA preparation. For the T7LAtPs library plants were grown in soil and infected with *P. syringae* pv. *tomato* by infiltration of bacteria into plant leaves; plants were infiltrated either with the virulent strain DC3000 (*Pst*) or its avirulent variant *Pst*(*avrRpt2*) (Chen et al., 2000) and cDNAs were pooled before the cloning step. In both cases an early defence response was observed in the plant as chlorotic lesions appearing on the infected tissues. The infection with *Pa* or *Pst* strains progressed until plant dead, whereas *Pst(avrRpt2)* only caused a hypersensitive reaction without further damage for the host.

Plant cDNAs were cloned into the T7Select10-3 vector. This system uses the T7-10B capsid protein of lytic phage to display foreign polypeptides of up to 1200 amino acids. cDNAs that are cloned in frame with the 3' end of the T7-10B-encoding gene (about 1/3 of the fusions) can be displayed as recombinant proteins

on the viral surface. The recombinant phage genomes generated after cDNA ligation into the vector were *in vitro* packaged to generate a primary library, which was next transfected into the *E .coli* host to allow for replication and translation of recombinant capsids. Transfected cultures were plated with molten agarose to determine the number of plate forming units (pfu) in the primary library and then amplified to 10^{10}-10^{11} pfu/ml. These amplified suspensions constituted our stock libraries for further biopanning experiments. To estimate the complexity of the libraries we analyzed the inserts contained in a representative fraction of amplified viral clones (Table 3.2). According to this estimation, the T7LATPa library contained at least $7.7x10^4$ different plant transcripts, whereas the T7LATPs library, where the size and the proportion of clones with insert were higher, contained $2.3x10^7$. Considering that only 1/3 of the cloned cDNAs are expected to be in-frame fusions, both libraries cover virtually the entire genome of *A. thaliana* (about $2.5x10^4$ protein-encoding genes). Further hybridizations of the labelled cDNA inserts with Arabidopsis microarrays confirmed detectable signals ($\geq$ 2 x background) for more than 50% of the spotted genes.

Table 3.2 Characteristics of the phage-display libraries constructed for this study. (*) Total number of distinct inserts = Total pfus x inserts (%) X non-redundant inserts (%).

Parameter	Library	
	T7LAtPa	T7LAtPs
Microorganism infected in *A. thaliana*	*P. aeruginosa*	*P. syringae*
Total pfus	7×10^5	6×10^7
Clones with insert	20%	70%
Insert sizes (Kb)	0.2 - 1.5	0.2 - 2.2
Non-redundant inserts	55%	55%
Most redundant insert	5.6%	16%
Total number of distinct cDNA inserts (*)	7.7×10^4	2.3×10^7

3.3.2 Selection of *Pseudomonas*-bound clones by biopanning

Both libraries were panned to select for phage clones displaying candidate targets for MAMPs or virulence effectors. Since some effectors might be expressed by the pathogen only upon contact with the host, bacterial cells were handled as in the root infection model described for PA14 strain (Walker et al., 2004). Bacteria were incubated together with *A. thaliana* plantlets in liquid MS medium and recovered alive from plant surfaces before using them as the ligands for selection. Bio-panning was performed by incubating the amplified phage libraries ($6.3x10^9$ pfu) with these infective cells of *Pa* (T7LAtPa library), *Pst* or *Pst*(*avrRpt2*) strains (T7LAtPs library in both cases). *Pseudomonas*-bound phage were recovered by elution from bacterial pellets and re-amplified to $6.3x10^9$ pfu for successive rounds of selection. Eluates were titred after each round to assess for the enrichment in specific-binding clones (Figure 3.1). Biopanning of the T7LAtPa library with *Pa* cells produced eluates with an initial titre of $1.5x10^5$ pfu/ml (in the first round), which increased up to a maximum of $5x10^7$ pfu/ml in the 5th round. Biopanning of the T7LAtPs library with *Pst* or *Pst*(*avrRpt2*) cells produced titres similar to those of *Pa* in the first round, but reached a maximum more rapidly (after 3 rounds $4.3x10^7$ pfu/ml for *Pst*(*avrRpt2*) and $2.8x10^7$ pfu/ml for *Pst*). However, biopanning with agarose beads as a control for non-specific binding failed to produce high eluate titres, with the number of eluted phage falling down to 10^3 pfu/ml in the 5th round. These data suggested that enrichment in specific-binding clones already happened in rounds 5th (*Pa*) and 3rd (*Pst*(*avrRpt2*) and *Pst*), and additional rounds of amplification and selection were not required.

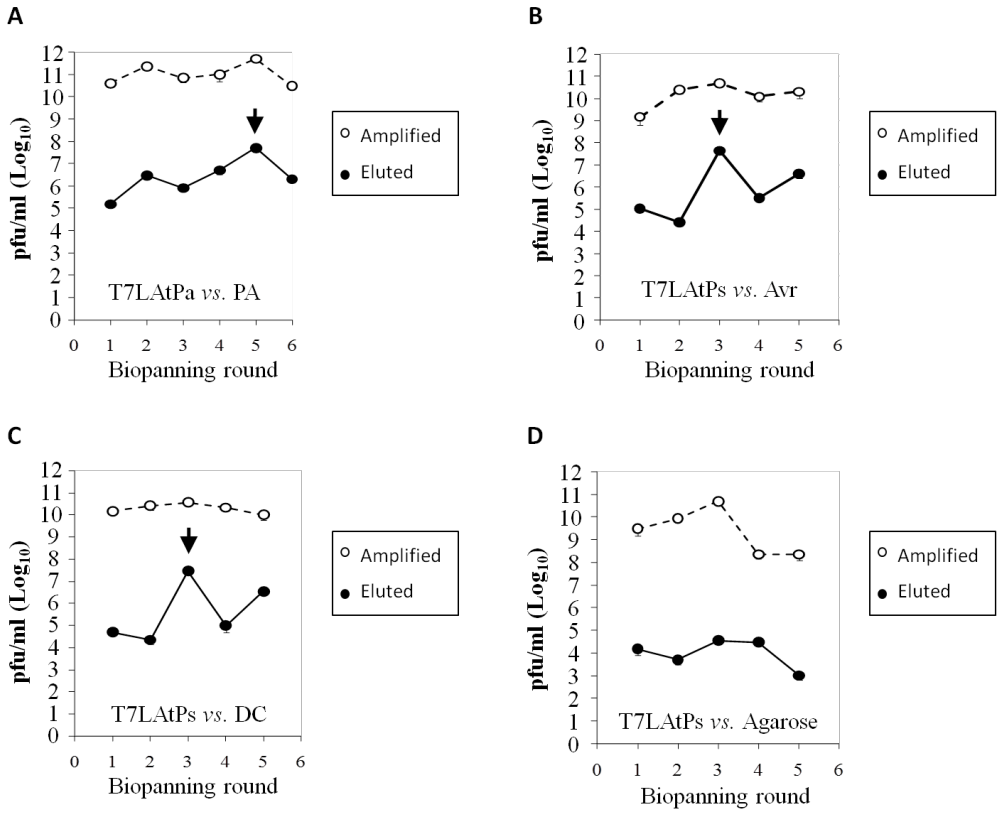


Figure 3.1 Titers of eluates and amplified phages obtained after successive rounds of biopanning. $6.3x10^9$ pfus from T7LAtPa or T7LAtPs libraries were panned against 1 OD of *P. aeruginosa* (A), *P. syringae pto avrRpt2* (B), *P. syringae pto* DC3000 (C) or agarose (D). Bacteria were pre-incubated with 10-days old plants growing in liquid MS, recovered from plant roots and incubated with the phage solution in MS/LB media. After washes, bound-phages were eluted and amplified for the next round of biopanning. The titers of eluted (black dots, continuous line) or amplified (white dots, dashed line) phages were calculated by pfu-counting. The arrows indicate the peak of maximal titer for each biopanning.

3.3.3 Sequence analysis of dominant clones selected by biopanning

The pool of cDNA inserts contained in the eluates from maximum-titre rounds (the 5th round for *Pa* or 3rd round for *Pst* and *Pst*(*avrRpt2*) strains) was PCR-amplified using oligonucleotides in the flanking regions of the T7-10B gene. The cDNA amplicon from the panned eluates was enriched in specific bands when compared to the "smear" from non-panned libraries (not shown). Thus, the increase of the eluate titres after biopanning correlated with enrichment in certain types of cDNA inserts. To identify the dominant clones in the eluates, individual pfu were randomly selected and sequenced. A total of 166 clones from the 3 biopannings were analyzed (Figure 3.2). As expected, most inserts were present as redundant copies and only 27 different sequences were found. The study of their fusion sites resulted in the identification of 10 different polypeptides in frame with the T7-10B minor coat protein. Their description and the fraction of the full-length protein that is fused to T7-10B are shown in Table 3.3. The most abundant clone rescued from the *Pa* biopanning encoded for the defence-related protein ATERF-1 (At4g17500), a member of the AP2/ERF-family of transcription factors which is highly induced upon infection with different pathogens (Fujimoto et al., 2000; Thilmony et al., 2006; On et al., 2002); the ATERF-1 fusion to T7-10B (amino acids 176 to 268) comprises the DNA-binding and defence-related domains. The PSAN subunit of photosystem I (At5g64040) and the anti-silencing protein AtSP7 (At1g66740) were the dominant clones identified from *Pst*(*avrRpt2*) biopanning and both contained significant fragments of the full-length proteins (82% and 83% respectively). The major in-frame clone rescued from *Pst* biopanning encoded for the ubiquitin-activating enzyme ATUBA1 (At2g30110). This protein has a role in defence, since a 15-bp deletion in its C-terminus (*mos5* mutant) is able to revert the constitutive defence response phenotype of *snc1* mutant (Goritschnig et al., 2007). Interestingly,

the fusion with the T7-10B protein in the rescued clone covers the fragment of ATUBA1 that is deleted in *mos5* (amino acids 1040 to 1080).

A

Biopanning substrate	*Pa*	*avr*	*Pst*
Biopanning round	5B	3B	3B
Total pfu in the eluates	$4x10^6$	$8.6x10^7$	$4.8x10^7$
Analyzed clones	56	48	52
Distinct sequences	10	10	7
Non-identified sequences	0	1	7
Distinct T7-10B in-frame fusions	3	5	2

B

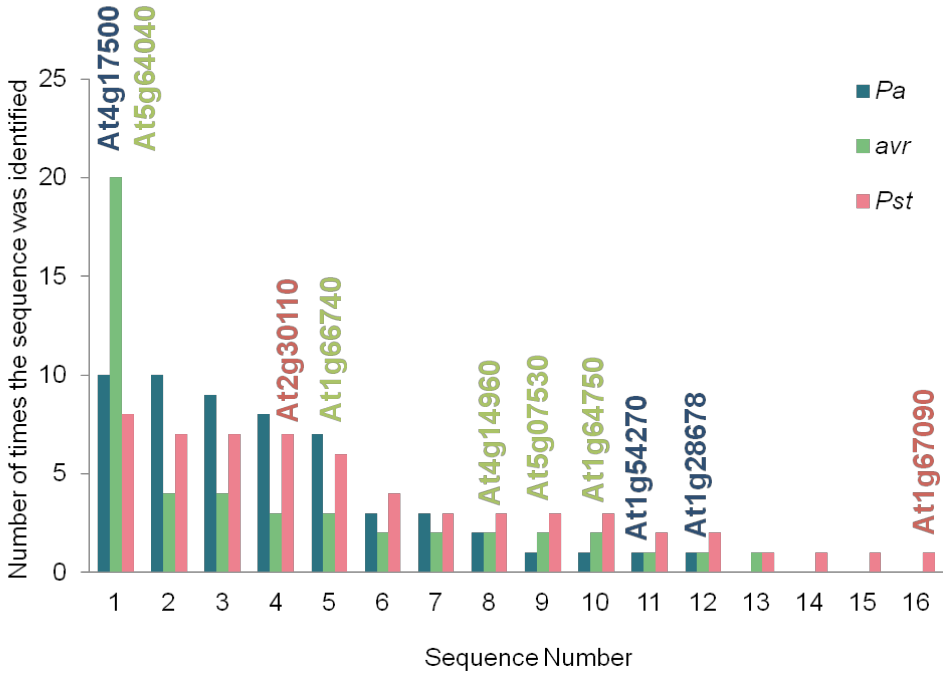


Figure 3.2 Sequence analysis of 166 phage clones eluted after bio-panning with *Pa*, *Pst*(*avrRpt2*) (*avr*) or *Pst* strains. (A) Number of phage clones analyzed from each selection. (B) Bar graph shows the redundancy of the sequences that were identified. The 10 sequences containing T7-10B in-frame fusions are labelled with their TAIR accession on the top of the corresponding bars.

Table 3.3 Sequence analyisis of the 10 in-frame fusions selected after biopanning with Pa, Avr or DC strains of *Pseudomonas sp*.

Biopanning substrate	Gene ID	Description	Aminoacids fussed to T7-10b	Percentage of the full-length	Fre-quency
Pa	At4g17500	ATERF-1; Ethylene Responsive Element Binding Factor 1	176-268	35%	10/56
	At1g54270	EIF4A-2; Eukaryotic Translation Initiation Factor 4A-2	175-412	58%	1/56
	At1g28670	ARAB-1; Carboxylesterase/ Hydrolase, acting on ester bonds	316-384	18%	1/56
Avr	At5g64040	PSAN; Calmodulin Binding	30-171	82%	20/48
	At1g66740	AtSP7; Anti Silencing Protein 7	34-196	83%	3/48
	At1g64750	ATDSS1(I); *Arabidopsis thaliana* Deletion of SUV3 Supressor 1 (I)	40-74	46%	2/48
	At5g07530	GRP17; Glycine Rich Protein 17	161-512	69%	2/48
	At4g14960	TUA6; Structural Constituent of Cytoskeleton	391-427	8%	2/48
DC	At2g30110	ATUBA1; Ubiquitin Activating Enzyme/ubiquitin-protein ligase	1040-1080	4%	7/52
	At1g67090	RBCS1A; Ribulose Bisphosphate Carboxylase Small Chain 1A	101-136	26%	1/52

3.3.4 T7-ATERF-1 clone binds competitively to *Pseudomonas* cells.

To confirm the Pseudomonas-binding capacity of the proteins selected by our biopanning method, we performed competitive biopanning assays for two dominant clones from Table 3.3 (T7-ATERF-1 and T7-ATUBA1). Phage displaying the ATERF-1 polypeptide (T7-ATERF-1 clone) or a competing, non-related peptide (T7-C1 clone), were bio-panned against bacterial cells. The T7-ATERF-1:T7-C1 phage clones were mixed in three different input proportions (1:1, 1:6 and 1:17) and Both phage clones were mixed in three different input ratios containing decreasing proportions of T7-ATERF-1 (55.1%, 16.2% or 5.9% inputs). The input mixtures were biopanned against the three bacterial strains, against agarose-coupled LPS (a common elicitor for the three strains) or against agarose as a control for non-specific binding (Figure 3.3 A). In the four cases after a single round of biopanning the mixture recovered from the eluates contained T7-ATERF-1 as the major clone, representing up to 100% of the rescued phage. In contrast, the proportion of this clone recovered after biopanning against agarose remained similar to the input mixture. Using the minimal input (1:17) as the baseline, the ratio between input and rescued phage was used to assess for the maximal enrichment in T7-ATERF-1 that could be detected after one round of selection. The three strains resulted in significant ($p<0.05$) and more-than-10-fold enrichment. Agarose-coupled LPS produced a maximun enrichment of 5.9-fold,, but statistical analysis determined that the percentage of rescued phage was not significantly different ($p=0.075$ in the best case) from the agarose control. These results support the assertion that the polypeptide displayed in the T7-ATERF-1 clone binds selectively to *Pseudomonas* cells. This binding is likely through a bacterial component common to the three strains, since panning against all of them resulted in a significant increase of the T7-ATERF-1 clone in rescued phage. A similar competition assay was performed for the clone T7-ATUBA1 (Figure 3.3 B), but the maximal enrichment that could be

measured in this case was 1.7-fold. Thus, we focused our next studies in the *in vivo* interactions of ATERF-1 protein during bacterial infection.

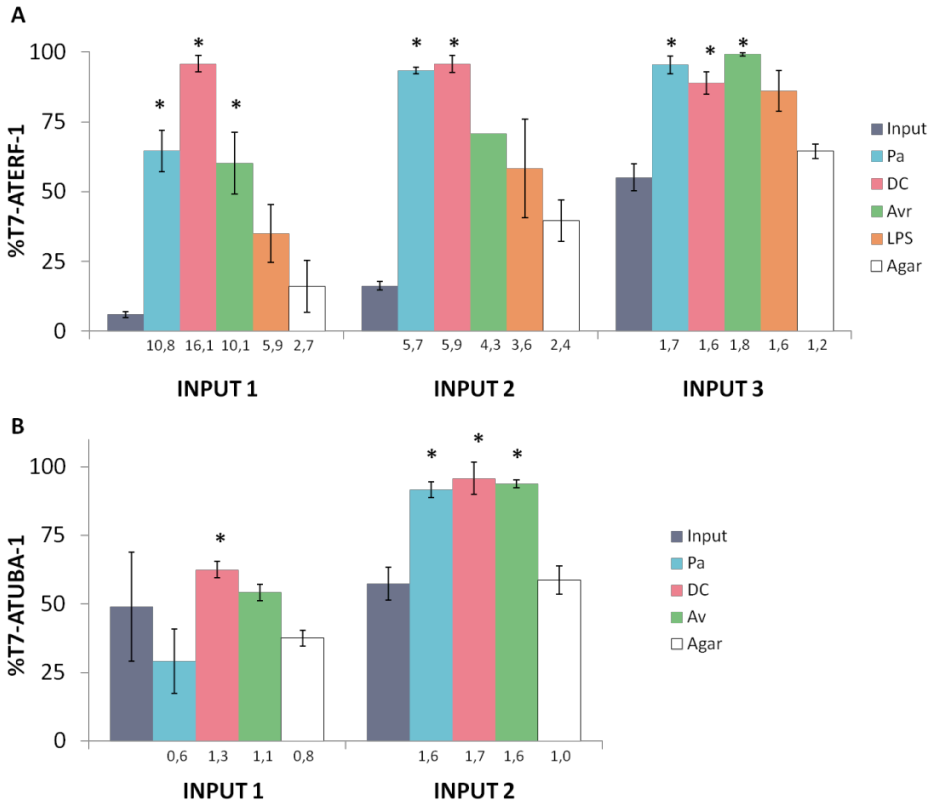


Figure 3.3 Competitive biopanning assay shows specific binding of T7-ATERF-1 clone to the three strains of *Pseudomonas*. (A) Competition between T7-ATERF-1 phage (which expresses ATERF-1 polypeptide) and T7-C1 (competing phage randomly selected). Mixtures of both phage clones containing 55.1% (input 1), 16.2% (input 2) and 5.9% (input 3) of T7-ATERF-1 were prepared and 6.3×10^9 pfu of each mixture were panned against 1 OD of *Pa*, *Pst*, *Pst*(*avrRpt2*) (*avr*), agarose-coupled lipopolysaccharide (LPS) or agarose (Agar). The bar series show the percentage of T7-ATERF-1 clone recovered after each selection round. The numbers below the bars represent the fold enrichment for T7-ATERF-1, calculated as the ratio between eluate and input percentages. (B) Competition between T7-ATUBA1 and T7-C1 phage. Input 1 contains 57.3% of T7-ATUBA1 clone, whereas input 2 contains 48.9%. Asterisks indicate significant differences (*t*-test, $p<0.05$) respect to the agarose (non-specific binding) control.

3.3.5 ATERF-1 is translocated from the nucleus to the cytosol after challenge with *Pseudomonas*.

Since the *in vivo* localization of ATERF-1 is predicted to be nuclear, the binding to bacterial components as was suggested by our competition assay is expected to take place in the nucleus as well. Bacterial MAMPs and effectors are usually perceived by plant receptors located in the plasma-membrane or internalized through the endocytotic pathway to signal pathogen presence from different subcellular localisations (Altenbach and Robatzek, 2007); however, very few nuclear proteins have been involved in bacterial MAMP/effector-binding (Deslandes et al., 2003; Shen et al., 2007). To determine the subcellular localization of ATERF-1, a translational fusion to the Green Fluorescent Protein (*35S:GFP-ATERF-1*) was transformed into *Agrobacterium tumefaciens* C58C1 (pGV2260) and infiltrated into *N. benthamiana* leaves, allowing transient expression of *GFP-ATERF1*. The *RFP-H2B* reporter line, which expresses nuclear Red Fluorescent Protein, was used in this study to visualize co-localization with *GFP-ATERF-1*. As expected, the localization of ATERF-1 in non-treated plants was clearly nuclear (Figure 3.4). However, when leaves were infiltrated with *Pa*, *Pst* or *Pst*(*avrRpt2*) strains, *GFP-ATERF-1* was translocated within 3 h to the cytoplasm. Mock treatment failed to induce cytoplasmic localisation of *GFP-ATERF-1*, which indicates that the translocation is due to the presence of bacteria rather than the damage by wounding. Moreover, infiltration with heat-killed strains also resulted in cytoplasmic localisation (only shown for *Pst*(*avrRpt2*) strain), suggesting that translocation to the cytoplasm does not require metabolically active bacteria.

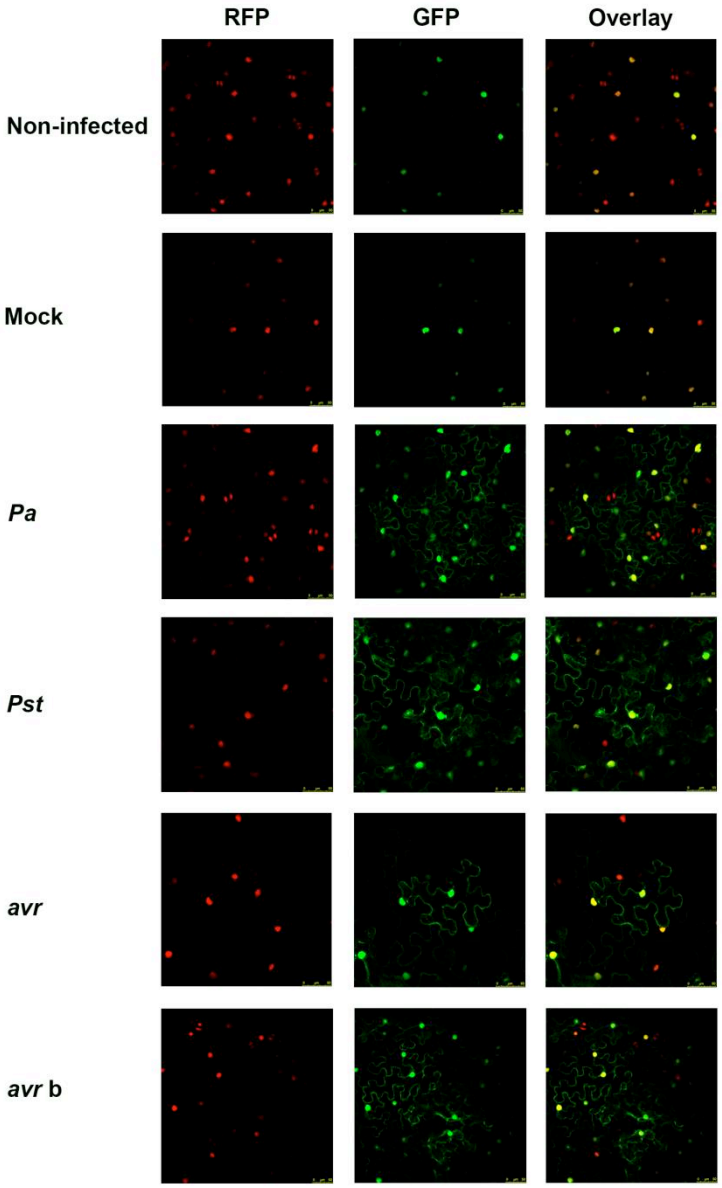


Figure 3. 4 GFP-ATERF-1 localisation after inoculation with *Pseudomonas* strains. Confocal microscopy of *N. benthamiana* leaves over-expressing GFP-ATERF-1 together with a RFP-tagged nuclear marker (RFP-H2B transgenic line). Non-infected control and plants inoculated with an sterile solution of $MgS0_4$ (Mock) or with a bacterial suspension of *Pa*, *Pst*, *Pst*(*avrRpt2*) (*avr*) or boiled *avr* (*avr* b) strains. Photographs were taken 3-5 h. after the inoculation.

To confirm the nuclear translocation of ATERF-1 in Arabidopsis, transgenic plants able to constitutively express the *35S:GFP-ATERF-1* construct were generated. Homozygous T3 lines with a single insertion were selected (Table 3.1) and gain-of-function was confirmed by RT-Q-PCR comparing the *35S:GFP-ATERF-1* with a loss-of-function mutant *aterf1-1* and the wild type (Figure 3.5). GFP fluorescence was observed in the roots from one homozygous T3 line, although no cytoplasmic localization could be imaged upon *Pst* infection (Figure 3.6) under the conditions assayed. The localization of fluorescence was always nuclear, in spite of the nuclear florescence from plants exposed to *Pa* (Figure3.6 D) looked expanded and different to florescence from non infected plants (Figure3.6 C). However, western-blot analysis of subcellular fractions from transgenic plants was successful to show cytoplasmic localisation of GFP-ATERF-1 upon *Pst* infiltration in plants grown under more physiological conditions (Figure 3.7).

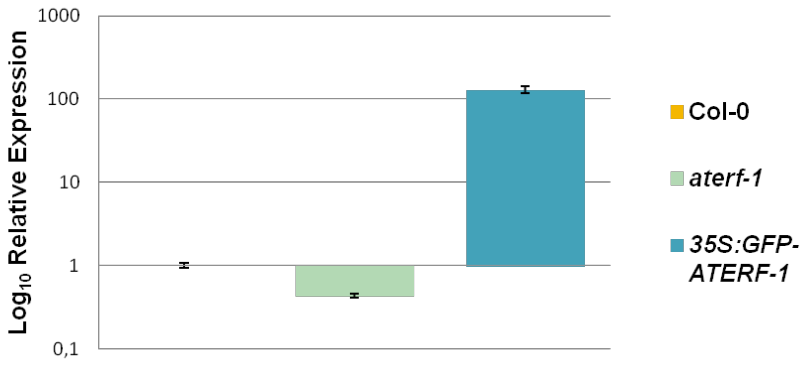


Figure 3.5 *ATERF-1* relative expression levels in lose-of-function plants *aterf1-1* and transgenic plants *35S:ATERF-1* compared with the wild type (Col-0). The expression levels were firstly normalized with the endogenous gene *Actin8* and later calibrated with gene expression levels obtained in Col-0.

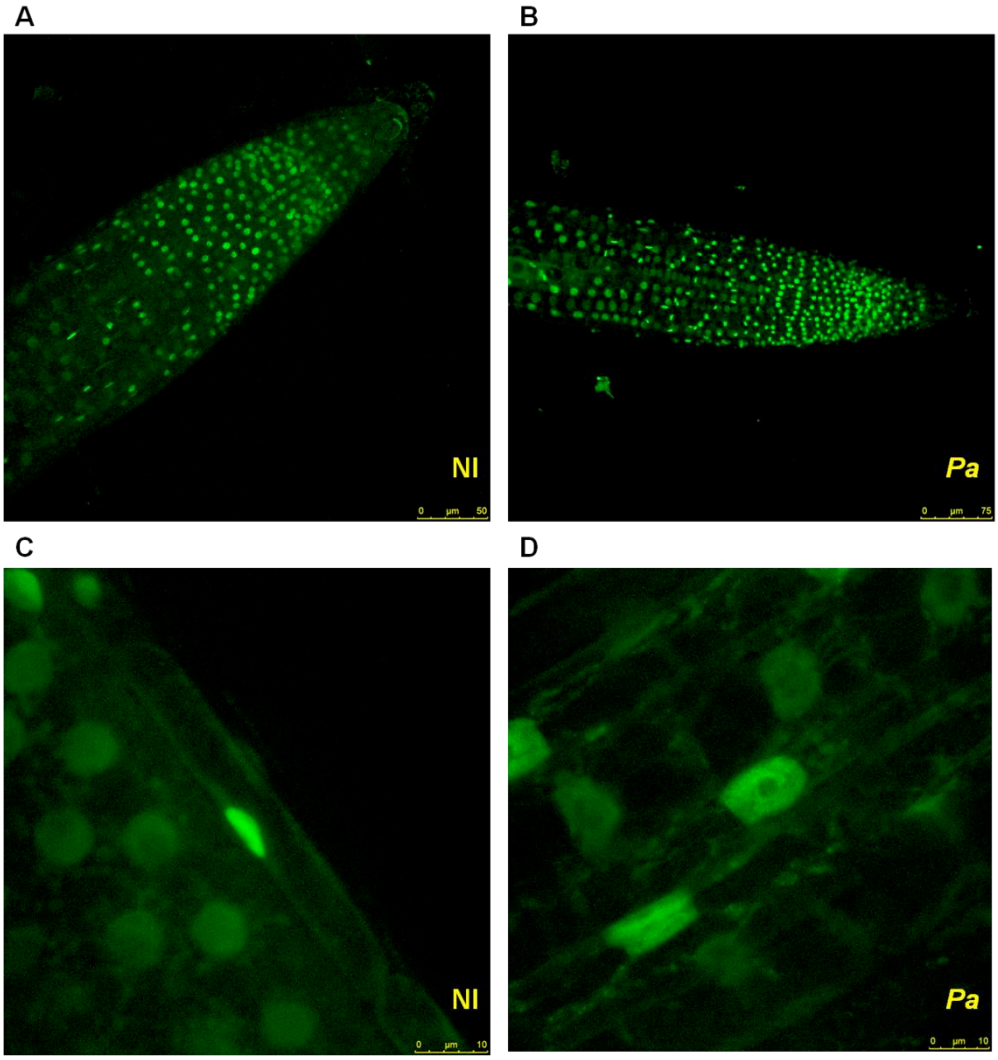
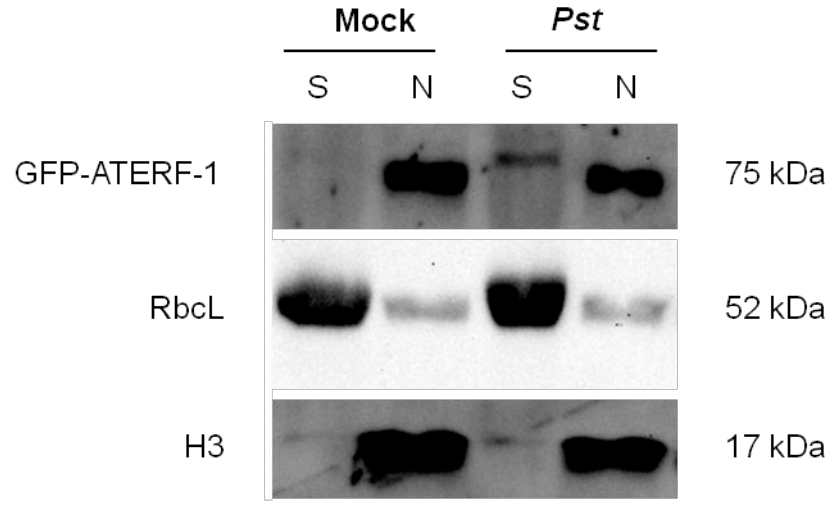


Figure 3.6 Confocal imaging of GFP-ATERF-1 localization in transgenic roots of Arabidopsis. Seedlings growing in liquid medium during 10 days were non-infected (A, C) or infected with 0.0002 ODs of *Pst* (B) or *Pa* (D). Infected seedlings were incubated for 10 hours in a growth chamber without shaking and were mounted and observed in water without previous fixation. Images show the maximal projection from stacks of 10-20 sections.



3.7 Western-blot detection of GFP protein in cytoplasmic and nuclear fractions from *35S:GFP-ATERF-1* transgenic plants of *A. thaliana*. Plants were grown in the greenhouse and leaves were infiltrated with a mock or *Pst* suspension. Protein extracts from the whole plants were separated in nuclear (N) and cytoplasmic (S) fractions. Fractions were resolved in SDS-PAGE gels, blotted and probed against anti GFP antibodies to detect the 75 KDa, GFP-ATERF-1 fusion protein. Membranes were re-probed with anti-H3 histone (H3, nuclear) and anti RuBisCo (RbcL, cytoplasmic) antibodies.

3.3.6 Mutant *aterf-1* shows increased susceptibility to *Pst* infection.

To gain further insight into the role of ATERF-1 in plant defence, we examined the susceptibility phenotype of a lack-of-function, *aterf-1* mutant upon infection with *P. syringae*. Leaves of Col-0 wild-type and *aterf-1* plants were infiltrated with the *Pst* strain and 3 days later disease symptoms and bacterial growth *in planta* were determined. Mutant *aterf-1* displayed more extensive chlorosis after infection compared to Col-0 (Figure 3.8 A,B). In accordance, *in planta* growth of the *Pst* bacteria was significantly higher in *aterf-1* mutant compared to Col-0 plants (Figure 3.8 C). These results demonstrate that the *ATERF-1* gene product is required *in vivo* for a proper defence response to *Pst* infection.

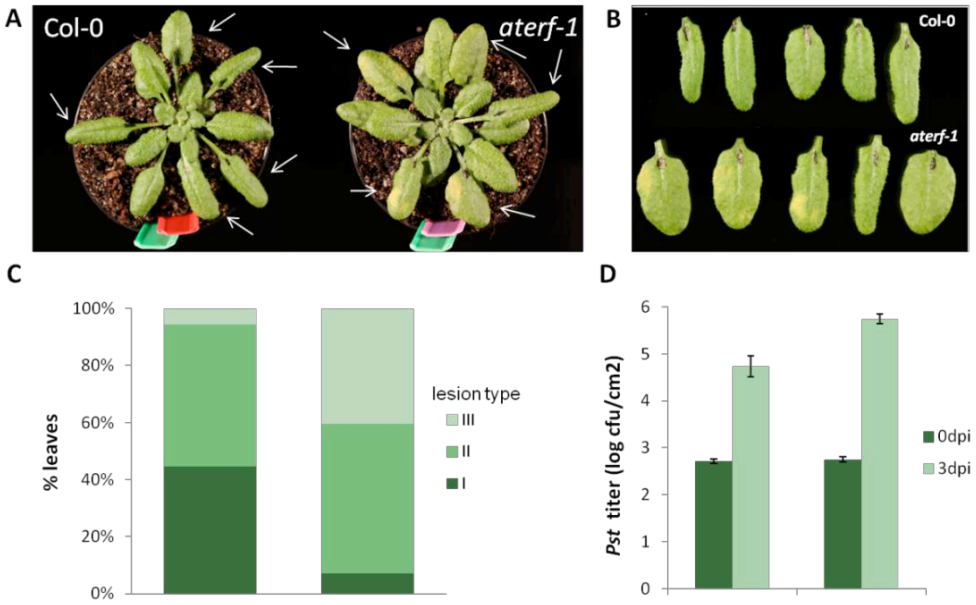
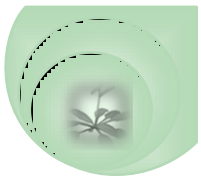


Figure 3.8 *aterf-1* mutant shows increased susceptibility to bacterial infection. (A,B) Macroscopic symptoms of disease caused by inoculation of *Pst* strain into the leaves of Col-0 and *aterf-1* mutant. (C) Disease symptoms (3 days post-inoculation) were rated on 36-42 leaves/genotype (8 plants), with the following disease severity classes: I, 0-10% of leaf surface as a chlorotic lesion, II, 10-50% of leaf surface, and III, >50% of leaf surface. (D) Bacterial titre in *Pst*-inoculated leaves, at 0 and 3 days post-inoculation (dpi). Bars represent the average cfu/cm^2 leaf surface (n=8 plants) and error bars represent SD. Asterisk indicates significant difference between genotypes (Student's *t*-test, $p<0.01$), which was observed in two independent bioassays.

3.3.7 Systematic identification of selected clones by microarrays

Next, we developed a microarray-based strategy to allow systematic identification and quantification of clones selected by biopanning. Clones shown in table 3.3 were easily isolated since they were present at high frequency in the bio-panned eluates, which contained as much as $8.6x10^7$ pfu (Figure 3.2). However, the identification of less frequent but still valuable clones requires large-scale analysis. The isolation of individual pfu and the sequencing of their cDNA inserts is a time-consuming and expensive procedure when large number of clones needs to be



analysed. On the other hand, although redundancy of a clone after biopanning suggests affinity selection, a clone that was already very frequent in the initial library would be also expected at high frequency after a random selection. Therefore, the abundance of a clone before and after biopanning should be compared to determine the enrichment yield after selection.

To this end, we used Arabidopsis, two-colour microarray probes to rapidly quantify the copy number of each cDNA insert before and after biopanning. The inserts contained in the libraries (L) or in the bio-panned eluates (B, rounds 1 to 5) were PCR-amplified, labelled with Alexa 555 or 647 fluorochromes and hybridized to Quiagen AROS Version 3 microarrays. The signals from the T7-LAtPa or T7-LAtPs libraries were used as the reference colour channel and 4 replicas were performed for each B *versus* L comparison in order to provide error estimations for the statistical analysis. Normalized values from all the probes are provided in supplemental datasets S1-S3.

3.3.7.1 Significance analysis of *P. aeruginosa* biopanning

Significance analysis of microarrays was performed with GeneSpring software using the *t*-test against 0. This analysis provided p- and fold-change values for each gene spotted on the microarray, which are fully listed in S4 dataset. The dataset is represented as a plot in Figure 3.9. To select for genes with maximal fold-change after selection but significant p-values we used a cut-off plus multiple testing correction. Since stringency of multiple testing corrections depends on how many genes are tested, a first cut-off on fold-change ($> |\pm 1.45|$) and p-value (<0.1) was used to reduce the number of tests, and 806 genes were pre-selected for correction (Table 3.4). From here genes with negative fold-change (genes that were selected against during the biopanning) were removed and then False Discovery Rate (FDR)

correction was applied to define a final set of 101 candidates with corrected p<0.05 (S5, S8).

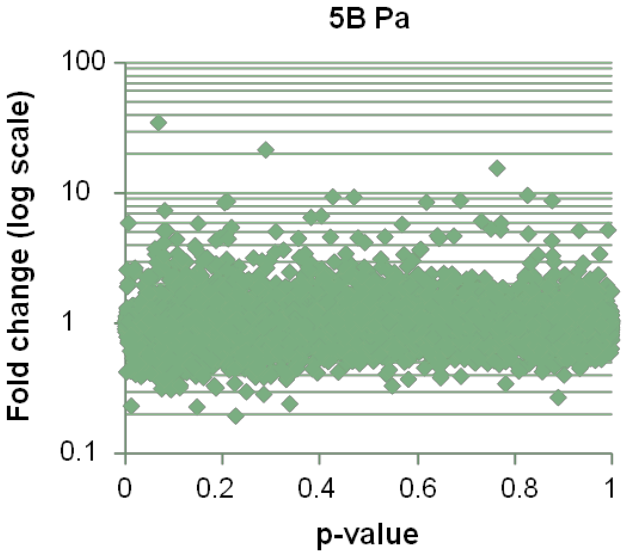


Figure 3.9 Microarray analysis of the *P. aeruginosa* biopanning, significance *vs.* fold-change plot. cDNA from the LAtPa library (L) and the clones selected after biopanning (B) was hybridised to AROS microarrays. For each gene spotted in the microarray the B/L ratio and the statistical significance of B/L≠1 were determined with GeneSpring. The plot represents the B/L ratio as a fold-change in the Y-axis (log values centered around 1) and the p-values for the statistical test in the X-axis. Genes with maximum fold-change and minimum p-value are the best candidates as microbia ligands.

Table 3.4 Microarray selection of three sets of 101 (*Pa*), 153 (*avr*) and 318 (*Pst*) genes with significant fold-change after biopanning.

Bio-panned library	Biopanning substrate	Biopanning round	Genes with FC>1.45 and p<0.1	Genes that pass FDR correction	Significant genes in either of the rounds
T7LAtPa	*Pa*	5B	806	101	101
T7LAtPs	*avr*	1B	121	31	31∪122∪9 = 153
		2B	488	122	
		3B	10	9	
	Pst	1B	531	171	171∪142∪17 = 318
		2B	374	142	
		3B	24	17	
	TOTAL				101∪153∪318 = 472

FC fold change, FDR False Discovery Rate

3.3.7.2 Significance analysis of *P. syringae* biopanning

The fold-change and p-value for each gene were calculated from the *t*-test as described for *P. aeruginosa* microarrays (full list in S4). In this case we observed that in rounds 1 and 2 the genes with highest fold-changes were concentrated around the lowest p-values, whereas in the 3rd round fold-changes were more evenly distributed along the p-value axe (Figure 3.10). In addition, fold-changes in the 1st and 2nd rounds were overall higher than in the 3rd round of selection. Thus, the best candidates (maximal fold-change with the minimal p-value) were already defined in rounds 1 and 2. Consequently, we delimited the final set of candidates taking into account microarray data generated during the 3 rounds of selection. The genes that passed the FDR correction in at least one round of biopanning defined two sets of 153 and 318 genes from *Pst*(*avrRpt2*) and *Pst* strains respectively (Table 3.4 and S6-S8).

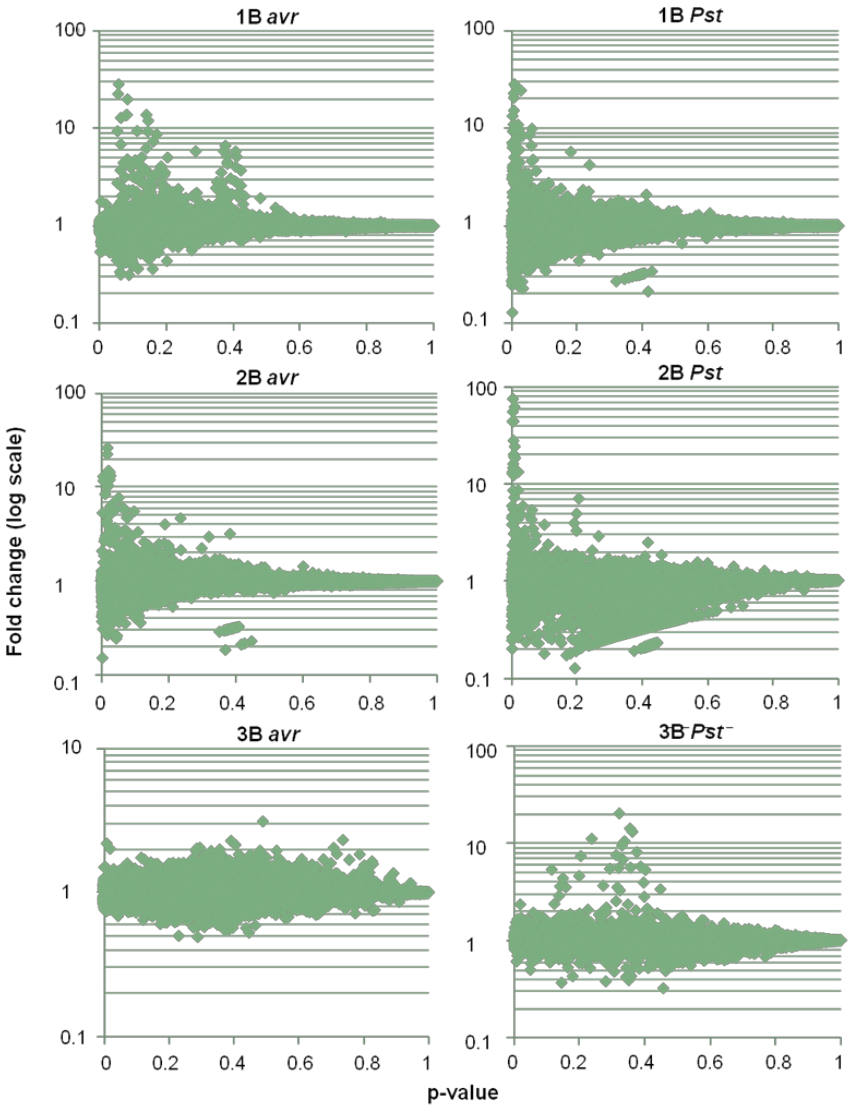


Figure 3.10 Microarray analysis shows significant fold-changes in rounds 1 and 2 of *Pst and Pst(avrRpt2) (avr)* bio-pannings. cDNA from the T7LAtPs library (L) and the clones selected after bio-panning rounds 1, 2 or 3 (1B, 2B or 3B) were hybridized to microarrays. For each gene spotted in the microarray the B/L ratio and the statistical significance of B/L≠1 were determined. The plot represents the B/L ratio as a fold-change in the Y-axis (log values centred around 1) and the p-values for the statistical test in the X-axis. *Pseudomonas*-bound clones should show maximum fold-change with minimum p-values, and are located on the top-left area of the graph.

3.7.3 Overlapping of significant gene sets

The 101-, 153- and 318-gene sets define three groups of phage clones for which copy number was significantly increased after biopanning with *Pa*, *Pst*(*avrRpt2*) and *Pst* strains respectively. The union of the three sets contains 472 genes which represent potential MAMP/effector-interacting proteins. The overlapping among sets is shown in Figure 3.11. As expected, the two strains of *P. syringae* shared a large number of genes (95), whereas *Pa* shared only 5 genes with the *Pst* strain, 2 of them common to *Pst*(*avrRpt2*). Three subsets of 96, 58 and 220 genes remained specific for *Pa*, *Pst*(*avrRpt2*) and *Pst* strains respectively. The genes in each subset are listed in S9-S14. We inspected the lists to identify in-frame clones already sequenced in table 3.2. Since the microarray probe for *ATERF-1* is located at the very N-terminal of the predicted protein, this probe does not cover the fragment that is present in the T7-ATERF-1 clone, and therefore the clone could not be identified by hybridisation to microarrays. However the lists includes *AtSP7* (*At1g66740*) as a top-represented gene (see complete list of fold-change values in S4) and *RBCS1A* (*At1g67090*). Both genes were identified by sequence analysis of the dominant clones rescued after biopanning. Thus, our significance analysis confirmed in-frame proteins identified through the first approach to characterize *Pseudomonas*-bound clones. The 472-gene set also contained 23 targets (S15) that produced immune interactions in the Plant-Pathogen Immune Network-1 (PPIN-1) (Mukhtar et al., 2011), including *RBCS1A*.

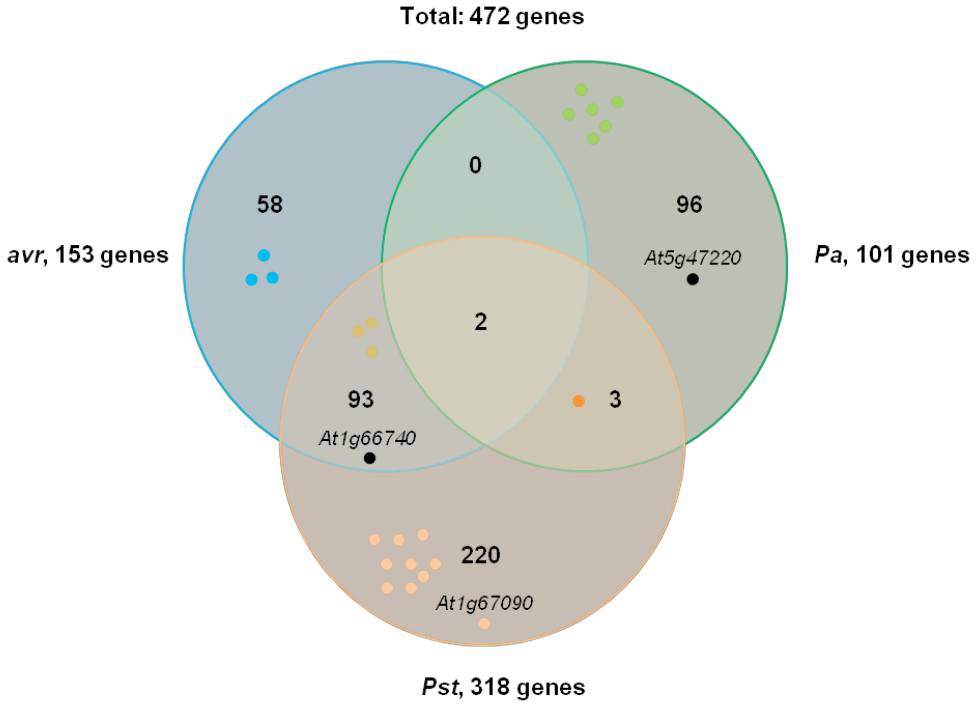


Figure 3.11 Overlapping of the gene sets defined by significance analysis of microarray data. The analysis identified 3 sets of genes: 101, 153 and 318 genes were selected after bio-panning against *Pa*, *Pst*(*avrRpt2*) (avr) and *Pst* strains respectively. The sets are represented as overlapping Venn diagrams and the number of shared genes is shown in the intersections. The union of the 3 sets produces a total of 472 genes as candidate MAMP/effector ligands. The positions of *At1g66740* (*AtSP7*), *At1g67090* (*RBCS1A*) and *At5g47220* (*ATERF-2*) are shown in each set. Gene products that produced immune interactions in PPIN-1 are represented by coloured dots.

The 472 genes were grouped into broad functional categories based on the GO hierarchy. The percentage of genes falling in each category was compared between this set and the whole set of genes in the microarray (26.303 genes, Figure 3.12). The 472 gene-set was significantly enriched in genes falling in the categories of

response to abiotic/biotic stimulus and response to stress (12% and 10% in the 472-gene set compared to 5% and 6% in the microarray respectively). This analysis confirmed that the 472 genes are not a random selection from the microarray gene-set and include a significantly increased number of representatives for biological categories consistent with a role as immune targets.

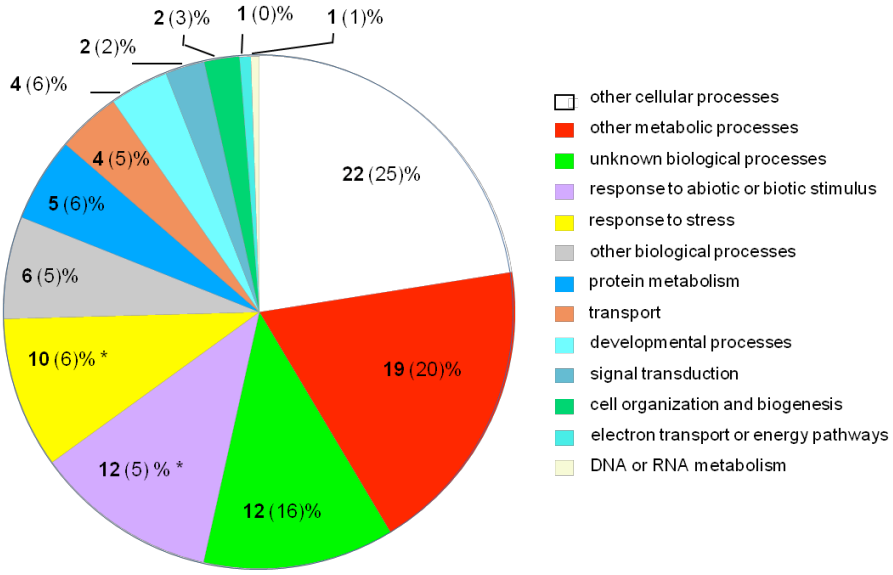


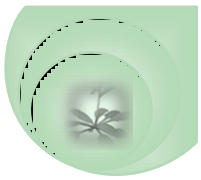
Figure 3.12 Categorisation of the gene sets defined by significance analysis of microarray data. Genes in the 472-gene set were annotated according to the GO categories for Biological Process and the percentage of annotations falling in each category (in bold) was compared to the percentage produced by the 26.303 genes spotted on the microarray (in brackets). Categories with significant ($p<0.01$) over-representation are shown by asterisks.

3.8 Defence-related genes in the significant sets identified by microarays.

Genes falling in the broad categories of response to biotic/abiotic stimulus or stress response were inspected to find the best candidates for MAMPs/effector targets. The genes annotated as defence-related in the fine GOslim classification were listed apart (Table 3.5). This list includes different molecular families involved in plant immunity, some of which are discussed next.

The most represented family of proteins in Table 3.5 is the group of LRR receptors (Kobe and Kajava, 2001), with 5 representatives. From them, *At2g31880* has been previously reported as a flagellin-responsive gene. *At2g31880* expression is elicited by flagellin or *Pseudomonas* infection and activates defence responses through a signalling pathway that is repressed by *BIR1*, a negative regulator of *BAK1* (Navarro et al., 2004; Gao et al., 2009). BAK1 and its partner FLS2 are themselves LRR-containing receptors that signal pathogen presence by interacting with flagellin (Gómez-Gómez and Boller, 2000; Chinchilla et al., 2007) but also with pathogen-derived effectors like AvrPto (Shan et al., 2008; He et al., 2006). Our data suggest that the mechanism by which *At2g31880* activates defence involves the binding between the encoded protein and a Pseudomonas-derived molecule, which could also be in the basis of *BIR1* inhibitory action.

The table also includes 2 genes encoding for nuclear proteins: *At5g47220* (*ATERF-2*) and *At4g23810* (*WRKY53*). *ATERF-2* is the closer homologe to *ATERF-1* and both transcription factors share extensive similarity within and outside the ERF domain (Fujimoto et al., 2000; On et al., 2002). *WRKY53* is a positive regulator of basal resistance triggered by virulent pathogens/MAPMs and is tightly regulated at various levels, *i.e.,* its interaction with the MEKK1 kinase that signals pathogen infection (Murray et al., 2007; Miao et al., 2007). This mechanism is a short cut to directly activate WRKY53-controlled genes upon pathogen infection, which shows



that transcription factors involved in plant defence have evolved several check-points to sense pathogen attack.

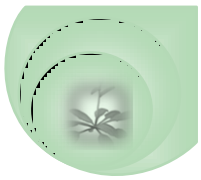
At3g28930 (*AIG2*) and *At5g06320* (*NHL3*) are in the subset of genes that were specifically identified after biopanning with *Pst*(*avrRpt2*) strain (58-gene subset). *AIG2* was one of the first genes isolated that exhibited *RPS2*- and *avrRpt2*-dependent transcript induction early after infection with *P. syringae* strains carrying *avrRpt2* (Reuber and Ausubel, 1996). Although its function remains unknown, the protein structure suggests that AIG2 can bind small ligands in a hydrophilic cavity as a part of its active site (Lytle et al., 2006). *NHL3* encodes for a plasma membrane protein responsive to *P. syringae* infection which has been proposed to function as an R receptor for Avr proteins (Varet, 2003).

In the Pst-specific group (220-gene subset) there are 8 genes previously annotated as defence-related. *At1g79210* encodes for an endopeptidase which forms part of the 26S proteasome complex and accumulates differentially in response to inoculation with *Pst* or *avrRpm1* strains (Book et al., 2010). *At3g21220* encodes for the AtMKK5 kinase. This protein has been shown able to interact directly with the HopF2 effector of *P. syringae* and interfere with MAMP-triggered immunity (Asai et al., 2002; Wang et al., 2010; Wu et al., 2011). *At1g19610* (*PDF1.4*) and *At2g43530* are included in the defensin family of proteins (Silverstein et al., 2005). Defensins bind strongly to microbial surfaces as a first step to exert they antimicrobial function (Brogden, 2005), and so they are good candidates to be rescued by affinity biopanning.

Table 3.5 Significant genes falling in the GO fine category of "defence response".

Gene set	Gene ID	Subset	1B FC	2B FC	3B FC	5B FC	Description
101 *Pa*	At1g09340	96	-	-	-	5.4	CRB CHLOROPLAST RNA BINDING protein, putative
	At3g04720	96	-	-	-	5.1	PR4 PATHOGENESIS-RELATED 4
	At3g11010	96	-	-	-	3.0	RLP34 Receptor likeprotein 34 (LRR-containing N-terminal domain, type 2)
	At5g47220	96	-	-	-	2.9	ATERF-2 ETHYLENE RESPONSE FACTOR 2
	At4g23670	96	-	-	-	2.5	Major latex protein-related
	At2g31880*	96	-	-	-	1.7	LRR transmembrane protein kinase, putative
153 *Avr*	At1g24020	93	1.4	2.2	-1.2	-	MLP-like protein 423
	At3g28930	58	1.0	1.6	-1.3	-	AIG2 AVRRPT2-INDUCED GENE 2
	At1g63870*	58	-1.0	1.5	-1.1	-	Disease resistance protein (TIR-NBS-LRR class), putative
	At5g06320	58	-1.2	-1.0	1.5	-	NHL3 NDR1/HIN1-like 3
	At3g27850	93	-1.4	3.1	1.0	-	RPL12-C RIBOSOMAL PROTEIN
318 *Pst*	At1g24020	93	3.9	-1.1	-1.0	-	MLP-like protein 423
	At1g79210	220	2.5	-1.0	1.1	-	20S proteasome alpha subunit B
	At3g21220	220	2.1	-1.2	1.1	-	ATMKK5 MITOGEN-ACTIVATED PROTEIN KINASE KINASE 5
	At1g52660*	220	1.9	-1.4	1.4	-	Disease resistance protein, putative (NB-ARC, LRR type 3)
	At4g23810	220	1.9	-1.2	-1.0	-	WRKY53 transcription factor
	At2g15220	220	1.5	1.2	-1.0	-	Plant basic secretory protein (BSP) family protein
	At3g27850	93	1.5	-3.3	-1.5	-	RPL12-C RIBOSOMAL PROTEIN
	At5g53890*	220	1.0	1.9	1.3	-	LRR transmembrane protein kinase, putative
	At1g19610	220	-0.9	1.5	1.2	-	LCR78/PDF1.4 Low-MW-cysteine rich 78
	At2g43530	220	-2.5	3.8	2.0	-	Trypsin inhibitor, putative

FC Fold change, 1 to 5 rounds of bio-panning (1B to 5B). Predicted LRR-containing proteins are marked in bold, asterisks indicate immune-related baits tested by(Mukhtar et al., 2011)



3.4 DISCUSSION

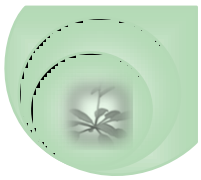
Phage display is a powerful technique that allows for the expression of a large number of proteins on viral particles and their selection on the basis of their binding affinity for a ligand. In this study we used the technique to perform a high-throughput selection of Arabidopsis proteins with affinity for *Pseudomonas* bacteria and therefore with a putative role in natural plant-microbe interactions. Phage-display-based strategies have been shown before suitable for the selection of plant proteins with physiological ligands (Willats, 2002; Chen et al., 2010), but to date no attempts to use them in a wide screening of plant immunity targets have been reported. To provide input for this selection we constructed two phage-displayed libraries covering different fractions of the plant immune transcriptome. To identify the output we took advantage of microarray development in Arabidopsis. This approach stood for the quantification of all putative binders in a genome-wide scale and provided a significant list of candidate targets for MAMPs and virulence effectors. Clone identification is the last and less efficient step of phage display, and only recently has been addressed by using high-throughput technologies (Dias-Neto et al., 2009).

Three different plant-pathosystems were used as a cDNA source for the construction of the libraries. In the Arabidopsis *vs. Pst* pathosystem, infection with the bacterium results in a compatible interaction and induces de transcription of a large number of plant genes (Thilmony et al., 2006). Infection with the *Pst*(*avrRpt2*) strain elicits the HR response in hosts that recognize the AvrRpt2 protein and results in incompatible interaction. The use of *P. aeruginosa* in the third pathosystem has additional interest since this bacterium is an opportunistic pathogen of humans with an extended range of hosts (Gopalan and Ausubel, 2008). *P. aeruginosa* PA14 strain is a hypervirulent isolate that produces pyonacin and other factors of virulence both for mammalian and plant hosts (Rahme et al., 2000, 1995; Walker et al., 2004). The

three strains share MAMPs common to most Gram-negative bacteria, like LPS or flagellin, and secrete virulence factors some of which are able to subvert host defences activated by MAMPs. Recently, it was shown that *P. aeruginosa* is able to evade immune recognition of flagellin through a similar mechanism in mammals and plants (Bardoel et al., 2011). Unlike *P. syringae*, this bacterium has not evolved to be nutritionally dependent of a plant host (Mithani et al., 2011). Thus, these 3 pathosystems represent different degrees in the specificity of the plant-microbe interaction during which the host response might involve a broad range of molecules that recognize, signal and neutralise MAMPs and/or virulence effectors.

As the vector for phage display expression we used T7, since display in the lytic phage can produce libraries of greater diversity than M13. According to our estimations in Table 1 the two libraries constructed in this study represent virtually the entire AtORFeome. Our determination of phage numbers in the primary library was based on pfu-counting; however quantification by real-time PCR provide estimations 5 to 10 times higher (Dias-Neto et al., 2009). Thus, the actual coverage of these libraries might be higher than reported here. Since the average size of cloned fragments ranges from 0.2 to 1.5 or 2.2 kb, full-length cDNAs should be represented to some extent, although N-terminal domains are under-represented as observed from Table 3.3. The screening of $6.3x10^9$ pfu that we performed for each pan represents every possible cDNA in the most complex, T7LatPs library with a multiplicity of 100. This allows sufficient sequence representation to find rare cDNAs during selection.

The next step in our strategy involved the selection of affinity clones by bio-panning against a living substrate. Living cells like zoospores or cultured cell lines (Bishop-Hurley et al., 2002; Lipes et al., 2008) have been bio-panned against peptide libraries. We used bacterial cells that were pre-incubated with plants to ensure the expression of effectors induced only upon host contact. To follow the enrichment in affinity clones we monitored the titres of eluates during successive



rounds of selection (Figure 1). When bio-panning high-affinity antibodies titres usually increase up to a maximum that indicates the round from which eluted clones should be analysed. The selection and amplification of the clones bound in the first round results in increasing titres in later rounds, but once the eluates are saturated for affinity-binding clones titres plateau since no further selection happens and clones amplify similarly. This is not necessary observed when selecting for low-affinity interactions where clones compete weakly for binding to the substrate. In our case, the iteration of pannings against bacteria but not against the agarose control resulted in titres that were increased 10^2-10^3 times respect to the first round. This is a significant increase, similar to values reported for antibody libraries. However, maximum titres of rounds 5th and 3rd did not remain stationary, suggesting that bio-panning was not driven to saturation. The point of maximum enrichment for affinity-binding clones depends on the complexity of the library and the requirements of each displayed protein *vs.* substrate interaction, since phage selected in one round compete to each other in different proportion during the next round. In our bio-panning we introduced an additional level of complexity because bacterial cells can display many different ligands for selection at the same time. The analysis of the dominant clones isolated from rounds with titration peaks (Figure 3.2 and Table 3.4) was relevant since the T7-ATERF-1 clone confirmed its binding properties in the competition assay. However, the isolation of a clone at high frequency in a particular round of bio-panning does not imply enrichment during selection. Microarrays allow for the quantification of the differences in the copy number of each clone during selection (B *vs.* L fold change), which can provide an absolute measurement for enrichment. In our study, B *vs.* L fold-changes, were monitored across the first 3 rounds of bio-panning with T7LAtPs library, and this provided additional data about the kinetics of selection. From significance *vs.* fold-change plots in Figure 3.10 the clones with lowest *p*-values and highest fold-changes appeared in the first and

second rounds of selection; therefore we included these data in the significance analysis (Table 3.4).

The S4 dataset summarizes the most relevant information produced by our genome-wide analysis: for each gene spotted in the microarray a fold-change value is provided plus its associated significance level. We choose significance analysis of microarrays as a supervised statistical procedure to define the best candidates, but a variety of methods are available for microarray selection that can be applied to our supplemental data. The significance analysis defined a total of 418 genes with significant fold-changes (Table 3.4), distributed in overlapping sets. The 101 gene-set (*Pa*) was selected from a different input (T7LAtPa library) and the low overlapping with the other two sets might be due to the lower coverage of this library. However, gene sets arising from *Pst* and *Pst*(*avrRpt2*) bio-pannings are comparable since both were selected from the same input (T7LAtPs). Thus the high overlapping between them (Figure 3.11, 95 shared genes) likely reflects common targets for the two *P. syringae* strains. Mining of data in S8 can provide additional information about the specificity of the interaction with the three bacterial strains tested in his study. Overall, the 418-gene set represents a microarray selection of candidate genes based on the microbe-binding properties of their phage-displayed proteins. Functional categorization indicates over-representation of biological processes that are consistent with a role of the selected genes as putative targets for MAMPs or virulence effectors (Figure 3.12). A finer inspection through GO hierarchy detected 19 defence-related genes with very different molecular functions, discussed in detail in section 8. At least one of them (*At3g21220*) encodes for an *in-vivo*-validated target of a bacterial effectors. Moreover, the 418-gene set contains 23 immune targets (S15) confirmed by interactome analysis (Mukhtar et al., 2011) which are also very diverse in their molecular roles. NB-LRR disease resistance proteins are represented (*At1g52660*), but also metabolic enzymes validated by sequence analysis (*At1g67090*) and transcription factors like SNZ (*At2g39250*), in

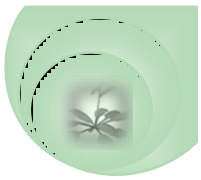
the family of AP2-ERF DNA-binding proteins, or AtTCP15 (*At1g69690*). The latter was able to interact with several groups of Avr and Hop effectors from *Pseudomonas syringae* used as baits for the PPIN-1 map.

These results demonstrated that microarray hybridization plus significance analysis provide an efficient method for wide identification of putative targets. Since hybridisation does not pre-require isolation of clones, this approach is fast and produces information for many genes. However, clone isolation facilitates frame analysis and further characterization of the binding properties of selected protein. We used the T7-ATERF-1 clone isolated from biopanned eluates to validate our selection strategy and investigated the role of ATERF-1 during plant-defence. The binding of T7-ATERF-1 to the 3 strains of *Pseudomonas* was compared and we found that, in addition to *Pa*, *Pst* strains also produced significant enrichments in recovered phage (Figure 3.3 A). This suggested that the bacterial ligand for ATERF-1 is a molecule present in the 3 strains rather than a strain-specific virulence factor. In accordance, the nuclear translocation of GFP-ATERF-1 protein was promoted equally by the 3 strains (Figure 3.4). Inoculation with heat-killed bacteria induced the same translocation effect, whereas a mock solution without bacteria failed to change subcellular location of the protein. Thus, translocation from the nucleus does not require the signals produced by living bacteria in the plant apoplast, and is not induced by wounding signals associated to inoculation. Although our results do not imply that the binding of a bacterial ligand to ATERF-1 causes the exportation from the nucleus, the two phenomena share common features. Bacterial effectors like PopP2 can interact with host proteins to modulate their nuclear localisation (Deslandes et al., 2003; Bernoux et al., 2008).

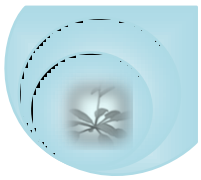
In an effort to go deeper into the physiological role of ATERF-1, we assessed the resistance of an *aterf-1* mutant against *P. syringae* infection (Figure 3.8). The sensitive phenotype indicates that *ATERF-1* is required for a proper immune response and other defence mechanisms induced during natural response to the

bacterium are not able to compensate for its effect. The ERF family of transcription factors is very redundant and loss-of-function mutants do not usually exhibit susceptible phenotypes (Solano et al., 1998). In contrast to other members of the family (Pré et al., 2008; Mcgrath et al., 2005; Lorenzo et al., 2003), the expression of *ATERF-1* is induced not only by ethylene, but also by flg22 treatment and *Pst* infection. However, the transcriptional response to *Pst* is stronger than that to flg22 or ethylene alone (Thilmony et al., 2006). According to these authors, this would indicate that ethylene basal response is insufficient to prevent *Pst* pathogenesis or that *Pst* is able to block ethylene signalling or responses downstream *ATERF-1* induction. Considering our results, it is tempting to speculate that a *Pseudomonas* ligand can be internalized into the nucleus where interacts with ATERF-1 to release transcriptional regulation by this factor. The finding that ATERF-1 activity is both sensitive to ethylene and pathogen-derived molecules suggests that the long-distance control of defence response by hormones and direct sensing of pathogen molecules can be integrated through the same transcription factor. Although classically transcription factors have not been considered as direct binders of the microbe-derived molecules that trigger immunity, there are recent examples of nuclear host proteins which are able to interact with bacterial factors (Deslandes et al., 2003; Kay et al., 2007). The RRS1-R protein from *A. thaliana* interacts with the *Ralstonia solanacearum* effector protein PopP2 in the nucleus of living plant cells (Tasset et al., 2010). RRS1-R is composed of two differentiated domains, with a DNA-binding motif which is characteristic of the Zin-finger class or WKRY transcription factors and a Toll/Interleukin receptor (TIR)-NBS-LRR-like domain for pathogen sensing. Similarly, *ATERF-1* (*At4g17500*) is composed of a C-terminal domain with homology to the ERF-1 family of transcription factors plus an N-terminal extension which is not present in any other member of the family.

The PPIN-1 interactome map revealed that transcriptional regulators represent the most enriched category in the subgroup of 165 putative effector targets (Mukhtar



et al., 2011). The identification of a variety of immune targets as a result of our microarray analysis underscores the possibility that pathogen-sensing is a capacity retained in very different families of proteins that are involved in plant defence, from surface receptors to transcriptional regulators. Typical R proteins have a modular structure composed of different terminal domains in addition to their central NB-LRR region. This structure facilitates a tight regulation of their activity, which is accomplished by intramolecular interactions between the various domains (Takken and Tameling, 2009). The acquisition of pathogen-sensing domains during the evolution of structurally unrelated proteins would facilitate the integration of defence responses in the complex immune system of the plant.



4. CHAPTER II:

Mechanism of action of ERF1 during abiotic stress through derepression of key transcription factors and crosstalk with ABA

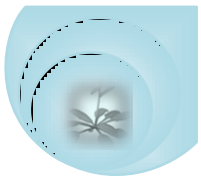
4.1 INTRODUCTION

Plants have evolved different physiological, biochemical and genetic mechanisms to tolerate environmental stress conditions such as drought, cold or high salinity. Under these challenging conditions drastic changes in plant hormone metabolism and signalling occur. The phytohormones abscisic acid (ABA) and ethylene (ET) play a major role in the regulation of plant responses to the different types of abiotic stresses. In addition, these hormones participate in certain developmental processes in non-stress situations, such as seed germination or early seedling growth.

ABA mediates the physiological response of vegetative tissues to osmotic stress and water-deficit, and triggers stomatal closure to reduce water loss through transpiration. The molecular mechanisms involved in this response are very diverse, and it has been shown that ABA regulates changes in the activity of ion channels and signalling molecules, as well as promotes changes in gene expression (Wasilewska et al., 2008). The products of these genes are thought to function, directly or indirectly, in protecting cells from dehydration (Gómez et al., 1988; Bartels and Sunkar, 2005). As an indication of the extent and complexity of ABA-mediated gene regulation, genome-scale analyses identified more than 2900 ABA-responsive genes in *Arabidopsis thaliana* (Nemhauser et al., 2006). Several ABA signalling components have been characterized, some of them associated with germination and involved in the removal of sensitivity to ABA. These include G proteins, secondary messengers, kinases, phosphatases, protein-degradation pathways and transcription factors (Finkelstein et al., 2002; Lopez-Molina et al., 2003; Nishimura et al., 2004, 2007; Verslues and Zhu, 2005; Zhang, Zhang, et al.,

2005; Pandey et al., 2006; Saez et al., 2006; Yoine et al., 2006; Holdsworth et al., 2008). Recently, the Regulatory Components of ABA Receptor / Pyrabactin Resistance Protein1 / PYR-Like proteins (RCAR/PYR1-PYL) family of proteins has been identified (Ma et al., 2009; Park et al., 2009; Cutler et al., 2010). These proteins function as ABA sensors and have been demonstrated to inhibit clade A type 2C protein phosphatases (PP2Cs) such as ABI1 and ABI2 (for ABA-Insensitive) , which are known negative regulators of ABA responses. Inhibition of PP2Cs triggers a reversible phosphorylation cascade (Ma et al., 2009; Park et al., 2009) to transmit ABA signal to downstream elements of the pathway, some of which had been previously identified by genetic screens (Santos-Mendoza et al., 2008). Plants mutated in downstream transcriptional regulators of ABA pathways present pleiotropic defects as a consequence of reduced ABA sensitivity, as well as precocious seed germination. Many of these transcription factors are known, including ABA-responsive element (ABRE)-binding proteins (ABA-INSENSITIVE5 [ABI5]/ABF/AREB/AtbZIP family), ABI3/VP1/B3, ABI4/APETALA2, MYC, MYB, and HD-ZIP domain proteins (Giraudat et al., 1992; Suzuki et al., 1997; Finkelstein et al., 1998; Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Bensmihen et al., 2002; Himmelbach et al., 2002; Abe et al., 2003). Most of them play a positive role in ABA signalling, but some are repressors of ABA response (Himmelbach et al., 2002; Pandey et al., 2005; Song et al., 2005)..It is widely accepted that ABA responses depend on coordinated interactions between positive and negative regulators of this complex signalling pathway.

The downstream transcriptional regulators involved in stress signalling were the focus of this work. Transcriptional regulation has been implicated as a crucial regulatory mechanism in the ABA, ET and stress-responsive pathways in plants. More than 30 families of transcription factors have been predicted in plants, from which members of APETALA2/ET-responsive element binding factor (AP2)/



(EREBP) and bZIP families, that includes the dehydration responsive element binding (DREB) and the ABRE-binding protein/ABRE-binding factor (AREB)/ (ABF) subfamilies, respectively, have been involved in the regulation of stress responses. Most of them regulate expression through the binding to their cognate *cis*-elements in the promoters of the stress-related genes. Two well characterized dehydration-stress-related *cis*-elements are the drought-responsible element (DRE), and the ABA- responsive element (ABRE), which are recognized by the DRE-binding (DREB) or CBF transcription factors (Kizis et al., 2001), and the bZIP domain transcription factors (Busk and Pagès, 1998), respectively. Regarding to the AP2/EREBP family (Riechmann and Meyerowitz, 1998), the ERF subfamily of these transcription factors has been widely studied in connexion with ET-mediated signalling (Ohme-Takagi and Shinshi, 1995). This subfamily of Arabidopsis proteins includes members that respond to abiotic stresses, such as drought or high salinity, and has orthologs in other plants like tomato or tobacco (Fujimoto et al., 2000; Jeong Mee Park1 et al., 2001; Zhang, Zhang, et al., 2005). Their overexpression in transgenic plants can confer tolerance to these stresses (Zhang et al., 2004; Seo et al., 2010). For example, overexpression of tomato *TERF1* confers drought tolerance and ABA hypersensitivity to tobacco plants (Zhang, Zhang, et al., 2005). AP2/ERF proteins also activate ABA-dependent gene expression in response to dehydration by binding to specific *cis*-acting elements (Zhang, Zhang, et al., 2005). Recently, a few AP2/ERF genes were found to be involved in the response to various environmental stresses. The overexpression of some of these stress-inducible transcription factors can increase the tolerance to drought, salinity or low temperature in Arabidopsis and other plant species (Tran et al., 2007). The identification of the transcription factors and the cis-acting elements that conform the transcriptional regulatory networks controlling stress tolerance and hormone sensitivity is crucial to better understand stress responses of plants. Since mutations in many of them have pleiotropic effects on the responses to different types of stress,

it is tempting to speculate that the same transcription factor can bind with more o less affinity to different *cis*-acting elements, depending on the stress conditions and the presence of other components of the network. .

Etylene Response Factor 1 (ERF1, At3g23240) was described previously as a downstream component of ET/jasmonic acid (JA) signaling pathways that might play a key role in the integration of both signals to activate the responses to pathogens in Arabidopsis (Lorenzo et al., 2003). *ERF1* is a close homologue to the *ATERF-1* gene (At4g17500) isolated in the screening performed in the chapter I of this Thesis. The integration of ET and JA signals relays in the capacity of ERF1 to regulate the expression of defence response genes through binding to the GCC-box (Ohme-Takagi and Shinshi, 1995; Solano and Ecker, 1998; Lorenzo et al., 2003; Alonso and Stepanova, 2004; Hao et al., 1998). The use of a protein-binding-microarray containing all the putative DNA contexts for the double-strand, *cis*-acting sequences, allowed to define target genes of ERF1 (Godoy et al., 2011). Among these targets, ERF1 itself and two transcription factors from the AP2/EREBP family (DEAR5 and AtERF4), potentially involved in the drought response, were selected thought this work for further analysis. The description of these genes and their promoter sequences recognized by ERF1 protein in a in vitro binding assay are shown in the Table 4.1.

Table 4.1 Selected genes containing promoter cis-elements targeted by ERF1, as defined by (Godoy et al., 2011).

Nº	Name	Description	Targeted *cis*-elements
At3g23240	ERF1	Encodes a member of the ERF (Ethylene Response Factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF1)	GCC 5'-GCCGCC-3'
At4g06746	DEAR5, RAP2.9	Encodes a member of the DREB subfamily A-5 of ERF/AP2 transcription factor family (DEAR5)	DRE 5'-TACCGACAT-3'
At3g15210	AtERF4, RAP2.5	Encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (AtERF4)	GCC and DRE

Both DEAR5 and AtERF4 (Sakuma et al., 2002) proteins have been described as transcriptional repressors containing the ERF-associated amphiphilic repressor (EAR) motif (Fujimoto et al., 2000; Ohta et al., 2001). ERF repressors, including AtERF4, are able to decrease the expression levels of a GCC box-containing reporter gene (Seo et al., 2010). Althought the *in vivo* function of DEAR5 is unknown, its closer homologue DEAR1 is involved in the drought response and both genes are able to bind to the DRE motif *in vitro* (Tsutsui et al., 2009). *AtERF4* expression is induced by cold, high NaCl concentration, and drought stress (Fujimoto et al., 2000) and down-regulates the expression of ABA- responsive genes upon saline stress (Yang et al., 2005). In addition, AtERF4 is able to bind specifically either GCC or DRE motives (Yang et al., 2009). Because the potencial involvement of these repressors in the drought response, the study of their cis-regulation through ERF1 is interesting to deepen the response to abiotic stress.

The general aim of this work was to uncover the function of ERF1 in abiotic stress through crosstalk with the ABA signal transduction pathway, and to assess the functional significance of a derepression mechanism through other transcription factors involved in development and stress tolerance.

4.2 MATERIALS AND METHODS

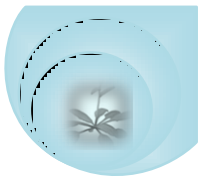
4.2.1 Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) are the genetic backgrounds for all the plants used in this work. The wild type (Col-0), and the *era3-1/ein2*, *abi1-1* and *abi5-1* mutants were obtained from ABRC. Transgenic *ERF1*-overexpressing and *hab2* mutant lines were described in (Lorenzo et al., 2003; Fernandez-Arbaizar et al., 2012). Transgenic *35S:ABI5* seeds were kindly provided by Dr. L. López-Molina. Different lines and their characteristics are listed below (Table 4.2). Plants were routinely grown in pots containing a 1:3 vermiculite:soil mixture and incubated in the greenhouse or in a growth chamber under controlled-enviromental conditions (50-60% humidity, 22°C and a 16-h light/8-h dark photoperiod at 80 to 100 µE m^{-2} s^{-1}).

Table 4.2 Mutants or overexpressor lines (OX) used in this chapter

Mutant or OX Line	Genetic background	Gene code	Characterisstics	References:
era3-1/ ein2	Col-0	At5g03280	ABA enhanced response and ET insensitive mutant of a farnesyltransferase	(Cutler et al., 1996; Alonso, 1999)
abi1-1	Ler	At4g26080	ABA insensitive mutant of ABI1, a negative regulator of ABA promotion of stomatal closure.	(Koornneef et al., 1998)
abi5-1	Col-0	At2g36270	ABA insensitive mutant of ABI5, involved in ABA signalling during seed maturation and germination	(Finkelstein, 1994)
35S:ABI5	Col-0	At2g36270	OX line of ABI5	(Lopez-Molina et al., 2001)
35S:ERF1	Col-0	At3g23240	OX line of ERF1	(Solano et al., 1998)

For *in vitro* culture, Arabidopsis seeds were surface-sterilized in 75% sodium hypochlorite and 0.01% Triton X-100 for 5 min and washed three times in sterile water before sowing. Seeds were stratified for 3 days at 4°C and then sowed on plates containing MS (Murashige and Skoog, 1962) basal salts, 2% (w/v) glucose and0.6% (w/v) agar. The pH of the medium was adjusted to 5.7 with KOH before



autoclaving. Plates were sealed and incubated in a controlled-environment growth chamber.

4.2.2 Germination assays

All the seeds that were compared in one experiment came from the same batch. Batches contained seeds harvested at the same day from individual plants that were grown under identical environmental conditions. To measure ABA and PAC sensitivity, seeds were sown on solid medium composed of MS basal salts, 2% (w/v) glucose, and the specified concentrations of ABA (0.1, 0.5 and 1 µM) or PAC (0.2, 0.6 and 1 µM). ABA [(2*Z*,4*E*)-5-[(1*S*)-1-hydroxy-2,6,6-trimethyl-4-oxocyclohex-2-en-1-yl]-3-methylpenta-2,4-dienoic acid] and PAC [2RS, 3RS)-1-(4-Chlorophenyl)-4,4-dimethyl-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol] were obtained from Sigma. Stratification of the seeds was conducted at 4°C for 3 d. Values plotted in the germination graphs represent the average of three assays where 80 to 100 seeds per assay were used to calculate the germination percentage. To determine the sensitivity to osmotic and salt stress during germination, MS medium was supplemented with 200 or 250 mM mannitol (Sigma) and 100 or125 mM NaCl (Sigma). For the germination assays, *sensu stricto* and seedling establishment, seeds were observed under a dissecting microscope (Leica) to determine the percentage of seeds with an emerged radicle or green cotyledons, respectively.

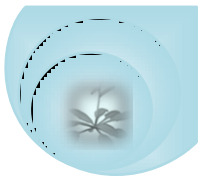
4.2.3 Quantification of hormones *in planta*.

Hormone extraction and analysis were carried out essentially as described in Durgbanshi et al. (2005) with slight modifications. Briefly, plant tissues were homogenised in distilled water using a tissue homogenizer (Ultra-Turrax, Ika-Werke). Tissue samples were spiked before homogenisation, with deuterated ABA (d_6-ABA) as internal standard. Homogenates were centrifuged and the supernatants were buffered to adjust the pH down to 3 and partitioned twice against diethylether.

The organic layers were combined and evaporated in a centrifuge vacuum evaporator (Jouan). The dry residue was thereafter resuspended in a water:methanol (9:1) solution, filtered, and injected into a HPLC system (Alliance 2695, Waters Corp.). ABA was separated in a reversed-phase C18 column using methanol and 0.01% acetic acid as solvents. A triple quadruple (Quattro LC, Micromass Ltd.) mass spectrometer, which was operated in negative ionization electrospray mode, was used for detecting ABA. ABA-Specific transitions were determined using a multiresidue mass spectrometric method.

4.2.4 Generation of *hab2;35S:ERF1* plants

The overexpressor line *35S:ERF1-3* was crossed with the dominant ABA-insensitive mutant *hab2* by transferring pollen from homozygous *35S:ERF1-3* plants to emasculate flowers of homozygous *hab2* plants. Plants from the F1 generated were sown on pots and homozygous *hab2* were selected after sequence analysis of the *hab2* locus in the F2 segregating population. To this end, leaf tissue from individual plants was collect and directly amplified as recommended in the Phire Plant kit. The primers *hab2* Forward/Reverse (F: 5'-ATGGAAGAGATTTCACCTGC-3'/ R: 5'-GCGCCTTGCCATTGTATAAC-3') were used for amplification and sequencing. The amplified fragment was purified with GeneClean (Q-BioGene), sequenced and analysed with BioEdit. Seeds from homozygous (F3 populations) were sown on MS supplied with Kanamicine 50µg/µl to select for kanamycin resistant, *35S:ERF1* transgenics. Siliques from F3 (*hab2* homozygous) 100% Kanamicine-resistant and 100% ERF1 overexpressor-phenotype (dwarf and larger cothyledons with respect to Col-0) were selected as homozygous double transgenic plants.



4.2.5 Gene expression analysis

RNA for quantitative Real Time PCR (q RT-PCR) was obtained from different plant samples. To determine the gene expression under drought conditions, leaf tissues were used for RNA extraction. Plants were grown in a growth chamber for 4 weeks and leaves were excised and exposed to an air stream into a laminar-flow hood during 3 hours. For gene expression measurement upon ABA addition, seeds were sterilized, stratified and sown on MS as described above. Twelve days after sown, plants were treated with water or 50µM ABA for 3 hours. Whole plants were then collected and frozen by inmersion in liquid N_2. RNA extraction was performed by using TRI Reagent (Ambion, Auxtin TX) as suggested by the manufacturer. Two µg of DNase-treated total RNA was used for reverse transcription (RT) with the SuperscriptIII Kit (Roche). The RT product was subjected to quantitative PCR using the primers designed with the aid of Primer Express software 1.0 (Applied Biosystems) and listed below (Table 4.3). The actin gene (*ACTIN8*) was used as control of endogenous expression levels. Real-time PCR was performed in ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Amplification was monitored by using the Brilliant® SYBR® Green QPCR MasterMix (Stratagene), following the manufacturer's instructions. The thermal profile followed in these experiments was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

To generate the standard curves, a range of serial dilution from a solution containing 50ng/µl of cDNA isolated from Arabidopsis seedlings was used. Each quantification was repeated three times to get representative values for the slope of the standard curves and the standard deviation. The concentration of samples was calculated with the ABI-Prism 7000 SDS software, which created threshold cycle values (C_t) and extrapolated relative levels of PCR product from the standard curve. The expression of all genes was normalized against the expression of the endogenous control gene (*ACTIN8*) and gene expression plots show the expression

level or fold-difference of the target sample relative to the calibrator (wild type in normal conditions) as log10 of the $2^{-\Delta\Delta Ct}$ value.

Table 4.3: Primers for Real Time Q-PCR assays used in this work.

Gene to amplify	Sense	Sequence
ERF1	Forward	5'-CTTCCCTTCAACGAGAACGA-3'
	Reverse	5'-GTTTGTTGCGTGGACTGCT-3'
DEAR5	Forward	5'-GTCTCCGACGTTGATACGG-3'
	Reverse	5'-AACTCTGCGTCGACTCTAGCTC-3'
ATERF4	Forward	5'-ATGGGGATCGGTAACGTAGG-3'
	Reverse	5'-CGATCTAAACGCCGATGTC-3'
RD29B	Forward	5'-ATGGAGTCACAGTTGACACGTCC-3'
	Reverse	5'-GAGATAGTCATCTTCACCACCAGG-3'
P5CS1	Forward	5'-TTTATGGTGCTATAGATCACA-3'
	Reverse	5'-GAATGTCCTGATGGGTGTAAAC-3'
ACTIN8	Forward	5'-AGTGGTCGTACAACCGGTATTGT-3'
	Reverse	5'-GAGGATAGCATGTGCAAGTGAGAA-3'

4.2.6 Drought Stress and Water-Loss Assays

To determinate the water loss different kind of experiments were performed in which the water loss was measured as a difference in the fresh weight. In long-term experiments individual pots containing 4-week-old plants that had been grown into a growth chamber were used for the quantification. To avoid soil evaporation, pot surface was covered with PARAFILM®. Irrigation was stopped after the 4th week and 6 pots per genotype were weighted at days 0, 7 and 14. In a second type of experiments the plant ability to take water after a drought stress was determined. Rosettes from drought-stressed plants were excised and weighted. After that, rosettes were rehydrated by immersing them in water for 3 hours and weight was determined again. In short-term experiments five leaves from 4-week-old plants (5 plants per genotype) were excided and exposed to an air stream into a laminar-flow hood during 6 hours. The weight of the 5 leaves from each plant was determined each hour along the drying treatment.

4.2.7 Protein analysis by Western blot

For Western blot analysis, approximately 500 seeds per genotype were sterilized, stratified and sown on filter paper soaked with 3µM ABA or water. After 48h seeds were frozen and ground with a pestle and mortar. Grinded tissue was incubated for 10 min in ice-coldextraction buffer [50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 15 mM EGTA, 15 mM MgCl2, 1 mM DTT, 0.1% Tween 20, 1 mM NaF, 0.2 mM Na vanadate, 2 mM Na-pyrophosphate, 60 mM β-glycerophosphate, and 1× proteases inhibitor mix (Roche)] and centrifuged for 10 min at 13,000 × g and 4 °C. The supernadants were used as total protein extracts. Protein concentration was determined by the Bradford method (1976) using the Bio-Rad Protein Assay . Thirty µg of protein extracts were used for SDS-PAGE analysis.. Proteins were electrophoretically transferred to Inmobilon^{TM}-P PVDF membrana (Millipore) using the Trans-Blot cell (Bio-Rad). Membranes were blocked in PBS-T with 5% ECL Advance Blocking Agent (Amersham) and probed with the indicated antibodies diluted in blocking buffer. Anti-ABI5 (kindly provided by Dr. L. Lopez-Molina) and anti-*ACTIN* (Sigma) were used as primary antibodies and ECL-Peroxidase-labelled anti-mouse or anti-rabbit (Amersham) antibodies were used as the secondary antibodies. Detection was performed using ECL Advance Western Blotting Detection Kit (Amersham). The chemiluminescence was detected using an Intelligent Dark-Box II, LAS-1000 scanning system (Fujifilm).

4.3 RESULTS

4.3.1 ERF1 transgenic lines are resistant to drought stress

To unravel the roles of ERF1 in abiotic stress responses we first determined the effect of *ERF1* over-expression in the response to drought stress. The phenotype of *35S:ERF1* transgenic plants was compared under water deprivation conditions to those of the Col-0 wild type, the drought-tolerant mutant *era3-1/ein2* (Enhance Response to ABA 3/ Ethylene Insensitive 2) and the drought-susceptible mutant *abi1-1*(ABA Insensitive 1). Because ABA regulates the stomata closure during drought stress to avoid the loss of water by transpiration, ABA insensitive mutants (*abi1-1*) are not able to integrate the ABA signal being more susceptible to the stress. Conversely, ABA hypersensitive mutants (*era3-1/ein2*) are more tolerant to drought. Photographs of 6-week-old plants 14 days after watering was stopped are shown in Figure 4.1A,B. Clearly, plants over-expressing *ERF1* looked greener and more turgid than the wild type Col-0, even more than the drought-resistant phenotype of *era3-1/ein2* plants. To quantify the magnitude of the drought response in the three genotypes classical water loss measurements were used (Figs. 4.1.C to E). A rehydration assay was performed to determine the capacity of these plants to recover from drought stress (Figure 1C). Rosette leaves from stressed plants were incubated in water to recover turgor and water loss was estimated by comparing fresh and turgid weight. Consistently with the phenotype shown in 4.1.A, Col-0 was able to absorb more water than the *ERF1* over-expressing lines, however, differences were not significant in a t-test ($p<0,05$). In a long-term assay with 4-week-old plants, irrigation was stopped for 14 days and the loss of water through transpiration was estimated by comparing the fresh weight of plant-containing pots before and after draught stress. Measurements were taken at 7 and 14 days after stopping irrigation (Figure 1D). The loss of water was significantly lower in *35S:ERF1* lines than Col-0 plants. In a short-term assay the leaves from 4-week-old plants were excised and exposed to an air stream into a hood. The leaves were

weighted every each hour for a total of 8 hours to produce a kinetics curve for water loss (Figure 1E). The curves for *35S:ERF1* transgenic plants are over the wild-type curve during the entire time course studied, with significant differences at time points of 1, 2, 3 and 8 hour for *35S:ERF1-3* line. All together these results demonstrate that over-expression of *ERF1* in a Col-0 background confers resistance to drought stress.

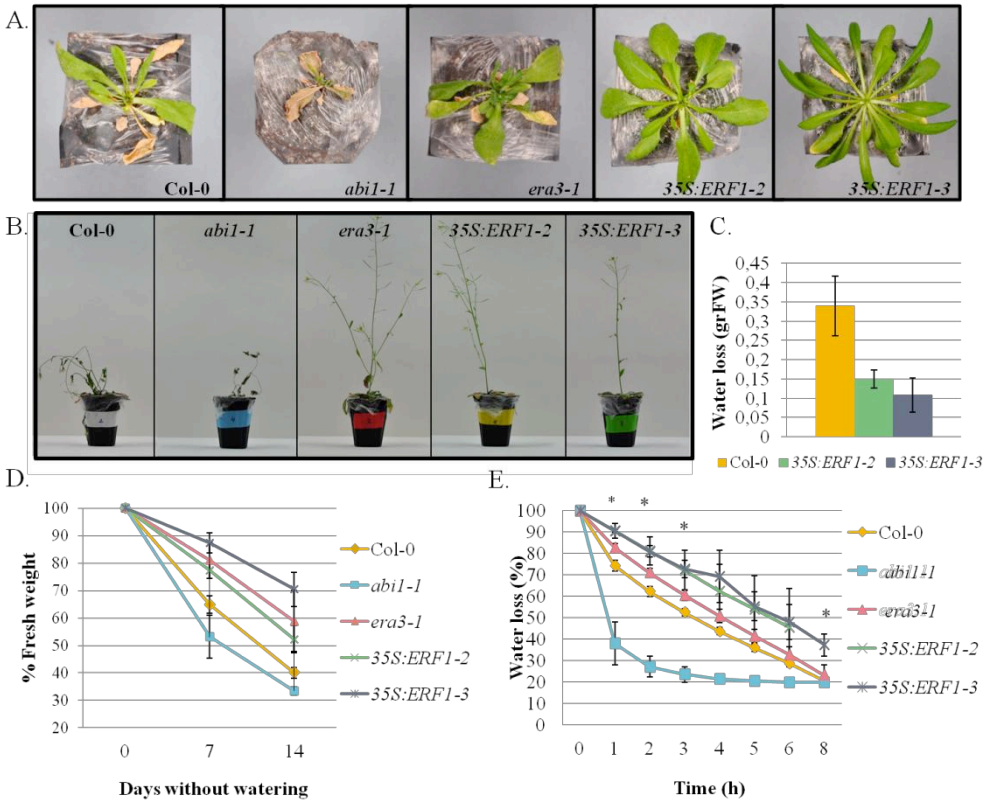


Figure 4.1 *35S:ERF1* transgenic plants are drought tolerant. A, B. Phenotypic assay showing the enhanced drought tolerance of *35S:ERF1-2* and *35S:ERF1-3* lines compared to wild type or *abi1-1* and *era3-1/ein2* controls. Photographs were taken 14 days after water was withheld. C. Rehydration assay with rosettes collected from 5-week-old plants after 14 days without irrigation. Leaves were weighted before and after incubation in water for 3 hours. Y-axe represents the difference in grams of fresh weight. Error bars indicate the standard deviation between two biological replicates. Significant differences were not found in a t-test ($p<0.05$). D. Water loss due to transpiration was stimated in a long-term assay, where six plants per genotype were weighted at 0, 7 and 12 days after stopping irrigation.Y-

axe represents the percentage of fresh weight calculated with respect day=0. Error bars represent standard deviation between the six plants. Significant differences between *35S:ERF1* lines and Col-0 were found at 7 and 14 days in a t-test (p-value<0.05). E. Kinetics of water loss in fresh leaves exposed to a drying air stream. Five leaves were excised from each 4-week-old plant and their loss of weight was determined after expossing them to a drying atmosphere into a laminar flow hood. Error bars represent the standard deviation between three replicates. Asterisks represent significant differences between *35S:ERF1-3* and Col-0 in a t-test (p-value<0.05). The experiments were repeated three times obtaining the same trend.

4.3.2 *ERF1* expression is increased after drought stress but decreased by ABA-treatment

The physiological adaptation to drought stress is mediated by different plant hormones. The phytohormone ABA is involved mainly in the regulation of abiotic stress responses such as drought or salt. In vegetative tissues, water stress produced by drought or high osmolarity conditionss increases ABA biosynthesis, which leads to a variety of adaptive responses including stomata closure and differential expression of ABA-responsive genes. Thus, we studied in detail *ERF1* expression upon drought-stress and ABA-treatment.

The transcript levels of *ERF1* upon drought treatment were compared by real time Q-PCR in the Col-0, the drought-resistant *era3-1/ein2* mutant and the *35S:ERF1* genetic backgrounds (Figure 2A). *ERF1* expression was highly induced by drought treatment in wild type plants. In comparison, gene expression decreased slightly in *era3-1/ein2* plants, whereas the *ERF1*-over-expressing lines exhibited high transcript levels independently of the drought treatment. This result together with the phenotypic analysis supports a role of ERF1 as a positive regulator of the plant response to drought. Since *ERA3/EIN2* is an upstream regulator of ERF1 in the ET signalling pathway, the lack of *ERF1* induction in *era3/ein2* mutants suggests that response to drought mediated by ERF1 is ET-dependent.

ERF1 transcription was also quantified in 12-day-old seedlings that were treated for 3h with 50μM ABA or with water (Figure 2B). *ERF1* expression was

down-regulated after ABA treatment in Col-0 seedlings. Interestingly, the *era3-1/ein2* and *abi1-1* mutants, which exhibit respectively ABA-hypersensitive and ABA-insensitive phenotypes, had opposite effects in the regulation of *ERF1* expression in response to ABA treatment. This result suggests that the components of ABA signal transduction pathway are required to modify *ERF1* expression.

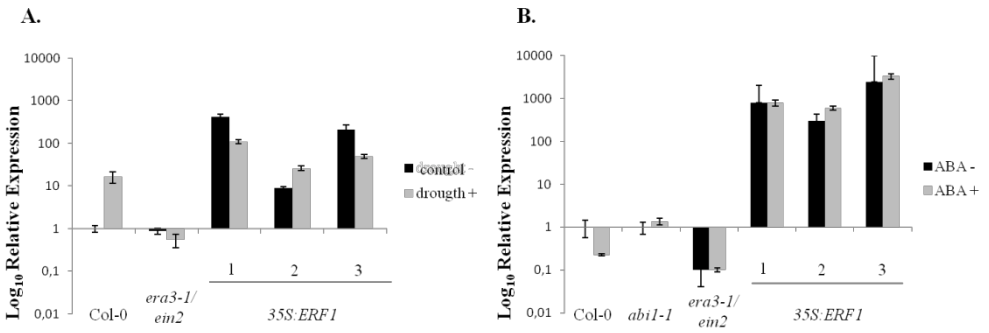


Figure 4.2 *ERF1* expression levels in response to drought and ABA treatments. Real time Q-PCR was used to quantify *ERF1* transcription in Col-0, *era3-1/ein2, abi1-1* and *35S:ERF1* (transgenic lines 1 to 3) genetic backgrounds. *ERF1* transcript levels were normalized respect to the *ACT8* control and expressed as a ratio relative to the Col-0, untreated reference. A. Relative expression of *ERF1* in leaves from 4-week-old plants before (control) or after 3h-exposure to a drying air stream (drought+). B. *ERF1* expression in 12-day-old seedlings after 3h-treatment with water (ABA-) or 50µM ABA (ABA+).

4.3.3 Over-expression of *ERF1* blocks the induction by drought-stress of *DEAR5 and AtERF4*

The drought tolerance of *35S:ERF1* lines (Figure 2.1) and the evidence that *DEAR5* and *AtERF4* are direct targets of ERF1 (Godoy et al., 2011) suggest a possible AP2/ERF regulation cascade through a derepression mechanism where ERF1 mediates drought responses.To test whether or not *AtERF4* and *DEAR5* are regulated by ERF1 in response to drought, their transcription was quantified in

35S:ERF1 transgenic plants and compared to Col-0 and *era3-1/ein2* control lines (Figure 3A, B). Drought stress induced over-expression of both genes in the wild-type background. By contrast, gene expression was down-regulated in the *35S:ERF1* transgenic plants. Thus, over-expression of *ERF1* results in a blockage of the transcriptional induction of *DEAR5* and *AtERF4.* These data suggests that *ERF1* functions as a transcriptional repressor of these genes during drought stress. Interestingly, the transcriptional induction of *DEAR5* (and to less extend of *AtERF4*) upon drought stress is decreased in the *era3-1/ein2* mutant respect to the wild-type background. This suggests that drought stress regulation requires the product of the *EIN2* gene. Since the ET pathway is impaired in the *era3-1/ein2* mutant, these data support the idea that drought induction of these genes needs an intact ET signalling cascade. In accordance with this, it has been shown that *AtERF4* transcript is induced during high salinity treatments through an ET-dependent pathway in which EIN2 plays a major role (Fujimoto et al., 2000).

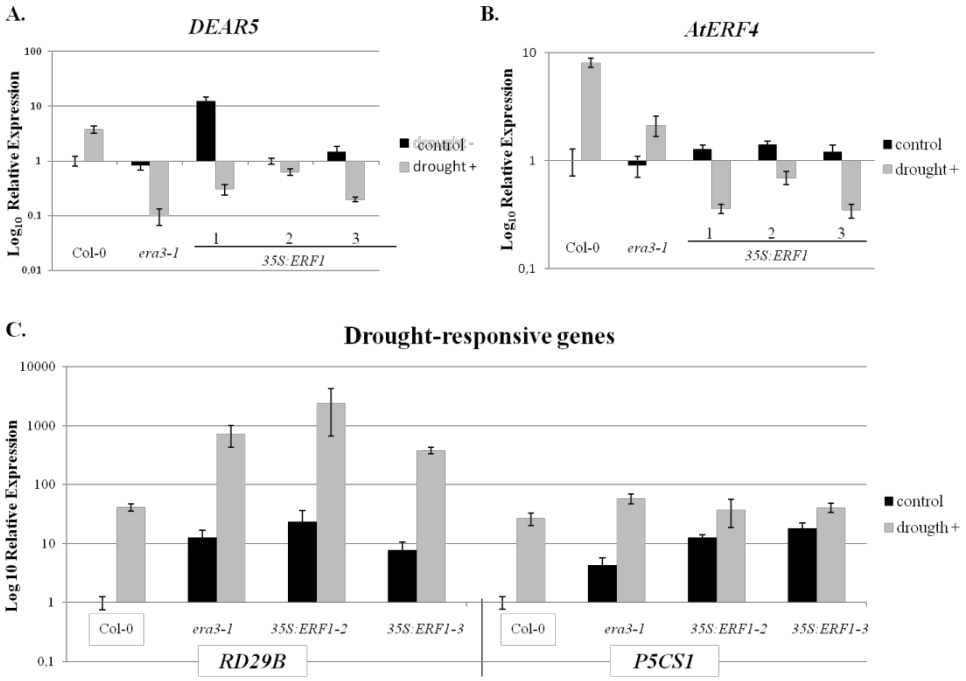


Figure 4.3 Expression of drought-responsive genes in *35S:ERF1* transgenic lines. RT Q-PCR was performed with cDNA extracted from leaves from 4-week-old plants before (control) or after (drought+) 3h. of exposure to a drying air stream.. Expression was quantified in the *35S:ERF1* plants (1-3), the wild type Col-0 and the *era3-1/ein2* mutant. A. *AtERF4* expression levels relative to the Col-0, untreated reference. B. *DEAR5* relative expression. C. Relative expression of the abiotic stress marker genes *RD29B* and *P5CS1.*

The effect of *ERF1* over-expression on the transcription of two classical targets for drought-stress regulation was also studied. RT Q-PCR was performed to analyze the expression of the ABA- and drought-responsive *P5CS1* and *RD29B* genes in wild type, *35S:ERF1* lines and the drought-resistant mutant *era3-1/ein2* (Figure 4.3 C). These genes have been widely used as the markers to monitor abiotic stress response in plants (Strizhov et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994), a condition which typically results in a dramatic increase of their transcript levels. Consistently, the expression of *P5CS1* and *RD29B* was enhanced after the drought treatment in the Col-0 background (Figure 3C). In the *35S:ERF1* lines the

basal expression of both genes was higher than in Col-0, although the drought treatment was still producing up-regulated gene expression. The *era3-1/ein2* mutant exhibited an expression pattern similar to the *35S:ERF1* transgenic plants. Since both genotypes result in drought-tolerant phenotypes, the resistance to abiotic stress in *ERF1* over-expressing plants might be a consequence of the increased transcription of stress-response marker genes. In addition, other ABA- and drought-responsive genes (*RD21*, *RD19*...) were identified to be up-regulated in *35S:ERF1* transgenics using a partial transcriptomic profiling (Lorenzo et al., 2003).

4.3.5. ERF1 over-expression confers resistance to salt and osmotic stresses during early seedling growth

The mechanisms evoked by drought stress in plants are similar to the ones involved in other abiotic stresses, such as salt or osmotic stimuli. To test whether over-expression of *ERF1* results in protection against other ABA-mediated stresses, as well as it does against drought stress, the germination of seeds from*35S:ERF1* plants was analysed in the presence of low concentrations of NaCl (Figure 4) or mannitol (Figure5). Germination is a crucial decision for plants and abiotic stress can compromise the growth and development of an early seedling. ABA regulates this developmental process and seeds of ABA-hipersensitive mutants do not germinate in low salt or mannitol concentrations, whereas seeds of ABA-insensitive mutants are osmotolerant. Here, the ability of *35S:ERF1* transgenic lines to develop green seedlings in NaCl (Figure 4) or mannitol-added (Figure 5A) media was analyzed. Seedling establishment in NaCl-added (100mM and 125mM) or mannitol-added (200mM and 250mM) media was clearly delayed in *35S:ERF1* lines when compared to Col-0. ABA-insensitive and ABA-hypersensitive mutants (*abi1-1* and *era3-1/ein2,* respectively) were used as the controls in the assay. Significant differences between *35S:ERF1* and wild type-lines were observed in both media

after day 7 (Figures 4.4 and 4.5 C and D). These results indicate that *ERF1* over-expressing seeds are hypersensitive to low salt or osmotic concentrations during the development of green cotyledons.

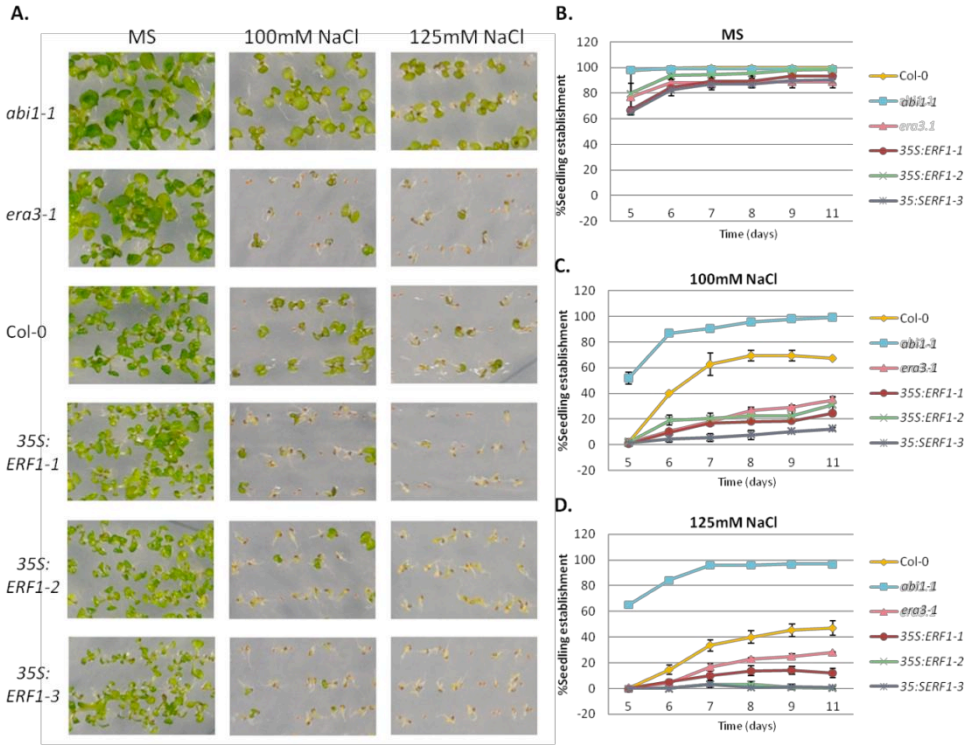


Figure 4.4 Early inhibition of seedling growth in *ERF1*-overexpressing plants upon saline stress. The phenotypes of the *35S:ERF1* transgenic lines (1 to 3), the wild type Col-0, the *abi1-1* ABA-insensitive mutant and the *era3-1/ein2* ABA-hypersensitive mutant are shown. A. Photographs of seedlines growing in MS medium or MS supplemented with 100mM or 125mM NaCl. Pictures were taken 11 days after sown. B, C and D. Time-course of seedling establishment inMS plates (B) or MS supplemented with 100mM (C) or125mM NaCl (D) Error bars represent the standard deviation from three biological replicates. Significance differences were contrasted with a t-test (p-value<0.05). Differences between the three independent *35S:ERF1* lines and Col-0 were significant after day 6 (C) and day 7 (D).

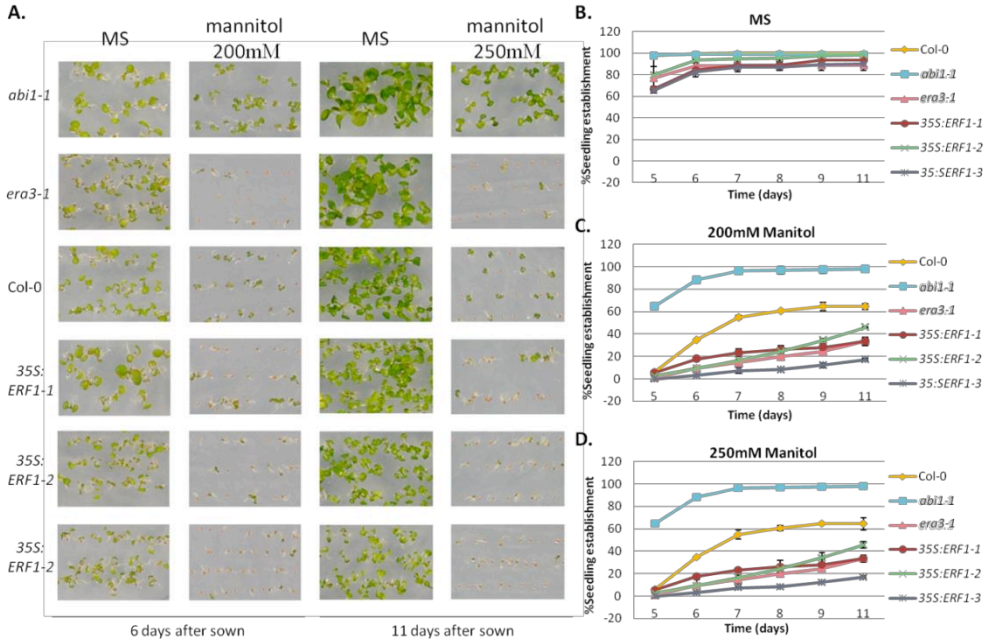


Figure 4.5 Early inhibition of seedling growth in *ERF1*-overexpressing plants upon osmotic stress. The phenotypes of the *35S:ERF1* transgenic lines (1 to 3), the wild type Col-0, the *abi1-1* ABA-insensitive mutant and the *era3-1/ein2* ABA-hypersensitive mutant are shown. A. Photographs of seedlines growing in MS medium or MS supplemented with 200mM or 250mM mannitol. Pictures were taken 6 and 11 days after sown. B, C and D. Time-course of seedling establishment in MS plates (B) or MS supplemented with 200 mM (C) or 250 mM mannitol (D) Error bars represent the standard deviation from three biological replicates. Significance differences were contrasted with a t-test (p-value<0.05). Differences between the three independent *35S:ERF1* lines and Col-0 were significant after day 7 (C and D)

4.3.6 *ERF1* over-expression confers hypersensitivity to ABA treatment

Drought and other abiotic stresses are regulated mainly by the plant hormone ABA. RT-Q-PCR analysis in Figure 4. 2A showed that *ERF1* gene expression was down-regulated in response to this hormone. To get further insights into the role of *ERF1* during abiotic stress, a germination assay was performed to test whether constitutive expression of *ERF1* would affect ABA sensitivity during early seedling growth.

To study the first stage of seed growth, the percentage of seeds able to germinate in ABA-supplemented medium was determined for the *35S:ERF1* transgenic lines and for the Col-0 wild-type, *era3-1/ein2* and *abi1-1* mutants (Figure 4.6). Three different concencentrations of ABA (0.1, 0.5 and 1 mM) were used in the experiment. Although a slight decrease in the germination *sensu stricto* percentage of *35S:ERF1* lines could be observed at the second day after sowing, no significant differences were found between the *ERF1*-over-expressing and Col-0 lines under the conditions tested in this experiment.

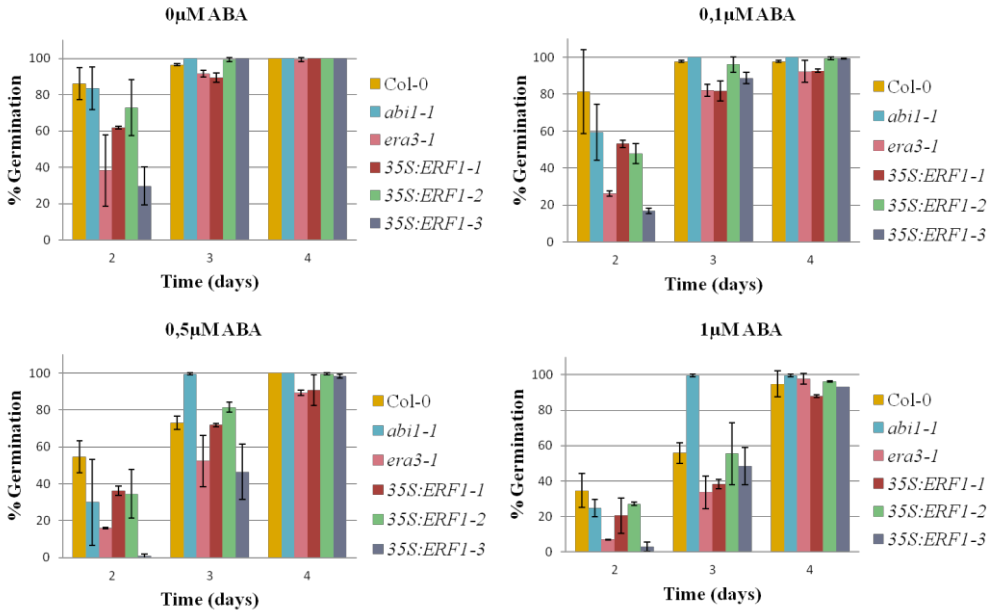


Figure 4.6 Inhibition of seed germination by ABA in *35S:ERF1* lines. Approximately 100 seeds per genotype were sown in MS or 0.1, 0.5 and 1 µM of ABA-supplemented media and scored for radicle emergence after 2, 3 and 4 days. The percentage of germinated seeds is shown for three independent *35S:ERF1* lines (1 to 3) and for Col-0, ABA-hypersensitive (*abi1-1*) and ABA-insensitive *era3-1/ein2* mutants.

Seedling establishment of *35S:ERF1* lines upon addition of ABA at low concentrations was also studied during the second stage of seed growth. The percentage of seeds able to develop green and expanded cotyledons was scored from

day 4 to 12 after sown (Figure 4.7A to D). A significant delay in the growth of *35S:ERF1* lines was found after the 4th (in 0.1µM ABA) or 6th day of the experiment (in 0.5µM ABA). *ERF1*-over-expressing lines behave similarly to the *era3-1/ein2* mutants, whereas the *abi1-1* mutant exhibited an increased rate of seedling establishment in 0.5 µM ABA, consistently with the ABA-hypersensitive phenotype. Considering data from the second stage of seed development, a main role for *ERF1* is envisaged as a repressor of ABA sensing.

To further substantiate this hypothesis, the response of *35S:ERF1* transgenic seeds to paclobutrazol (PAC) was analyzed. PAC is a synthetic plant growth regulator which inhibits GA biosynthesis and is able to cause several physiological changes in plants, including a decreased yield of seed germination. It is known that seeds of ABA-insensitive mutant are also PAC resistant, suggesting a reduced requirement of GA to germinate (Leon-kloosterziel et al., 1998). Seedling establishment in media supplemented with different PAC concentrations (0.2µM, 0.6µM and 1µM) was assayed (Figure 4.8). Emergence of green cotyledons was significantly delayed in *35S:ERF1* plants when compared with Col-0. Since *35S:ERF1* lines are hypersensitive to salt and osmotic stresses, a delay in cotyledon growth under PAC treatment is also expected in our transgenic plants.

These results demonstrate that *35S:ERF1* seeds exhibit an enhanced degree of sensitivity to inhibition of germination and seedling development by exogenous ABA and PAC, suggesting that ERF1 might be involved in the regulation of ABA responsiveness in seeds by itself or through an ABA signalling mediator.

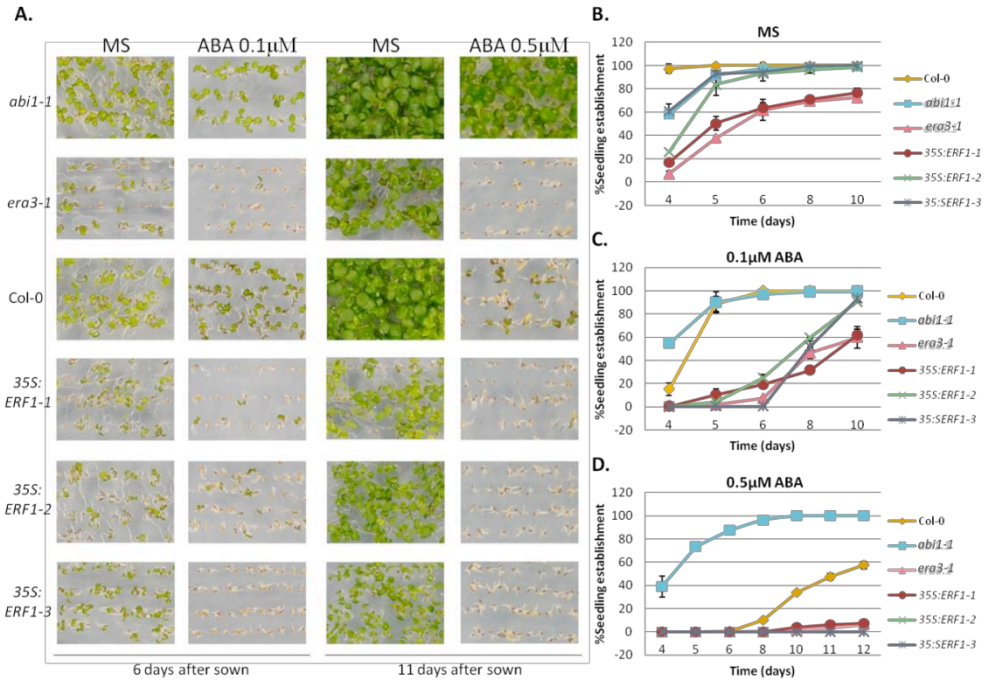


Figure 4.7 Inhibition of seed establichment by ABA in *35S:ERF1* lines. Approximately 100 seeds per genotype (Col-0, *abi1-1*, *era3-1/ein2* and 3 independent (1 to 3) *35S:ERF1* lines) were growth in MS or ABA-suplemented media(0.1 or 0.5µM of ABA). A. Photographs of the cotyledons developed 6 or 11 days after sown B to D. Percentage of seeds able to develop green and expanded cotyledons in MS (B), 0.1 µM (C) and 0.5 µM (D) of ABA-suplemented MS. Error bars represent the standard deviation in two biological replicates. Significant differences were checked with a t-test (p-value<0.05). Differences between the *35S:ERF1* lines and Col-0 were significant after the 4th day when 0.1 µM of ABA was added or after the 6th day when using 0.5 µM of ABA. The experiment was repeated for three times and results obtained follow the same trend.

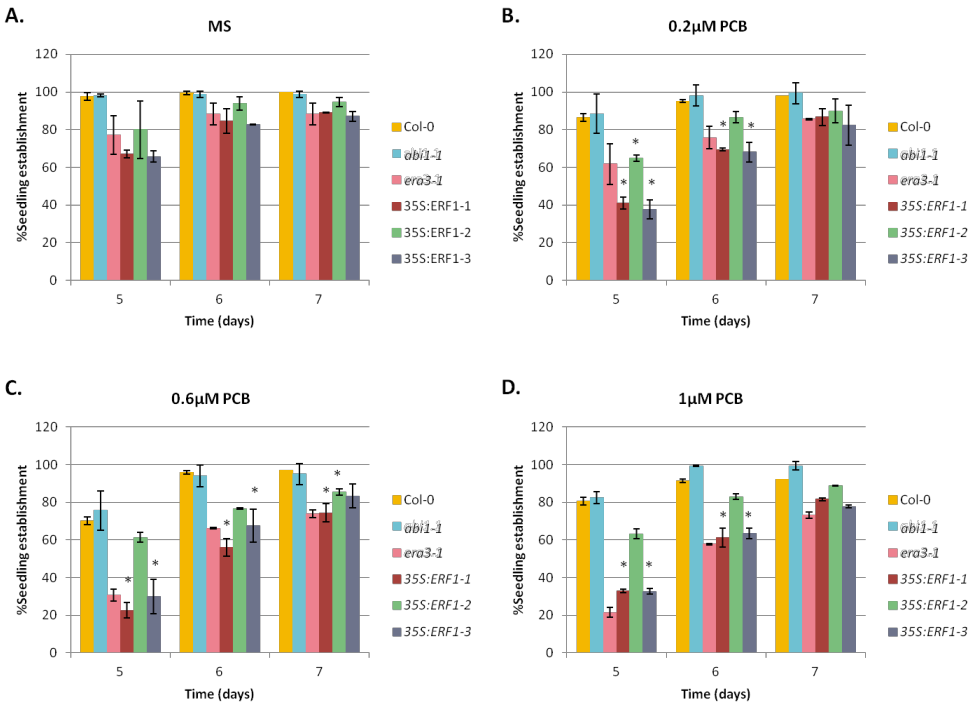
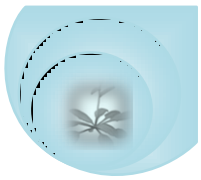


Figure 4.8 Seedling establishment in *35S:ERF1* plants under PAC treatments. Approximately 100 seeds were sown on (A) MS plates or MS plus (B) 0.2µM, (C) 0.6µM or (D) 1µM of the GA-inhibitor paclobutrazol (PAC). Mean values are represented by the bars with the standard deviation from three biological replicates on the top. Asterisks represent significant differences in comparison to Col-0, as determined with a t-test (p-value<0.05)

4.3.7 ERF1 acts downstream of ABA signal transduction pathway

The drought-resistance and ABA-hypersensitivity phenotypes displayed by *ERF1*-over-expressing lines suggest an involvement of *ERF1* the in the ABA signal transduction pathway. Alternatively, the phenotype can be explained by an increased biosynthesis of the hormone in these plants. To discard this alternative hypothesis, ABA levels were measured in vegetative tissue of *35S:ERF1* plants before and after exposure to drought stress (Table 4.3). The ABA levels in *35S:ERF1* lines did not significantly differ from the levels measured in the Col-0 wild type. Hence,



downstream involvement of *ERF1* in ABA signalling was studied next by epistatic analysis with other known components of the pathway.

Table 4.3 ABA levels in leafs from 4-week-old plants before (Control) and after 3 hours of exposition to an air stream into a hood (Drought). Mean values ±SD three biological replicates are shown. Student's *t*-test did not reveal any significant difference between ABA levels in *35S:ERF1* and Col-0 genotypes.

ABA levels (ng/g Fw)		
	Control	Drought
Col-0	9.47±1.45	115.56±28.22
era3-1/ein2	11.54±1.91	120.93±18.81
35S:ERF1	8.03±0.76	124.61±16.46

The ABA signalling mutant *hab2* had been identified by our group as a dominant mutant which exhibited resistance to high (1µM) ABA concentrations. The *HAB2* gene encodes for a cluster A PP2C involved with PYR1/PYL-RCAR proteins in the ABA perception complex. Thus, *HAB2* is a very upstream component of the transduction pathway. To further assess the real function of *ERF1 in planta* and its relevance in the ABA signal transduction pathway we crossed the *35S:ERF1-3* line with *hab2* mutant. The cross resulted in a first generation (F1) of heterozygotes with a hypersensitive response to ABA during germination, the same as the *35S:ERF1* parental line (Figure 4.9A). In the F3 generation from a cross between F1 heterozigotes, *hab2;35S:ERF1* homozygous lines turned out to be more sensitive to ABA than the *hab2* parental (Figure 4.9B). From this experiment it can be concluded that ERF1 acts downstream HAB2 in the ABA signalling pathway, since *ERF1* over-expression was able to revert the *hab2* phenotype.

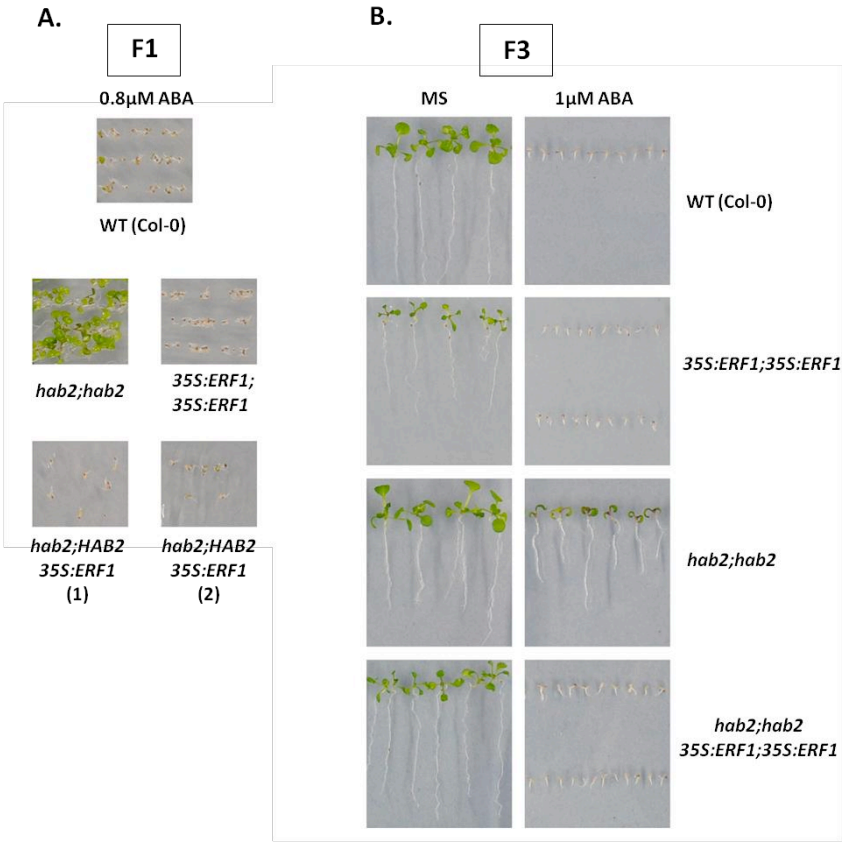
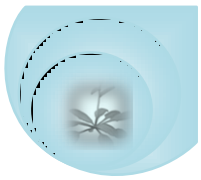


Figure 4.9 Phenotype of *hab2;35S:ERF* seedlings in response to ABA. A. ABA-hypersensitive inhibition of germination in *hab2,HAB2;35S:ERF1* heterozygous lines as compared to the wild type or *hab2* parental lines. Two different siliques (1 and 2) from the F1 were sown in MS 0.8µM ABA (F1). B. ABA-hypersensitive inhibition of germination in double homozygous lines *hab2;35S:ERF1*. Several siliques from the F3 were sown in MS or MS supplemented with 1µM ABA.

4.3.8 *35S:ERF1* plants accumulate ABI5 protein

ABI5 encodes a member of the bZIP family of transcriptional regulators which is expressed in both vegetative tissue and seeds and is regulated by ABA (Finkelstein and Lynch, 2000). This protein plays a key role in the developmental



stages from germination to seedling establishment , and prevents early development when ABA levels in the plant are high (Lopez-Molina et al., 2001).

Because *35S:ERF1* lines are highly hypersensitive to ABA during seedling establishment but not during germination *sensu stricto* (Figure 4.6), we wonder if ABI5 could be involved in a possible regulatory mechanism through ERF1. Stratified seeds were treated with 3 µM ABA for 48h and the accumulation of ABI5 protein was determined by Western blot in this early stage of seed development (Figure 4.10 A). Clearly, the levels of ABI5 protein were increased in the *35S:ERF1* lines respect to Col-0, independently of ABA treatment (Figure 4.10 A). The levels of ABI5 protein were also determined in the post-germinative developmental window. During this stage, ABI5 is usually accumulated upon abiotic stress conditions, and tends to disappear when conditions turn favourable to allow seedling establishment (Lopez-Molina et al., 2001). Stratified seeds were sown and treated for longer periods (6 days) with 0.5µM ABA before Western-blot analysis (Figure 4.10 B). In un-treated seedlings of Col-0 ABI5 totally disappeared, whereas in *35S:ERF1* lines retained low levels of protein. In ABA-treated seedlings, accumulation of ABI5 was higher in the *35S:ERF1* transgenic lines than in Col-0 (Figure 4.10 B).

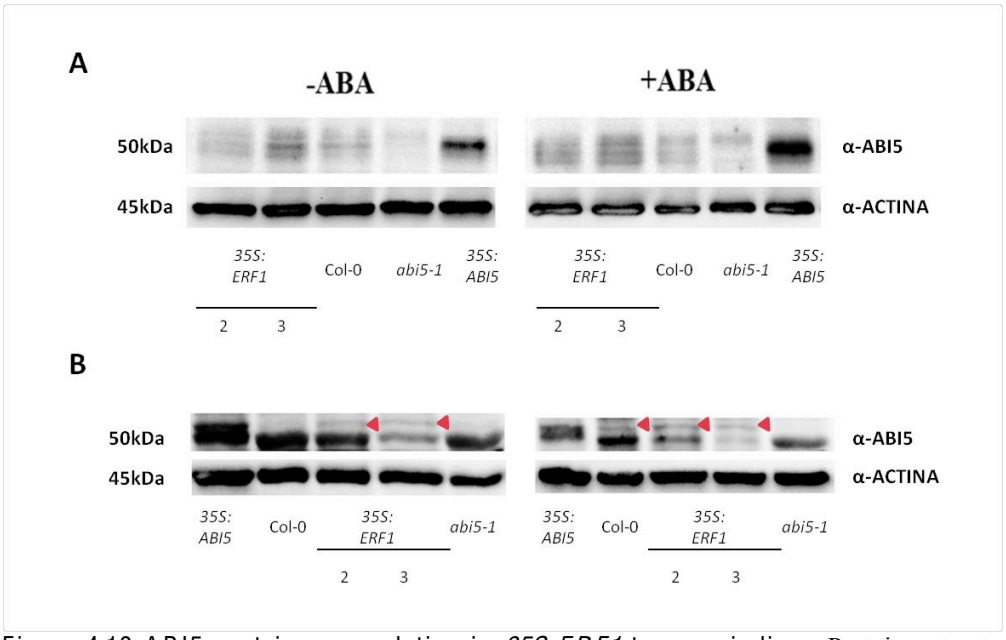
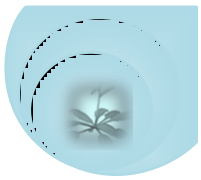


Figure 4.10 ABI5 protein accumulation in *35S:ERF1* transgenic lines. Protein extracts were obtained from stratified seeds of two (2 and 3) *35S:ERF1* lines and treated for A 48h with water (-ABA) or ABA 3µM (+ABA); B 6 days with water (-ABA) or ABA 0.5µM (+ABA). The ABA-insensitive *abi5-1* mutant and the *ABI5* over-expressing line were used as a negative and positive control, respectively. ABI5 protein was detected by Western-blot analyses using ABI5 antibody (α-ABI5). Western-blot against α-ACTIN) was performed to compare loads from different lines.



4.4 DISCUSSION

The use of Arabidopsis as a model has highlighted the relevance of the crosstalk between different hormone pathways to control the plant responses to stress (Lorenzo and Solano, 2005). Hormone signalling pathways target gene expression via transcription factors (TFs), which specifically bind short DNA sequences in the promoters of their target genes to regulate expression levels. TFs are regulatory proteins that have played a pivotal role in the evolution of eukaryotes and have great potential for biotechnological applications, and therefore this study was focussed on candidate TFs with a main role in the regulation of stress responses.

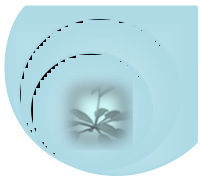
Different hormonal pathways seem to converge in the AP2/ERBP-type TFs, a large multigene family which containing 147 genes (Feng et al., 2005). Since AP2/ERF TFs recognize both the GCC and DRE *cis*-elements involved in the response to biotic and drought stress respectively, it has been suggested that these TFs play a crucial role in integration of stress responses (Zhang et al., 2004) (Lorenzo and Solano, 2005) ERF1 integrates signals from ET and JA pathways in the response to biotic stress, and therefore is a suitable target for hormonal crosstalk regulation. The specificity of the interactions between this TF and the *cis*-elements that are present in different types of stress-responsive genes has been studied in detail (Godoy et al., 2011), and provides indices of an additional role of ERF1 in the regulation of drought-stress response.

Most of the components of the hormonal signalling pathways have been uncovered by using loss of function mutants with a visible phenotype. However, although saturating T-DNA insertion libraries are available for Arabidopsis, a relatively small number of knock-out plants produce relevant information to define gene function. The high level of redundancy observed in the Arabidopsis genome might be an explanation (Riechmann and Ratcliffe, 2000). Gain–of-function approaches using transgenic plants able to over-express components of the pathways

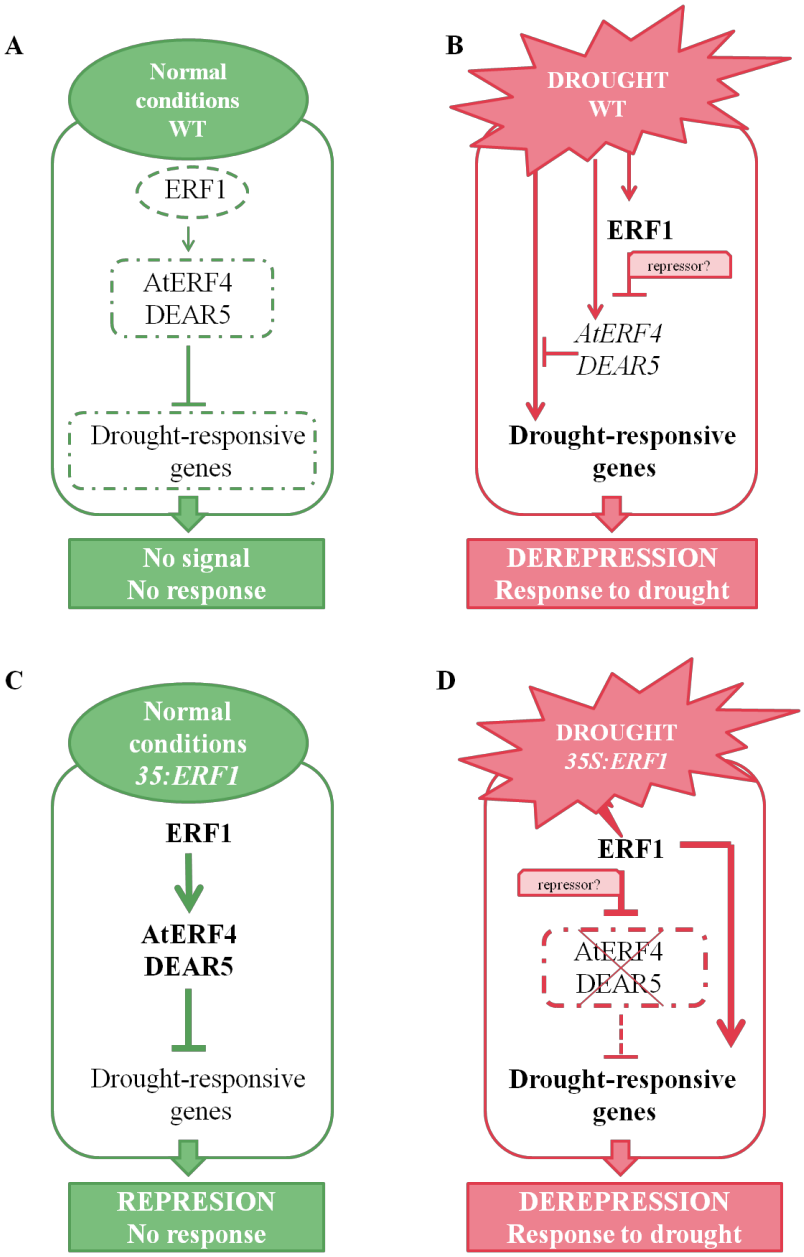
can overcome these limitations and complement knock-out strategies to further deep in the understanding of plant response to stress.

Here we demonstrated that plants over-expressing the transcription factor *ERF1* were more tolerant to drought than wild type (Figure 4.1). The measures of water loss in adult plants or excised leaves upon exposure to drying conditions indicated significant differences between *35S:ERF1* lines and wild type plants (Figure 4.1 C to E). As a further support for the involvement of ERF1 in the tolerance to desiccation, RT-Q-PCR experiments showed that the transcription of the *ERF1* gene in wild type plants is enhanced after drought stress (Fig 4.2 B). Interestingly, in *era3-1/ein2* plants *ERF1* expression was not induced by drought (Fig 4.2 B). Since *era3-1/ein2* is a mutant allele of *EIN2* (Beaudoin et al., 2000), the positive regulator of ET signalling upstream of *ERF1* (Solano and Ecker, 1998), these data suggest that the response to drought mediated by ERF1 is also ET-dependent.

The results of this work also advance a de-repression mechanism to explain the tolerance to drought conferred by *ERF1* ovexpression. Data mining of the protein-binding microarray performed by Godoy et al. pointed at the transcriptional repressors DEAR5 and AtERF4 as direct targets for ERF1, since they contain the promoter *cis*-elements targeted by this TF. DEAR5 and AtERF4 are themselves TFs from AP2/ERF family and are able to regulate the expression of other genes containing the DRE and/or GCC elements (Tsutsui et al., 2009; Yang et al., 2009). Both genes include an EAR active repressor motif in their encoded proteins, which has been involved in epigenetic reprogramming of gene expression as a mechanism to achieve a coordinate response to stress and hormonal signals. The EAR motif can inhibit transcription through different mechanisms that involve *i)* modification of chromatin structure to prevent binding of transcriptional activators to *cis*-elements or *ii)* interaction and inhibition of components of the basal transcription machinery (Hanna-Rose and Hansen, 1996; Pazin and Kadonaga, 1997; Kagale and



Rozwadowski, 2011). EAR repressors facilitate the histone deacetylase (HDA) mediated chromatin modification of target *loci* through recruitment of co-repressors such as AtSAP18 or TPL (TOPLESS) (Kagale and Rozwadowski, 2011). In particular for the case of AtERF4, it has been shown that the EAR domain physically interacts with AtSAP18, which it turn forms a repression complex with the AtHDA19 chromatin remodelling factor (Song and Galbraith, 2006). In addition, it has been demonstrated that AtERF4 is able to actively repress gene transcription both *in vitro* and *in vivo* (Ohta et al., 2001; Fujimoto et al., 2000; Mcgrath et al., 2005; Yang et al., 2005). Figure 4. 3 A, B shows that overexpression of *ERF1* increased basal transcription of *DEAR5* and *AtERF4*, but blocked their transcriptional induction by drought stress. Together with the evidences of physical binding of ERF1 to *DEAR5* and *AtERF4* promoters, results suggest that ERF1 negatively regulates the drought-induced expression of these genes at their transcription site. Since no repressor motif has been found in the *ERF1* coding sequence (Kagale and Rozwadowski, 2011), we hypothesized that the mechanism of action for ERF1-mediated transcriptional blockage requires the help of a yet-unknown repressor protein. The model in Figure 4.11 summarizes the different responses of ERF1 and drought-responsive genes under normal or drought-stress conditions. In ERF1-over-expressing plants the active repression of drought-responsive genes thought AtERF4/DEAR5 is released by the increased levels of ERF1, which blocks their transcriptional induction with the help of a repressor domain in a different protein.



4.11 Model for ERF1 action under normal or drought-stress conditions. The model describes four different responses depending on conditions and the genotypes of: wild type (WT) or ERF1-over-expressing (*35S:ERF1*) plants.

P5CS1 and *RD29B* encode for stress-regulated proteins which are induced upon drought and ABA treatments. Both genes contain the ABRE *cis*-element regulated by ABA (Strizhov et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). These genes are over-expressed in transgenic *35S:ERF1* plants both in normal and drought-stress conditions (Figure 4. 3. C). This argues in favour of an ERF1-mediated de-repression of drought-responsive genes. Despite both genes do not contain DRE or GCC motif and therefore they are not direct targets of DEAR5 or AtERF4, their enhanced expression in *era3-1/ein2* plants (Figure 4.3 C) suggest again an ET-dependent response to drought. .

Since ABA plays a pivotal role in the abiotic stress regulation the effect of exogenous ABA treatment in *ERF1* expression was studied (Figure 4.2). *ERF1* expression in wild-type plants was repressed upon ABA addition, which suggest that *ERF1* expression can be regulated by ABA in addition to ET and JA (Solano et al., 1998; Lorenzo et al., 2003). Moreover, *ERF1* expression was not affected by ABA treatment in the *abi1-1* ABA-insensitive mutant, indicating a requirement of ABA-signal transduction components to down-regulate *ERF1* expression. More importantly, *ERF1* expression levels in *era3-1/ein2* plants were down-regulated under normal conditions compared with Col-0 and its expression did not change after ABA treatment. This result suggests that ERF1 integrates the ABA signal in the crosstalk ABA-ET. In addition, other authors have showed than the plant defensin PDF1.2 is down-regulated after ABA treatment (Anderson et al., 2004). Since *PDF1.2* is a direct target of ERF1 (Godoy et al., 2011) we suggest ABA repress PDF1.2 through down-regulation of ERF1.

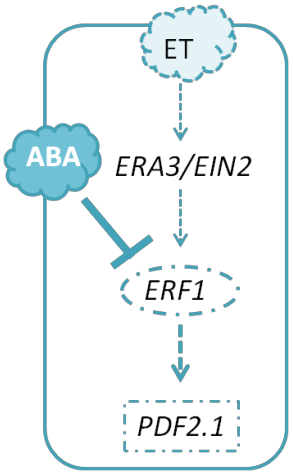


Figure 4.12 Hypothetical model describing the role of ERF1 in the integration of ABA and ET signalling to regulate *PDF1.2* gene expression.

The regulatory role of ABA is especially manifest during the germination and early development of seedlings. This hormone prevents seed germination under unfavourable water conditions such as drought, salt, or osmotic stress. When seeds detect water deficit, ABA signalling pathway is activated, which leads to a delay in germination and early development. ABA-insensitive plants such as *abi1-1* are able to germinate and develop under harsh conditions, while ABA-hypersensitive (*era3-1/ein2*) mutants have difficulties to germinate even in non-stressed conditions. ET and ABA have antagonist roles during germination (Gazzarrini and McCourt, 2003) and constitutive ET responses or application of the ET precursor, ACC, decrease the ABA sensitivity of the seeds (Ghassemian et al., 2000), whereas ET-arrest in the presence of AgNO3 increases ABA sensitivity during seedling establishment (Subbiah and Reddy, 2010). ET-insensitive mutants (*ein2* or *etr1*) are ABA-hypersensitive, and conversely, ET-enhanced production (*eto1*) and response (*ctr1*) mutants show reduced sensitivity to ABA (Ghassemian et al., 2000; Beaudoin et al., 2000). Here, when transgenic *35S:ERF1* seeds were sown in medium supplemented

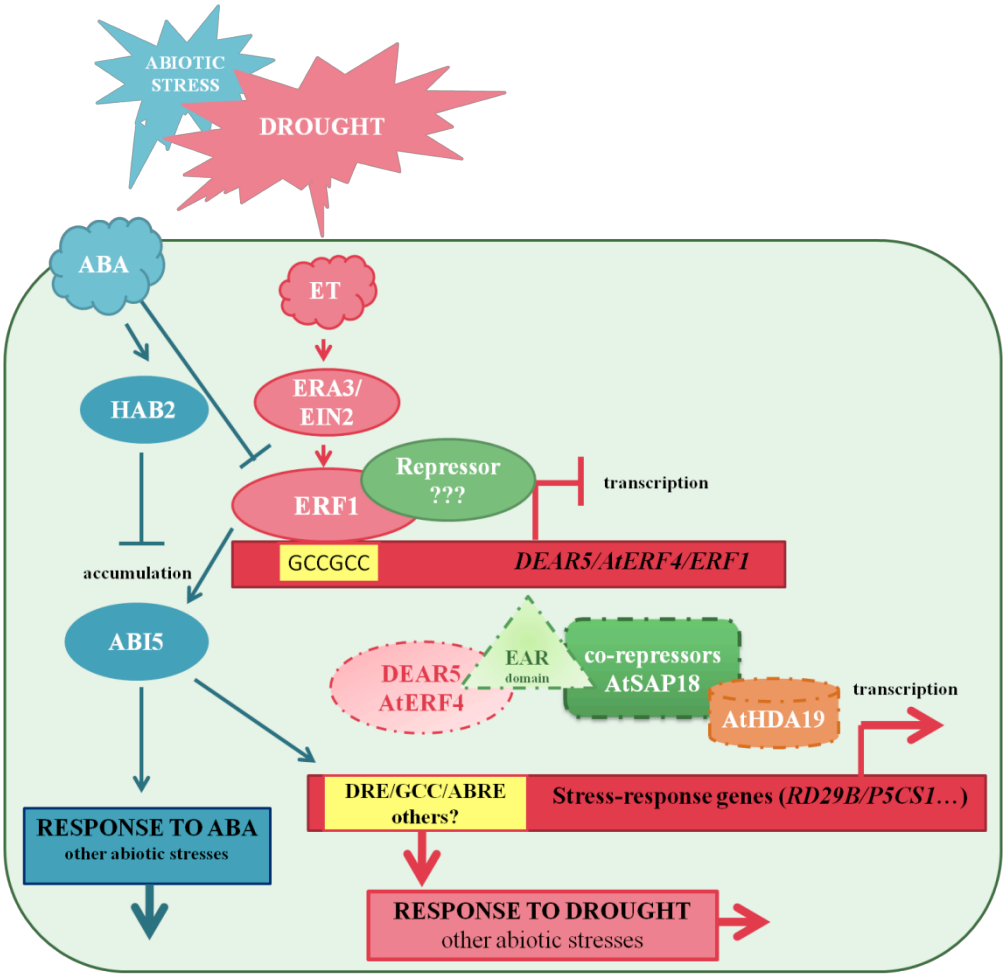
with NaCl or mannitol (Figure 4.4 and 4.5), a clear delay in the seedling establishment was observed. Furthermore, early development growth was significantly delayed in *35S:ERF1* plants as compared to Col-0 under low ABA concentrations. Considering that ABA biosynthesis did not result altered by ERF1 over-expression (Table 4.3), this data support the role of ERF1 in the signalling events downstream ABA biosynthesis during early development.

In order to determine ERF1 position in the ABA signalling pathway, epistatic experiments with other well-localized components of the pathway were performed. The ABA-resistant, *hab2* mutant encodes for an upstream component of the pathway involved in the ABA perception complex with PYR1/PYL-RCAR proteins. In the absence of ABA, PP2Cs proteins like HAB2 inhibit SnRKs (Snf1-related protein kinases) and the consequent transduction of ABA signal through ABFs (ABA-responsive element binding factors). In the presence of ABA, PYR1/PYL-RCAR proteins inhibit PP2Cs, which allows the accumulation of active SnRKs and subsequent phosphorylation and activation of ABFs (Cutler et al., 2010). Homozygous *hab2*;*35S:ERF1* mutants exhibited an ABA-hypersensitive phenotype similar to the *35S:ERF1* parental line (Figure 4.9). Thus, *ERF1* overexpression was able to revert the *hab2* phenotype in early seedling grown which strongly suggests than ERF1 acts downstream of HAB2.

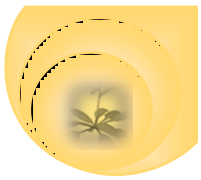
ABA and GA have antagonist effects during seed development, and the balance between both hormones determines the final outcome of germination, *i.e.,* growth arrest, delay or unabated growth (Finch-Savage and Leubner-Metzger, 2006). Plants treated with PAC, a synthetic inhibitor of GA biosynthesis, exhibit a delay in the establishment of green cotyledons. In our study, transgenic plants overexpressing *ERF1* showed delayed seedling establishment under PAC treatment as compared with wild type (Figure 4.8). Similarly to osmotic and salt stresses or exogenous ABA application, *35S:ERF1* transgenic plants behave like ABA-hypersensitive mutants under PAC treatment.

ABI3 and ABI5 are downstream components of the ABA-signalling pathway that encode for TFs expressed mainly in seeds and barely in vegetative tissues (Giraudat et al., 1992; Finkelstein and Lynch, 2000). Plants with mutations in these genes exhibit ABA insensitivity during seed germination and early seedling development (Ooms et al., 1993; Parcy et al., 1994). *ABI3* and *ABI5* mRNAs accumulate in dry seeds and, under normal growth conditions, germination is a period during which *ABI3* and *ABI5* mRNAs rapidly decay. However, germination can be delayed by osmotic stress or exogenous ABA treatments if performed 48 h after seed embedding, prior to cotyledon greening (Lopez-Molina et al., 2001). Such treatments stimulate and maintain *ABI3* and *ABI5* expression and lead to sustained germination arrest and increased osmotolerance. It has been suggested that the time window permitting ABA-induced growth arrest represents a last protective embryonic checkpoint, prior to irreversible commitment to autotrophy (Lopez-Molina *et al.*, 2001). According to this, the post-germinative arrest upon ABA addition was studied. *ERF1*-over-expressing lines were delayed during seedling establishment under low ABA concentrations, but were able to germinate at the wild type level when supplemented with increasing concentrations of ABA (Figure 4.6 and 4.7), which demonstrates a clear alteration in the post-germinative process. Remarkably, ABI5 accumulation was increased in *35S:ERF1* embedded seeds in comparison to the wild type and independently of ABA treatment (Figure 4.10), which suggest a position of ERF1 upstream the ABI5 protein in the ABA-signalling pathway that regulates early seedling-growth.

A diagram summarizing the action mode of ERF1 during abiotic stresses and its position in the crosstalk between ABA-ET pathways is shown in Figure 4.11. A possible mechanism to explain the ERF1-mediated derepression that was observed in *35S:ERF1* transgenic plants under drought conditions is drawn.



4.12 Schematic model representing the position of ERF1 in the ABA signalling pathway that mediate plant response to drought stress and the crosstalk with ET/JA route.



5. CONCLUSIONS

1. Phage-display technology can be used to express and select for Arabidopsis proteins with functional microbe-binding domains. In this work two libraries for phage-mediated display of Arabidopsis proteins have been constructed. One of them, the T7LATPs library, contained $2.3x10^7$ different plant transcripts, which virtually covers the entire protein-encoding genome of *A. thaliana*.

2. A biopanning procedure using living cell of *Pseudomonas sp*. can be used for selection of particular phage clones, such as T7-ATERF-1, which confirmed their microbe-binding capacity in competitive assays.

3. DNA-microarray hybridization as described in this work can be used to high-throughput quantify clone enrichment during the selection.

4. Significance analysis of microarray data produced upon hybridization of bio-panned libraries identified 418 genes as putative binders of bacterial molecules. From these, immune-related functions of 23 genes have been confirmed by a two-hybrid based approach to high-throughput identification of Plant-Pathogen Interaction Networks (*PPIN*-1).

5. *GFP-ATERF-1* translocates from the nucleus to the cytoplasm upon infiltration of *Pseudomonas sp*. components into the leaves of transgenic tobacco and Arabidopsis plants.

6. A loss-of-function, *aterf1-1* mutant is more sensitive to *Pst* than the wild type Col-0, which demonstrates that *ATERF-1* is required for a proper immune response *in vivo*.

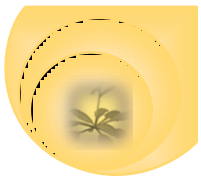
7. The overexpression of *Ethylene Response Factor 1* (*ERF1*) in Arabidopsis confers tolerance to drought, potentially through a derepression mechanism which involves TFs from AP2/ERF1 family. Specifically, under drought conditions ERF1 blocks the induction of the active drought-repressors *DEAR5* and *AtERF4* allowing the transcription of drought-responsive genes, and consequently an enhanced drought response.

8. *ERF1* expression is increased after drought stress and this expression is ET-dependent. Furthermore, *ERF1* expression is decreased by ABA-treatment through an ET-independent pathway, suggesting ERF1 is a key node in the interaction between ABA and ET signalling pathways during abiotic stress.

9. *ERF1* overexpression prevents early seedling growth during salt and osmotic stresses and confers hypersensitivity to exogenous addition of ABA and the GA biosynthesis inhibitor PAC.

10. ERF1 acts downstream of HAB2 in the ABA signalling pathway that regulates early seedling establishment. Furthermore, ERF1 is able to antagonize HAB2 function supporting new evidences of the ABA-ET crosstalk at this developmental cue.

11. The overexpression of *ERF1* induces ABI5 protein accumulation, both in absence and presence of ABA, early in the post-germinative period. Our results suggest that transgenic *35S:ERF1* plants show delayed seedling establishment in response to abiotic stress or exogenous ABA, due to these higher ABI5 levels.



1. La tecnología *phage-display* puede emplearse para expresar y seleccionar proteínas de Arabidopsis con dominios funcionales de unión a microorganismos. En este trabajo se han construido dos genotecas de Arabidopsis que se expresan a través de fagos. Una de ellas, L7LATPs, contiene $2.3x10^7$ transcritos vegetales diferentes, que cubren virtualmente el genoma completo que codifica proteínas de *A. thaliana.*

2. La técnica del *biopanning,* usando células vivas de *Pseudomonas sp.,* puede emplearse para la selección de clones determinados, como T7-ATERF-1, del que se ha confirmado su capacidad de unión a microorganismos mediante ensayos competitivos.

3. La hibridación de microarrays con DNA, descrita en este trabajo, puede emplearse para la cuantificación a gran escala del enriquecimiento de clones durante la selección.

4. El análisis significativo de los datos obtenidos de los *microarrays* tras su hibridación con las genotecas cribadas por *biopanning,* identificó 418 genes como posibles ligandos de moléculas bacterianas. Entre ellos, 23 genes relacionados con la inmunidad vegetal habían sido confirmados por una aproximación basada en la técnica de selección de dos híbridos para la identificación de la red de interacción planta-patógeno (*PPIN-1*).

5. *GFP-ATERF-1* se transloca del núcleo al citoplasma tras la infiltración de componentes de *Pseudomonas sp.* dentro de las hojas de tabaco y Arabidopsis.

6. El mutante de pérdida de función *aterf-1* es más susceptible a *Pst* que el ecotipo silvestre Col-0, demostrando que *ATERF-1* se requiere para una respuesta inmune correcta *in vivo.*

7. La sobreexpresión del Factor de Respuesta a Etileno 1 (ERF1) en Arabidopsis confiere tolerancia a sequía, potencialmente a través de un mecanismo de desrepresión que implica a TFs de la familia AP2/ERF. Concretamente, bajo condiciones de sequía ERF1 bloquea la inducción de los represores activos DEAR5 y AtERF4, permitiendo la transcripción de genes de respuesta a sequía y consecuentemente, una mayor respuesta a sequía.

8. La expresión de *ERF1* aumenta tras el estrés por sequía y es dependiente de ET. Además, la expresión de *ERF1* disminuye tras el tratamiento con ABA a través de una ruta independiente de ET, sugiriendo que ERF1 es un punto clave en la interacción entre las rutas de señalización de ABA y ET durante el estrés abiótico.

9. La sobreexpresión de *ERF1* previene el crecimiento y desarrollo temprano de plántulas durante el estrés salino y osmótico, y confiere hipersensibilidad a la adición exógena de ABA y al inhibidor de la biosíntesis de GAs, PAC.

10. ERF1 actúa aguas abajo de HAB2 en la ruta de señalización del ABA que regula el establecimiento temprano de plántulas. Del mismo modo, ERF1 es capaz de antagonizar la función de HAB2 aportando nuevas evidencias del *crosstalk* ABA-ET en este proceso del desarrollo.

11. La sobreexpresión de *ERF1* induce la acumulación de la proteína ABI5, en ausencia y en presencia de ABA, durante el comienzo del periodo post-germinativo. Nuestros resultados sugieren que en respuesta a estrés abiótico o tras la aplicación exógena de ABA, las plantas transgénicas *35S:ERF1* presentan un retraso en el establecimiento de plántula debido a este aumento en los niveles de ABI5.

6. BIBLIOGRAPHY

Abe, H. et al. (2003). Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *The Plant cell* 15: 63-78.

Abramovitch, R.B. et al. (2006). Bacterial elicitation and evasion of plant innate immunity. *Nature reviews. Molecular cell biology* 7: 601-11.

Ackermann, H.W. (1998). Tailed bacteriophages: the order caudovirales. *Advances in virus research* 51: 135-201.

Adie, B.A.T. et al. (2007). ABA Is an Essential Signal for Plant Resistance to Pathogens Affecting JA Biosynthesis and the Activation of Defenses in Arabidopsis[W]. *The Plant cell* 19: 1665-81.

Agrios, G.N. (1997). Plant Pathology. Ed. 4º

Alfano, J.R. and Collmer, A. (2004). Type III secretion system effector proteins: double agents in bacterial disease and plant defense. *Annual review of phytopathology* 42: 385-414.

Alonso, J.M. (1999). EIN2, a Bifunctional Transducer of Ethylene and Stress Responses in Arabidopsis. *Science* 284: 2148-2152.

Alonso, J.M. and Stepanova, A.N. (2004). The ethylene signaling pathway. *Science (New York, N.Y.)* 306: 1513-5.

Altenbach, D. and Robatzek, S. (2007). Pattern recognition receptors: from the cell surface to intracellular dynamics. *Molecular plant-microbe interactions : MPMI* 20: 1031-9.

Anderson, J.P. et al. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. *The Plant cell* 16: 3460-79.

Asai, T. et al. (2002). MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415: 977-83.

Bardoel, B.W. et al. (2011). Pseudomonas Evades Immune Recognition of Flagellin in Both Mammals and Plants. *PLoS Pathogens* 7: e1002206.

Bartels, D. and Sunkar, R. (2005). Drought and Salt Tolerance in Plants. *Critical Reviews in Plant Sciences* 24: 23-58.

Beaudoin, N. et al. (2000). Interactions between abscisic acid and ethylene signaling cascades. *The Plant cell* 12: 1103-15.

Bender, C.L. et al. (1999). Pseudomonas syringae phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. *Microbiology and molecular biology reviews : MMBR* 63: 266-92.

Bensmihen, S. et al. (2002). The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. *The Plant cell* 14: 1391-403.

Bent, A.F. et al. (1994). RPS2 of Arabidopsis thaliana: a leucine-rich repeat class of plant disease resistance genes. *Science (New York, N.Y.)* 265: 1856-60.

Bent, A.F. and Mackey, D. (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. *Annual review of phytopathology* 45: 399-436.

Bernoux, M. et al. (2008). RD19, an Arabidopsis cysteine protease required for RRS1-R-mediated resistance, is relocalized to the nucleus by the Ralstonia solanacearum PopP2 effector. *The Plant cell* 20: 2252-64.

Bishop-Hurley, S.L. et al. (2002). Phage-Displayed Peptides as Developmental Agonists for Phytophthora capsici Zoospores. *Applied and Environmental Microbiology* 68: 3315-3320.

Blatt, M.R. (2000). Cellular signaling and volume control in stomatal movements in plants. *Annual review of cell and developmental biology* 16: 221-41.

Boller, T. (1995). Chemoperception of Microbial Signals in Plant Cells. *Annual Review of Plant Physiology and Plant Molecular Biology* 46: 189-214.

Boller, T. and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual review of plant biology* 60: 379-406.

Boller, T. and He, S.Y. (2009). Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. *Science (New York, N.Y.)* 324: 742-4.

Book, A.J. et al. (2010). Affinity purification of the Arabidopsis 26 S proteasome reveals a diverse array of plant proteolytic complexes. *The Journal of biological chemistry* 285: 25554-69.

Bratkovič, T. (2009). Progress in phage display: evolution of the technique and its applications. *Cellular and molecular life sciences : CMLS* 3: 749-767.

Brogden, K.A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature reviews. Microbiology* 3: 238-50.

Brooks, D.M. et al. (2005). The Pseudomonas syringae phytotoxin coronatine promotes virulence by overcoming salicylic acid-dependent defences in Arabidopsis thaliana. *Molecular plant pathology* 6: 629-39.

Buchanan, Gruissem y Jones (2000). Biochemistry and Molecular Biology of Plants. *American Society of Plant Physiology.*

Busk, P.K. and Pagès, M. (1998). Regulation of abscisic acid-induced transcription. *Plant molecular biology* 37: 425-35.

Caplan, J. et al. (2008). Plant NB-LRR immune receptors: from recognition to transcriptional reprogramming. *Cell host & microbe* 3: 126-35.

Chang, J.H. et al. (2005). A high-throughput, near-saturating screen for type III effector genes from Pseudomonas syringae. *Proceedings of the National Academy of Sciences of the United States of America* 102: 2549-54.

Charlton, K. et al. (2001). The isolation of super-sensitive anti-hapten antibodies from combinatorial antibody libraries derived from sheep. *Biosensors & bioelectronics* 16: 639-46.

Chen, H. et al. (2009). ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 repress SALICYLIC ACID INDUCTION DEFICIENT2 expression to negatively regulate plant innate immunity in Arabidopsis. *The Plant cell* 21: 2527-40.

Chen, T. et al. (2010). Substrates of the Arabidopsis thaliana protein isoaspartyl methyltransferase 1 identified using phage display and biopanning. *The Journal of biological chemistry* 285: 37281-92.

Chen, Z. et al. (2007). Pseudomonas syringae type III effector AvrRpt2 alters Arabidopsis thaliana auxin physiology. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20131-6.

Chen, Z. et al. (2000). The Pseudomonas syringae avrRpt2 gene product promotes pathogen virulence from inside plant cells. *Molecular plant-microbe interactions : MPMI* 13: 1312-21.
Chinchilla, D. et al. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448: 497-500.
Chinnusamy, V. et al. (2007). Cold stress regulation of gene expression in plants. *Trends in plant science* 12: 444-51.
Chisholm, S.T. et al. (2006). Host-microbe interactions: shaping the evolution of the plant immune response. *Cell* 124: 803-14.
Choi, H. et al. (2000). ABFs, a family of ABA-responsive element binding factors. *The Journal of biological chemistry* 275: 1723-30.
Christie, P.J. et al. (2005). Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annual review of microbiology* 59: 451-85.
Cicchini, C. et al. (2002). Searching for DNA-protein interactions by lambda phage display. *Journal of molecular biology* 322: 697-706.
Clark, J.R. and March, J.B. (2006). Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends in biotechnology* 24: 212-8.
Collmer, A. et al. (2000). Pseudomonas syringae Hrp type III secretion system and effector proteins. *Proceedings of the National Academy of Sciences of the United States of America* 97: 8770-7.
Costerton, J.W. et al. (1999). Bacterial biofilms: a common cause of persistent infections. *Science (New York, N.Y.)* 284: 1318-22.
Curtis, M.D. and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant physiology* 133: 462-9.
Cutler, S. et al. (1996). A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. *Science (New York, N.Y.)* 273: 1239-41.
Cutler, S.R. et al. (2010). Abscisic acid: emergence of a core signaling network. *Annual review of plant biology* 61: 651-79.
Danner, S. and Belasco, J.G. (2001). T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. *Proceedings of the National Academy of Sciences of the United States of America* 98: 12954-9.
Daszkowska-Golec, A. (2011). Arabidopsis seed germination under abiotic stress as a concert of action of phytohormones. *Omics : a journal of integrative biology* 15: 763-74.
Deblaere, R. et al. (1985). Efficient octopine Ti plasmid-derived vectors for Agrobacterium -mediated gene transfer to plants. *Nucleic Acids Research* 13: 4777-4788.
Deng, S.J. et al. (1994). Selection of antibody single-chain variable fragments with improved carbohydrate binding by phage display. *The Journal of biological chemistry* 269: 9533-8.
Deslandes, L. et al. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. *Proceedings of the National Academy of Sciences of the United States of America* 100: 8024-9.
Dias-Neto, E. et al. (2009). Next-generation phage display: integrating and comparing available molecular tools to enable cost-effective high-throughput analysis. *PloS one* 4: e8338.

Dong, X., Mindrinos, M., Davis, K.R., et al. (1991). Induction of Arabidopsis Defense Genes by Virulent and Avirulent Pseudomonas syringae Strains and by a Cloned Avirulence Gene. *The Plant Cell* 3: 61.

Dong, X., Mindrinos, M., D, K.R., et al. (1991). lnduction of Arabídopsís Defense Genes by Virulent and Avirulent Pseudomonas syrngae Strains and by a Cloned Avirulence Gene. *Society* 3: 61-72.

Eitas, T.K. and Dangl, J.L. (2010). NB-LRR proteins: pairs, pieces, perception, partners, and pathways. *Current opinion in plant biology* 13: 472-7.

Faix, P.H. et al. (2004). Phage display of cDNA libraries: enrichment of cDNA expression using open reading frame selection. *BioTechniques* 36: 1018-22, 1024, 1026-9.

Felix, G. et al. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant journal : for cell and molecular biology* 18: 265-76.

Feng, J.-X. et al. (2005). An annotation update via cDNA sequence analysis and comprehensive profiling of developmental, hormonal or environmental responsiveness of the Arabidopsis AP2/EREBP transcription factor gene family. *Plant molecular biology* 59: 853-68.

Fernández-Arbaizar, A. et al. (2012). Isolation and characterization of novel mutant loci suppressing the ABA hypersensitivity of the Arabidopsis coronatine insensitive 1-16 (coi1-16) mutant during germination and seedling growth. *Plant & cell physiology* 53: 53-63.

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

Finkelstein, R.R. et al. (2002). Abscisic acid signaling in seeds and seedlings. *The Plant cell* 14 Suppl: S15-45

.

Finkelstein, R.R. (1994). Mutations at two new Arabidopsis ABA response loci are similar to the abi3 mutations. *The Plant Journal* 5: 765-771.

Finkelstein, R.R. et al. (1998). The Arabidopsis abscisic acid response locus ABI4 encodes an APETALA 2 domain protein. *The Plant cell* 10: 1043-54.

Finkelstein, R.R. and Lynch, T.J. (2000). The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. *The Plant cell* 12: 599-609.

Fonseca, S. et al. (2009). (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. *Nature chemical biology* 5: 344-50.

Fujimoto, S.Y. et al. (2000). Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. *The Plant cell* 12: 393-404.

Gabriel, D.W. and Rolfe, B.G. (1990). Working Models of Specific Recognition in Plant-Microbe Interactions. *Annual Review of Phytopathology* 28: 365-391.

Gao, M. et al. (2009). Regulation of cell death and innate immunity by two receptor-like kinases in Arabidopsis. *Cell host & microbe* 6: 34-44.

Garcia-Mata, C. and Lamattina, L. (2007). Abscisic acid (ABA) inhibits light-induced stomatal opening through calcium- and nitric oxide-mediated signaling pathways. *Nitric oxide : biology and chemistry / official journal of the Nitric Oxide Society* 17: 143-51.

García-Sánchez, S. et al. (2005). Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen, Candida albicans. *Molecular biology of the cell* 16: 2913-25.
Gargir, A. et al. (2002). Single chain antibodies specific for fatty acids derived from a semi-synthetic phage display library. *Biochimica et biophysica acta* 1569: 167-73.
Gazzarrini, S. and McCourt, P. (2003). Cross-talk in plant hormone signalling: what Arabidopsis mutants are telling us. *Annals of botany* 91: 605-12.
Gelvin, S.B. (2000). AGROBACTERIUM AND PLANT GENES INVOLVED IN T-DNA TRANSFER AND INTEGRATION. *Annual review of plant physiology and plant molecular biology* 51: 223-256.
Gera, N. et al. (2012). Protein selection using yeast surface display. *Methods (San Diego, Calif.)*.
Ghassemian, M. et al. (2000). Regulation of abscisic acid signaling by the ethylene response pathway in Arabidopsis. *The Plant cell* 12: 1117-26.
Gimenez-Ibanez, S. and Rathjen, J.P. (2010). The case for the defense: plants versus Pseudomonas syringae. *Microbes and infection / Institut Pasteur* 12: 428-37.
Giraudat, J. et al. (1992). Isolation of the Arabidopsis ABI3 gene by positional cloning. *The Plant cell* 4: 1251-61.
Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual review of phytopathology* 43: 205-27.
Glickmann, E. et al. (1998). Auxin production is a common feature of most pathovars of Pseudomonas syringae. *Molecular plant-microbe interactions : MPMI* 11: 156-62.
Godoy, M. et al. (2011). Improved protein-binding microarrays for the identification of DNA-binding specificities of transcription factors. *The Plant journal : for cell and molecular biology*.
Gopalan, S. and Ausubel, F.M. (2008). A metasystem of framework model organisms to study emergence of new host-microbe adaptations. *PloS one* 3: e3891.
Goritschnig, S. et al. (2007). The ubiquitin pathway is required for innate immunity in Arabidopsis. *The Plant journal : for cell and molecular biology* 49: 540-51.
Govan, J. and Deretic, V. (1996). Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. *Microbiol. Rev.* 60: 539-574.
Grant, M.R. and Jones, J.D.G. (2009). Hormone (dis)harmony moulds plant health and disease. *Science (New York, N.Y.)* 324: 750-2.
Gómez, J. et al. (1988). A gene induced by the plant hormone abscisic acid in response to water stress encodes a glycine-rich protein. *Nature* 334: 262-4.
Gómez-Gómez, L. and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular cell* 5: 1003-11.
Hamilton, D.W. et al. (2000). Ca2+ channels at the plasma membrane of stomatal guard cells are activated by hyperpolarization and abscisic acid. *Proceedings of the National Academy of Sciences of the United States of America* 97: 4967-72.
Hanin, M. et al. (2011). Plant dehydrins and stress tolerance: versatile proteins for complex mechanisms. *Plant signaling & behavior* 6: 1503-9.
Hanna-Rose, W. and Hansen, U. (1996). Active repression mechanisms of eukaryotic transcription repressors. *Trends in genetics : TIG* 12: 229-34.

Hao, D. et al. (1998). Unique mode of GCC box recognition by the DNA-binding domain of ethylene-responsive element-binding factor (ERF domain) in plant. *The Journal of biological chemistry* 273: 26857-61.

Haq, I.U. et al. (2012). Bacteriophages and their implications on future biotechnology: a review. *Virology journal* 9: 9.

Hauck, P. et al. (2003). A Pseudomonas syringae type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. *Proceedings of the National Academy of Sciences of the United States of America* 100: 8577-82.

Hauser, M.-T. et al. (2011). Transgenerational epigenetic inheritance in plants. *Biochimica et biophysica acta* 1809: 459-68.

He, P. et al. (2007). Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cellular microbiology* 9: 1385-96.

He, P. et al. (2006). Specific bacterial suppressors of MAMP signaling upstream of MAPKKK in Arabidopsis innate immunity. *Cell* 125: 563-75.

Hetherington, A.M. and Woodward, F.I. (2003). The role of stomata in sensing and driving environmental change. *Nature* 424: 901-8.

Hilhorst, H.W.M. et al. (2007). Definitions and hypotheses of seed dormancy. *Seed Development, Dormancy and Germination. Annual Plant Reviews* 27: 50-71.

Himmelbach, A. et al. (2002). Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. *The EMBO journal* 21: 3029-38.

Hirano, S.S. and Upper, C.D. (2000). Bacteria in the leaf ecosystem with emphasis on Pseudomonas syringae-a pathogen, ice nucleus, and epiphyte. *Microbiology and molecular biology reviews : MMBR* 64: 624-53.

Ho, M. and Pastan, I. (2009). Mammalian cell display for antibody engineering. *Methods in molecular biology (Clifton, N.J.)* 525: 337-52, xiv.

Holdsworth, M.J. et al. (2008). Molecular networks regulating Arabidopsis seed maturation, after-ripening, dormancy and germination. *The New phytologist* 179: 33-54.

Hoth, S. et al. (2002). Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. *Journal of cell science* 115: 4891-900.

Hsieh, T.-H. et al. (2002). Tomato plants ectopically expressing Arabidopsis CBF1 show enhanced resistance to water deficit stress. *Plant physiology* 130: 618-26.

Huang, G.-T. et al. (2012). Signal transduction during cold, salt, and drought stresses in plants. *Molecular biology reports* 39: 969-87.

Hundertmark, M. et al. (2011). The reduction of seed-specific dehydrins reduces seed longevity in Arabidopsis thaliana. *Seed Science Research* 21: 165-173.

Inal, J.M. (2003). Phage therapy: a reappraisal of bacteriophages as antibiotics. *Archivum immunologiae et therapiae experimentalis* 51: 237-44.

Ito, Y. et al. (2006). Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice. *Plant & cell physiology* 47: 141-53.

Jacobsson, K. et al. (2003). Shotgun Phage Display - Selection for Bacterial Receptins or other Exported Proteins. *Biological procedures online* 5: 123-135.

Jander, G. et al. (2000). Positive Correlation between Virulence of Pseudomonas aeruginosa Mutants in Mice and Insects. *Journal of Bacteriology* 182: 3843-3845.
Janská, A. et al. (2010). Cold stress and acclimation - what is important for metabolic adjustment? *Plant biology (Stuttgart, Germany)* 12: 395-405.
Jelenska, J. et al. (2007). A J domain virulence effector of Pseudomonas syringae remodels host chloroplasts and suppresses defenses. *Current biology : CB* 17: 499-508.
Jeong Mee Park1, C.-J.P., Suk-Bae Lee, Byung-Kook Ham, Ryoung Shin, and Kyung-Hee Paek et al. (2001). Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *THE PLANT CELL* 13: 1035-46.
Jha, G. et al. (2005). Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. *Molecular plant-microbe interactions : MPMI* 18: 891-8.
Jones, J.D.G. and Dangl, J.L. (2006). The plant immune system. *Nature* 444: 323-9.
Kagale, S. and Rozwadowski, K. (2011). EAR motif-mediated transcriptional repression in plants: An underlying mechanism for epigenetic regulation of gene expression. *Epigenetics* 6: 141-146.
Kasuga, M. et al. (1999). Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. *Nature biotechnology* 17: 287-91.
Katagiri, F. (2004). A global view of defense gene expression regulation--a highly interconnected signaling network. *Current opinion in plant biology* 7: 506-11.
Katagiri, F. et al. (2002). The Arabidopsis Thaliana-Pseudomonas Syringae Interaction. *The Arabidopsis Book* 20: 1.
Kay, B.K. et al. (2000). Convergent evolution with combinatorial peptides. *FEBS letters* 480: 55-62.
Kay, S. et al. (2007). A bacterial effector acts as a plant transcription factor and induces a cell size regulator. *Science (New York, N.Y.)* 318: 648-51.
Keen, N.T. (1990). Gene-for-gene complementarity in plant-pathogen interactions. *Annual review of genetics* 24: 447-63.
Kehoe, J.W. and Kay, B.K. (2005). Filamentous phage display in the new millennium. *Chemical reviews* 105: 4056-72.
Khraiwesh, B. et al. (2012). Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants. *Biochimica et biophysica acta* 1819: 137-48.
Kizis, D. et al. (2001). Role of AP2/EREBP transcription factors in gene regulation during abiotic stress. *FEBS letters* 498: 187-9.
Kobe, B. and Kajava, A.V. (2001). The leucine-rich repeat as a protein recognition motif. *Current opinion in structural biology* 11: 725-32.
Koornneef, A. et al. (2008). Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. *Plant physiology* 147: 1358-68.
Kosova, K., Vyzkumny Ustav Rostlinne Vyroby et al. (2007). The role of dehydrins in plant response to cold. A review. *Biologia Plantarum* v. 51(4) p.
Koutsoudis, M.D. et al. (2006). Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in Pantoea stewartii subspecies stewartii. *Proceedings of the National Academy of Sciences of the United States of America* 103: 5983-8.

Krasensky, J. and Jonak, C. (2012). Drought, salt, and temperature stress-induced metabolic rearrangements and regulatory networks. *Journal of experimental botany* 63: 1593-608.

Krüger, D.H. and Schroeder, C. (1981). Bacteriophage T3 and bacteriophage T7 virus-host cell interactions. *Microbiological reviews* 45: 9-51.

Kunkel, B.N. et al. (1993). RPS2, an Arabidopsis disease resistance locus specifying recognition of Pseudomonas syringae strains expressing the avirulence gene avrRpt2. *The Plant cell* 5: 865-75.

Kunze, G. et al. (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. *The Plant cell* 16: 3496-507.

Köhler, B. and Blatt, M.R. (2002). Protein phosphorylation activates the guard cell Ca2+ channel and is a prerequisite for gating by abscisic acid. *The Plant journal : for cell and molecular biology* 32: 185-94.

Leigh, J.A. and Coplin, D.L. (1992). Exopolysaccharides in plant-bacterial interactions. *Annual review of microbiology* 46: 307-46.

Leon-kloosterziel, K.M. et al. (1998). The genetic and molecular dissection of abscisic acid biosynthesis and signal transduction in Arabidopsis. *Plant Physiology and Biochemistry* 36: 83-89.

Li, X.-B. et al. (2006). Molecular addresses of tumors: selection by in vivo phage display. *Archivum immunologiae et therapiae experimentalis* 54: 177-81.

Lim, A.P.C. et al. (2008). Neutralizing human monoclonal antibody against H5N1 influenza HA selected from a Fab-phage display library. *Virology journal* 5: 130.

Lipes, B.D. et al. (2008). An entirely cell-based system to generate single-chain antibodies against cell surface receptors. *Journal of molecular biology* 379: 261-72.

Liu, Y. and Zhang, S. (2004). Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. *The Plant cell* 16: 3386-99.

Loake, G. and Grant, M. (2007). Salicylic acid in plant defence--the players and protagonists. *Current opinion in plant biology* 10: 466-72.

Lopez-Molina, L. et al. (2001). A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 98: 4782-7.

Lopez-Molina, L. et al. (2003). AFP is a novel negative regulator of ABA signaling that promotes ABI5 protein degradation. *Genes & development* 17: 410-8.

Lorenzo, O. et al. (2003). ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. *The Plant cell* 15: 165-78.

Lorenzo, O. and Solano, R. (2005). Molecular players regulating the jasmonate signalling network. *Current opinion in plant biology* 8: 532-40.

Lytle, B.L. et al. (2006). Solution structure of Arabidopsis thaliana protein At5g39720.1, a member of the AIG2-like protein family. *Acta crystallographica. Section F, Structural biology and crystallization communications* 62: 490-3.

López-Pérez, L. et al. (2009). Changes in plasma membrane lipids, aquaporins and proton pump of broccoli roots, as an adaptation mechanism to salinity. *Phytochemistry* 70: 492-500.

Ma, Y. et al. (2009). Regulators of PP2C phosphatase activity function as abscisic acid sensors. *Science (New York, N.Y.)* 324: 1064-8.
Mahajan, S. and Tuteja, N. (2005). Cold, salinity and drought stresses: an overview. *Archives of biochemistry and biophysics* 444: 139-58.
Mahajan-Miklos, S. et al. (1999). Molecular mechanisms of bacterial virulence elucidated using a Pseudomonas aeruginosa-Caenorhabditis elegans pathogenesis model. *Cell* 96: 47-56.
Martin, K. et al. (2009). Transient expression in Nicotiana benthamiana fluorescent marker lines provides enhanced definition of protein localization, movement and interactions in planta. *The Plant journal : for cell and molecular biology* 59: 150-62.
McCafferty, J. et al. (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* 348: 552-4.
McEwan, D.L. et al. (2012). Host Translational Inhibition by Pseudomonas aeruginosa Exotoxin A Triggers an Immune Response in Caenorhabditis elegans. *Cell host & microbe* 11: 364-74.
McGregor, D.P. and Robins, S.P. (2001). External surface display of proteins linked to DNA-binding domains. *Analytical biochemistry* 294: 108-17.
Mcgrath, K.C. et al. (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. *Plant physiology* 139: 949-59.
Miao, Y. et al. (2007). Arabidopsis MEKK1 can take a short cut: it can directly interact with senescence-related WRKY53 transcription factor on the protein level and can bind to its promoter. *Plant molecular biology* 65: 63-76.
Michelfelder, S. et al. (2007). Vectors selected from adeno-associated viral display peptide libraries for leukemia cell-targeted cytotoxic gene therapy. *Experimental hematology* 35: 1766-76.
Mindrinos, M. et al. (1994). The A. thaliana disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. *Cell* 78: 1089-99.
Mithani, A. et al. (2011). Comparative analysis of metabolic networks provides insight into the evolution of plant pathogenic and nonpathogenic lifestyles in Pseudomonas. *Molecular biology and evolution* 28: 483-99.
Mizoi, J. et al. (2012). AP2/ERF family transcription factors in plant abiotic stress responses. *Biochimica et biophysica acta* 1819: 86-96.
Mudgett, M.B. (2005). New insights to the function of phytopathogenic bacterial type III effectors in plants. *Annual review of plant biology* 56: 509-31.
Mukhtar, M.S. et al. (2011). Independently Evolved Virulence Effectors Converge onto Hubs in a Plant Immune System Network. *Science* 333: 596-601.
Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. *Annual review of plant biology* 59: 651-81.
Murashige, T. and Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* 15: 473-497.
Murray, S.L. et al. (2007). Basal resistance against Pseudomonas syringae in Arabidopsis involves WRKY53 and a protein with homology to a nematode resistance protein. *Molecular plant-microbe interactions : MPMI* 20: 1431-8.

Mäkelä, A.R. and Oker-Blom, C. (2008). The baculovirus display technology--an evolving instrument for molecular screening and drug delivery. *Combinatorial chemistry & high throughput screening* 11: 86-98.

Nakai, Y. et al. (2005). Isolation of a Drosophila gene coding for a protein containing a novel phosphatidylserine-binding motif. *Journal of biochemistry* 137: 593-9.

Nambara, E. and Marion-Poll, A. (2005). Abscisic acid biosynthesis and catabolism. *Annual review of plant biology* 56: 165-85.

Navarro, L. et al. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. *Science (New York, N.Y.)* 312: 436-9.

Navarro, L. et al. (2008). DELLAs control plant immune responses by modulating the balance of jasmonic acid and salicylic acid signaling. *Current biology : CB* 18: 650-5.

Navarro, L. et al. (2004). The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defense responses and bacterial pathogenesis. *Plant physiology* 135: 1113-28.

Nemhauser, J.L. et al. (2006). Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. *Cell* 126: 467-75.

Nishimura, N. et al. (2007). ABA-Hypersensitive Germination1 encodes a protein phosphatase 2C, an essential component of abscisic acid signaling in Arabidopsis seed. *The Plant journal : for cell and molecular biology* 50: 935-49.

Nishimura, N. et al. (2004). Isolation and characterization of novel mutants affecting the abscisic acid sensitivity of Arabidopsis germination and seedling growth. *Plant & cell physiology* 45: 1485-99.

Nomura, K. et al. (2005). Suppression of host defense in compatible plant-Pseudomonas syringae interactions. *Current opinion in plant biology* 8: 361-8.

Ohme-Takagi, M. and Shinshi, H. (1995). Ethylene-inducible DNA binding proteins that interact with an ethylene-responsive element. *The Plant cell* 7: 173-82.

Ohta, M. et al. (2001). Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *The Plant cell* 13: 1959-68.

On, L. et al. (2002). Identification of Arabidopsis ethylene-responsive element binding factors with distinct induction kinetics after pathogen infection. *Plant physiology* 128: 1313-22.

Ooms, J. et al. (1993). Acquisition of Desiccation Tolerance and Longevity in Seeds of Arabidopsis thaliana (A Comparative Study Using Abscisic Acid-Insensitive abi3 Mutants). *Plant physiology* 102: 1185-1191.

O'Brien, P.M. et al. (1999). Generation of native bovine mAbs by phage display. *Proceedings of the National Academy of Sciences of the United States of America* 96: 640-5.

Pandey, G.K. et al. (2005). ABR1, an APETALA2-domain transcription factor that functions as a repressor of ABA response in Arabidopsis. *Plant physiology* 139: 1185-93.

Pandey, S. et al. (2006). G-protein complex mutants are hypersensitive to abscisic acid regulation of germination and postgermination development. *Plant physiology* 141: 243-56.

Parcy, F. et al. (1994). Regulation of gene expression programs during Arabidopsis seed development: roles of the ABI3 locus and of endogenous abscisic acid. *The Plant cell* 6: 1567-82.
Park, J.M. et al. (2001). Overexpression of the tobacco Tsi1 gene encoding an EREBP/AP2-type transcription factor enhances resistance against pathogen attack and osmotic stress in tobacco. *The Plant cell* 13: 1035-46.
Park, S.-Y. et al. (2009). Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science (New York, N.Y.)* 324: 1068-71.
Paschke, M. (2006). Phage display systems and their applications. *Applied microbiology and biotechnology* 70: 2-11.
Pasqualini, R. and Ruoslahti, E. (1996). Organ targeting in vivo using phage display peptide libraries. *Nature* 380: 364-6.
Pazin, M.J. and Kadonaga, J.T. (1997). What's up and down with histone deacetylation and transcription? *Cell* 89: 325-8.
Pei, Z.M. et al. (2000). Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. *Nature* 406: 731-4.
Pepper, L.R. et al. (2008). A decade of yeast surface display technology: where are we now? *Combinatorial chemistry & high throughput screening* 11: 127-34.
Pieterse, C.M. et al. (1996). Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *The Plant cell* 8: 1225-37.
Pieterse, C.M.J. et al. (2009). Networking by small-molecule hormones in plant immunity. *Nature chemical biology* 5: 308-16.
Plotnikova, J.M. et al. (2000). Pathogenesis of the human opportunistic pathogen Pseudomonas aeruginosa PA14 in Arabidopsis. *Plant physiology* 124: 1766-74.
Plückthun, A. (2012). Ribosome display: a perspective. *Methods in molecular biology (Clifton, N.J.)* 805: 3-28.
Preston, G.M. et al. (2005). Profiling the secretomes of plant pathogenic Proteobacteria. *FEMS microbiology reviews* 29: 331-60.
Prithiviraj, B. et al. (2005). Plant models for animal pathogenesis. *Cellular microbiology* 7: 315-24.
Pré, M. et al. (2008). The AP2/ERF domain transcription factor ORA59 integrates jasmonic acid and ethylene signals in plant defense. *Plant physiology* 147: 1347-57.
Radutoiu, S. et al. (2003). Plant recognition of symbiotic bacteria requires two LysM receptor-like kinases. *Nature* 425: 585-92.
Rahme, L.G. et al. (1995). Common virulence factors for bacterial pathogenicity in plants and animals. *Science (New York, N.Y.)* 268: 1899-902.
Rahme, L.G. et al. (2000). Plants and animals share functionally common bacterial virulence factors. *Proceedings of the National Academy of Sciences of the United States of America* 97: 8815-8821.
Rahme, L.G. et al. (1997). Use of model plant hosts to identify Pseudomonas aeruginosa virulence factors. *Proceedings of the National Academy of Sciences of the United States of America* 94: 13245-50.
Reuber, T.L. and Ausubel, F.M. (1996). Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. *The Plant cell* 8: 241-9.

Ribiero, D.M. et al. (2009). Differential requirement for NO during ABA-induced stomatal closure in turgid and wilted leaves. *Plant, cell and environment* 32: 46-57.

Riechmann, J.L. and Meyerowitz, E.M. (1998). The AP2/EREBP family of plant transcription factors. *Biological chemistry* 379: 633-46.

Riechmann, J.L. and Ratcliffe, O.J. (2000). A genomic perspective on plant transcription factors. *Current opinion in plant biology* 3: 423-34.

Rosenberg, A. et al. (1996). T7Select ® Phage Display System: A powerful new protein display system based on bacteriophage T7. *DNA Sequence*.

Russel, M. (1991). Filamentous phage assembly. *Molecular microbiology* 5: 1607-13.

Saez, A. et al. (2006). Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1. *Plant physiology* 141: 1389-99.

Sakuma, Y. et al. (2002). DNA-binding specificity of the ERF/AP2 domain of Arabidopsis DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. *Biochemical and biophysical research communications* 290: 998-1009.

Santini, C. et al. (1998). Eficient display of an HCV cDNA expression library as C-terminal fusion to the capsid protein D of bacteriophage lambda. *Journal of Molecular Biology* 282: 125-135.

Santos-Mendoza, M. et al. (2008). Deciphering gene regulatory networks that control seed development and maturation in Arabidopsis. *The Plant journal : for cell and molecular biology* 54: 608-20.

Sawyer, C. (1997). Methodology for selection of human antibodies to membrane proteins from a phage-display library. *Journal of Immunological Methods* 204: 193-203.

Schroeder, J.I. et al. (2001). GUARD CELL SIGNAL TRANSDUCTION. *Annual review of plant physiology and plant molecular biology* 52: 627-658.

Seki, M. et al. (2002). Monitoring the expression pattern of around 7,000 Arabidopsis genes under ABA treatments using a full-length cDNA microarray. *Functional & integrative genomics* 2: 282-91.

Seo, Y.J. et al. (2010). Overexpression of the ethylene-responsive factor gene BrERF4 from Brassica rapa increases tolerance to salt and drought in Arabidopsis plants. *Molecules and cells* 30: 271-7.

Shan, L. et al. (2008). Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell host & microbe* 4: 17-27.

Shao, H.-B. et al. (2007). Understanding molecular mechanism of higher plant plasticity under abiotic stress. *Colloids and surfaces. B, Biointerfaces* 54: 37-45.

Sharma, M.K. et al. (2010). Identification, phylogeny, and transcript profiling of ERF family genes during development and abiotic stress treatments in tomato. *Molecular genetics and genomics : MGG* 284: 455-75.

Sharoni, A.M. et al. (2011). Gene structures, classification and expression models of the AP2/EREBP transcription factor family in rice. *Plant & cell physiology* 52: 344-60.

Shen, Q.-H. et al. (2007). Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses. *Science (New York, N.Y.)* 315: 1098-103.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Current opinion in plant biology* 3: 217-23.

Shiu, S.H. and Bleecker, A.B. (2003). Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in Arabidopsis. *Plant physiology* 132: 530-43.
Sidhu, S.S. (2001). Engineering M13 for phage display. *Biomolecular engineering* 18: 57-63.
da Silva, A.C.R. et al. (2002). Comparison of the genomes of two Xanthomonas pathogens with differing host specificities. *Nature* 417: 459-63.
Silverstein, K.A.T. et al. (2005). Genome organization of more than 300 defensin-like genes in Arabidopsis. *Plant physiology* 138: 600-10.
Smith, G. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 228: 1315-1317.
Smith, R.S. and Iglewski, B.H. (2003). P. aeruginosa quorum-sensing systems and virulence. *Current opinion in microbiology* 6: 56-60.
Smothers, J.F. et al. (2002). Tech.Sight. Phage display. Affinity selection from biological libraries. *Science (New York, N.Y.)* 298: 621-2.
Solano, R. et al. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. *Genes & Development* 12: 3703-3714.
Solano, R. and Ecker, J.R. (1998). Ethylene gas: perception, signaling and response. *Current opinion in plant biology* 1: 393-8.
Song, C.-P. et al. (2005). Role of an Arabidopsis AP2/EREBP-type transcriptional repressor in abscisic acid and drought stress responses. *The Plant cell* 17: 2384-96.
Song, C.-P. and Galbraith, D.W. (2006). AtSAP18, an orthologue of human SAP18, is involved in the regulation of salt stress and mediates transcriptional repression in Arabidopsis. *Plant molecular biology* 60: 241-57.
Spaepen, S. and Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harbor perspectives in biology* 3.
Spoel, S.H. et al. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. *The Plant cell* 15: 760-70.
Spoel, S.H. et al. (2007). Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proceedings of the National Academy of Sciences of the United States of America* 104: 18842-7.
Sternberg, N. and Hoess, R.H. (1995). Display of peptides and proteins on the surface of bacteriophage lambda. *Proceedings of the National Academy of Sciences of the United States of America* 92: 1609-13.
Strizhov, N. et al. (1997). Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis. *The Plant journal : for cell and molecular biology* 12: 557-69.
Subbiah, V. and Reddy, K.J. (2010). Interactions between ethylene, abscisic acid and cytokinin during germination and seedling establishment in Arabidopsis. *Journal of biosciences* 35: 451-8.
Suzuki, M. et al. (1997). Use of a 3D structure data base for understanding sequence-dependent conformational aspects of DNA. *Journal of molecular biology* 274: 421-35.

Taguchi, F. et al. (2003). Post-translational modification of flagellin determines the specificity of HR induction. *Plant & cell physiology* 44: 342-9.

Taiz, L and Zeiger, E. (2010). Plant Physiology. *Sinauer Associates, Inc.*

Takahashi, S. et al. (2004). Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in Arabidopsis cell culture using a full-length cDNA microarray. *Plant molecular biology* 56: 29-55.

Takken, F.L.W. and Tameling, W.I.L. (2009). To nibble at plant resistance proteins. *Science (New York, N.Y.)* 324: 744-6.

Tasset, C. et al. (2010). Autoacetylation of the Ralstonia solanacearum effector PopP2 targets a lysine residue essential for RRS1-R-mediated immunity in Arabidopsis. *PLoS pathogens* 6: e1001202.

Thilmony, R. et al. (2006). Genome-wide transcriptional analysis of the Arabidopsis thaliana interaction with the plant pathogen Pseudomonas syringae pv. tomato DC3000 and the human pathogen Escherichia coli O157:H7. *The Plant journal : for cell and molecular biology* 46: 34-53.

Thomma, B.P.H.J. et al. (2011). Of PAMPs and effectors: the blurred PTI-ETI dichotomy. *The Plant cell* 23: 4-15.

de Torres Zabala, M. et al. (2009). Antagonism between salicylic and abscisic acid reflects early host-pathogen conflict and moulds plant defence responses. *The Plant journal : for cell and molecular biology* 59: 375-86.

de Torres-Zabala, M. et al. (2007). Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. *The EMBO journal* 26: 1434-43.

Toth, I.K. and Birch, P.R.J. (2005). Rotting softly and stealthily. *Current opinion in plant biology* 8: 424-9.

Tran, L.-S.P. et al. (2007). Functional analysis of AHK1/ATHK1 and cytokinin receptor histidine kinases in response to abscisic acid, drought, and salt stress in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* 104: 20623-8.

Traw, M.B. et al. (2003). Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of Arabidopsis thaliana. *Molecular ecology* 12: 1125-35.

Tsuda, K. et al. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. *The Plant journal : for cell and molecular biology* 53: 763-75.

Tsuda, K. et al. (2009). Network properties of robust immunity in plants. *PLoS genetics* 5: e1000772.

Tsutsui, T. et al. (2009). DEAR1, a transcriptional repressor of DREB protein that mediates plant defense and freezing stress responses in Arabidopsis. *Journal of plant research* 122: 633-43.

Tzfira, T. et al. (2004). Involvement of targeted proteolysis in plant genetic transformation by Agrobacterium. *Nature* 431: 87-92.

Uno, Y. et al. (2000). Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity conditions. *Proceedings of the National Academy of Sciences of the United States of America* 97: 11632-7.

Uppalapati, S.R. et al. (2007). The phytotoxin coronatine contributes to pathogen fitness and is required for suppression of salicylic acid accumulation in tomato inoculated with Pseudomonas syringae pv. tomato DC3000. *Molecular plant-microbe interactions : MPMI* 20: 955-65.

Van der Biezen, E.A. and Jones, J.D. (1998). Plant disease-resistance proteins and the gene-for-gene concept. *Trends in biochemical sciences* 23: 454-6.

Von Bodman, S.B. et al. (2003). Quorum sensing in plant-pathogenic bacteria. *Annual review of phytopathology* 41: 455-82.

Valadon, P. et al. (2006). Screening phage display libraries for organ-specific vascular immunotargeting in vivo. *Proceedings of the National Academy of Sciences of the United States of America* 103: 407-12.

Valliyodan, B. and Nguyen, H.T. (2006). Understanding regulatory networks and engineering for enhanced drought tolerance in plants. *Current opinion in plant biology* 9: 189-95.

Valls, M. et al. (2006). Integrated regulation of the type III secretion system and other virulence determinants in Ralstonia solanacearum. *PLoS pathogens* 2: e82.

Varet, A. (2003). The Arabidopsis NHL3 Gene Encodes a Plasma Membrane Protein and Its Overexpression Correlates with Increased Resistance to Pseudomonas syringae pv. tomato DC3000. *PLANT PHYSIOLOGY* 132: 2023-2033.

Verslues, P.E. and Zhu, J.-K. (2005). Before and beyond ABA: upstream sensing and internal signals that determine ABA accumulation and response under abiotic stress. *Biochemical Society transactions* 33: 375-9.

Voinnet, O. et al. (2003). An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *The Plant journal : for cell and molecular biology* 33: 949-56.

Walker, T.S. et al. (2004). Pseudomonas aeruginosa-Plant Root Interactions . Pathogenicity , Biofilm Formation , and Root Exudation 1. *Society* 134: 320-331.

Wang, D. et al. (2007). Salicylic acid inhibits pathogen growth in plants through repression of the auxin signaling pathway. *Current biology : CB* 17: 1784-90.

Wang, Y. et al. (2010). A Pseudomonas syringae ADP-ribosyltransferase inhibits Arabidopsis mitogen-activated protein kinase kinases. *The Plant cell* 22: 2033-44.

Wasilewska, A. et al. (2008). An update on abscisic acid signaling in plants and more... *Molecular plant* 1: 198-217.

Van Wees, S.C.M. et al. (2008). Plant immune responses triggered by beneficial microbes. *Current opinion in plant biology* 11: 443-8.

Weingart, H. et al. (1999). Comparison of Ethylene Production by Pseudomonas syringae and Ralstonia solanacearum. *Phytopathology* 89: 360-5.

Weingart, H. et al. (2001). The Role of Ethylene Production in Virulence of Pseudomonas syringae pvs. glycinea and phaseolicola. *Phytopathology* 91: 511-8.

Whalen, M.C. et al. (1991). Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *The Plant cell* 3: 49-59.

Willats, W.G.T. (2002). Phage display: practicalities and prospects. *Plant molecular biology* 50: 837-54.

Winter, G. et al. (1994). Making antibodies by phage display technology. *Annual review of immunology* 12: 433-55.

Wu, S. et al. (2011). Bacterial effector HopF2 suppresses arabidopsis innate immunity at the plasma membrane. *Molecular plant-microbe interactions : MPMI* 24: 585-93.

Xiong, L. et al. (2002). Cell Signaling during Cold, Drought, and Salt Stress. *PLANT CELL* 14: S165-183.

Yamaguchi-Shinozaki, K. (2007). [Molecular mechanisms of abiotic stress responses: water and temperature stress conditions--overview]. *Tanpakushitsu kakusan koso. Protein, nucleic acid, enzyme* 52: 515-6.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. *The Plant cell* 6: 251-64.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annual review of plant biology* 57: 781-803.

Yang, S. et al. (2009). Four divergent Arabidopsis ethylene-responsive element-binding factor domains bind to a target DNA motif with a universal CG step core recognition and different flanking bases preference. *The FEBS journal* 276: 7177-86.

Yang, Z. et al. (2005). Arabidopsis ERF4 is a transcriptional repressor capable of modulating ethylene and abscisic acid responses. *Plant molecular biology* 58: 585-96.

Yoine, M. et al. (2006). The lba1 mutation of UPF1 RNA helicase involved in nonsense-mediated mRNA decay causes pleiotropic phenotypic changes and altered sugar signalling in Arabidopsis. *The Plant journal : for cell and molecular biology* 47: 49-62.

Yu, G.L. et al. (1993). Arabidopsis mutations at the RPS2 locus result in loss of resistance to Pseudomonas syringae strains expressing the avirulence gene avrRpt2. *Molecular plant-microbe interactions : MPMI* 6: 434-43.

Zeevaart, J.A.D. and Creelman, R.A. (1988). Metabolism and Physiology of Abscisic Acid. *Annual Review of Plant Physiology and Plant Molecular Biology* 39: 439-473.

Zeidler, D. et al. (2004). Innate immunity in Arabidopsis thaliana: lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. *Proceedings of the National Academy of Sciences of the United States of America* 101: 15811-6.

Zhang, G. et al. (2008). Phylogeny, gene structures, and expression patterns of the ERF gene family in soybean (Glycine max L.). *Journal of experimental botany* 59: 4095-107.

Zhang, H. et al. (2004). The ethylene-, jasmonate-, abscisic acid- and NaCl-responsive tomato transcription factor JERF1 modulates expression of GCC box-containing genes and salt tolerance in tobacco. *Planta* 220: 262-70.

Zhang, X., Zhang, Z., et al. (2005). Expressing TERF1 in tobacco enhances drought tolerance and abscisic acid sensitivity during seedling development. *Planta* 222: 494-501.

Zhang, X. et al. (2001). K+ channels inhibited by hydrogen peroxide mediate abscisic acid signaling in Vicia guard cells. *Cell research* 11: 195-202.

Zhang, Y., Davis, J.L., et al. (2005). Identification of tribbles homolog 2 as an autoantigen in autoimmune uveitis by phage display. *Molecular immunology* 42: 1275-81.

Zhang, Y. et al. (2007). Panning and identification of a colon tumor binding peptide from a phage display peptide library. *Journal of biomolecular screening* 12: 429-35.
Zhuang, J. et al. (2008). Genome-wide analysis of the AP2/ERF gene family in Populus trichocarpa. *Biochemical and biophysical research communications* 371: 468-74.
Zipfel, C. et al. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428: 764-7.
Zipfel, C. et al. (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. *Cell* 125: 749-60.