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GENES RELACIONADOS A AUXINAS E RIZOGÊNESE ADVENTÍCIA EM *Arabidopsis*

Porto Alegre – Brasil

Março de 2015

GENES RELACIONADOS A AUXINAS E RIZOGÊNESE ADVENTÍCIA EM *Arabidopsis*

Cibele Tesser da Costa

Tese de doutorado apresentada no Programa de Pós-Graduação em Biologia Celular e Molecular da Universidade Federal do Rio Grande do Sul (UFRGS) e desenvolvida no Laboratório de Fisiologia Vegetal do Centro de Biotecnologia da UFRGS e no "*Department of Molecular and Developmental Genetics, Institute of Biology Leiden*" sob orientação do professor doutor

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Tese submetida ao Programa de Pós-Graduação em Biologia Celular e Molecular do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul como parte dos requisitos necessários para a obtenção do grau de Doutor em Biologia Celular e Molecular.

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Dedico esta tese aos meus pais,
Volmar e Teresinha (*in memorian*),
que sempre me ensinaram que
o aprendizado é o maior bem que
pode ser adquirido.

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"A ciência nunca resolve um problema

sem criar pelo menos outros dez".

(George Bernard Shaw)

SUMÁRIO

LISTA DE TABELAS E FIGURAS

INTRODUÇÃO

CAPÍTULO 1

CAPÍTULO 2

ANEXO 1

ANEXO 2

LISTA DE ABREVIATURAS

2,4-D – Ácido 2,4-diclorofenoxiacético

aa – Aminoácidos

ABA – Ácido abscísico

ABCB/MDR/PGP – *ABCB/Multidrug Resistant/P-Glycoprotein*

ABP1 – *Auxin Binding Protein1*

ACT – Actina

AIA ou IAA – Ácido indol-3-acético

AIB ou IBA – Ácido indol-3-butírico

ANA ou NAA – Ácido naftaleno-acético

ARF – Fator responsivo a auxinas

IAA28 – Aux/IAA28

AUX1/LAX1 – *AUXIN1/LIKE AUX1*

Col-0 – Columbia

CRISPRs – Repetições palindrômicas curtas agrupadas e regularmente espaçadas

CT – Citocinina

CYCB1;1 – *Cyclin B1;1*

DRA ou ARD – desenvolvimento de raízes adventícias

EF-1 – *Elongation Factor 1*

GA – Giberelina

GH3 – *Gretchen Hagen 3-like proteins*

JA – Jasmonato

MP ou PM – Membrana plasmática

NPA – Ácido-1-N-naftilftalâmico

O.N. ou NO – Óxido Nítrico

PAT ou TPA – Transporte polar de auxinas

Picloram – Ácido 4-amino-3,5,5-tricloropicolínico

PID – *PINOID*

PILS – *PIN-LIKES*

PIN – *PINFORMED*

PINHL – Alça hidrofílica central de PIN

PP2A – proteínas fosfatases 2 A

QC – Centro quiescente

RA ou AR – Raízes adventícias

RE ou ER – Retículo endoplasmático

Real-Time RT-PCR – Reação em Cadeia da Polimerase em Tempo Real via Transcriptase Reversa

RL ou LR – Raízes laterais

ROPs – *Rho-like guanosine triphosphatases*

SCR – *SCARECROW*

TIR1 – *Transport Inhibitor Response 1*

WAG – *WAVING AGRAVITROPIC ROOT*

RESUMO

Enquanto as raízes laterais (RL) se desenvolvem a partir da raiz primária, as raízes adventícias (RA) são geralmente formadas em órgãos da parte aérea da planta. As RA podem ser formadas como uma resposta adaptativa a estresses, como ferimentos ou alagamentos e a sua formação também é importante para a propagação vegetativa de espécies economicamente relevantes, que frequentemente dependem da propagação clonal de genótipos elite. A aplicação de hormônios pode estimular o desenvolvimento das RA (DRA), e as auxinas são consideradas os principais hormônios envolvidos nesse processo. Neste estudo, o sistema de plântulas estioladas foi usado em *Arabidopsis thaliana* para analisar diversos aspectos do DRA. Diferentes tipos de auxinas, naturais ou sintéticas, foram testadas e verificou-se que AIA causou um aumento no número de raízes sem afetar seu comprimento, ANA foi efetivo para o DRA, mas as raízes ficaram pequenas, e altas concentrações de 2,4-D causaram a formação de calos. Através de imunolocalização, um nível elevado de AIA foi detectado nos tecidos do hipocótilo que deram origem ao primórdio radicular. O padrão de expressão de genes potencialmente envolvidos com o enraizamento adventício foi testado por PCR em Tempo Real. O DRA foi marcado essencialmente por aumento na expressão de *PIN1*, *SUR2*, *GH3.3*, *GH3.6*, *ARF8* e *IAA28*. A expressão dos genes induzidos foi mais estimulada por ANA, seguida de AIA. A expressão de *IAA28* aumentou com o DRA, diferente do que foi observado no desenvolvimento de RL. Os receptores de auxinas TIR1/AFB e ABP1 iniciam a sinalização de auxinas na célula pelo controle da expressão gênica, proteólise seletiva e afrouxamento da parede celular. Verificou-se que TIR1 e as proteínas AFBs são importantes para o DRA, mas que estes receptores devem estar exercendo funções redundantes no processo e que ABP1 pode agir complementando a sua ação. Durante a organogênese das RA, TIR1 e AFB2 parecem exercer uma maior influência. As auxinas são transportadas de maneira polar, célula a célula e geralmente dependem de transportadores. Analisamos o DRA em diferentes mutantes deficientes no transporte de influxo e efluxo de auxinas juntamente com construções com genes repórteres, na presença ou ausência de auxina exógena. Uma função essencial foi estabelecida para AUX1 no enraizamento adventício e, embora LAX3 *per se* não tenha sido chave no processo, este parece agir em conjunto com AUX1. Também observamos que a formação eficiente de RA depende dos transportadores de efluxo PIN, principalmente PIN1, 3 e 7. A adequada fosforilação dos PINs pelas quinases PID, WAG1 e WAG2 e, consequentemente, a direção do transporte, foi igualmente essencial para o estabelecimento das RA.

Palavras-chave: enraizamento adventício, auxina, *Arabidopsis thaliana*, expressão gênica, transporte de auxinas, fosforilação de PIN

ABSTRACT

Lateral roots (LR) develop from the primary root, whereas adventitious roots (AR) are generally formed from above-ground organs. AR can be formed as an adaptive response to stresses, like wounding or flooding, and their formation is also important for efficient vegetative propagation of economically relevant species, which often depend on clonal propagation of elite genotypes. Hormonal application can stimulate AR development (ARD) and auxins are recognized as major hormones involved in this process. Here, the etiolated seedlings system was used in *Arabidopsis thaliana* to study several aspects of ARD. Different auxin types, natural or synthetic, were tested and it was found that IAA caused an increase in root number without affecting root length, NAA was effective for ARD, but roots remained short and higher levels of 2,4-D caused callus formation. Through immunolocalization, a higher level of IAA was detected in hypocotyl tissues from which the root primordia differentiated. The expression pattern of genes potentially involved in adventitious rooting was tested by Real-Time PCR. ARD was essentially marked by increased expression of *PIN1*, *SUR2*, *GH3.3*, *GH3.6*, *ARF8* and *IAA28*. The magnitude of expression of induced genes was much stimulated by NAA, followed by IAA. *IAA28* expression increased with ARD, differently from what is known for lateral root development. The auxin receptors TIR1/AFB and ABP1 initiate auxin signaling in the cell through changes in gene expression, selective proteolysis and cell wall loosening. We observed that TIR1/AFB are important in ARD but might be playing redundant roles in the process, whereas ABP1 could be complementing their action. During AR organogenesis, TIR1 and AFB2 seemed to exert greater influence. Auxins are transported in a polar, cell to cell way and depend on several transporters. We analyzed ARD in different mutants affected in auxin influx and efflux transporters, coupled with reporter gene constructs, in presence or absence of exogenous auxin. An essential role was established for AUX1 in AR. Although LAX3 *per se* was not a key player in the process, it seemed to act in conjunction with AUX1. We also observed that efficient formation of AR depends on the PIN efflux transporters, mainly PIN1, 3 and 7. The proper phosphorylation of PINs by the kinases PID, WAG1 and WAG2, and hence the direction of auxin transport, was equally essential for AR establishment.

Keywords: adventitious rooting, auxin, *Arabidopsis thaliana*, gene expression, auxin transport, PIN phosphorylation

INTRODUÇÃO

O sistema radicular exerce vários papéis importantes na planta, como a captura de água e nutrientes e a sua fixação no solo (Smith e De Smet et al., 2012). Em condições ambientais adversas, a arquitetura do sistema radicular pode ser ajustada como uma estratégia de adaptação e sobrevivência. Esse ajuste pode incluir o crescimento radicular e a formação de raízes laterais (RL) e adventícias (RA) (Franco et al., 2011). As RL e RA são formadas durante o período pós-embrionário, sendo que as RL são tipicamente formadas a partir do periciclo de raízes pré-existentes, enquanto as RA normalmente emergem do caule, folhas ou, ocasionalmente, do periciclo de raízes mais velhas. RA são naturalmente formadas a partir de células com potencial meristemático e podem, igualmente, ter origem em condições de estresse ambiental. Por exemplo, no caso de alagamento ou hipoxia, a indução de RA regulada por etileno e espécies reativas de oxigênio, é um mecanismo utilizado pelas plantas para sobreviver, pois permite que as raízes originais danificadas pela deficiência de oxigênio extrema sejam substituídas (Morgan & Malcolm, 1997; Bailey-Serres et al., 2012). Além disso, devido ao seu ângulo de crescimento, as RA podem aumentar a captura de fósforo nas camadas mais superficiais do solo (Smith e De Smet et al., 2012; Bellini et al., 2014).

As RA também exercem papel fundamental na propagação vegetativa de plantas, sendo o enraizamento adventício estratégico para a indústria florestal brasileira, especialmente no setor de celulose e papel, no qual as florestas comerciais são clonais. No entanto, os clones com características ideais de celulose e baixa lignina muitas vezes têm dificuldades no enraizamento, limitando a produção (Negishi et al., 2014; . As bases moleculares do desenvolvimento e regeneração radiculares vêm sendo estudadas em plantas modelo como *Arabidopsis thaliana*, passíveis de avaliações mais precisas e com as facilidades de um genoma completamente conhecido, com baixa redundância e uma infinidade de mutantes. Entretanto, o conhecimento sobre as respostas das plantas ao nível molecular e da influência de diferentes tipos de auxinas no enraizamento adventício ainda é limitado.

RA e RL compartilham alguns mecanismos e etapas comuns em seu desenvolvimento. O enraizamento compreende três fases, quais sejam: indução, com a ocorrência de eventos bioquímicos e moleculares; iniciação, com divisões celulares e organização dos primórdios radiculares; e expressão, com o crescimento dos primórdios radiculares internos através de

tecidos do caule e emergência da raiz (Li et al., 2009). Os hormônios vegetais auxinas (Garrido et al., 2002; De Klerk et al., 1999, Ramírez-Carvajal et al., 2009), etileno (Negi et al., 2010; Lewis et al., 2011), poliaminas (Neves et al., 2002; Arena et al. 2003; Naija et al. 2008), ácido abscísico, giberelinas (Steffens et al., 2006), citocininas (Ramírez-Carvajal et al., 2009), jasmonato (Gutierrez et al., 2012), estrigolactonas (Rasmussen et al., 2012) e óxido nítrico (Lanteri et al., 2009; Abied et al., 2012) têm sido descritos como participantes em diferentes etapas do processo de enraizamento adventício (Fig. 1).

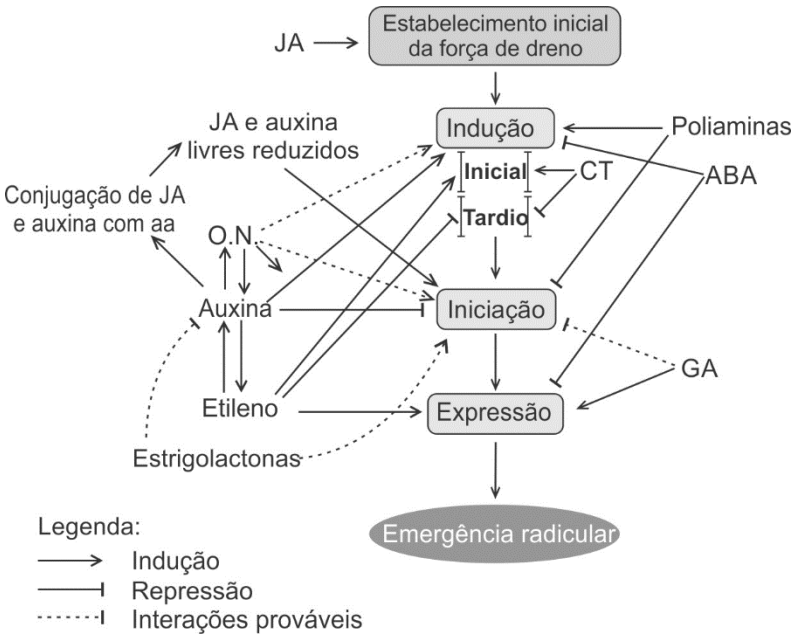

Figura 1. Possíveis interações hormonais durante fases distintas do processo de enraizamento adventício. JA, jasmonato; CT, citocinina; ABA, ácido abscísico; GA, giberelina; O.N., óxido nítrico; aa, aminoácidos. Adaptado de da Costa et al. (2013).

O desenvolvimento de RA é um processo fisiológico complexo, influenciado tanto por fatores endógenos (hormônios, carboidratos, flavonóides) como ambientais (luz, temperatura, nutrientes minerais). Nesse sentido, as auxinas são os hormônios que parecem exercer maior influência na indução de RA (da Costa et al., 2013; Pacurar et al., 2014; . No entanto, os mecanismos fisiológicos subjacentes à capacidade de indução de RA ainda não são completamente compreendidos. As plantas apresentam respostas distintas frente a diferentes tipos de auxinas, especialmente no que diz respeito ao enraizamento. O grupo de auxinas compreende tanto hormônios que ocorrem naturalmente na planta como tipos sintéticos. O primeiro hormônio a ser utilizado para estimular o enraizamento foi a auxina endógena ácido indol-3-acético (AIA) e esta é a auxina natural mais abundante (Cooper, 1935). Outra auxina

endógena, o ácido indol-3-butírico (AIB), promove o enraizamento mais eficientemente do que o AIA (De Klerk et al., 1999; Choffe et al., 2000; Mansseri-Lamrioui et al., 2011), mas a resposta é espécie-dependente e acredita-se que AIB seja um precursor importante de AIA (Strader et al., 2010). O ácido naftaleno acético (ANA) é uma forma sintética de auxina que também promove o enraizamento e tem maior estabilidade metabólica (De Klerk et al., 1999). AIA, AIB e ANA induziram a formação de RA em segmentos de inflorescências de *A. thaliana*, mas o ácido 2,4-diclorofenoxiacético (2,4-D) e o ácido 4-amino-3,5,5-tricloropicolínico (picloram) induziram a formação de calos nas mesmas concentrações (Verstraeten et al., 2013).

Assim como a concentração de auxinas, a sensibilidade das células aos hormônios também é importante para as respostas desencadeadas. Após a percepção da auxina na célula, a expressão gênica é modificada, regulando as divisões celulares e o desenvolvimento radicular. Neste processo, os membros da família de proteínas F-box TIR1/AFB atuam como receptores de auxina. A auxina promove o recrutamento dos repressores transcricionais Aux/AIA pelo complexo ubiquitina ligase SCF^{TIR1}/AFB (Skp1/Cullin/F-box) E3, levando à sua posterior ubiquitinação e degradação (Dharmasiri et al. 2005; Parry et al., 2009; Maraschin et al., 2009). Isso libera os fatores responsivos à auxina (*Auxin Response Factors* - ARFs) da ação repressiva dos Aux/AIAs e permite promover a transcrição dos genes responsivos à auxina (Santner e Estelle, 2009).

Além dos receptores nucleares de auxina TIR1/AFB, uma proteína de ligação à auxina (*Auxin Binding Protein 1* - ABP1) foi identificada como receptor de auxina extracelular. ABP1 está localizada tanto no retículo endoplasmático (RE) quanto no apoplasto próximo à membrana plasmática (MP) e vários relatos indicam que ABP1 também está envolvida na regulação da expressão dos genes responsivos à auxina (Tromas et al., 2009, revisado por Tromas et al., 2010, Effendi et al., 2011). A cascata de sinais desencadeada por ABP1 inclui a ativação das bombas de próton ATPase, a acidificação do meio extracelular e a ativação de canais de entrada de K^+, o que pode eventualmente levar a alterações na expressão gênica (revisado por Tromas et al., 2010 e por Scherer et al., 2012). Mutantes nulos de ABP1 foram identificados por Chen et al. (2001) e foram letais para o embrião, indicando que ABP1 tem função importante na embriogênese. Entretanto, recentemente Gao et al. (2015) identificaram alelos nulos de ABP1 pelo método CRISPRs (*clustered regularly interspaced short palindromic repeats*), que são indistinguíveis de plantas do tipo selvagem, incluindo sinalização de auxinas e desenvolvimento da planta. Essa publicação revela um

contraste de resultados e evidencia a falta de unanimidade em relação ao real papel de ABP1 na planta.

As auxinas têm ação rizogênica e estimulam a formação de meristemóides na fase de indução, mas tornam-se inibitórias após 96 horas e impedem o crescimento dos primórdios radiculares como, por exemplo, em macieiras (De Klerk et al., 1999). Portanto, a homeostase de auxinas é importante para o desenvolvimento das RA e alguns membros da família de genes *Gretchen Hagen 3* (*GH3*) estão envolvidos neste processo, codificando sintetases que atuam na conjugação de excesso de AIA livre fisiologicamente ativo com aminoácidos (Staswick et al., 2005; Chapman e Estelle, 2009). Os genes *GH3.3*, *GH3.5* e *GH3.6* são induzidos por auxinas e atuam na modulação da homeostase de jasmonato (Gutierrez et al., 2012). ARF6 e ARF8 são reguladores positivos e ARF17 é um regulador negativo desses genes, que controlam a conjugação de jasmonato com aminoácidos e reprimem a ação de COI1, um inibidor do enraizamento (Fig. 2). A expressão de ARF17 é controlada por AGO1, que age no *crosstalk* entre auxinas e luz no enraizamento adventício (Sorin et al., 2005). O gene *SUR2* também está envolvido na homeostase de auxinas, codificando a proteína CYP83B1, uma monoxigenase P450 dependente do citocromo que modula a biossíntese de AIA em *Arabidopsis* (Barlier et al., 2000).

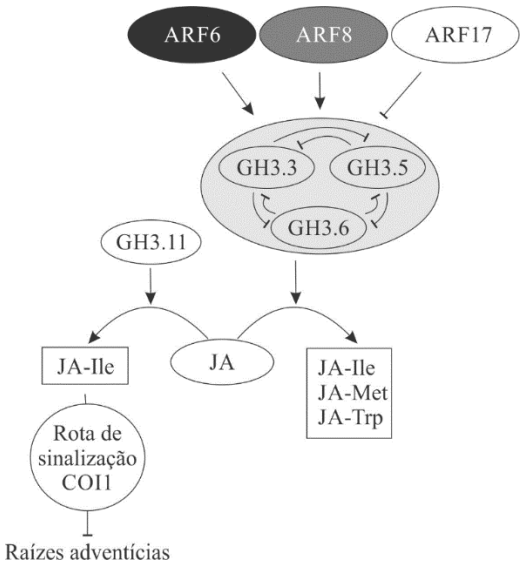

Figura 2. Modelo de rota de regulação proposto para a iniciação das raízes adventícias em *Arabidopsis*. Adaptado de Gutierrez et al. (2012).

Outro mecanismo de regulação da homeostase de auxinas é o transporte polar de auxinas célula a célula. Na parte aérea, as auxinas movem-se a partir do ápice para a base,

enquanto que nas raízes o transporte ocorre em duas direções: i) acrópeta, para a ponta de raiz e ii) basípeta, do ápice da raiz para a sua base (Muday e DeLong, 2002, Morris et al., 2010). O transporte polar de auxinas (*Polar Auxin Transport* - PAT) é mediado por três classes de proteínas transportadoras. As moléculas de auxinas podem passar pela MP por difusão quando estão no estado protonado (IAAH). Entretanto, dentro das células, a auxina fica no estado desprotonado (IAA^-) e a sua entrada nessa forma é facilitada por transportadores de influxo chamados *AUXIN1/LIKE AUX1* (AUX1/LAX1) (Swarup e Péret, 2012). AUX1 exerce papel importante no desenvolvimento das RL por estar envolvido no transporte de AIA (Marchant et al., 2002). O transportador de influxo LAX3 parece ser induzido por auxinas e promover a emergência das raízes laterais (Swarup et al., 2008). Os mutantes *lax1* e *lax2* apresentaram fenótipos semelhantes ao selvagem, enquanto *lax3* mostrou redução de cerca de 40% na emergência de raízes laterais (Swarup et al., 2008).

Duas classes de transportadores de efluxo de auxinas foram identificados até o momento, a família de proteínas *PINFORMED* (PIN) (revisado por Nodzyński et al., 2012) e os transportadores *ABC-B/MULTI-DRUG RESISTANT/P-GLYCOPROTEIN* (ABCB/MDR/PGP) (Geisler and Murphy, 2006). Outra família que tem sido considerada como contendo proteínas que provavelmente atuam no efluxo de auxinas é a de *PIN-LIKES* (PILS). Dentre estas classes, os transportadores que exercem papel mais importante na polaridade do transporte de auxinas são os membros da família PIN. Em *Arabidopsis*, a família de proteínas PIN compreende oito membros, dos quais PIN1, 2, 3, 4 e 7 (do tipo PIN1) medeiam o efluxo polar pela sua distribuição assimétrica na MP; enquanto PIN5, 6 e 8 (do tipo PIN5) estão localizados no RE e, provavelmente, estão envolvidos na regulação da homeostase intracelular de auxinas. A função de transporte de efluxo foi vista em plantas e sistemas heterólogos assim como por interferência genética e utilização de inibidores de transporte (revisado por Nodzyński et al., 2012). Além disso, a distribuição polar dos transportadores do tipo PIN1 e a direção de PAT são determinadas pela fosforilação da alça hidrofílica central de PIN através da ação antagonista das quinases *PINOID* (PID)/*WAVING AGRAVITROPIC ROOT* (WAG) e das proteínas fosfatases 2A (PP2A) (Friml, 2004, Michniewicz et al., 2007, Huang et al., 2010).

A segunda família de transportadores é composta de transportadores *ATP-binding cassette* (ABC) (Kamimoto et al., 2012), entre os quais a P-glicoproteína (PGP)/subclasse ABCB é relevante para o transporte de auxinas, tanto no nível celular como de longa distância (revisado por Geisler e Murphy, 2006; Verrier et al., 2008; Kamimoto et al., 2012).

O ácido-1-N-naftilftalâmico (*N-naphthylphthalamic acid* - NPA) é um inibidor do PAT e liga-se aos transportadores ABCB. A identificação de inibidores de transporte, como o NPA e proteínas que ligam esses inibidores é importante para a pesquisa do transporte de auxinas (revisado por Geisler e Murphy, 2006). Uma maneira do transporte de auxinas ser regulado é por intermédio do receptor de auxinas ABP1, que funciona promovendo a endocitose mediada por clatrina dos transportadores de efluxo PIN. A ligação de auxinas inibe a ação de ABP1, estabilizando os PINs na MP e proporcionando um mecanismo pelo qual as auxinas aumentam o seu próprio efluxo (Robert et al., 2010). A família de prováveis transportadores de auxina *PIN-LIKES* (PILS) foi recentemente identificada *in silico* por Barbez et al. (2012). Os autores mostraram a importância destas proteínas para a homeostase de auxinas e para a regulação do crescimento das plantas dependente deste fitormônio.

Vários sistemas de enraizamento têm sido utilizados em estudos de rizogênese adventícia com *Arabidopsis* e, frequentemente, são considerados equivalentes. Contudo, sistemas de enraizamento adventício, considerados equivalentes em *Arabidopsis*, parecem apresentar regulação distinta. Três sistemas diferentes foram propostos por Corrêa et al. (2012): folhas destacadas, plantas com raízes destacadas e plântulas estioladas e inundadas. O enraizamento adventício teve origem a partir de tecidos diferentes em todos os casos, sem passar por uma fase de formação de calos. Constatou-se também que as respostas fisiológicas e de desenvolvimento foram diferentes nos três sistemas estudados, indicando a importância de não generalizar os dados de diferentes sistemas experimentais, mesmo dentro da mesma espécie modelo. Dentre os sistemas citados, o que é mais utilizado em estudos de rizogênese adventícia é o de plântulas estioladas, geralmente sem alagamento (Fig. 3).

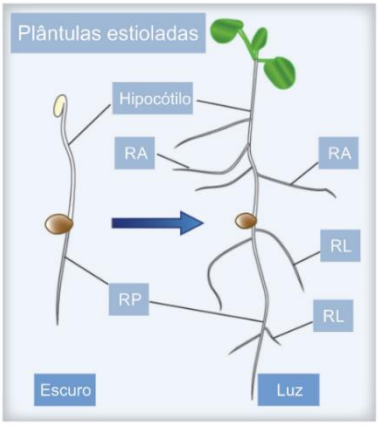

Figura 3. Sistema de plântulas estioladas. As RA são induzidas devido à transição do escuro para a luz em *Arabidopsis*. Adaptado de Bellini et al. (2014).

Os enraizamentos lateral e adventício compartilham alguns elementos-chave das rotas de regulação genética e hormonal, mas as diferenças entre os mecanismos regulatórios ainda não são bem compreendidas. A complexidade do processo e as muitas lacunas que ainda existem no conhecimento do enraizamento adventício exigem abordagens experimentais integradas que reúnam estrutura e função, a partir de planta inteira em nível molecular, com uma perspectiva atenta a aspectos cinéticos. Apesar da importância das auxinas no enraizamento adventício, ainda não se tem um conhecimento completo sobre as repostas das plantas em nível molecular, assim como sobre a ação de diferentes tipos de auxinas. Um foco no metabolismo e localização dos genes e proteínas relacionados às auxinas em tecidos e tipos celulares envolvidos no desenvolvimento de RA, assim como a utilização de mutantes ou de plantas transgênicas afetadas em etapas específicas do processo, são abordagens promissoras para avançar significativamente na capacidade de controlar o enraizamento adventício.

Objetivos

Objetivo geral:

Realizar análises moleculares e morfológicas do enraizamento adventício em plântulas estioladas de *Arabidopsis thaliana*, comparando controles livres de auxinas e RA induzidas por aplicação de auxina exógena.

Objetivos específicos:

1. Avaliar os efeitos de diferentes tipos de auxina como AIA, ANA e 2,4-D sobre o número e o comprimento de RA em diferentes etapas do seu desenvolvimento.

2. Verificar a distribuição de AIA nos tecidos do hipocótilo de plântulas estioladas de *Arabidopsis* durante o desenvolvimento de RA por imunolocalização de AIA.

3. Monitorar a expressão de genes potencialmente envolvidos em diferentes etapas do enraizamento adventício, relacionados à sinalização, homeostase e transporte de auxinas em *Arabidopsis*, por PCR em Tempo Real.

4. Investigar a participação de receptores e transportadores de influxo e efluxo de auxinas, bem como o estado de fosforilação dos últimos, durante o desenvolvimento de RA em ecotipos selvagens e em diferentes mutantes de *Arabidopsis*.

5. Analisar a expressão *in vivo* dos genes *AUX1/LAX1*, *TIR/AFBs*, *PIN1s* e *PID/WAGs* por meio da fusão destes com genes que codificam proteínas fluorescentes (*green fluorescent protein* – GFP, *yellow fluorescent protein* – YFP).

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CONTEÚDOS ABORDADOS

Os resultados obtidos durante o doutorado estão organizados nesta tese em capítulos e anexos, na forma de artigos científicos publicados ou a serem submetidos à publicação.

O **Capítulo 1** apresenta um manuscrito a ser submetido ao periódico *Physiologia Plantarum*. Este capítulo aborda a influência de diferentes tipos de auxinas no enraizamento adventício, bem como a imunolocalização de AIA e análises de expressão gênica por PCR em Tempo Real de genes relacionados com a sinalização, metabolismo e transporte de auxinas na presença ou ausência AIA ou ANA exógenas.

O **Capítulo 2** apresenta um manuscrito a ser submetido à publicação futuramente. Este capítulo trata da influência dos transportadores de influxo de auxinas no enraizamento adventício.

O **Capítulo 3** apresenta um manuscrito a ser submetido à publicação futuramente. Este capítulo aborda a participação dos receptores de auxinas e dos transportares de efluxo de auxinas PINs, bem como o estado de fosforilação de PINs durante o desenvolvimento de RA.

O **Anexo 1** foi publicado no periódico *Frontiers in Plant Science*. Trata-se de uma revisão de literatura acerca do estado da arte do enraizamento adventício e os mecanismos envolvidos em diferentes espécies. A revisão engloba uma análise dos principais hormônios, o *crosstalk* entre estes e os genes e proteínas que têm alguma participação no processo de enraizamento, bem como o envolvimento de outros componentes, como o óxido nítrico e os miRNAs.

O **Anexo 2** está apresentado na sua formatação preliminar e traz os resultados da dinâmica molecular do receptor de auxinas ABP1, que foi desenvolvida em parceria com o Grupo de Bioinformática Estrutural da UFRGS.

O **Anexo 3 (artigo fora do escopo da Tese publicado durante o período de Doutorado)** apresenta uma revisão sobre a biossíntese de saponinas triterpenóides em plantas. Apesar de o tema da revisão não estar relacionado com o tema da tese, esta foi desenvolvida durante este doutorado. A minha contribuição está relacionada com a modelagem comparativa da proteína AsCyp51H10.

CAPÍTULO 1

Adventitious rooting in *Arabidopsis* hypocotyls: a comparative analysis of spontaneous and exogenously induced development with natural or synthetic auxins

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Manuscrito a ser submetido ao periódico *Physiologia Plantarum*.

Adventitious rooting in *Arabidopsis* hypocotyls: a comparative analysis of spontaneous and exogenously induced development with natural or synthetic auxins

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Keywords: Adventitious rooting, auxin, *Arabidopsis*, auxin-related genes, gene expression

Abstract

Adventitious roots (ARs) emerge from the stem, leaves or the pericycle of older roots and are strategic for forestry and horticulture. ARs may develop spontaneously or be stimulated under environmental stress or by hormonal application and auxins are the hormones that exert greater influence in AR development (ARD). We analyzed the effect of different auxin types in the number and length of AR, gene expression profiles, and IAA immunolocalization. NAA and 2,4-D effects were dose-dependent; at higher concentrations NAA inhibited root growth and 2.4-D caused callusing. IAA increased the number of roots with less interference in elongation, proving to be the best auxin type tested using the etiolated seedlings system. Immunolocalization of IAA indicated a higher concentration of this auxin close to the tissues where the roots originate, underlining the key role of this phytormone in adventitious rooting. Expression of genes potentially involved in rooting was monitored, with a focus on auxin homeostasis, signaling and transport. Several genes were induced by exogenous auxin, mainly NAA, followed by IAA, while others remained unchanged. ARD was marked by increased expression of *PIN1*, *SUR2*, *GH3.3*, *GH3.6*, *ARF8*, *IAA28*. Increased *PIN1* expression indicated the relevance of auxin efflux transport for focusing in target cells, whereas *GH3.3* and *GH3.6* could be relevant for auxin homeostasis. *ARF8* and *IAA28* would be important for auxin action. *SUR2* induction may cause a diversion of indole rings away from endogenous auxin biosynthesis in favor of glucosinolates. *IAA28* expression increased with ARD, unlike what was reported on lateral root development.

Abbreviations

2,4-D, 2,4-dichlorophenoxyacetic acid; AR, adventitious roots; ARD, adventitious root development; ARF, AUXIN RESPONSE FACTOR; Aux/IAA28, IAA28; ABP1, AUXIN BINDING PROTEIN 1; CYCB1;1, CYCLIN B1;1; GH3, Gretchen Hagen 3-like proteins; IAA, auxin indole-3-acetic acid; IBA, indole-3-butyric acid; JA, jasmonate; NAA, naphthalene-acetic acid; PIN, PINFORMED; Real-Time RT-PCR, Real-time quantitative reverse transcription-Polymerase Chain Reaction; SCR, SCARECROW; SUR2, SUPERROT2; TIR1, TRANSPORT INHIBITOR RESPONSE 1

Introduction

Adventitious roots (ARs) normally emerge from the stem, leaves or, occasionally, the pericycle of older roots. ARs may develop spontaneously from stem cells or be stimulated under environmental stress. AR are strategic for forestry and horticulture, especially when commercial materials are clonal. AR develop in three phases: induction, initiation and expression (Li et al. 2009) and most phytormones have been shown to participate in different steps of AR development (reviewed by da Costa et al., 2013). The endogenous auxin indole-3-acetic acid (IAA) was first shown to stimulate ARs (Cooper, 1935). Another endogenous auxin, indole-3-butyric acid (IBA), often promotes rooting more efficiently than IAA (De Klerk et al., 1999; Choffe et al., 2000; Mansseri-Lamrioui et al., 2011). IBA is also thought to be a transport intermediate of IAA (Strader et al., 2010). Naphthalene-acetic acid (NAA) is a synthetic form of auxin which promotes rooting and has higher metabolic stability (De Klerk et al., 1999). Different auxin types were investigated in *A. thaliana* inflorescence segments and IAA, IBA and NAA induced ARs formation, but 2,4-dichlorophenoxyacetic acid (2,4-D) and picloram induced callus formation at the same concentration (Verstraeten et al., 2013).

TRANSPORT INHIBITOR RESPONSE 1 (TIR1) and AUXIN RECEPTOR F-BOX PROTEINS (AFB) family of F-box proteins act as auxin receptors (Dharmasiri et al., 2005a; Dharmasiri et al., 2005b; Kepinski et al., 2005). Auxin promotes recruitment of the Aux/IAA transcriptional repressors by the $SCF^{TIR1/AFB}$ (Skp1/Cullin/F-box) E3 ubiquitin ligase complex, leading to their subsequent ubiquitination and degradation (Dharmasiri et al. 2005a; Parry et al., 2009; Maraschin et al., 2009). This releases the Auxin Response Factors (ARFs) from the repressive action of Aux/IAAs, allowing the transcription of auxin responsive genes (Santner and Estelle, 2009). Auxin Binding Protein 1 (ABP1) has been identified as an extracellular auxin receptor too, located both in the ER and outside of the plasma membrane. ABP1 signaling includes the activation of ATPase proton pumps, apoplast acidification and activation of input K^+ channels, possibly followed by changes in gene expression (reviewed by Tromas et al., 2010 and by Scherer, 2011).

Members of the Gretchen Hagen 3-like proteins (GH3) family are involved in auxin homeostasis, encoding IAA-amido synthetases which conjugate physiologically active excess free IAA with amino acids (Staswick et al., 2005; Chapman and Estelle, 2009). Jasmonate (JA) homeostasis is also modulated by the auxin-inducible *GH3.3*, *GH3.5* and *GH3.6* genes, fine-tuning AR initiation (Gutierrez et al., 2012). *ARF6* and *ARF8* are positive regulators and *ARF17* is a negative regulator of these three *GH3* genes that control JA conjugation with amino acids, downregulating the rooting inhibitor COI1 signaling pathway (Gutierrez et al., 2012). ARF17 expression is controlled by AGO1, which acts in the cross talk between auxin and light in adventitious rooting (Sorin et al.,

2005). *SUPERROT2* (*SUR2*) gene, encoding CYP83B1 protein, a cytochrome P450-dependent monooxygenase, modulates IAA and glucosinolate biosynthesis in *Arabidopsis* (Barlier et al., 2000; Bak et al., 2001).

In shoots auxin moves basipetally, whereas in roots its transport occurs in both directions (Muday and DeLong, 2002, Morris et al., 2010). Auxin uptake by cells is facilitated by influx transporters called AUXIN1/LIKE AUXIN1 (AUX1/LAX1) (Péret et al., 2012). Polar auxin transport (PAT) is mediated efflux transporter proteins and the most important for auxin polar transport are the PINFORMED (PIN) auxin efflux carriers. The auxin efflux function of PIN proteins was clearly shown both in plants and heterologous systems (reviewed by Nodzyński et al., 2012). Another group of auxin transporters includes ATP-binding cassette (ABC) transporters (Kamimoto et al., 2012), among which the P-glycoprotein (PGP)/ABCB subclass is relevant for auxin transport both at cellular and long-distance levels (reviewed by Geisler and Murphy, 2006; Verrier et al. 2008; Kamimoto et al., 2012). Auxin transport may be regulated by ABP1, which functions to promote clathrin-mediated endocytosis of PIN auxin efflux carriers. Binding of auxin inhibits ABP1 action, thereby stabilizing PINs at the plasma membrane, causing auxin to enhance its own efflux (Robert et al., 2010). The role of ABP1, however, was recently found out as controversial, since Gao et al. (2015) obtained abp1 null alleles that are not different from wild type-plants.

Establishment of the root meristem and the control of cell division, differentiation and cell homeostasis involve transcriptional factors of the GRAS family, including SCARECROW (SCR) and SHORTROOT (SHR) (Sabatini et al., 2003; Heidstra et al., 2004; Legué et al., 2014). Expression of *SCR* is necessary for quiescent center identity and to maintain the surrounding stem cells (Sabatini et al., 2003). B-type cyclins play roles in the regulation of mitotic and meiotic cell cycles (Lee et al. 2003; Malapeira et al. 2005; Pérez-Hidalgo et al. 2008; Guo et al. 2010). *CYCB1;1* has been used as a marker gene to monitor cell mitotic activity along the root initiation process through Real Time PCR analysis or fusion with reporter systems (Ferreira et al., 1994; Himanen et al., 2002; Vanneste et al., 2005; Yin et al., 2014).

Despite the importance of auxin in adventitious rooting, knowledge about plant responses at the molecular level and the role of different auxin types is still limited. The present work was undertaken to investigate the adventitious rooting process induced by different auxin types. Expression patterns of genes potentially involved with ARD were evaluated in *Arabidopsis* seedlings treated or not with natural and synthetic auxins. Moreover, the auxin distribution in the hypocotyl during the AR formation was visualized through IAA immunolocalization.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana seeds were obtained from ABRC – The Arabidopsis Biological Resource Centre (Ohio, USA) and the ecotype Col-0 was used in all experiments. Seed sterilization was carried out according to Correa et al. (2012). Surface sterilized seeds of Col-0 were placed on medium 0.1x MS supplemented with 2% sucrose and solidified with agar (0.6% w/v) in 24 well microplates of 2 mL each. The plates were wrapped with aluminum foil and maintained in the dark for 5 days at 20 ± 2º C to allow for germination and etiolation. Subsequently, 0.5 mL of liquid 0.3% agar medium with or without auxins was aseptically added into each well, covering part of the hypocotyl. All media pH were adjusted to 5.8 prior to autoclave sterilization. Seedlings were then transferred to light, with a photoperiod of 16 h per day and 45 µmol m^{-2} s^{-1} of photosynthetically active radiation (PAR) (Correa et al., 2012).

Auxin treatments

To evaluate the influence of different auxin types and concentrations in adventitious rooting, etiolated seedlings were treated with 57 µM of IAA (previously described by Correa et al., 2012); 2, 10, 40 or 57 µM of NAA or 500 nM, 1, 2 or 5 µM of 2,4-D. Stocks were freshly prepared, dissolved in NaOH 10 mM, diluted in water and added to the media at indicated concentrations before autoclaving. To access whether autoclaving auxins could yield different responses compared to filter sterilization of these phytohormones, the number of adventitious roots and the root length of the longest adventitious root were compared by treating with auxins sterilized by each one of the methods. No difference was observed for the parameters monitored (Fig. S1). All media pH were adjusted to 5.8 prior to autoclave sterilization. The ARs were counted and root length of the longest root was measured four and eight days after treatment with auxins and transfer to light (Fig 1). Each experiment was repeated three times and each biological replicate was composed of 7 plants. After normality check, a *t-test* comparing each treatment to the respective control free of exogenous auxin was applied ($P \leq 0.05$).

Immunohistological analysis

Etiolated seedlings were treated with 57 µM of IAA or with media devoid of auxins and monitored for AR development. Seedling samples were harvested at the end of etiolation period (day zero) and at two and four days after exposure of seedlings to light and auxins (Fig 1). The samples were fixed in 4% formaldehyde [w/v] in 0.1 M sodium phosphate buffer pH 7.2 at 4ºC. After 24 h, they were washed three times with sodium phosphate buffer pH 7.2 and kept in this solution for 48 h. The seedlings were then dehydrated in an ethanol series, placed in gelatin capsules, embedded in resin (LR

white resin – Sigma Aldrich) and polymerized at 50 ºC for 24 h. Samples were transversally sectioned in a microtome with glass blades (2 µm thick) and placed in slides covered with gelatin. The antibody incubation was done following Sakata et al. (2010), using the anti-IAA monoclonal antibody (Sigma-Aldrich, St Louis, USA) and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody. Prior to mounting in anti-fade reagent, one drop of calcofluor white was applied on the slices for 10 minutes and washed three times with distilled water.

RNA extraction and cDNA synthesis

Plants were grown and etiolated as described above. After etiolation, 57 µM of IAA, 57 µM of NAA or media devoid of auxins were added to the wells to induce AR initiation. The whole seedlings were collected at the end of etiolation period (day zero) and at two, four, six, eight and ten days after exposure of seedlings to light and auxins (Fig. 1). Samples were frozen in liquid nitrogen and stored at -80º C. Total RNA was extracted using Concert Plant RNA Reagent (Invitrogen®, Carlsbad, CA, USA), treated with DNAseI (Invitrogen®, Carlsbad, CA, USA) and the primary cDNA was synthesized from approximately 3 µg of total RNA with oligo dT and reverse transcriptase (M-MLV, Invitrogen®, Carlsbad, CA, USA).

Real Time RT-PCR

The cDNA from each time point was diluted 100 times. Real-time quantitative reverse transcription-PCR was performed in an ABI 7500 Real-Time PCR System (Applied Biosystems). Primers were designed using Primer3 tool, version 0.4 (Rozen and Skaletsky, 2000) or selected based on the literature and are listed on Table S1. Reactions were done with SYBR green (1:10,000, Molecular Probe) for detection, with total volume of 20 µl and a cDNA fraction of 50%. *ACTIN* (*ACT*) and *ELONGATION FACTOR 1* (*EF1*) were selected as reference genes to normalize Real-Time RT-PCR data due to their stability under the experimental conditions, based on GeNorm and NormFinder softwares. A melting curve analysis was performed for every PCR program to ensure that the fluorescence signal was being generated by the desired amplification product. PCR efficiency was determined for each one of the amplifications using LinRegPCR software (Ramakers et al., 2003) and the average PCR efficiency for each gene was used in the calculations. To confirm the specificity of the amplified sequences, the amplicons were sequenced. The relative expression was calculated by the comparative $\Delta\Delta C_t$ method. After normality check, a *t-test* comparing each normalized expression data against the condition prior to the transfer to light and flooding was applied ($P \leq 0.05$). In addition, ANOVA followed by Tukey test were used to compare expression levels within time points between treatments ($P \leq 0.05$). Each experiment was repeated three times and each biological replicate was composed of 15-22 plants.

Results

Effects of different auxin types on adventitious rooting

To assess the effects of different auxin types on AR formation, three auxins were tested, IAA, NAA and 2,4-D and compared with treatment without auxins. Seedlings were evaluated for number of AR per explant and length of the longest root on days four and eight after transfer to light and treatment with auxins. The ideal concentration of IAA was previously set to the etiolated seedlings system used in this work (Correa et al., 2012). On day four, it was possible to observe an increased number of AR with the application of exogenous IAA at 57 µM and NAA at 40 and 57 µM, whereas no effect was seen with the use of 2,4-D at 500 nM and 1 µM (Fig. 2A). The application of 2 or 5 µM of 2,4-D caused callus development instead of roots in all the seedlings (Fig. 2 and 5). In general, root length was reduced in the presence of auxin when compared with the treatment without auxins on day four (Fig. 2B).

On day eight, the distinct effects of auxin types on rooting became more evident. IAA caused an increase in number of ARs without significantly affecting root length. The response of adventitious rooting to NAA appeared to follow a dose dependent profile. When a concentration of 10 µM or higher of NAA was applied, ARs were induced at a higher efficiency than with IAA (Fig. 2A); however, roots remained short, as their extension was inhibited by NAA (Fig. 2B). In presence of 2 µM of NAA, the development of ARs did not differ from the control without auxins, except for a transient inhibition of root growth on day four. When treated with 500 nM or 1 µM of 2,4-D, the number of ARs increased on day 8 (Fig. 2A). Root length was not affected at 500 nM of 2,4-D, but it was inhibited at 1 µM on day eight (Fig. 2B).

IAA accumulation coincided with AR initiation tissues

Immunolocalization was used to visualize IAA distribution in the hypocotyl of the seedlings, using anti-IAA antibodies. Fixed samples were treated first with anti-IAA monoclonal antibody and then with Alexa 488-conjugated goat anti-mouse IgG antibody. No fluorescence was detected when the slices were treated only with Alexa 488-conjugated goat anti-mouse IgG antibody. We checked the immunolocalization of IAA in seedlings treated or not with IAA. The images suggested a higher concentration of IAA in the regions of the vascular cylinder and the pericycle of the hypocotyl (Fig. 3). On day two with exogenous IAA and day four without supply of auxins higher accumulation of auxin was detected in points around the vascular cylinder, including the pericycle, apparently where ARs are formed (Fig. 3C and D). On day four with external application of IAA it was possible to see the longitudinal section of an AR (Fig. 3E). There was intense accumulation signal of IAA along the root formed, the vascular cylinder and the pericycle of the hypocotyl.

Expression of auxin related genes

Gene expression analyses were conducted with seedlings treated with 57 µM of IAA, 57 µM of NAA or media devoid of auxins (control). Increased expression of *PIN1*, *SUR2*, *IAA28*, *ARF8*, *GH3.3* and *GH3.6* was observed with auxin, especially in seedlings treated with NAA (Fig. 4). *PGP1*, *ABP1*, *AGO1* and *ARF6* had similar profiles of higher expression with NAA, although not statistically significant compared to control (Fig. S2). The *TIR1* level of expression remained constant over the periods analyzed and *ABP1* didn't show statistically significant differences when compared with the control (Figs. 4B and S1A). *PIN1* increased the expression at later stages of the rooting process and in presence of IAA or NAA, which was not the case of *AUX1* (Fig. 4C and D). Surprisingly, *SUR2* and *IAA28* expression increased in presence of exogenous auxin, particularly with NAA (Fig. 4A and F). *ARF6* and *ARF8* showed similar results, IAA caused a slight rise in expression, whereas NAA caused a more pronounced increase (Figs 4G and S1C). Both IAA and NAA had an important effect of upregulating *GH3.3* in relation to auxin-free media (Fig. 4H). *GH3.3* and *GH3.6* showed increased expression after the transfer to light even without exogenous auxins supply (Fig. 4H and I). *GH3.6* had a major transcript accumulation in the presence of NAA in all time points evaluated (Fig. 4I). *CYCB1* increased expression mainly on days two and four after transfer to light in the presence of exogenous auxins, with a distinct induction by NAA on day four (Fig. 4E). After this period, *CYCB1* expression decreased and there was no difference between media with or without auxins. In general, *SCR* showed lower expression in the presence of exogenous auxins compared to the control (Fig. S2E).

Discussion

We tested three different types of auxin regarding the ability to promote adventitious rooting. The application of NAA induced different responses depending on the concentrations used and the same was observed with 2,4-D. In the concentrations of 10, 40 and 57 µM, NAA induced AR formation with higher efficiency than IAA, but root growth was inhibited (Fig. 2). It is known that auxins have a stimulatory effect on adventitious rooting in the first stages of the process, but repress later developmental stages (De Klerk et al., 1999; Verstraeten et al., 2014). The effect of NAA in increasing the number, but reducing the length of the AR is probably related to its higher metabolic stability, being less oxidized (De Klerk et al., 1999; Fleck et al., 2009), and capable of forming conjugates (glucosyl esters) (Hosek et al., 2012). In this sense, NAA has a longer persistence than IAA in the tissues, probably becoming inhibitory to root elongation. In *Eucalyptus globulus* and *E. saligna*, 49.3 µM of NAA promoted rooting percentage and number of roots in efficiency comparable to that of IAA, although yielding shorter roots (Fogaça and Fett-Neto, 2005). The ideal concentration of NAA in the etiolated seedling system was 10 µM, yielding similar responses to IAA (Fig. 2).

2,4-D was less efficient than IAA or NAA to induce AR formation. Among the concentrations tested of 2,4-D, 500 nM was the most effective in this system because, although the induction of roots was lower than that of IAA, it did not affect subsequent root growth (Fig. 2). Callus formation was observed with 2 or 5 µM of 2,4-D (Fig. 2 and 5), what was also noticed by Verstraeten et al. (2013) in stem segments of *A. thaliana* with 10 µM of 2,4-D and by Martínez-de la Cruz (2015) in maize. 2,4-D is poorly transported by auxin efflux (Delbarre et al., 1996), which could cause an accumulation of this auxin type in the cells inhibiting AR initiation and leading to callus formation. In addition, 2,4-D has been shown to promote cell division, but not cell elongation in tobacco cells (Campanoni and Nick, 2005), which could contribute to callus formation. IAA had the best effects on adventitious rooting, probably because it stays in the tissues enough time to induce ARs, but not to inhibit them, since its conjugation as well as transport, may happen rapidly in the seedling (Kramer and.Ackelsberg, 2015.

The immunolocalization of auxins allowed us to detect accumulation of auxin in the vascular cylinder and pericycle of the hypocotyl (Fig. 3), where a higher demand of this hormone is expected, since ARs are formed from these tissues in hypocotyls of etiolated seedlings (Correa et al., 2012). Expression of PIN1 was observed by Della Rovere et al. (2013) in the same regions and was stimulated by exogenous auxin supply, suggesting that PIN1 might transport auxins from the hypocotyl vasculature to pericycle cells. This idea is also supported by *PIN1* mRNA expression observed in etiolated seedlings (Fig. 4C), especially with provided IAA and NAA. When the root is already formed (Fig. 3E) there is accumulation of IAA not only in the vascular cylinder and pericycle,

but throughout the root. This finding further supports the key role of this phytohormone in root development.

We checked the relative mRNA accumulation of some genes potentially involved in rooting of etiolated seedlings, especially those related with auxin homeostasis, signaling and transport. The seedlings were treated with the same concentration (57 μM) of IAA or NAA, as well as without auxins (control). Several genes such as *PIN1*, *PGP1*, *SUR2*, *IAA28*, *ARF6*, *ARF8*, *GH3.3*, *GH3.6*, *ABP1* and *AGO1* seemed to be upregulated by NAA (Fig. 4 and S1). These increases in gene expression may explain the higher number and smaller size of AR that are formed with NAA in relation to IAA at the same concentration or to auxin-free media (Fig. 2). The pronounced stimulatory effects of NAA in AR meristem differentiation and gene expression may reflect not only its metabolic stability (De Klerk et al., 1999), but also the fact that it may enter cells by diffusion, being also recognized and transported by auxin efflux carriers (Marchant et al., 1999).

Transcript levels of the auxin receptor *TIR1* decreased after the transfer to light and the application of media with or without exogenous auxins and did not change over the subsequent period (Fig. 4B). IAA and 1-NAA compete efficiently for binding with TIR1, but IAA has a higher affinity for the receptor than 1-NAA (Dharmasiri et al., 2005a). However, it seems that receptor affinity differences did not affect gene expression in this case. *ABP1* also didn't show statistically significant differences. Thus it was not possible to infer which receptor plays a major role in AR with this experimental system. Other members of the TIR1/AFB-family of F-box proteins might be involved in the adventitious rooting process as well.

PIN1 was induced by IAA and more intensely by NAA (Fig. 4C), while the relative mRNA expression of *PGP1* was not as evident as that of *PIN1*. PGP1 was apparently downregulated after the transfer to light without auxins or with IAA (Fig. S2B). PGP1 acts in the mediation of cellular efflux of IAA and NAA and its expression is auxin responsive; however, other auxin efflux proteins are involved in transport too (Geisler et al., 2005). PINs and PGPs have distinct transport mechanisms, playing different developmental roles that cooperate to mediate and enhance auxin transport (Blakeslee et al., 2007; Mravec et al., 2009). The expression pattern of *PIN1* suggests a major participation of *PIN1* compared to *PGP1* in the adventitious rooting process promoted by auxins. The influx carrier *AUX1* did not show a clear pattern of expression, and as expected, it was not induced by NAA (Fig. 4D), since 1-NAA enters the cells via passive diffusion and does not depend on influx carriers (Delbarre et al., 1996). The control of NAA accumulation is mediated by efflux carriers (Delbarre et al., 1996), what could explain the prominent *PIN1* mRNA levels of expression in the presence of this auxin type.

SUR2 is involved in the control of IAA and indole glucosinolate synthesis in *Arabidopsis* (Bak et al., 2001). The *sur2* mutant overproduces IAA and displays a higher number of AR primordia (Delarue et al., 1998; Barlier et al., 2000). Surprisingly, *SUR2* expression increased in presence of exogenous auxin, particularly with NAA, indicating that auxin synthesis controls may be coupled with exogenous auxin uptake. The increased *SUR2* expression may have resulted in lower endogenous IAA production in favor of glucosinolates.

Another interesting observation was the increased expression of the Aux/IAA gene *IAA28*, particularly in presence of exogenous auxin (Fig. 4F). Previously, Rogg et al. (2001) found that auxin was not able to induce expression of *IAA28*, in fact its mRNA levels decreased in response to IAA. This pattern was different from the ones observed for other Aux/IAA genes that are induced by auxins and show a biphasic dose response to IAA (Abel et al., 1995). In lateral roots, it was observed that the gain-of-function mutant *iaa28-1* prevented lateral root formation and was resistant to auxin, suggesting that IAA28 could function in transcriptional repression of auxin-responsive genes involved in lateral root development (Rogg et al., 2001). Later, it was shown that lateral root founder cells are regulated by a GATA-type transcription factor, *GATA23* that depends on the *IAA28-ARF7-ARF19* regulatory module (De Rybel et al., 2010). However, it is not clear how this regulation happens on AR formation and the role of *IAA28* in this process requires further investigation. The expression profile of this gene may constitute a difference in the developmental pathways leading to lateral versus adventitious roots.

NAA appears to have induced expression of *ARF6* and *ARF8*, whereas the levels of transcripts in the control and with exogenous IAA kept low (Fig. 4G and S1C). Expression of *GH3.3* was induced by IAA and NAA, whereas *GH3.6* was mainly induced by NAA (Fig. 4H and I). Gutierrez et al. (2012) proposed a model in which the auxin response factors *ARF6* and *ARF8* are positive regulators of the conjugation genes *GH3.3*, *GH3.5* and *GH3.6* that regulate jasmonic acid (JA) homeostasis and control AR initiation through the *COI1* signaling pathway. JA is a negative regulator of AR initiation and its conjugation to amino acids by *GH3.3*, *GH3.5* and *GH3.6* proteins may regulate its concentration. Expression of *GH3.3* and *GH3.6* with exogenous auxins increased until day eight, decreasing afterwards (Fig. 4H and I), possibly when most of the free JA was already conjugated to amino acids. In this regulation, *AGO1* is also involved, by modulating ARF17 expression which negatively regulates expression of *GH3* genes (Sorin et al., 2005; Gutierrez et al., 2009). Indeed, *AGO1* mRNA expression was similar to that of *ARF6* and *ARF8*, suggesting that auxin induces its expression in the etiolated seedlings system (Fig. S2D). These observations corroborate the idea that JA homeostasis during AR development is under the control of auxins, as proposed by Gutierrez et al. (2012).

Steady state transcript levels of *SCR* were lower in presence of exogenous auxins in relation to auxin-free medium in almost all of the periods analyzed (Fig. S2E). Both in *Pinus radiata* D. Don and *Castanea sativa* Mill., an increase in mRNA expression of *SCR-LIKE* genes was observed in root cuttings treated with exogenous auxin within the first 24 hours, indicating that this gene plays a role at the earlier stages of AR development, prior to cell divisions and the appearance of AR primordia (Sánchez et al., 2007). In the present work, we checked SCR expression after 48 hours of auxin exposure, probably coinciding with decreased levels of SCR transcripts, as observed by Sánchez et al. (2007) in *P. radiata* and *C. sativa*. On the other hand, the mitotic cyclin *CYCB1;1* showed a peak of mRNA level mainly on days two and four in presence of NAA (Fig. 4E). Levels of mRNA also increased with IAA from days two to eight compared to day zero, although to a lower extent. B-type cyclins play a role in the cell cycle and, more specifically, CYCB1;1 acts on G2 to M transition (Hemerly et al., 1991; Ferreira et al., 1994). The increase in cyclin mRNA levels is closely related to the first cellular divisions that give rise to root primordia, indicating a correlation between induction of AR by auxin and cell cycle activation. Without exogenous auxin supply, a small increase in *CYCB1;1* transcript levels was observed after day eight in relation to day zero (Fig. 4E), indicating that transcriptional programs of the adventitious rooting process are accelerated by exogenous auxin.

The effects of different auxins in adventitious root development and gene expression are summarized in Fig. 5. We observed that IAA increased the number of adventitious roots per explant without affecting the root length, whereas NAA inhibited root growth at high concentrations and 2,4-D induced callusing. ARD was essentially marked by increased expression of *PIN1, SUR2, GH3.3, GH3.6, ARF8* and *IAA28*. The magnitude of expression of induced genes was much stimulated by NAA, followed by IAA, suggesting that auxin metabolic stability and transport rates may significantly change transcription profiles leading to ARD. *IAA28* expression increased with ARD, unlike reports on lateral root development, possibly representing a differential feature between these two developmental programs.

Author Contributions

Cibele Tesser da Costa helped to design and performed the experiments, analysed and interpreted data, and drafted the manuscript.

Jorge Ernesto de Araujo Mariath and Marcos Letaif Gaeta helped with the immunolocalization experiments and their interpretation.

Remko Offringa and Arthur Germano Fett-Neto devised the experiments, helped with data interpretation, supervised the project and finalized the manuscript.

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Supporting Information

Gene name	Forward Primer 5' → 3'	Reverse Primer 5' → 3'
PIN1	TGGTCCCTCATTTCCTTCAA	GGCAAAGCTGCCTGGATAAT
PGP1	TCTGGCGACTAGCTAAAATGAACTC	CCACAAATGACAGAGCCTACTGA
AUX1	AAGGGCTTTGGCTAGATTGCC	CAAGAAGAGCACCGACAGCG
CYP83B1/SUR2	ACTCTTGACCCTAACCGCCCTAAAC	TGCAGCCGCCGTGTCAGT
ARF6 uncleaved	CAAAGTTTAGCAGCTACCACGA	ACGTCGTTCTCTCGGTCAAC
ARF8 uncleaved	TTTGCTATCGAAGGGTTGTTG	CATGGGTCATCACCAAGGA
GH3.3	ACAATTCCGCTCCACAGTTC	ACGAGTTCCTTGCTCTCCAA
GH3.5	GTCTTCGAGGACTGCTGCTT	ATGTCCCTGGCTCAACAATC
GH3.6	CCTTGTTCCGTTTGATGCTT	CGTGTTACCGTTCAAGCAGA
ABP1	ACTGCTGCAAGGCTGAAGTT	GGTTGTGCTTGCTTTTAGCC
AGO1	AAGGAGGTCGAGGAGGGTATGG	CAAATTGCTGAGCCAGAACAGTAGG
SCARECROW	TCTGATCACGGTGGTGGAGCAAG	TCACCCAATCCATCTCCTAGCGC
IAA28	TCAAAGCCAAACCCCATTAG	TAAAGTTCTGGTCGGGGATG
TIR1	CCTAAACTGCAGCGCCTCT	GGTTGAAGCAAGCACCTCA
CYCB1;1	CTCAAAATCCCACGCTTCTTGTGG	CACGTCTACTACCTTTGGTTTCCC
Actin	GCACCCTGTTCTTCTTACCG	AACCCTCGTAGATTGGCACA
EF-1	TGAGCACGCTCTTCTTGCTTTCA	GGTGGTGGCATCCATCTTGTTACA

Table S1. Gene specific primers used for Real Time RT-PCR.

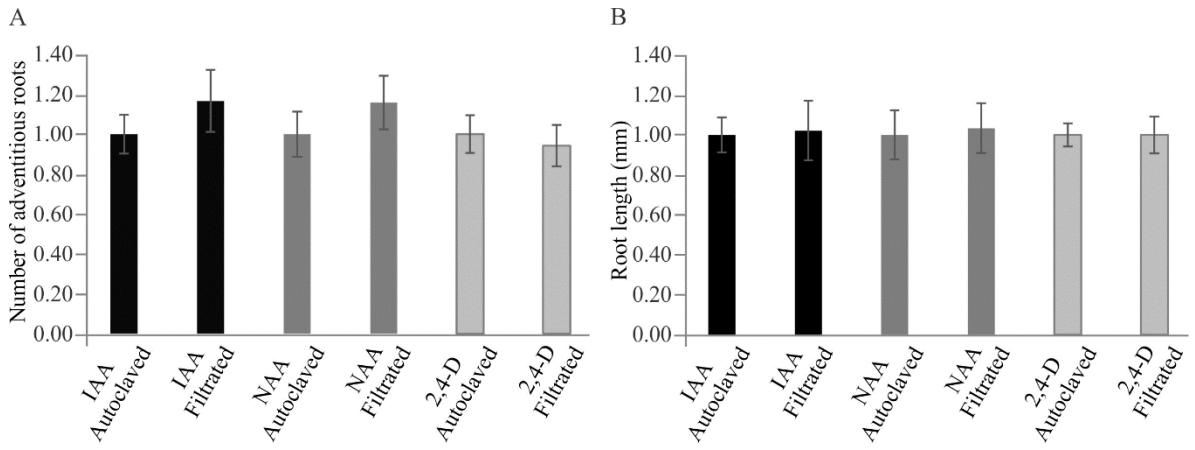


Figure S1. Effects of autoclaved or filtrated auxins on adventitious root number and length in *Arabidopsis thaliana* hypocotyls. The concentrations used were 57µM of IAA, 10µM of NAA and 500nM of 2,4-D. The data of different filtrated auxins were normalized in relation to the different autoclaved auxins (autoclaved auxins = 1.00). A) Average number of roots per explant. B) Average length of the longest root (mm). The error bars represent the standard error. No difference was observed between auxins autoclaved or filtrated (*t*-test, $P \leq 0.05$).

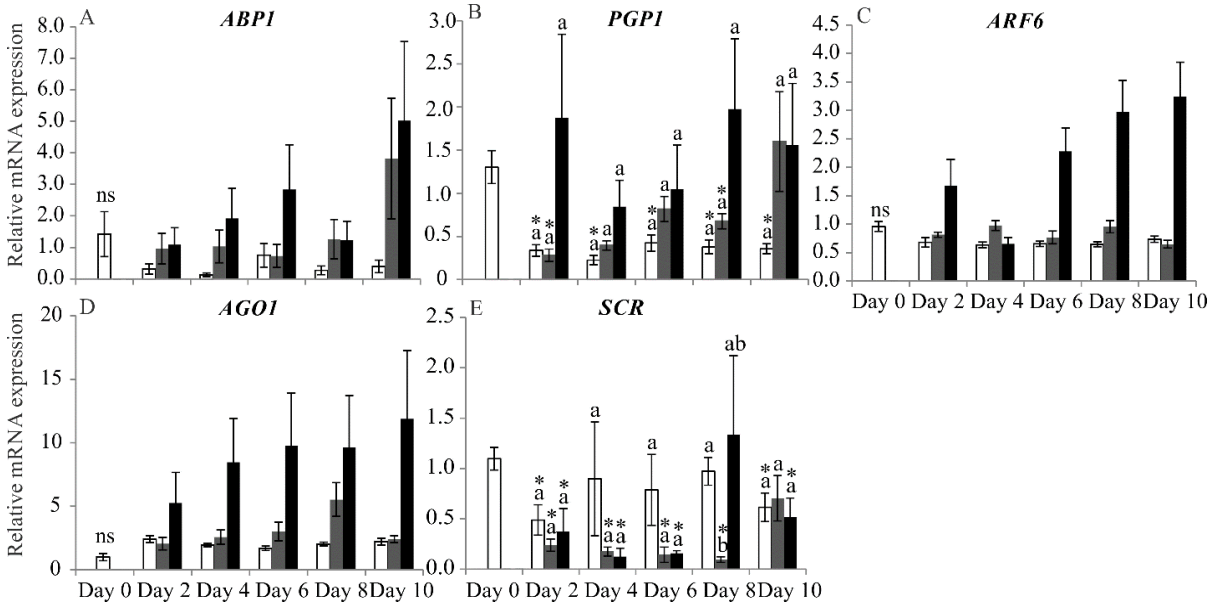


Figure S2. Relative mRNA expression of auxin-related genes during adventitious rooting in etiolated seedlings without exogenous auxin (empty bars), with 57µM of IAA (grey bars) or 57µM of NAA (black bars). A. *AUXIN BINDING PROTEIN* 1 (*ABP1*); B. *P-GLYCOPROTEIN 1* (*PGP1*); C. *AUXIN RESPONSE FACTOR 6 (ARF6)*; D. *ARGONAUTE 1 (AGO1)*; E. *SCARECROW* (*SCR*). The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to the respective expression on day zero (*t*-test, $P \leq 0.05$). Bars sharing letter are not significantly different within each time point (ANOVA followed by Tukey, $P \leq 0.05$).

Figure legends

Figure 1. Scheme of the etiolated seedling system for rooting, indicating time points used for sampling.

Figure 2. Effects of different auxin types and concentrations on adventitious root number and length in *Arabidopsis thaliana* hypocotyls. Using 2,4-D in the concentrations of 2µM and 5µM there was formation of callus instead of roots. A) Average number of roots or callus per explant. B) Average length of the longest root or callus (mm). The error bars represent the standard error. Different letters represent means statistically different ($P \leq 0.05$) in ANOVA followed by Dunnett's C test.

Figure 3. Imunolocalization of IAA on adventitious rooting in *Arabidopsis thaliana*. A) Day zero without auxins. B) Day two without auxins. C) Day two with exogenous supply of IAA. D) Day four without auxins. E) Day four with exogenous supply of IAA. White arrows indicate the region of vascular cylinder and pericycle. VC: vascular cylinder; CP: cortical parenchyma.

Figure 4. Relative mRNA expression of auxin-related genes during adventitious rooting in etiolated seedlings without exogenous auxin (empty bars), with 57µM of IAA (grey bars) or 57µM of NAA (black bars). A. *SUPERROT2* (*SUR2*); B. *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*); C. *PINFORMED 1* (*PIN1*); D. *AUXIN1* (*AUX1*); E. *CYCLIN B1;1* (*CYCB1;1*); F. *AUX/IAA 28* (*IAA28*); G. *AUXIN RESPONSE FACTOR 8* (*ARF8*); H. *GRETCHEN HAGEN 3.3 (GH3.3)* and *GRETCHEN HAGEN 3.6 (GH3.6).* The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to the respective expression on day zero (*t*-test, $P \leq 0.05$). Bars sharing letter are not significantly different within each time point (ANOVA followed by Tukey, $P \leq 0.05$).

Figure 5. Summary of different auxin types in ARD and gene expression. Seedlings in the images are in day 8 after the transfer to light and application of auxins.

Figures

Figure 1:

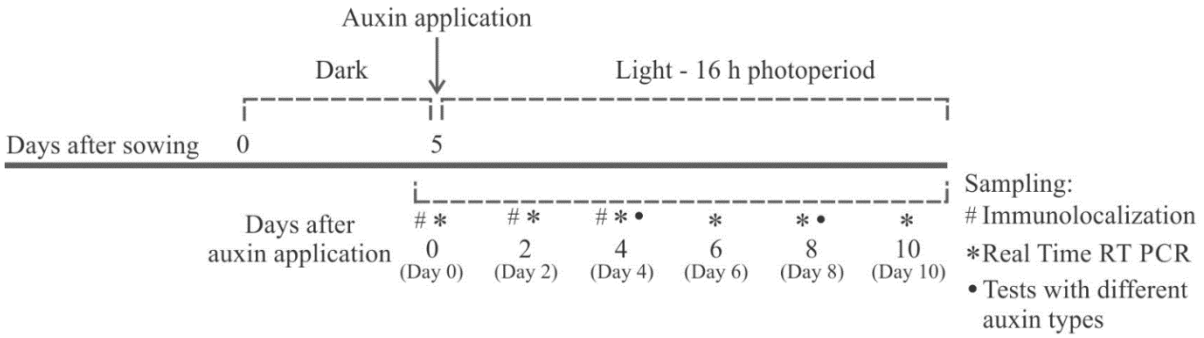


Figure 2:

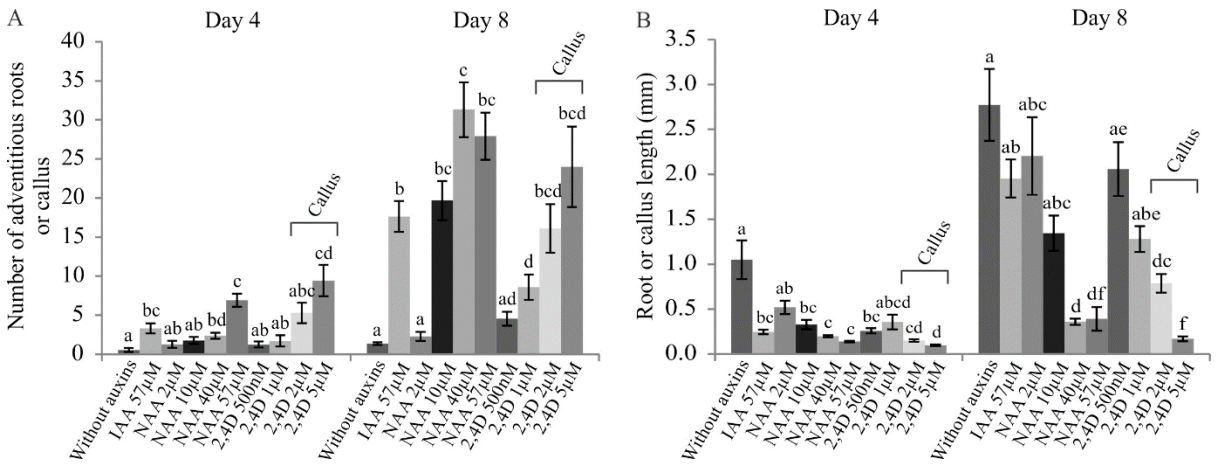


Figure 3:

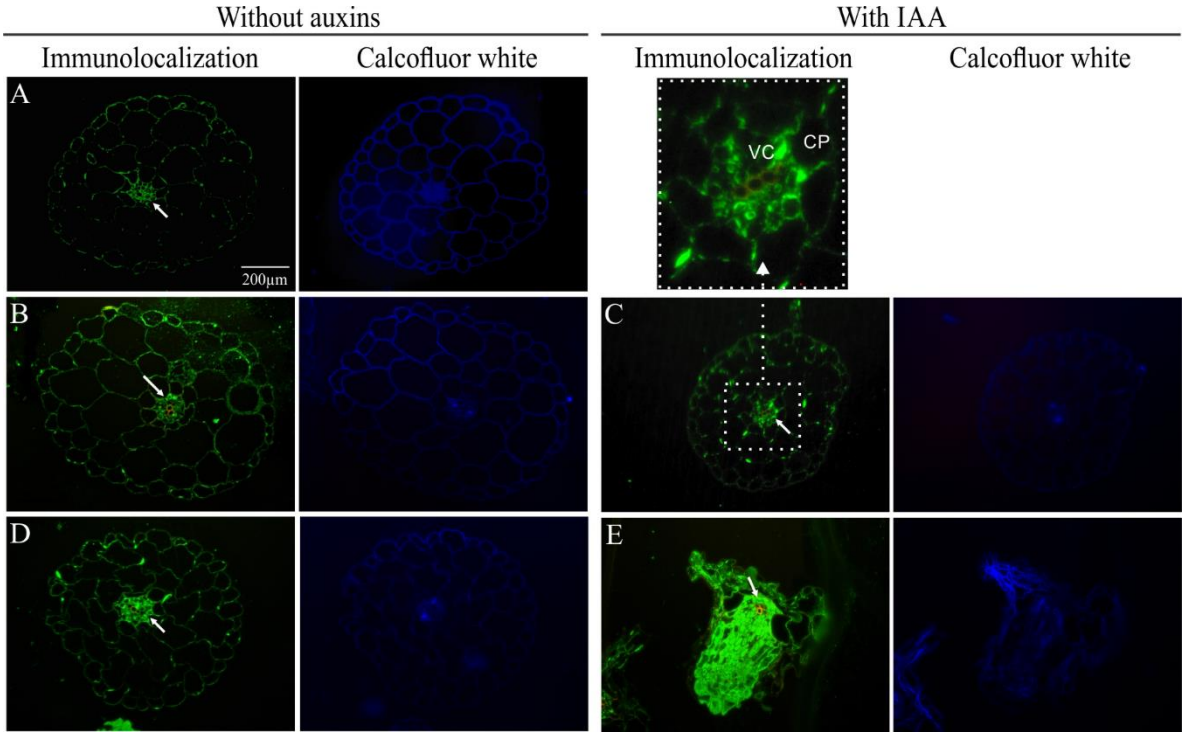

Without auxins
With IAA
Immunolocalization
Calcofluor white
Immunolocalization
Calcofluor white
A
200µm
VC
CP
B
C
D
E

Figure 4:

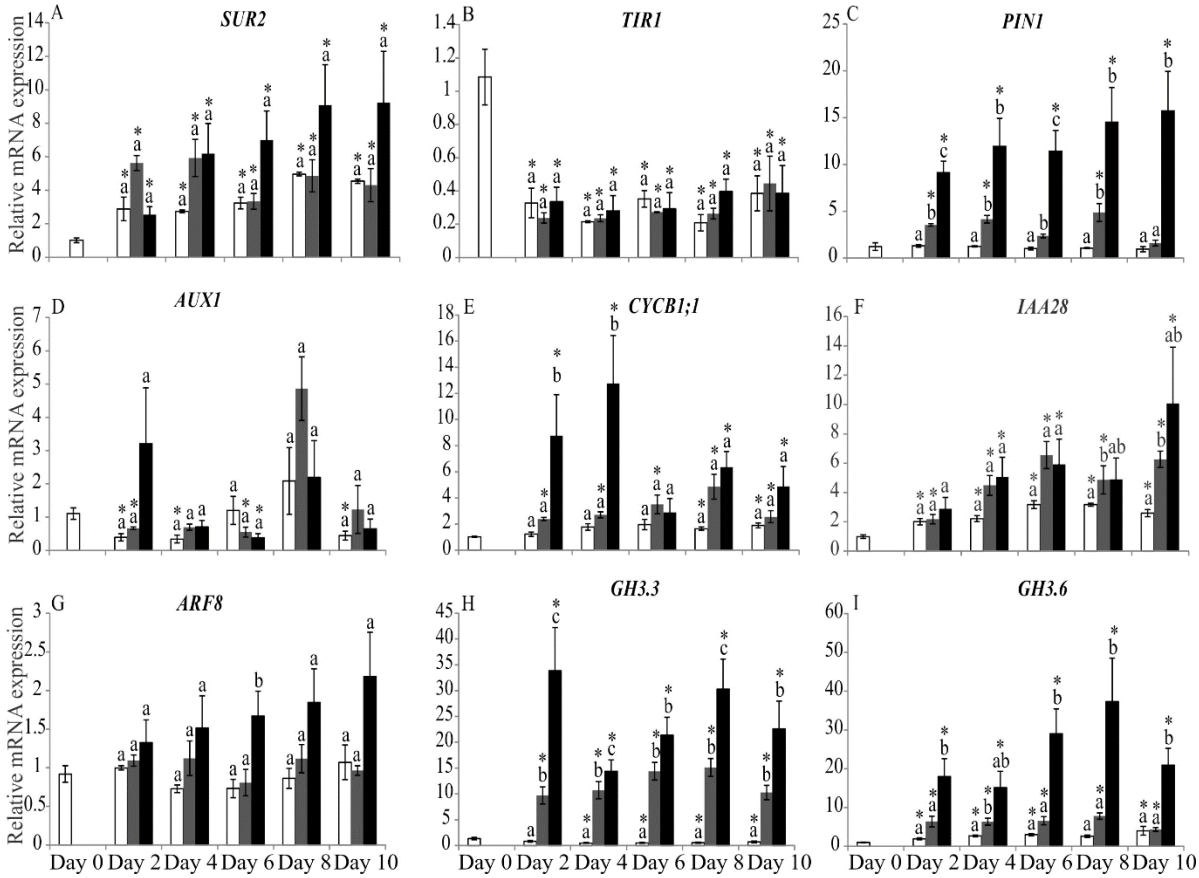
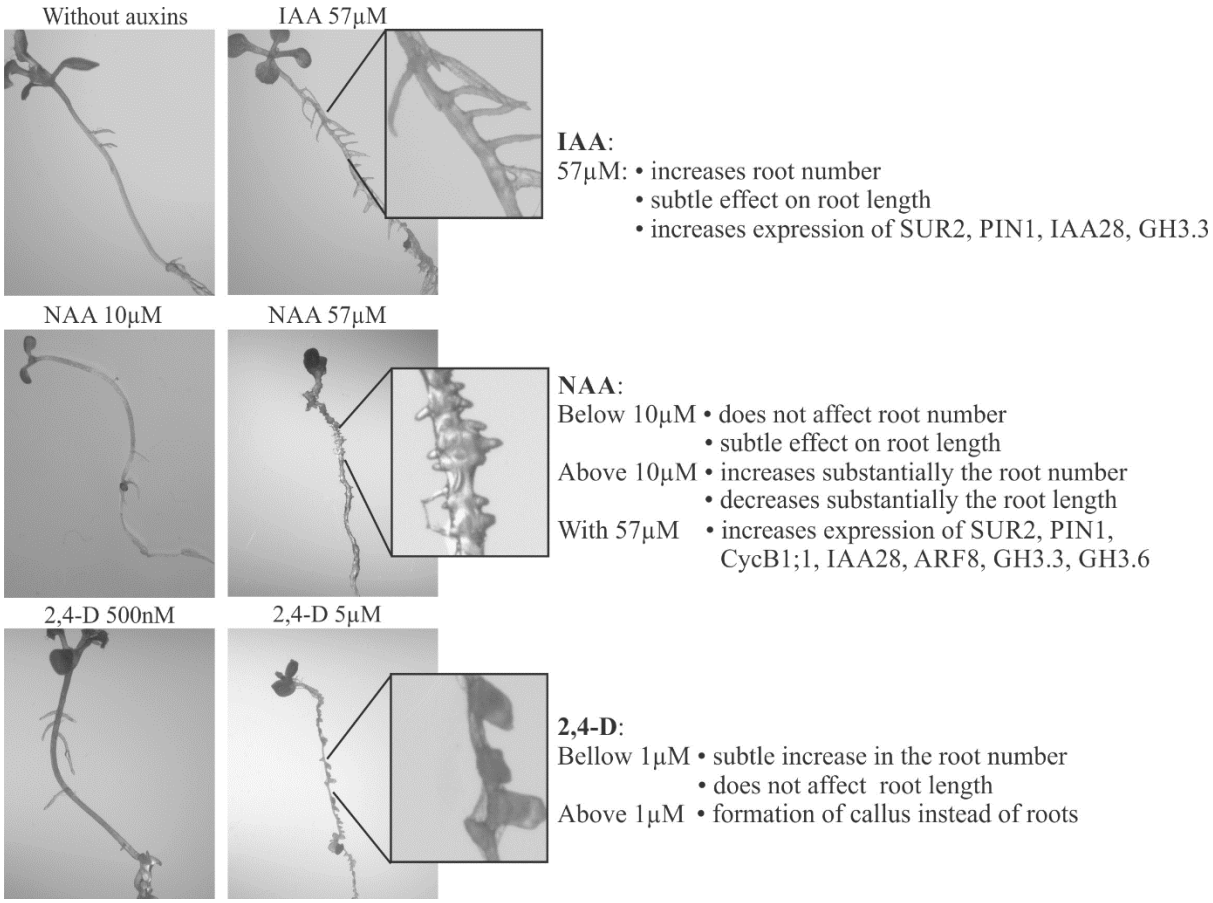

A
SUR2
B
TIR1
C
PIN1
D
AUX1
E
CYCB1;1
F
IAA28
G
ARF8
H
GH3.3
I
GH3.6
Relative mRNA expression
Day 0 Day 2 Day 4 Day 6 Day 8 Day 10

Figure 5:


Without auxins
IAA 57µM
IAA:
57µM: • increases root number
• subtle effect on root length
• increases expression of SUR2, PIN1, IAA28, GH3.3
NAA 10µM
NAA 57µM
NAA:
Below 10µM • does not affect root number
• subtle effect on root length
Above 10µM • increases substantially the root number
• decreases substantially the root length
With 57µM • increases expression of SUR2, PIN1, CycB1;1, IAA28, ARF8, GH3.3, GH3.6
2,4-D 500nM
2,4-D 5µM
2,4-D:
Bellow 1µM • subtle increase in the root number
• does not affect root length
Above 1µM • formation of callus instead of roots

CAPÍTULO 2

The role of auxin influx transporters in adventitious rooting of *Arabidopsis thaliana* etiolated seedlings

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Manuscrito a ser submetido à publicação futuramente

The role of auxin influx transporters in adventitious rooting of *Arabidopsis thaliana* etiolated seedlings

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Keywords: Adventitious rooting, auxin influx transport, Arabidopsis, mutant, gene expression

Abstract

Root system architecture modulation can include root growth and development of lateral roots (LRs) and adventitious roots (ARs). Whereas LRs develop from the primary root, ARs are generally formed from above-ground organs. ARs may be stimulated by wounding or hormonal application. Auxins are recognized as major hormones involved in AR development. Auxins are produced in the shoot apex of the plant and need to be transported to the base. The transport of auxins occurs in two different modes: the non-polar transport, a rapid and non-directional pathway through the phloem or the polar auxin transport (PAT), a slower and directional pathway, which involves specific transporters. The polarity of intracellular auxin transport is mostly controlled by the PINFORMED (PIN) and ABC-B/MULTI-DRUG RESISTANT/P-GLYCOPROTEIN (ABCB/MDR/PGP) auxin efflux carriers, together with the AUXIN1/LIKE AUXIN1 (AUX1/LAX1) influx carriers. AUX/LAX is a small family composed of *AUX1*, *LAX1*, *LAX2* and *LAX3* genes in *A. thaliana*, related to amino acid permeases. To further understand this relationship, we analyzed AR development in different mutants affected in auxin influx transporters, coupled with reporter gene constructs, in presence or absence of exogenous auxin. Loss of AUX1 function reduced the number of ARs in *A. thaliana* etiolated seedlings and this effect was not reversed by exogenous auxin supply. Single mutations in *LAX1*, *LAX2* and *LAX3* had no impact in AR, but different combinations of double, triple and quadruple mutations in *AUX1*, *LAX1*, *LAX2* and *LAX3* caused significant reductions in ARs. This effect, however, was reversed by auxin. Surprisingly, mutation in *LAX3* did not affect AR number and the visualization of LAX3pro:LAX3-YFP was stronger in the region of the stele. Therefore, an essential role was established for AUX1 in AR. Although LAX3 *per se* was not essential to the process, it seemed to act in conjunction with AUX1.

Introduction

The root system of a plant has several essential roles, such as uptake of water and nutrients and anchorage in soil (Smith and De Smet et al., 2012). Root system architecture can be modulated in response to environmental cues as a plant strategy to adapt and survive. This modulation can include root growth and development of lateral roots (LRs) and adventitious roots (ARs) (reviewed by Franco et al., 2011). The main difference between LRs and ARs is that the former develop from the primary root and ARs are generally formed from above-ground organs, such as stem, hypocotyls and leaves. ARs may naturally form from stem cells or their development can be stimulated by wounding or hormonal application. ARs can develop post-embryonically in monocots and dicots, improving nutrient acquisition. Their shallow growth angle can increase phosphorus uptake in upper soil strata, for example (Smith, De Smet et al., 2012; Bellini et al., 2014). ARs are also important for vegetative propagation of plants and this is a method frequently used to produce clones of economically important genotypes of a vast number of plant species. However, some clones have problems in developing ARs, limiting their propagation potential. ARs and LRs share some common mechanisms and steps in their development, but differences between them are expected and not fully understood to date, despite recent progresses (Bellini et al., 2014; Verstraeten et al., 2014).

Several hormones can participate in different steps of AR formation, and auxins are recognized as major hormones involved in the process (reviewed by da Costa et al., 2013). Auxins are produced in the shoot apex of the plant and need to be transported to the base. The transport of auxins occurs in two different modes: the non-polar transport, a rapid and non-directional pathway through the phloem or the polar auxin transport (PAT), a slower and directional pathway, which involves specific transporters (Friml and Palm, 2002). In root development, membrane diffusion seems to be a supplemental form of transport to influx of auxin mediated by carriers (Kramer and Bennett, 2006). PAT is active, polar and basipetal in *A. thaliana* inflorescence stems (reviewed in da Costa et al., 2013; Habets and Offringa, 2014). In roots, transport occurs in two directions: i) acropetal, towards the apex of the root and ii) basipetal, from root apex to its base (Muday and DeLong, 2001, Morris et al., 2010).

The polarity of intracellular auxin transport is mostly controlled by the PINFORMED (PIN) and ABC-B/MULTI-DRUG RESISTANT/P-GLYCOPROTEIN (ABCB/MDR/PGP) auxin efflux carriers, together with the AUXIN1/LIKE AUXIN1 (AUX1/LAX1) influx carriers (Péret et al., 2012; Band et al., 2014; Habets and Offringa, 2014). AUX/LAX is a small family composed of *AUX1*, *LAX1*, *LAX2* and *LAX3* genes in *A. thaliana*, which is highly conserved and is similar to amino acid permeases (Marchant et al., 2002).

AUX1 was identified in a screening for auxin resistant mutants in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) (Maher and Martindale, 1980) and this was the first auxin influx transporter identified (Marchant et al., 1999). The *aux1* mutant exhibits an agravitropic phenotype (Bennet et al., 1996), which can be rescued when the seedlings are grown in the presence of the membrane-permeable auxin analogue 1-naphthalene-acetic acid (1-NAA), but not 2,4-D (Marchant et al., 1999). This finding provided insights about the function of AUX1 as an auxin influx transporter. AUX1 was then identified as a high-affinity auxin influx transporter recognizing auxin indole-3-acetic acid (IAA) and 2,4-D as substrates, but not NAA (Yang et al., 2006). The *aux1* mutant has a reduced number of lateral roots and it was shown that AUX1 plays important roles in lateral root development through IAA transport between source and sink tissues (Marchant et al., 2002). In rice, besides defects in gravitropism, mutations in OsAUX1 disturbed free auxin distributions and caused a reduction in the number of primordia and LRs. As expected, OsAUX1 overexpression lines showed increased number of primordia and LRs (Zhao et al., 2014).

The influx carrier LAX3 is induced by auxins and promotes LR emergence (Swarup et al., 2008). The knockout mutants *lax1* and *lax2* have phenotypes comparable to wild-type, while *lax3* showed reduction of about 40% in the number of emerged LRs. The spatial pattern of expression of LAX3 is affected by the efflux auxin transporter PIN3 in LRs, contributing to the direction of auxin flux to cortical and epidermal cells overlying LR primordia (Péret et al., 2013). The network generated by the sequential induction of PIN3 and LAX3 causes softening of some cells over the LR primordia and contributes to LR emergence (Péret et al., 2013). In AR, PIN1 and LAX3 establish an auxin maximum in the root tip, where the expression of WOX5 is restricted, delimiting the quiescent center (QC) (Della Rovere et al., 2013).

Knowledge on the role of influx carrier transporters in adventitious rooting is still limited. To further understand this relationship, we analyzed AR development in different mutants affected in auxin influx transporters, coupled with reporter gene constructs, in the presence or absence of exogenous auxin. Mutation in *AUX1* gene caused a reduction in the number of ARs in *A. thaliana* etiolated seedlings and this effect was not reversed by exogenous auxin supply. On the other hand, single mutations in *LAX1*, *LAX2* and *LAX3* did not affect AR, but different combinations of double, triple and quadruple mutations in *AUX1*, *LAX1*, *LAX2* and *LAX3* caused reductions in the number of ARs. This effect, however, was reversed by exogenous IAA application. Interestingly, mutation in *LAX3* did not reduce AR and the visualization of *LAX3pro:LAX3-YFP* was stronger in the region of the stele, both features that have not been described for LR.

Material and Methods

Plant material, growth conditions and treatments

The ecotype Col-0 of *Arabidopsis thaliana* was used in all experiments as a control. Seeds of the single mutants *lax2* and *lax3* were kindly provided by Malcolm J. Bennett (University of Nottingham, Nottingham LE12 5RD, UK) and *aux1-T* (SALK_020355) and *lax1* (SALK_039003C) were obtained from the Nottingham Arabidopsis Stock Centre (NASC - UK). Seeds of the double and triple mutants *aux1 lax1*, *aux1 lax2*, *aux1 lax3* and *aux1 lax1 lax2* and the lines proLAX1:LAX1:VENUS and proLAX2:LAX2:VENUS were kindly provided by Ranjan Swarup (University of Nottingham, Nottingham LE12 5RD, UK). The lines proLAX1:LAX1:VENUS and proLAX2:LAX2:VENUS are heterozygous for the insertion. *AUX1pro:AUX1-YFP* (Swarup et al., 2004); *aux1 lax1 lax2 lax3* (Bainbridge et al., 2008) and *LAX3pro:LAX3-YFP* (Swarup et al., 2008) and all the other mutants and lines have been described previously (Bainbridge et al., 2008; Swarup et al., 2008). The growth conditions and rooting assays were performed as previously reported (Corrêa et al., 2012, Chapter 1). The treatment with exogenous auxin was carried out with 57 μM of IAA.

Characterization of the mutants

To check the *aux1* mutation in double and triple mutants, the seedlings were tested as previously described (Marchant and Bennett, 1998) and all the tested seedlings were auxin-resistant. The mutations in LAX1, LAX2 and LAX3 in the double and triple mutants and in the single mutants *lax2* and *lax3* were verified by PCR according to Bainbridge et al. (2008). The single mutant *lax1* was analyzed by PCR for the absence of the wild-type band using the following primers: LB 5' CTTGGACCAATCATTAATGGC 3' and RB 5' TCCATGGTCAGGTATGTCCTC 3'. To verify the presence of the T-DNA in *lax1*, the RB primer listed above was used with LBb1.3 5' ATTTTGCCGATTTCGGAAC 3'.

Confocal microscopy

For expression analysis of YFP and VENUS the signal was visualized in water without fixation. Signals were detected with confocal laser scanning microscopy (Zeiss LSM 5 confocal microscope). The images were processed in the free software Icy (de Chaumont et al., 2012).

Statistical analysis

All the data were normalized in relation to Col-0. The data without normalization can be found in Figures 1 and 2 of the supplemental material. *t-test* was used to compare the mutants with Col-0 ($p <$

0.05). Each experiment was repeated at least three times and each biological replicate was composed by approximately 20 seedlings.

Results and discussion

As previously observed for LR (Marchant et al., 2002), the activity of the auxin influx carrier AUX1 was crucial for adequate AR induction (Fig. 1). The mutant *aux1-T* showed important reductions in the number of ARs in both of the time points analyzed, even with exogenous auxin supply (Fig. 1B). Surprisingly, its homolog LAX3 did not seem to be essential in this process, since *lax3* mutant did not show reduction in the number of AR when compared to WT. In fact, on day eight without exogenous IAA supply, *lax3* had an increase in AR number in relation to WT (Fig. 1A). These results differ from those observed for LR development, in which *lax3* mutant showed a reduction of 40% in the number of emerged LRs in relation to the control and the result was comparable to *aux1* (Swarup et al., 2008). LAX1 and LAX2 did not yield differences in AR number either with or without exogenous IAA (Fig1A and B). Previous findings indicated that LAX2 regulates vascular development (Péret et al., 2012) and LAX1 and LAX2 are necessary for leaf phyllotactic patterning (Bainbridge et al., 2008), but none of them has been related to lateral root development (Swarup et al., 2008; Péret et al., 2012). The exogenous supply of IAA did not cause any effects on AR development in the single mutants in relation to Col-0 (Fig 1B).

The double mutants *aux1 lax1* and *aux1 lax2* showed that mutations in LAX1 and LAX2 were not able to enhance the *aux1* phenotype, unlike *aux1 lax3* (Fig. 2A).The double mutant *aux1 lax3* yielded the most severe reductions in AR development, blocking ARs formation on day four and reaching reduced numbers in the control on day eight (Fig. 2A). Similar results were obtained by Swarup et al. (2008) and Péret et al. (2012) in LR analysis. The quadruple mutant *aux1 lax1 lax2 lax3* also showed severe impact in adventitious rooting in the absence of exogenous auxin (Fig. 2A). Generally the double, triple and quadruple mutations caused reductions in the number of roots without exogenous auxin, as clearly observed on day eight. However, mutations in LAX1,2,3 could compensate for *aux1* loss-of-function in the presence of exogenous IAA (Fig. 2B). This was unexpected, since exogenous supply of IAA was unable to rescue the phenotype of the single mutant *aux1-T*. In the presence of exogenous auxin, the diffusion component of transport may prevail, in which case the absence of AUX1 and one or more of the LAX functional influx transporters would demand less energy spending in assembly the influx machinery, allowing diversion of this surplus to growth and differentiation of ARs. An exception to the compensation phenotype was *aux1 lax1 lax2* on day four, showing that LAX3 alone is not sufficient to compensate for the missing *aux1,* at least in presence of exogenous auxin and in the first days (Fig. 2B). In this sense, a conjunct action of *AUX1* and *LAX3* is apparently needed for proper auxin influx transport and AR development. When both genes are mutated, auxin may be unable to move down from the source tissues to the lower hypocotyl target tissues where ARs develop or its distribution could be disrupted. In the case of *aux1-T*, auxin could be moving down slower than in Col-0. To check for this possibility, the measurement of IAA in the roots of mutants

would be helpful. Moreover, mRNA expression of the mutated genes in the mutants by RT Real Time PCR or another approach to ensure that their expression is decreased or absent could also be carried out, since we were able to identify mutations only at the DNA level through PCR.

To gain further insight about the role of *AUX1* in adventitious rooting development, the expression of *AUX1pro:AUX1-YFP* was examined in the presence or absence of exogenous auxin. *AUX1* seemed to be expressed in the AR primordia from the first cellular divisions until later stages of root development, independently of exogenous IAA supply (Fig. 3). Besides the expression in root primordia, AUX1 signal was also observed in the pericycle (Fig. 3). The expression appeared to become stronger in the root tip at later stages, although it seems to remain weaker in the other cells of the root primordia (Fig. 3). Differences in expression between the control and the treatment with exogenous IAA were not observed.

Expression of *LAX3* was also checked, using the construct *LAX3pro:LAX3-YFP* (Swarup et al., 2008). Unlike *AUX1*, *LAX3* is expressed in stele, cortex and epidermis, but not in primordia (Fig. 4). It is possible to see some expression even in the dividing pericycle cells (Fig 5). This pattern of stronger expression of *LAX3* in the stele differs from that observed in LR development, in which expression of the same gene was restricted to a small group of cortical and epidermal cells facing the LR primordia (Swarup et al., 2008). LAX3 controls some cell-wall remodeling enzymes and leads to the softening and separation of epidermal and cortical cells overlying primordia to allow LR emergence (Swarup et al., 2008). The auxin efflux carrier PIN3 acts in coordinated fashion with LAX3 to allow the softening of only a few cells that overlay the primordia (Péret et al., 2013), thereby minimizing damage to the outer cell layers. Auxin derived from the LR primordia causes a sequential expression of PIN3 and LAX3 and this network enables the expression of LAX3 in only two cortical cell files to induce cell wall loosening, cell separation and facilitate LR emergence (Péret et al., 2013). As previously reported (Swarup et al., 2008), exogenous IAA supply caused a stronger expression of LAX3 (Fig. 4), confirming that this gene is auxin inducible also during AR.

Therefore, data suggests that the process of flooding and etiolation that triggers AR in the hypocotyl system employed in the present investigation (Corrêa et al., 2012) activates expression of LAX3 in inner tissues and at stronger levels than reported for LR. The auxin signaling components ARF7, ARF19 and IAA14/SLR mediate LAX3 expression induced by auxins (Swarup et al., 2008). Expression patterns of these genes and of LAX3 in mutants affected in these genes could be investigated during AR development in an attempt to address if adventitious and lateral rooting differ at levels downstream of LAX3 induction.

The constructs *proLAX1:LAX1:VENUS* and *proLAX2:LAX2:VENUS* yielded a weak signal (Fig. S3), similar to the one observed in LR development (Péret et al., 2012). Furthermore, no signal was

detected in the control, detection being restricted to seedlings treated with exogenous IAA. An auxin induced expression was also observed by Péret et al. (2012) in LAX1, but not in LAX2. The expression of LAX1 was concentrated in the stele region of the AR, whereas LAX2 expression was restricted to the central part of the AR primordia (Fig. S3).

In conclusion, these findings support a fundamental role of AUX1 in adventitious rooting, and provide insights on possible molecular differences between adventitious and lateral rooting. Further investigations on LAX3 participation during adventitious rooting could help to elucidate these possibilities. Monitoring expression patterns of the cell wall remodeling enzymes AIR3, XTR6 and AtPLA2 involved in LR emergence, as well as of the auxin signaling components ARF7, ARF19 and IAA14/SLR (Swarup et al., 2008), could be interesting future targets to compare during LR and AR development.

Figures:

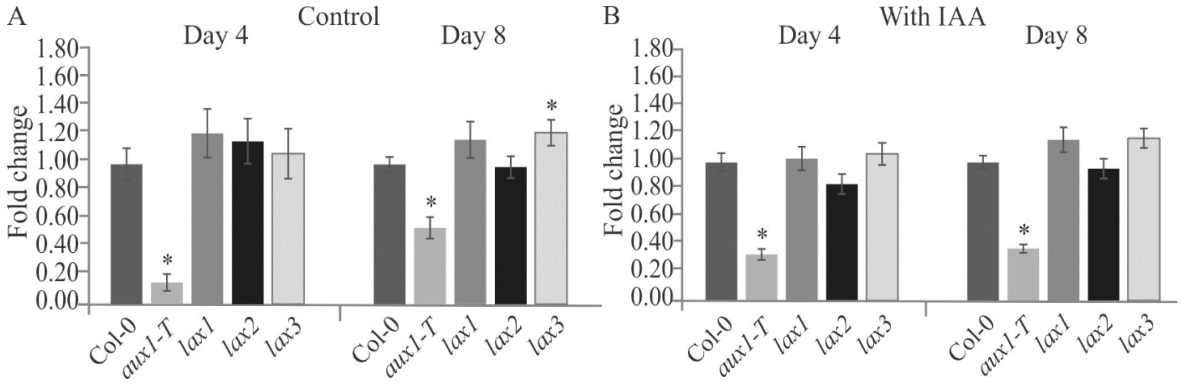


Figure 1. Adventitious roots on single mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM IAA. Data were normalized against the average number of roots in Col-0 for every treatment. The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to Col-0 (*t*-test, $P \leq 0.05$).

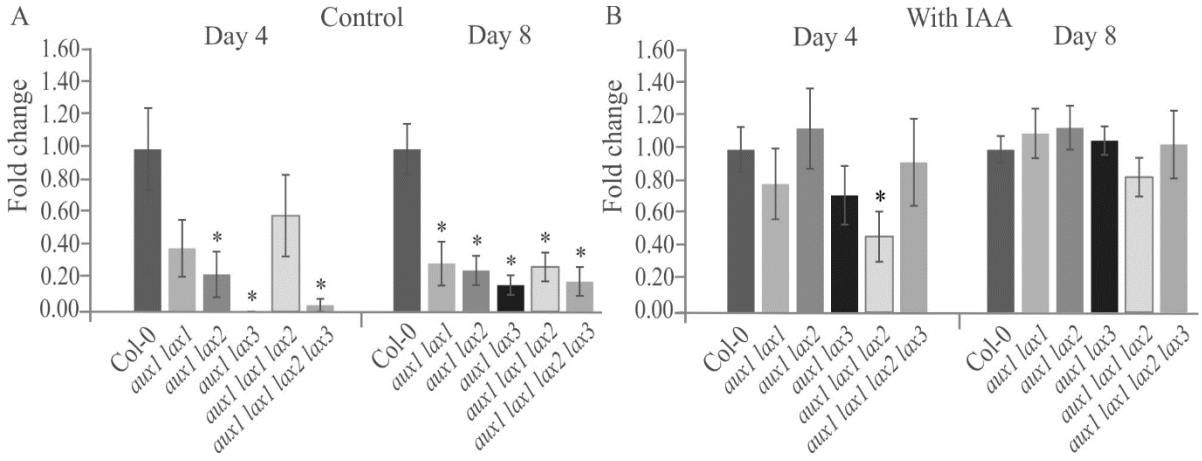


Figure 2. Adventitious roots on double, triple and quadruple mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM IAA. Data were normalized against the average number of roots in Col-0 for every treatment. The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to Col-0 (*t*-test, $P \leq 0.05$).

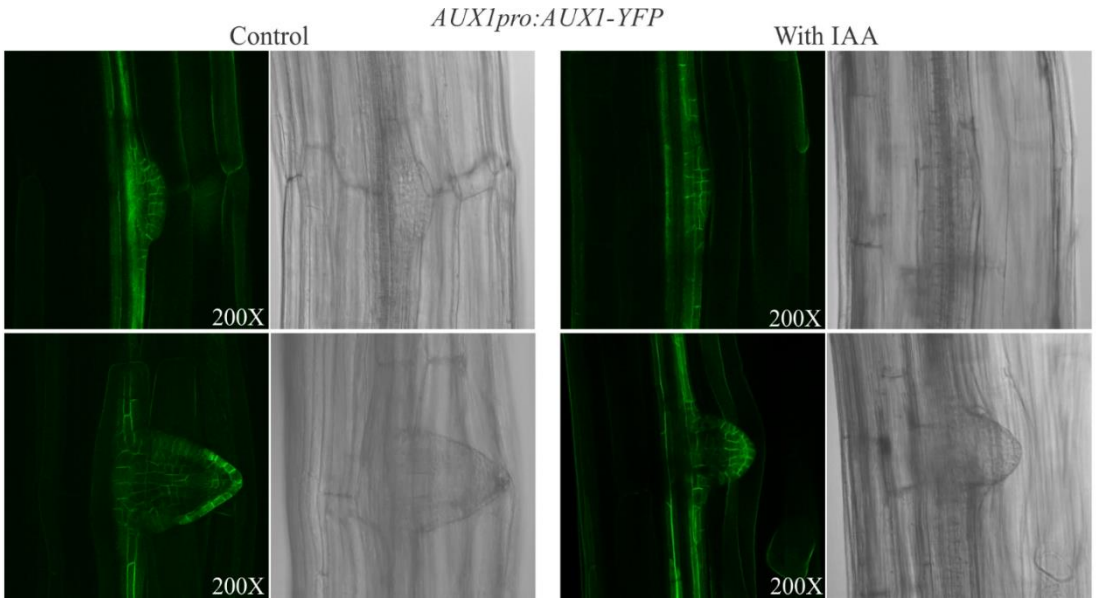


Figure 3. Confocal imaging of etiolated seedlings expressing *AUX1pro:AUX1-YFP* during AR development with or without exogenous auxin application 3 days after transfer to light.

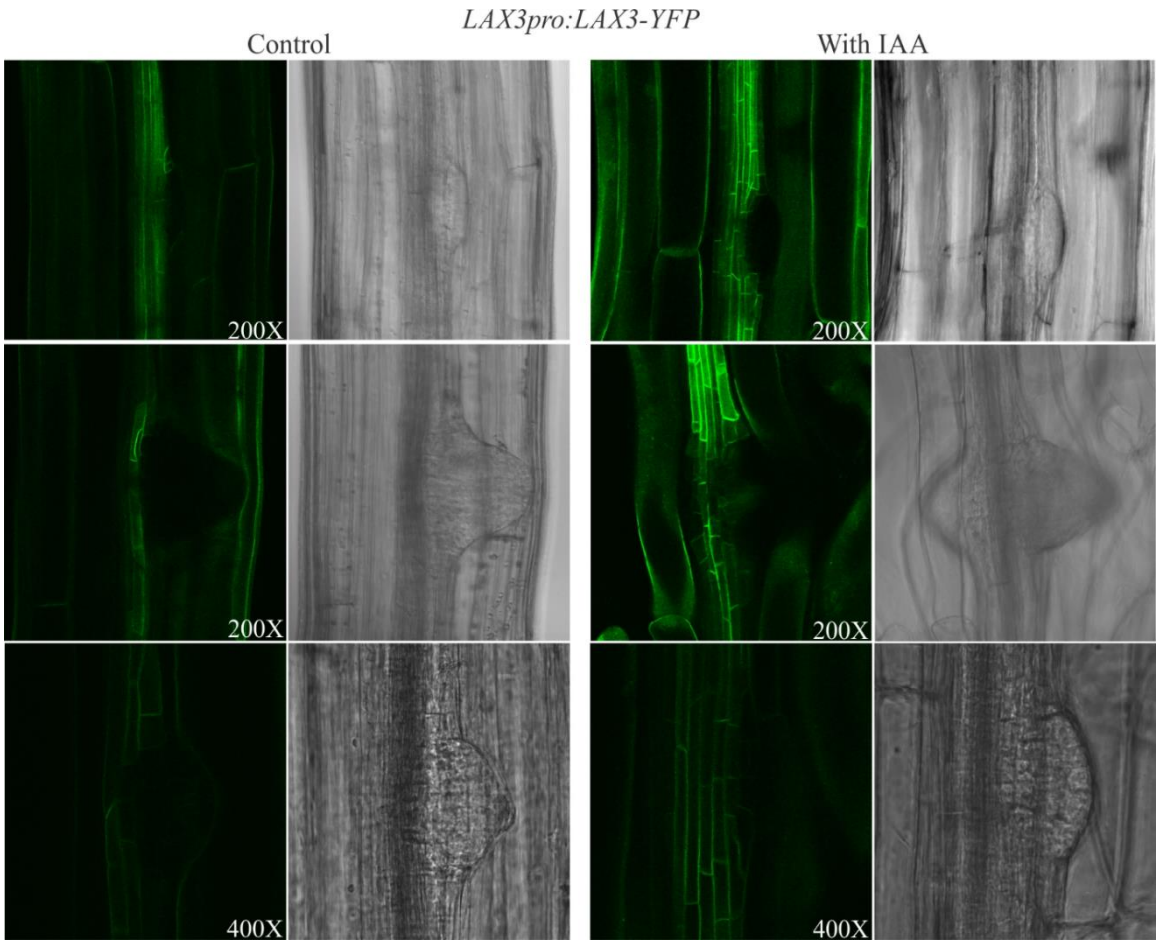


Figure 4. Confocal imaging of etiolated seedlings expressing *LAX3pro:LAX3-YFP* during AR development with or without exogenous auxin application 3 days after transfer to light.

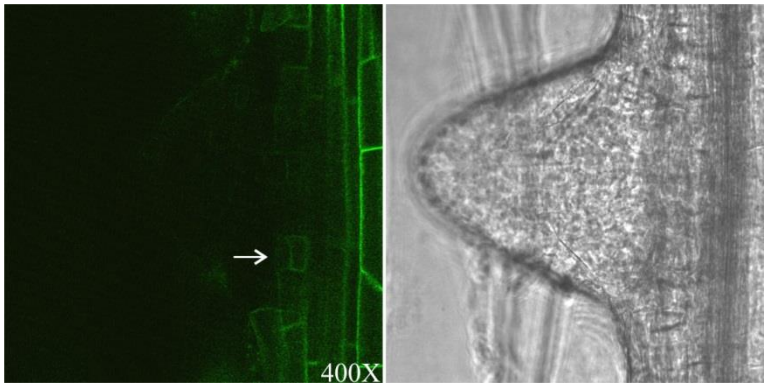
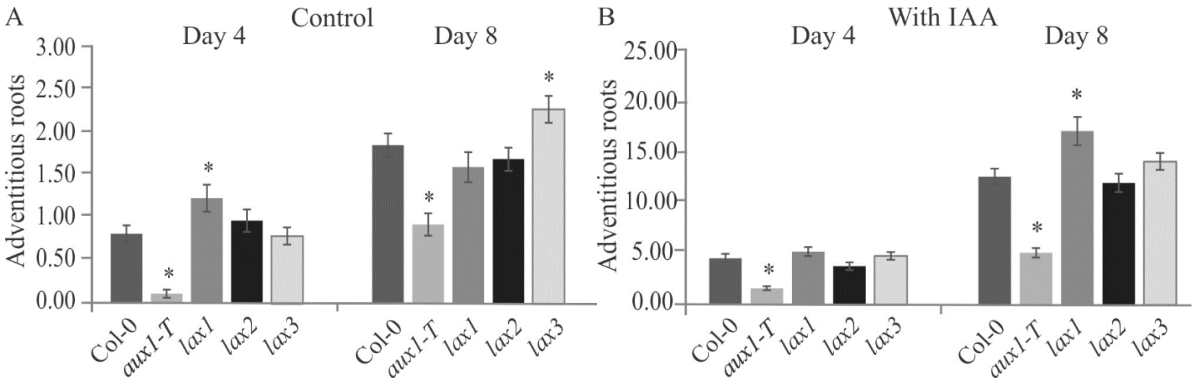
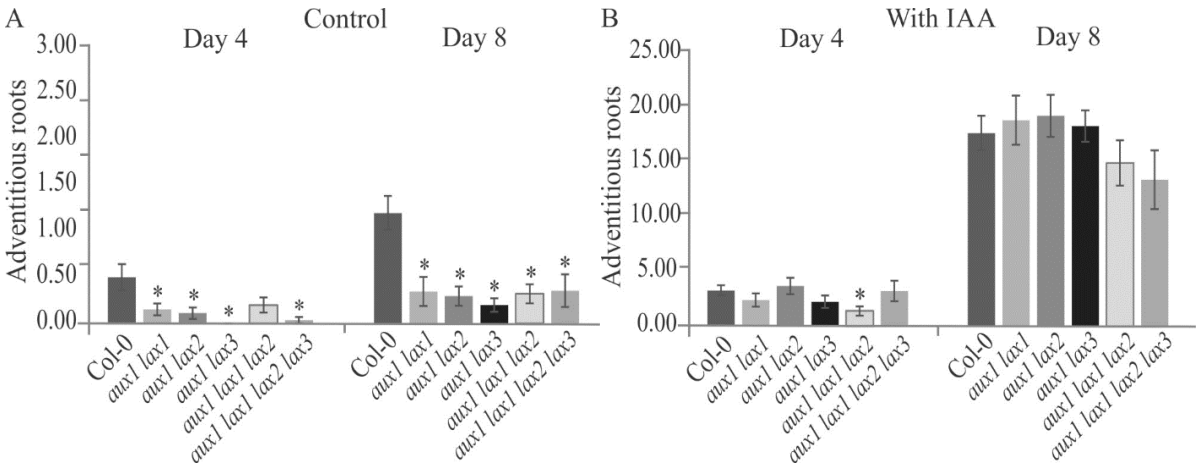


Figure 5. Expression of *LAX3pro:LAX3-YFP* in stele and pericycle dividing cells (arrow) during AR development with exogenous IAA supply 3 days after transfer to light.

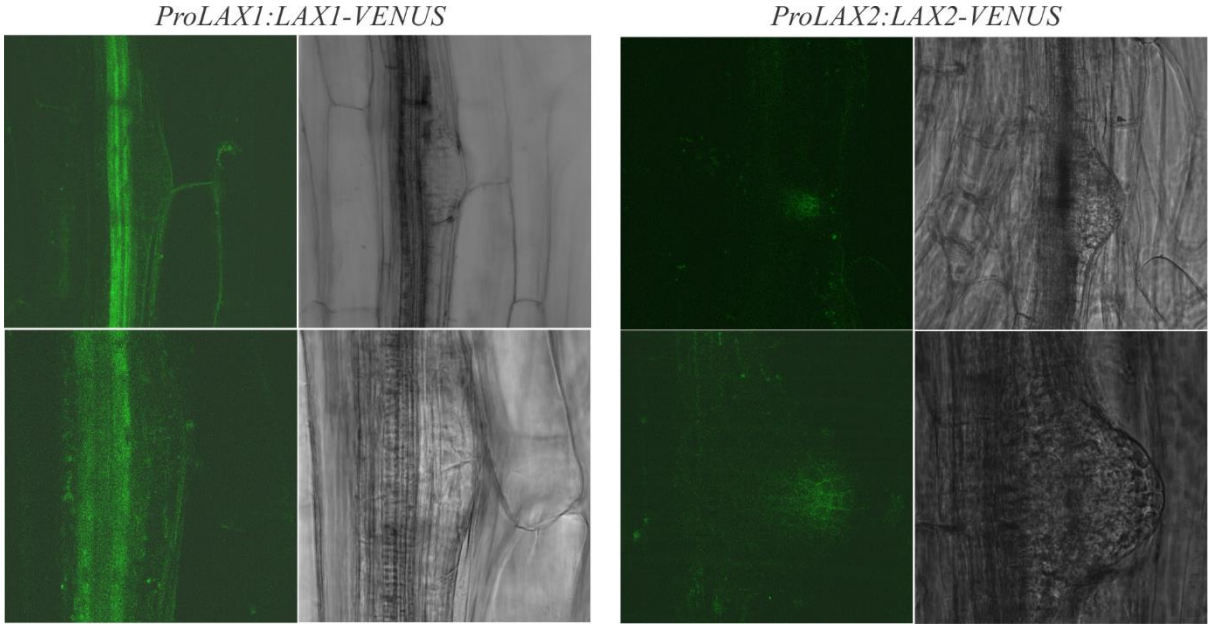
Supplemental material



Supplemental figure 1. Average number of adventitious roots on single mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to Col-0 (*t*-test, $P \leq 0.05$).



Supplemental figure 2. Average number of adventitious roots on double, triple and quadruple mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to Col-0 (*t*-test, $P \leq 0.05$).



Supplemental figure 3. *ProLAX1:LAX1-VENUS* and *ProLAX2:LAX2-VENUS* expression in adventitious roots of etiolated seedlings with exogenous IAA application 3 days after transfer to light.

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CAPÍTULO 3

Auxin receptors and PIN-driven polar auxin transport during adventitious root formation in *Arabidopsis thaliana* etiolated seedlings

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Auxin receptors and PIN-driven polar auxin transport during adventitious root formation in *Arabidopsis thaliana* etiolated seedlings

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Keywords: Adventitious rooting, auxin receptors, PIN phosphorylation, Arabidopsis, mutant, gene expression

Abstract

Adventitious root (AR) development is a complex physiological process in which auxins play a major role. The TIR1/AFB family of F-box proteins and the Auxin Binding Protein 1 (ABP1) act as auxin receptors and initiate the auxin signaling in the cell. The polar auxin transport (PAT) depends on the participation of the PIN efflux carriers and the distribution of PIN1-type proteins is regulated by phosphorylation of the PIN central hydrophilic loop (PINHL) through action of the Ser/Thr kinases PINOID (PID)/WAVING AGRAVITROPIC ROOT (WAG). Here, we investigated the participation of auxin receptors, efflux carrier transporters and phosphorylation of PINs during the adventitious rooting process in presence or absence of exogenous auxin in *Arabidopsis thaliana* etiolated seedlings. Analysis of TIR1/AFBs mutants and GUS expression indicated that these proteins are important in AR development but might be playing redundant roles in the process, whereas ABP1 could be complementing their action. During AR organogenesis, TIR1 and AFB2 seemed to exert greater influence. We also observed that efficient formation of AR depends on PIN mediated auxin transport, mainly by the auxin efflux carriers PIN1, 3 and 7. The proper phosphorylation of PINs by the kinases PID, WAG1 and WAG2 and hence the direction of PAT was equally essential for AR establishment.

Introduction

Auxins play several roles during plant development, regulating both cell division and cell expansion. During root development auxins are involved in several aspects, from cell fate acquisition to elongation in both mono and dicotyledons (Bellini et al., 2014). Adventitious roots (AR) are developed post embryonically from organs other than roots and are fundamental for vegetative propagation of plants (Pacurar et al., 2014).

After the perception of auxin by the cell, gene expression is modified. In this process, TIR1/AFB family of F-box proteins and the Auxin Binding Protein 1 (ABP1) act as auxin receptors. The TIR1/AFB family is composed of 6 members in *Arabidopsis* (TIR1, AFB1, AFB2, AFB3, AFB4 and AFB5) (Mockaitis and Estelle, 2008) and form the $SCF^{TIR1/AFB}$ (Skp1/Cullin/F-box) E3 ubiquitin ligase complexes that promote the degradation of the Aux/IAA transcriptional repressors (Dharmasiri et al., 2005a, Maraschin et al., 2009). When the repressors are degraded, the Auxin Response Factors (ARFs) are free to bind the auxin-responsive genes and activate or inhibit transcription (Santner and Estelle, 2009). ABP1 proteins act in the activation of ATPase proton pumps, acidification of the extracellular space and activation of input K^+ channels, which may eventually lead to changes in gene expression (reviewed by Tromas et al., 2010 and by Scherer et al., 2012). ABP1 is also important for the establishment of pavement cells interdigitation, mediated by Rho-like guanosine triphosphatases-GTPases (ROPs) (Xu et al., 2010). Transmembrane kinase (TMK) members of the receptor-like kinase family can serve as docking proteins to ABP1 on the cell surface, regulating pavement cell arrangement (Xu et al., 2014). Reports of null mutations of ABP1 in *Arabidopsis* proved embryo-lethal (Chen et al., 2001) indicating that ABP1 plays a crucial role in embryogenesis. However, the exact role of ABP1 remains controversial. A recent report describing two null alleles of *abp1* obtained through CRISPRs (clustered regularly interspaced short palindromic repeats) found no phenotype in development, growth or response to auxin under non-stressful standard growth conditions (Gao et al., 2015).

The transport of auxins in the plant happens in a polar, cell to cell way and is orchestrated through the action of three protein families, the AUXIN RESISTANT1/LIKE AUX1 (AUX/LAX) influx carriers, the PIN-FORMED (PIN) efflux carriers and the P-GLYCOPROTEIN (PGP/ABCB) transporters (Péret et al., 2012; Habets and Offringa, 2014). The participation of the PIN efflux carriers in polar auxin transport (PAT) has been well characterized. Eight PIN proteins constitute the PIN protein family in *Arabidopsis* and are divided in two groups: (1) PIN1-type proteins (PIN1,2,3,4, and 7) that are asymmetrically distributed in the plasma membrane (PM) and the PIN5-type proteins (PIN5, 6 and 8) that are localized in the endoplasmic reticulum (ER) and are probably involved in auxin homeostasis (Mravec et al., 2009).

Moreover, the polar distribution of the PIN1-type carriers, and thus the direction of PAT, was shown to be determined by reversible phosphorylation of the PIN central hydrophilic loop (PINHL) through the antagonistic action of the Ser/Thr kinases PINOID (PID)/WAVING AGRAVITROPIC ROOT (WAG) and protein phosphatase 2A (PP2A) (Michniewicz et al., 2007, Huang et al., 2010). PID regulates PAT through PIN protein localization, redirecting auxin flow (Friml et al., 2004) and seems to act as a binary switch, when PID is overexpressed there is a change of basal to apical PIN polarity in the root cells, causing agravitropic root growth and collapse of the primary root meristem. In *pid* loss-of-function mutants, there is an apical to basal reversion in PIN1 polarity that causes a failure in the establishment of the auxin maxima required for normal apical organ formation and leads to the development of pin-like inflorescences (Friml et al., 2004). The mechanism is the result of a post-transcriptional modification in which PIN polarity is determined by reversible phosphorylation of the PINHL (Michniewicz et al., 2007). PID belongs to the AGCVIII kinase family and forms a subgroup together with WAG1, WAG2 and AGC3-4 (still uncharacterized) (Galván-Ampudia & Offringa, 2007).The phosphorylation sites of PID, WAG1 and WAG2 were identified in central serine residues of three conserved TPRXS(N/S) motifs in the PINHL (Huang et al., 2010).

The auxin efflux transporter proteins are involved in several plant developmental processes. PIN3 and PIN7 redirect auxin flux in response to gravity stimulation and establish the asymmetric growth of plants (Friml et al., 2002a, Kleine-Vehn et al., 2010). In lateral root development PIN3 works together with LAX3 to help root emergence by facilitating cell wall loosening and separation (Péret et al., 2013). PIN3 and PIN7 had their expression increased by ethylene application on *Arabidopsis* seedlings and LR formation was blocked (Lewis et al., 2011). The higher availability of PIN3 and PIN7 proteins may have caused an elevated transport of auxins, preventing the auxin maxima formation that is necessary for LR development. PIN1 plays a role in the establishment of an auxin maxima in the root tip together with LAX3, positioning the quiescent center in adventitious roots (Della Rovere et al., 2013). The efflux protein PIN4 is important for auxin homeostasis and patterning of the root tip (Friml et al., 2002b), whereas its participation in AR development is still unknown.

Here we investigated the participation of the auxin receptors TIR1, AFB1, 2, 3 and ABP1, the auxin efflux carriers PIN1,3,4 and 7 and their phosphorylation during adventitious root development in *Arabidopsis* etiolated seedlings. Although *tir1-1* and *afb2-3* had stronger rooting phenotypes, our results pointed to a mechanism in which the TIR1/AFB proteins seem to play redundant roles in AR development, with possible cooperation of ABP1 in the process. The efficient formation of AR also depended on the proper phosphorylation of PINs by the kinases PID, WAG1 and WAG2. In the etiolated seedlings system used, the auxin efflux carriers PIN1, 3 and 7 were important in adventitious rooting as well.

Material and Methods

Plant material, growth conditions and treatments

The ecotypes Col-0 and Ler of *Arabidopsis thaliana* were used in the experiments as a control. The mutants *tir1-1* (Ruegger et al., 1998), *PIN1:GFP S1A*, *PIN1:GFP S3A*, *PIN1:GFP S1,3A* (Huang et al. 2010), *pid14/+ wag1 wag2*, *pid14 wag2* (Dhonukshe et al., 2010), *pin3-3* (Friml et al., 2002a), *pin4-3* (Friml et al., 2002b), *pin4 pin7* and *pin7-1* (Friml et al., 2003) and the line *PIN1:GFP* (Benková et al., 2003) were previously described. The mutants *afb1-3* (SALK_070172C), *afb2-3* (SALK_137151) and *afb3-5* (SALK_016356C) were ordered from SALK. The constructs *AFBs:GUS* (Dharmasiri et al., 2005b), *PIN1::PIN1-GFP* (Benková et al., 2003), *PIN3::PIN3-GFP* (Žádníková et al., 2010), *PIN4::PIN4-GFP*, *PIN7::PIN7-GFP* (Blilou et al., 2005), *PID::PID-YFP* (Michniewicz et al., 2007) and WAG1::WAG1-GFP (Dhonukshe et al., 2010) have also been described. The growth conditions were as reported in Chapter 1. The treatment with exogenous auxin was carried out with 57 µM of IAA. ARs were scored at four and eight days after auxin treatment and transfer to light.

Characterization of the mutants

To select for the triple homozygous mutant seedlings in the progeny of *pid14/+ wag1 wag2* only seedlings without cotyledons were considered in the evaluations (Dhonukshe et al., 2010). The *pin3-3*, *pin4-3*, *pin4 pin7*, *pin7-1* and *afb1-3*, *afb2-3*, *afb3-5* mutants were analyzed by PCR for the absence of the wild-type band and presence of the T-DNA band. The primers sequences are listed on Sup. table 1. *PIN1:GFP S1A*, *PIN1:GFP S3A*, *PIN1:GFP S1,3A* (Huang et al. 2010) and *pid14/+ wag1 wag2* (Dhonukshe et al., 2010) were already genotyped as previously described.

Confocal microscopy

For expression analysis of YFP and GFP, the signal was visualized in water without fixation. Signals were detected with confocal laser scanning microscopy in a Zeiss LSM 5 confocal microscope. The images were processed in the free software Icy (de Chaumont et al., 2012).

GUS staining and analysis

To detect GUS expression, the seedlings were collected, fixed in 90% acetone at -20ºC for 20 min, washed two times for 10 min each in 10mM EDTA, 0.1M sodium phosphate (pH 7.0), 2 mM $K_3Fe(CN)_6$ under vacuum and stained for up to 5 hours in 10mM EDTA, 0.1M sodium phosphate (pH 7.0), 2 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6.3H_2O$ containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-

D-glucuronide at 37 ºC in the dark. The staining was stopped with acetic acid/ethanol (3:1) for 60 min, the seedlings were then rehydrated with an ethanol series, prepared in slides with chloral hydrate solution for clearance and visualized in a Zeiss Axioplan2 imaging microscope with DIC optics.

Statistical analysis

All the data were normalized in relation to Col-0. The data without normalization can be found in the figures 1, 2 and 3 of the supplemental material. *t-test* was used to compare the mutants with the respective wild type (WT) ($p < 0.05$). Each experiment was repeated at least three times and each biological replicate was composed by approximately 20 seedlings.

Results

TIR/AFB proteins play a role in AR development

To investigate the participation of the auxin receptor proteins in AR development, we checked the *afb1-3*, *afb2-3*, *afb3-5*, *tir1-1* and *abp1-5* mutants for alterations in the number of ARs at days 4 and 8 after transfer to light with or without exogenous IAA application. The mutation in AFB1 only caused reduction in ARs number at day 8 with exogenous auxin in relation to Col-0 (Fig. 1B). On the other hand, AFB2 seemed to be important in all of the cases, since the mutant had reduced adventitious rooting in both time points analyzed (Fig. 1). This reduction became more evident with exogenous auxin, reaching statistically significant differences (Fig. 1B). Surprisingly, the mutation in AFB3 caused an increase in the number of roots in the control, but this was reversed when exogenous IAA was provided, causing even a reduction at day 4 (Fig 1). *tir1-1* mutant had the most constant response, decreasing AR formation in all the points analyzed (Fig. 1). Since null mutations in ABP1 of *Arabidopsis* are embryo lethal (Chen et al., 2001), we used the *abp1-5* mutant which has a point mutation in the auxin binding pocket and is defective in auxin binding (Xu et al., 2010). No difference was observed in the control in relation to the WT (Fig. 1A). Upon exogenous IAA supply the mean number of roots in *abp1-5* increased at day 4 and decreased at day 8 in relation to Col-0 (Fig. 1B).

The expression patterns of *TIR1:GUS*, *AFB1:GUS*, *AFB2:GUS* and *AFB3:GUS* were also assessed during AR development. Imaging and analysis of the roots were done 3 days after transfer to light and auxin in seedlings treated with exogenous IAA. In every case, a strong GUS signal was observed in AR primordia (Fig. 2). The signal of TIR1 and AFB2 (Fig. 2A and C, respectively) was mainly concentrated in the root primordia. Nevertheless, when primordia were smaller, some signal in the pericycle and vascular cylinder of *AFB2:GUS* seedlings was also visible (Fig. 2C). AFB1 was expressed not only in the root primordia but in the other tissues of the hypocotyl as well (Fig. 2B). Besides being visible in the primordia, the signal of *AFB3:GUS* was also found in the pericycle and vascular cylinder (Fig. 2D). In spite of the similar expression profile of *AFB2:GUS* and *AFB3:GUS*, the latter was expressed in higher intensity and in a more even fashion.

Phosphorylation of PIN efflux transporters is important for AR development

To check the significance of the phosphorylation in PIN efflux transporters during adventitious rooting we tested some previously described loss-of-phosphorylation mutants. The *pid14 wag1 wag2* triple mutant lacks the three kinases PID, WAG1 and WAG2 which phosphorylates the central serine in the PINHL and determines PIN polarity (Dhonukshe et al., 2010). This mutant has a fully penetrant no-cotyledon phenotype in the triple homozygous seedlings (Dhonukshe et al., 2010) so the seedlings without cotyledons were selected for the evaluations. In our analysis, *pid14 wag1 wag2* failed to

produce AR in the control at day 4 and showed a severe reduction in the number of roots at day 8 (Fig. 3A and Suppl. Fig1A). Even when exogenous auxin was provided, the reduced root phenotype was strong. In this case, a few AR were produced and the phenotype of Col-0 at days 4 and 8 could not be recovered (Fig 3B and Suppl. Fig 1B).

We wondered if this marked reduction in the AR production was caused by the lack of cotyledons. To test this hypothesis, we selected seedlings without cotyledons in the progeny of the double mutant *pid+ wag2*, in which the penetrance for cotyledon defects is about 50%; of these, around 14% lack cotyledons (Dhonukshe et al., 2010). Without exogenous auxin there was no production of roots, differently from Col-0 (data not shown). However, with IAA supply, the number of roots did not differ from the WT (Suppl. fig. 4). This observation indicated that the reduction observed in *pid14 wag1 wag2* was caused by the triple mutation and not by the absence of cotyledons.

The kinases PID, WAG1 and WAG2 phosphorylate the middle serine in three conserved TPRXS(N/S) motifs in the PINHL (Huang et al., 2010). In some mutants, one or two serines were substituted by alanines on these phosphosites in a complementing PIN1: green fluorescent protein (GFP) construct, previously described by Huang et al. (2010). The loss-of-phosphorylation PIN1:GFP (Ser to Ala) mutants *PIN1:GFP S1A*, *PIN1:GFP S3A* and *PIN1:GFP S1,3A* were checked for adventitious rooting. The line *PIN1:GFP* (Benková et al., 2003) was used as a control. The mutants had adventitious rooting phenotypes compared to PIN1:GFP in at least one time point. In the case of *PIN1:GFP S1A* there was an increase in the number of ARs at days 4 and 8 in the control, as well as for *PIN1:GFP S3A* on day 8 (Fig. 3A). When exogenous IAA was added, the number was reduced, becoming lower than PIN1:GFP (Fig. 3B). The double mutant *PIN1:GFP S1,3A* showed a reduction in the amount of AR in the control, albeit not statistically significant; however, with exogenous auxin supply this difference became significant (Fig. 3).

The expression of PID and WAG1 was verified using the constructs *PID::PID-YFP* and *WAG1::WAG1-GFP* during AR development in 5 day-old etiolated seedlings. The expression of *PID::PID-YFP* was weak in the control and a bit stronger with exogenous IAA supply (Fig. 4). *WAG1::WAG1-GFP* expression did not show differences between the seedlings treated with IAA and those without auxin (Fig. 5). PID and WAG1 were predominantly expressed in the epidermis and root cap, similar to the pattern previously observed in lateral roots (Dhonukshe et al., 2010). Before the root protrusion, it was possible to see some expression of WAG1 in the epidermis and at the base of the primordia, when the root is being formed (Fig. 6), only in the seedlings with exogenous IAA treatment.

PIN1, 3, 4 and 7 efflux carriers and their involvement in the adventitious rooting process

To understand whether mutations in the PIN efflux carriers PIN3, PIN4 and PIN7 could interfere with AR development, we assessed the AR number in *pin3-3*, *pin4-3, pin7* and *pin4pin7* with or without exogenous auxin supply. *pin3-3*, *pin4-3* and *pin4pin7* mutants are in Col-0 ecotype and *pin7* is in Ler ecotype. The only mutant that significantly reduced the number of AR in relation to the WT was *pin4-3*, when treated with exogenous auxin at day 8 (Fig. 7B). Without exogenous auxin supply, *pin4-3* developed more roots than the WT at day 8 and the same could be observed with the *pin3-3* mutant (Fig.7A). The mutation in PIN7 was stable along the evaluations, developing more AR than WT in all time points. This increase in AR is stronger at day 4 without IAA supply and at day 8 with exogenous IAA provided. (Fig. 7A and B). The double mutant *pin4pin7* had a profile similar to *pin7*, forming more AR than Col-0 in some points.

In an attempt to verify which PIN proteins are involved in auxin distribution during the initial steps of AR development, the expression patterns of PIN1, 3, 4 and 7 were compared. PIN1 was expressed at very early stages on AR primordia and kept being expressed in the primordia tissues afterwards (Fig. 8). At later stages of primordia development, the expression was present in the stele and root cap (Fig. 8). *PIN3::PIN3-GFP* signal was stronger in the vascular cylinder and pericycle during early steps of the primordia development (Fig. 9). As the primordia became larger, the signal became stronger in the columella precursors as well (Fig. 9). In both *PIN1::PIN1-GFP* and *PIN3::PIN3-GFP,* differences between seedlings treated or not with exogenous IAA were not observed (Fig. 8 and 9). *PIN4::PIN4-GFP* and *PIN7::PIN7-GFP* did not exhibit a strong expression in the checked time points. No signal was detected in *PIN4::PIN4-GFP* (data not shown). *PIN7::PIN7-GFP* yielded a weak signal in the hypocotyl at early developmental stage and in the region of provascular cells at more advanced stages of primordia development (Fig. 10).

Discussion

The auxin dynamics during AR development has been investigated in the last few decades, but a complete understanding of the process is far from being reached. The influence of auxin receptors in AR development is not fully understood to date, although TIR1/AFB and ABP1 proteins are considered true auxin receptors. To investigate which of these could play a major role in this process, we searched for alterations in adventitious rooting of etiolated seedlings in *tir1-1*, *afb1-3*, *afb2-3*, *afb3-5* and *abp1-5* mutants. Furthermore, the expression patterns of *TIR1:GUS*, *AFB1:GUS*, *AFB2:GUS* and *AFB3:GUS* were also analyzed.

The analysis of adventitious rooting in the TIR1-, AFBs- and ABP1-defficient seedlings indicated that all of the proteins play a role at some point during the process. However, TIR1 and AFB2 seem to have a more significant participation in the process. *tir1-1* and *afb2-3* mutants showed reduced rooting in etiolated seedlings and their expression was restricted to the root primordia (Fig. 1 and 2). Some lines of evidence indicate that TIR1/AFBs have overlapping and redundant functions in plant development (Dharmasiri et al., 2005b), and this seems to apply to AR as well. The involvement of ABP1 in AR development might be underestimated because the *abp1-5* mutant contains a point mutation in the auxin-binding pocket (His59 > Tyr), which reduces its affinity for auxin (Xu et al., 2010), but is not a knockdown mutant. ABP1 could be complementary to the action of the TIR1/AFBs, since the triggered responses by each of them often involve different changes in the cell. A possible cooperation between ABP1 and TIR1/AFB protein has been suggested at the level of gene expression regulation (Grones and Friml, 2014). The root promoting effect of the absence of AFB3 in control conditions is of potential practical significance to improve rooting rates in commercial species. Genetic deletion of AFB3 may result in higher binding of auxin to AR-relevant receptors, such as TIR1 and AFB2.

Polar auxin transport is required for AR initiation in hypocotyl or stem cuttings and depends on the coordinated action of auxin influx and efflux carrier proteins. The auxin efflux transporter ABCB19 (ATP-binding cassette B19) plays a role driving AR formation in *Arabidopsis* hypocotyls (Sukumar et al., 2013). We wondered if the participation of the PIN efflux carriers and its phosphorylation status would be important for AR development. The phosphorylation of PIN1-type protein is essential for its proper apical localization and activity in PAT (Dhonukshe et al., 2010; Huang et al., 2010). In this process PID, WAG1 and WAG2 are the three kinases which phosphorylate the central Ser in the PINHL, determining PIN polarity. In *pid wag1 wag2* triple mutant, root development was strongly affected in both wavy and gravitropic growth (Dhonukshe et al., 2010).

In our observations, *pid14 wag1 wag2* had a severe AR phenotype, with absence of ARs or production of only a few roots even with exogenous auxin supply (Fig. 3). The loss-of-

phosphorylation PIN1:GFP (Ser to Ala) mutants *PIN1:GFP S1A*, *PIN1:GFP S3A* and *PIN1:GFP S1,3A* also exhibited changes in AR development in relation to the control PIN1:GFP (Fig. 3). PID and WAG1 were expressed predominantly in the root epidermis in AR primordia, similar to the expression pattern observed in lateral roots by Dhonukshe et al. (2010). The results of lower AR development response for most of the loss-of-phosphorylation mutants indicate that the proper phosphorylation of PINs by the AGC kinase PINOID (PID) and its homologs WAG1 and WAG2 contributes to efficient AR formation. Interestingly, the higher AR response of *PIN1:GFP S1A* mutant under control conditions suggests that different degrees of PIN phosphorylation changes may yield distinct AR phenotypic outcomes. In addition, the mutation *PIN1:GFP S1A* may become an interesting target for improving AR rates in economically important species.

The central role of auxins and auxin transport in AR development has been frequently a topic of investigation. PIN-driven polar auxin transport seems to be required for adequate root formation. *PIN1::PIN1-GFP* expressing seedlings showed strong signal in the root primordia, initially in the pericycle cells of the hypocotyl and later in the provascular and columella cells of the primordia (Fig. 8). In a different adventitious rooting system, Sukumar et al. (2013) observed that the *pin1-1* mutant had a reduction of 40% in AR formation in relation to the WT. This result is in good agreement with the expression pattern observed in our system, indicating that PIN1 efflux transporter is important for primordia establishment and development.

Analysis of the *pin3-3* mutant did not tell much about its participation in AR development in our experimental system (Fig. 7). However, *PIN3::PIN3-GFP* was robustly expressed in AR primordia observed in etiolated seedlings. Initially the signal was observed in the pericycle cells of the hypocotyl where AR is being formed and later in the columella cells as well (Fig. 9). The visualization of PIN3 in important cells during the primordia formation and initial development suggests that this protein is playing a role in auxin efflux transport and helping in the adventitious rooting process. We could not observe clear differences between the expression of PIN3 in seedlings treated or not with exogenous auxin, unlike what has been reported by Péret et al. (2013) in lateral roots cortical cells. This could be due to differences in the experimental conditions or represent a difference between lateral and adventitious root development.

In spite of the reduction in AR number at day 8 with exogenous IAA supply in *pin4-3* mutant, PIN4 expression was absent in the root primordia of *PIN4::PIN4-GFP* expressing seedlings. PIN4 did not seem to have an important participation in AR development at the time points evaluated using the etiolated flooded seedling system to induce AR. Expression of *PIN7::PIN7-GFP* was weak in the hypocotyl during primordia formation, but became more perceptible when the primordia was larger, in provascular cells. In LRs, PIN3 is expressed in young emerging LRs, but has weaker expression in

older roots and this happens in opposite fashion for PIN4 and PIN7 which have stronger expression in older roots (Rosquete et al., 2013). Thus the inability of LRs to grow vertically downward was attributed to an early repression of PIN4 and 7, causing a deficiency in auxin transport. It is possible that in the case of adventitious rooting, PIN4 and PIN7 display a later pattern of expression. However, the mutants *pin 4-3* and *pin 7-1* had higher AR development in absence of exogenous auxin, indicating that they may act as inhibitors of this developmental process, possibly by disrupting auxin concentration gradients and focus relevant for AR. Such observations may also make these efflux transporters interesting targets for improving AR in species of economic relevance.

To sum up, although the participation of auxin receptors seems to be of fundamental importance to AR and their action is redundant and complementary, a possible major role of TIR1 and AFB2 became apparent. For adequate auxin transport and efficient formation of ARs, phosphorylation of PINs (particularly PIN1) by the kinases PID, WAG1 and WAG2 seems to be essential. Finally, potential gene targets for silencing in order to improve AR in *Arabidopsis* (and potentially their orthologues in economically relevant species) have emerged and deserve further investigation: AFB3, PIN4, and PIN7.

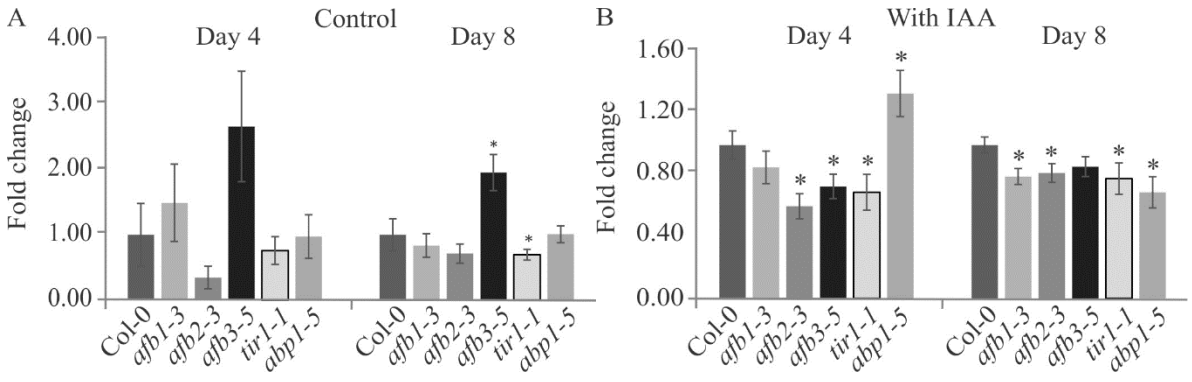


Figure 1. Adventitious roots on auxin receptor mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The data were normalized against the average number of roots in Col-0 for every treatment. The error bars represent the standard error. The presence of an asterisk on top of a bar indicates significant difference compared to the respective Col-0 values (*t*-test, $P \leq 0.05$).

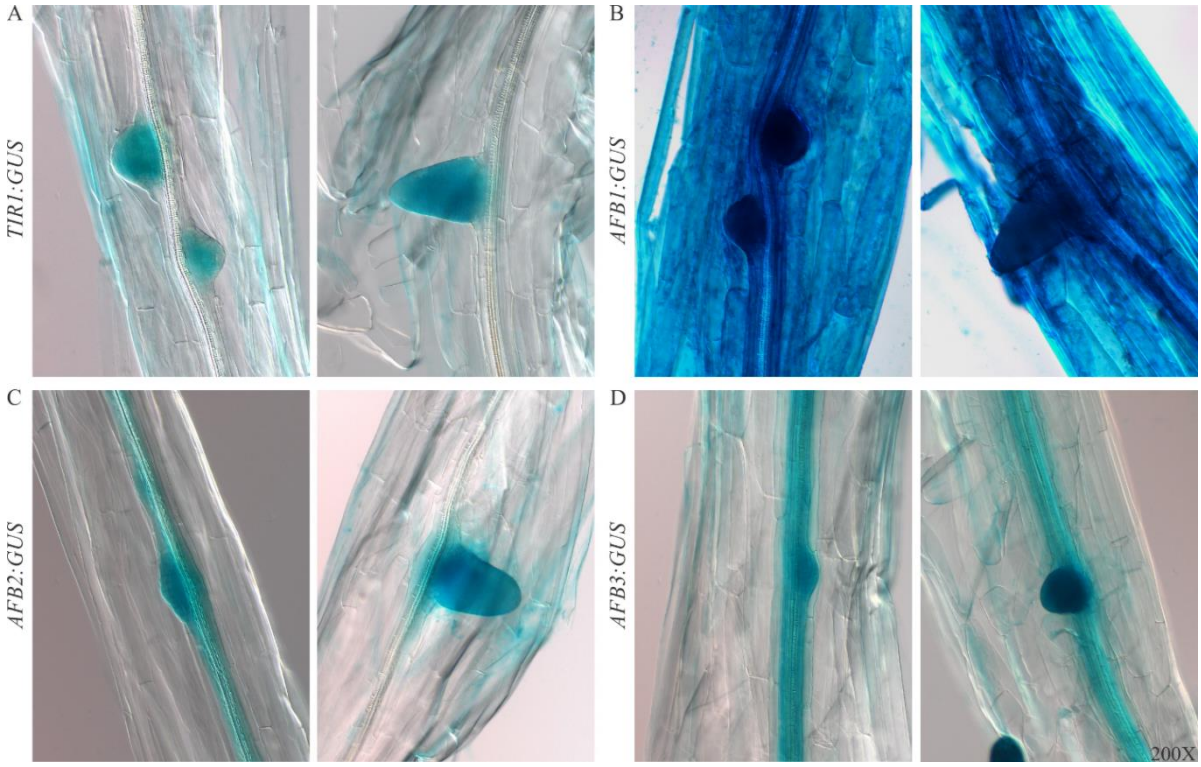


Figure 2. GUS expression in ARs of 3 day-old etiolated seedlings containing *TIR1:GUS*, *AFB1:GUS*, *AFB2:GUS* and *AFB3:GUS* in the presence of 57µM of IAA.

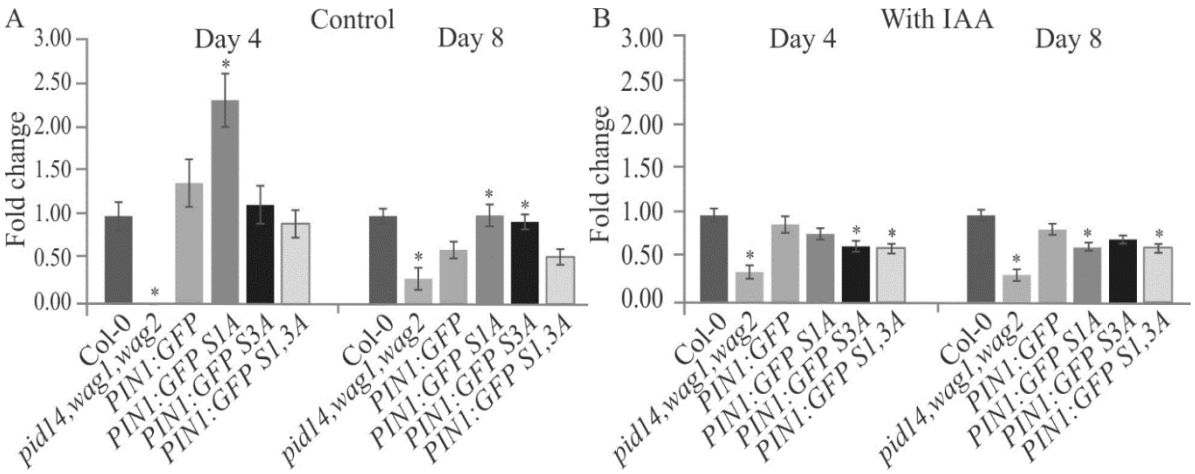


Figure 3. Adventitious roots on PIN phosphorylation mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** Presence of exogenous IAA (57 µM). The data were normalized against the average number of roots in Col-0 for every treatment. The error bars represent the standard error. The presence of asterisk on top of a bar indicates significant difference compared to the respective ecotype values (t-test, P≤ 0.05).

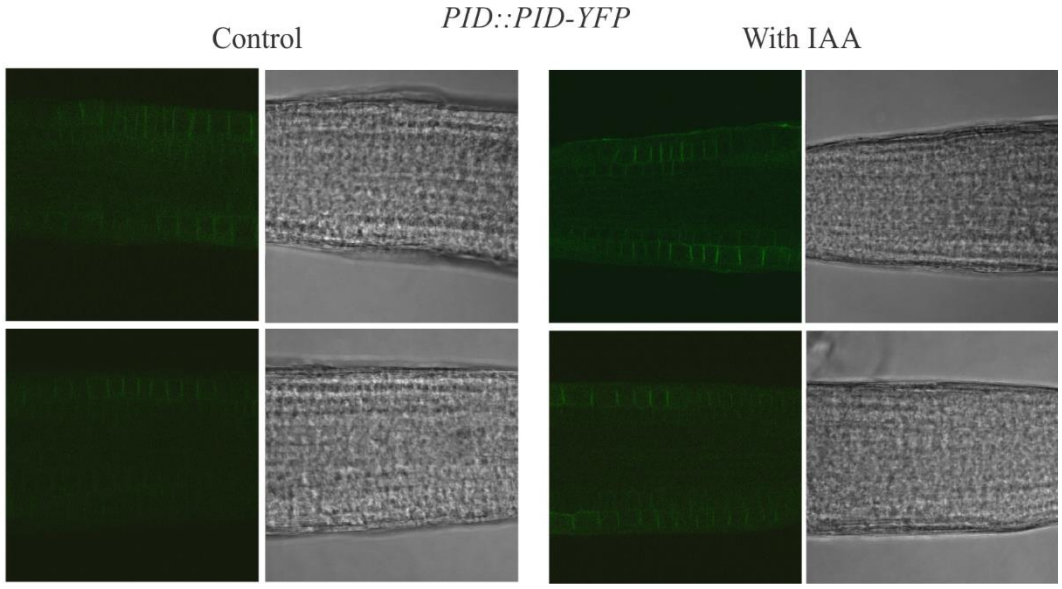


Figure 4. Expression of *PID::PID-YFP* in ARs of *Arabidopsis* 5 day-old etiolated seedlings without exogenous auxin (control) or with 57µM of IAA.

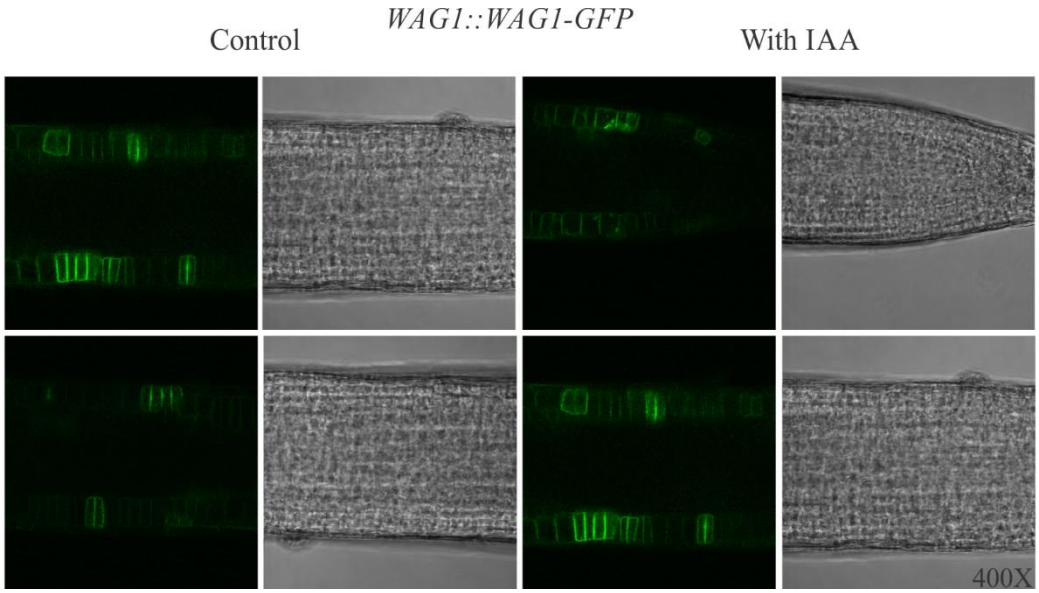


Figure 5. Expression of *WAG1::WAG1-GFP* in ARs of *Arabidopsis* 5 day-old etiolated seedlings without exogenous auxin (control) or with 57μM of IAA.

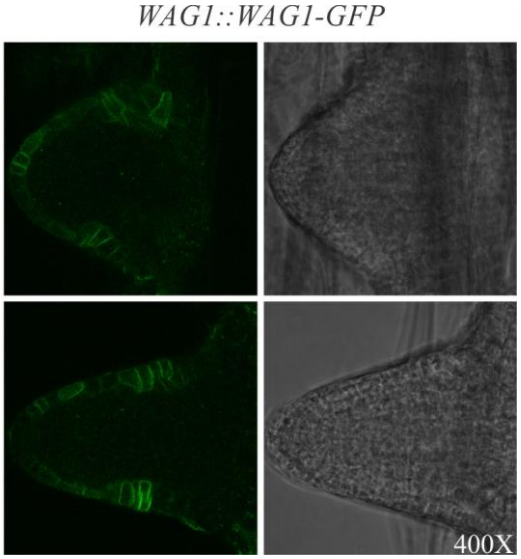


Figure 6. Expression of *WAG1::WAG1-GFP* in AR of *Arabidopsis* 3 day-old etiolated seedlings with 57μM of IAA.

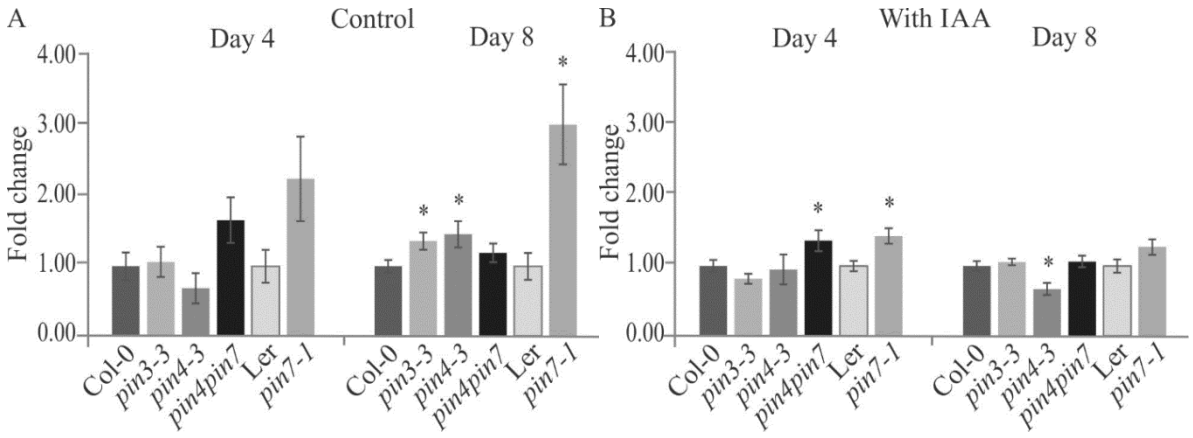


Figure 7. Adventitious roots on PIN efflux transport mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous IAA (57 µM). The ecotype of *pin3-3*, *pin4-3* and *pin4pin7* is Col-0 and of *pin7-1* is Ler. The data were normalized against the average number of roots in the WT for every treatment. The error bars represent the standard error. The presence of an asterisk on top of a bar indicates significant difference compared to the respective ecotype (*t*-test, $P \leq 0.05$).

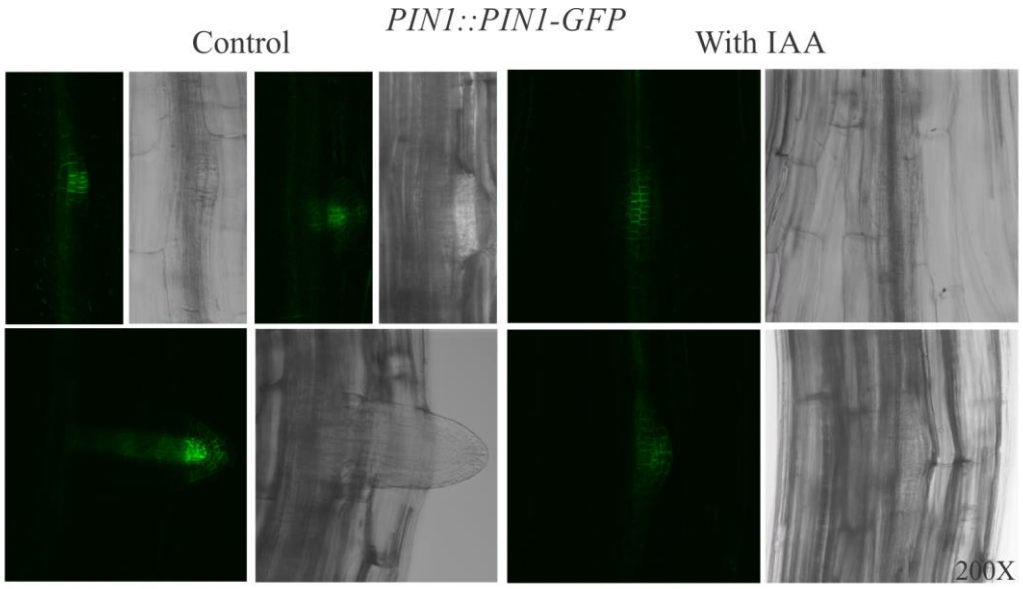


Figure 8. Expression of *PIN1::PIN1-GFP* in ARs of *Arabidopsis* 3 day-old etiolated seedlings without auxin (control) or with 57µM of IAA.

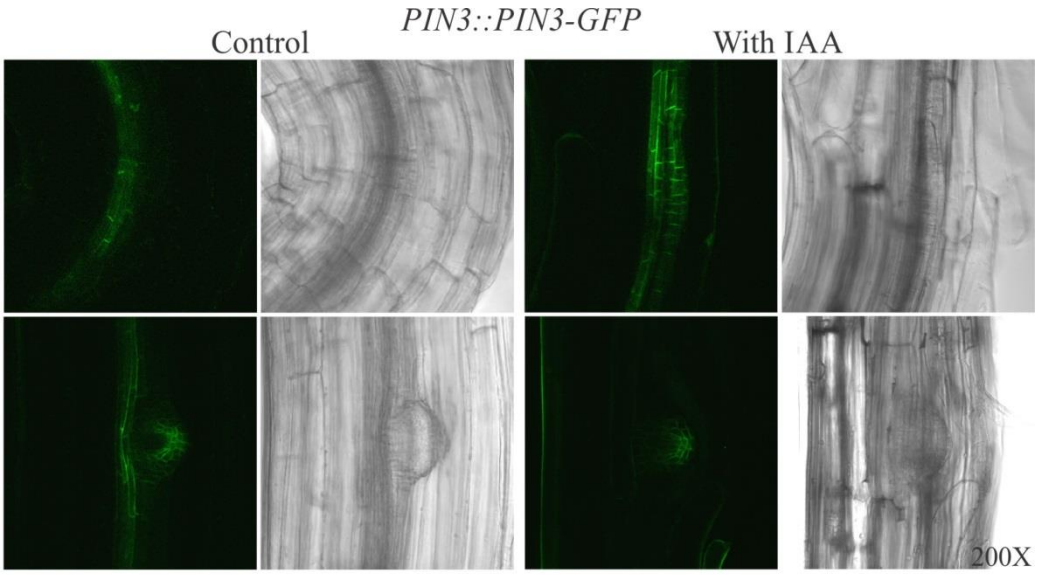


Figure 9. Expression of *PIN3::PIN3-GFP* in ARs of *Arabidopsis* 3 day- old etiolated seedlings without exogenous auxin (control) or with 57µM of IAA.

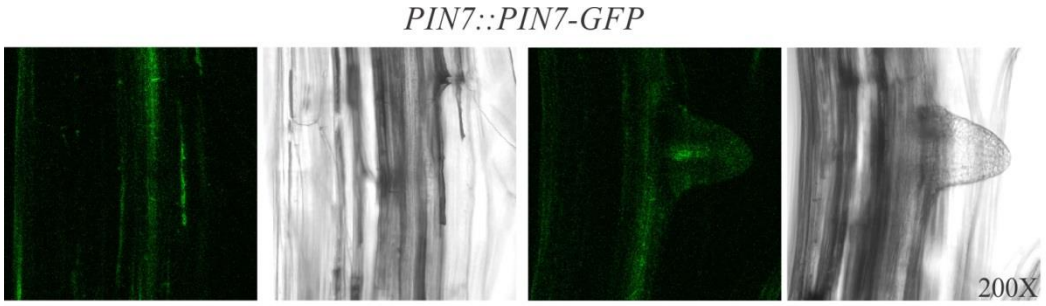
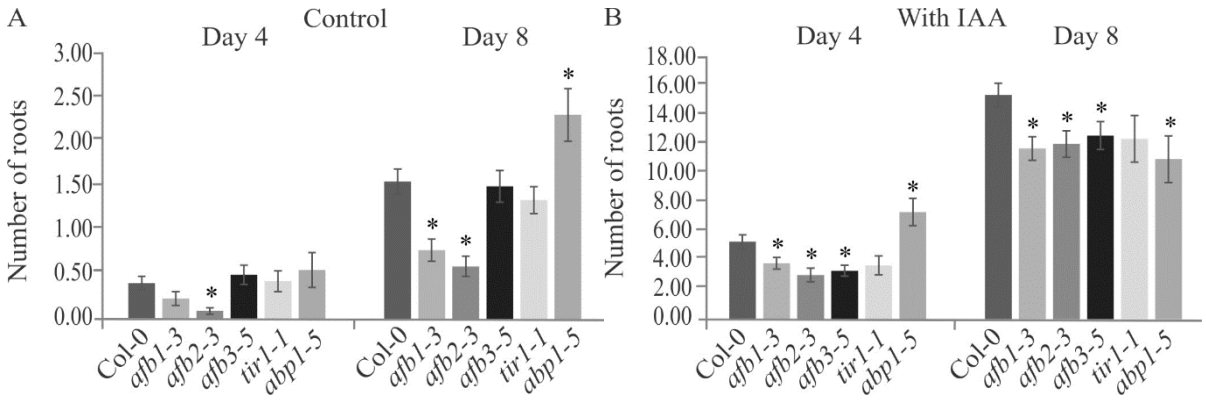


Figure 10. Expression of *PIN7::PIN7-GFP* in ARs of *Arabidopsis* 3 day-old etiolated seedlings with 57µM of IAA.

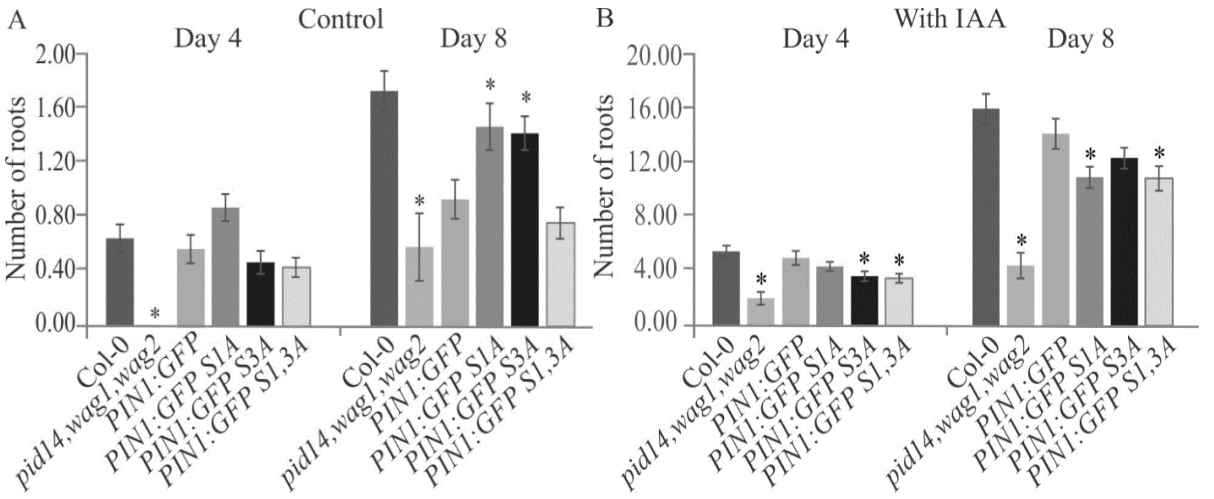
Supplemental material

Mutant	LP 5' → 3'	RP 5' → 3'
*pin3-3**	GGAGCTCAAAGGGTCACC	TAACCGGAAAGCGACGAGA
pin4-3	CCTAAAGAAAACCAACAGCA	AATTAAACACACAGACCCAC
pin7-1	AAATCCGATCAAGGCGGTG	CGTCGAATTTCCGCAAGC
*tir1-1**	AGCGACGGTGATTAGGAGGT	CAGGAACAACGCAGCAAAA
afb1-3	AACGGAAGACTAGGAAGCGAG	GCAACAGCTTCAAGACCTTTG
afb2-3	TCAACGGTCAAGATCCATCTC	CTGCAATTAGCGGCAATAGAG
afb3-5	TGCTTTGCTGATCTTCTAAGG	AGGGTCTAAGACGGCTCTCTG

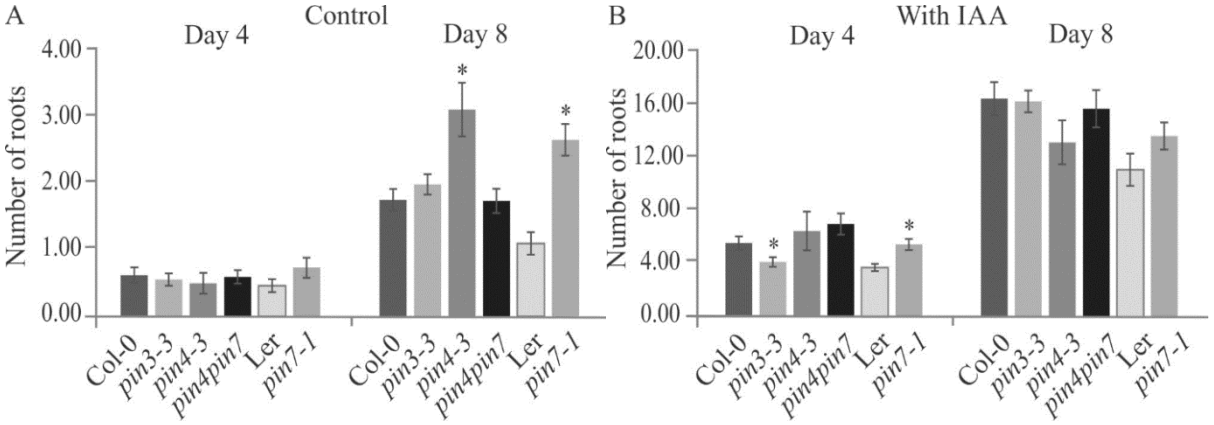
Supplemental table 1. Gene specific primers used for genotyping. **pin3-3* fragment was digested with StyI and *tir1-1* was tested by CAPs (Zenser et al., 2001). Border primers: En8130 5' GAGCGTCGGTCCCCACACTTCTATAC 3' (*pin4-3*); DS5 5' ACGGTCGGGAAACTAGCTCTA 3' (*pin7-1*) and LBb1.3 5'ATTTTGCCGATTTCGGAAC 3' (*afb* mutants).



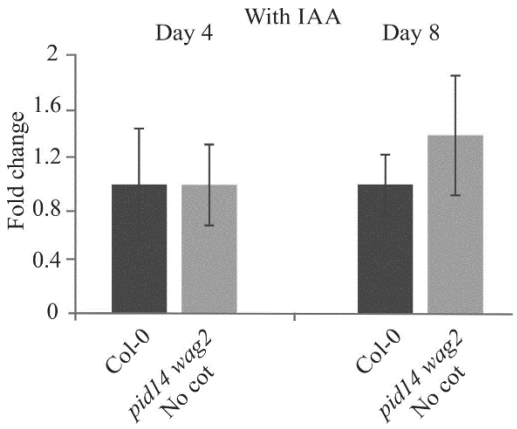
Supplemental figure 1. Average number of adventitious roots on receptor mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The error bars represent the standard error. The presence of an asterisk on top of a bar indicates significant difference compared to Col-0 (*t*-test, $P \leq 0.05$).



Supplemental figure 2. Average number of adventitious roots on PIN phosphorylation mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The error bars represent the standard error. The presence of an asterisk on top of a bar indicates significant difference compared to the respective ecotype (*t*-test, $P \leq 0.05$).



Supplemental figure 3. Average number of adventitious roots on PIN mutants at day 4 and day 8. **A)** Control, without exogenous IAA. **B)** With exogenous 57 µM of IAA. The ecotype of *pin3-3*, *pin4-3* and *pin4pin7* is Col-0 and of *pin7-1* is Ler. The error bars represent the standard error. The presence of an asterisk on top of a bar indicates significant difference compared to the respective ecotype (*t*-test, $P \leq 0.05$).



Supplemental figure 4. Adventitious root number in PIN phosphorylation double mutant *pid14 wag2* seedlings without cotyledons at day 4 and day 8 with exogenous auxin (IAA 57 µM). Col-0 = WT. The error bars represent the standard error. Treatments are not significantly different (*t*-test, $P \leq 0.05$).

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PRINCIPAIS RESULTADOS E PERSPECTIVAS

Os resultados obtidos durante o desenvolvimento deste doutorado e apresentados nesta tese trazem uma série de dados que contribuem para um maior entendimento do processo de enraizamento adventício em plântulas de *Arabidopsis thaliana*. Três diferentes auxinas foram testadas em plântulas crescidas em sistema de estiolamento: AIA, ANA e 2,4-D. Tanto ANA quanto 2,4-D apresentaram respostas que variaram de acordo com a concentração utilizada. ANA foi mais eficiente do que AIA na indução de RA, mas o crescimento das raízes foi inibido, provavelmente devido à maior estabilidade metabólica de ANA, que pode ter reprimido estágios mais avançados do desenvolvimento das raízes, como o alongamento. Isso deve ter ocorrido porque as auxinas têm um efeito estimulatório nas primeiras fases do processo de rizogênese, mas tornam-se inibitórias nas fases finais do seu desenvolvimento. 2,4-D induziu a formação de RA menos eficientemente do que ANA e AIA e, em concentrações mais altas, causou a formação de calos ao invés de raízes. Dentre os tipos testados, a auxina que teve melhores efeitos na rizogênese adventícia foi AIA. AIA deve ficar tempo suficiente nos tecidos para induzir a formação das RA sem inibir seu crescimento, uma vez que sua degradação e transporte ocorrem rapidamente na plântula. Através da imunolocalização de auxinas foi possível verificar maior acúmulo de AIA nos tecidos que dão origem às raízes adventícias, enfatizando o importante papel deste fitormônio no desenvolvimento das raízes.

A influência de diferentes tipos de auxinas no enraizamento adventício também pôde ser observada através da análise de expressão gênica por PCR em Tempo Real. De maneira geral, a magnitude da expressão dos genes induzidos foi mais estimulada por ANA, seguido de AIA. Esse efeito indica que a estabilidade metabólica e as taxas de transporte de auxinas devem mudar os padrões de expressão gênica durante o desenvolvimento das RA. O padrão de expressão da ciclina mitótica *CYCB1;1* indicou uma relação importante entre a indução das RA por auxina exógena e a ativação do ciclo celular, uma vez que um aumento na expressão de *CYCB1;1* coincidiu com as primeiras divisões celulares que dão origem ao primórdio radicular. Além disso, a rizogênese adventícia foi marcada pelo aumento de expressão dos genes *PIN1*, *SUR2*, *GH3.3*, *GH3.6*, *ARF8* e *IAA28*, especialmente na presença de ANA. Dentre os genes analisados, *IAA28* chamou atenção por ter sua expressão induzida durante o desenvolvimento das raízes adventícias, diferente do que foi anteriormente observado para o enraizamento lateral. Esse padrão observado pode representar uma

diferença entre as rotas de desenvolvimento que levam à formação de raízes laterais ou adventícias. Dessa forma, análises mais aprofundadas são necessárias para tentar elucidar o papel de *IAA28* no enraizamento adventício.

Outra diferença importante entre os enraizamentos lateral e adventício observada durante o desenvolvimento desta tese está relacionada com o papel do transportador de influxo de auxinas LAX3. O mutante *lax3* não apresentou redução na quantidade de RA desenvolvidas nos períodos analisados, ao contrário do que foi anteriormente verificado no enraizamento lateral. Ademais, a visualização de *LAX3pro:LAX3-YFP* foi mais forte na região do estelo, enquanto que nas RL a expressão de LAX3 se restringiu a um pequeno grupo de células do córtex e epiderme em frente ao primórdio radicular. A participação dos outros membros da família AUX/LAX no desenvolvimento de RA também foi investigada. Embora mutações individuais em LAX1, LAX2 e LAX3 não tenham afetado a formação de RA, combinações de mutações duplas e triplas em AUX1, LAX1, LAX2 e LAX3 e uma mutação nos quatro genes causaram reduções significativas na quantidade de RA. Entretanto, quando auxina exógena foi aplicada, esse efeito de redução foi revertido. AUX1 parece exercer um papel fundamental na rizogênese adventícia, e, embora LAX3 não seja o regulador chave do processo, sua atuação em conjunto com AUX1 parece ser importante para que o transporte de influxo de auxinas ocorra corretamente.

Os resultados obtidos também apontam para novos papéis dos transportadores de efluxo de auxinas e da fosforilação destes na rizogênese adventícia. Para que as RA sejam formadas adequadamente, o transporte de efluxo de auxinas depende da ação dos transportadores PIN, especialmente PIN1, 3 e 7. Além disso, a fosforilação dos transportadores PIN pelas quinases PID, WAG1 e WAG2 e, consequentemente, a localização polar destes transportadores, é um mecanismo essencial para que o transporte polar de auxinas ocorra corretamente, permitindo o estabelecimento das RA.

A participação dos receptores de auxinas no desenvolvimento das raízes adventícias tem sido pouco investigada e ainda não é bem compreendida. A família de proteínas TIR1/AFB e a proteína ABP1 são consideradas receptores de auxinas, embora o papel de ABP1 ainda seja controverso. As investigações apresentadas nesta tese indicam que os receptores são importantes para a rizogenêse adventícia, embora atuem de maneira parcialmente redundante e complementar. Apesar disso, um possível papel mais importante no processo parece ser exercido por TIR1 e AFB2.

Os resultados obtidos nesta tese ressaltam a importância das auxinas e dos genes envolvidos na sua sinalização, metabolismo e transporte durante a rizogênese adventícia em *Arabidopsis*. Diversos genes que têm potencial para serem silenciados a fim de melhorar a produção de RA em *Arabidopsis* foram identificados. Como perspectivas desta pesquisa, mutações em ortólogos de AFB3, PIN4 e PIN7 em espécies de potencial interesse econômico podem ser investigadas visando identificar possíveis benefícios à sua propagação vegetativa. Isso seria especialmente importante em árvores, que são a base de atividades chave, como a indústria florestal que depende da propagação clonal de genótipos elite. Outra abordagem interessante seria testar mutações nos genes que parecem exercer um papel importante na rizogênese em outros sistemas de enraizamento adventício de *Arabidopsis*, como o sistema de folhas destacadas ou de plantas com o sistema radicular extirpado. Nesses sistemas, as raízes adventícias se originam de diferentes tecidos e podem apresentar respostas que se aproximem mais dos processos observados em espécies economicamente relevantes.

ANEXOS

ANEXO 1

When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings

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When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings

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Adventitious rooting (AR) is a multifactorial response leading to new roots at the base of stem cuttings, and the establishment of a complete and autonomous plant. AR has two main phases: (a) induction, with a requirement for higher auxin concentration; (b) formation, inhibited by high auxin and in which anatomical changes take place. The first stages of this process in severed organs necessarily include wounding and water stress responses which may trigger hormonal changes that contribute to reprogram target cells that are competent to respond to rooting stimuli. At severance, the roles of jasmonate and abscisic acid are critical for wound response and perhaps sink strength establishment, although their negative roles on the cell cycle may inhibit root induction. Strigolactones may also inhibit AR. A reduced concentration of cytokinins in cuttings results from the separation of the root system, whose tips are a relevant source of these root induction inhibitors. The combined increased accumulation of basipetally transported auxins from the shoot apex at the cutting base is often sufficient for AR in easy-to-root species. The role of peroxidases and phenolic compounds in auxin catabolism may be critical at these early stages right after wounding. The events leading to AR strongly depend on mother plant nutritional status, both in terms of minerals and carbohydrates, as well as on sink establishment at cutting bases. Auxins play a central role in AR. Auxin transporters control auxin canalization to target cells. There, auxins act primarily through selective proteolysis and cell wall loosening, via their receptor proteins TIR1 (transport inhibitor response 1) and ABP1 (Auxin-Binding Protein 1). A complex microRNA circuitry is involved in the control of auxin response factors essential for gene expression in AR. After root establishment, new hormonal controls take place, with auxins being required at lower concentrations for root meristem maintenance and cytokinins needed for root tissue differentiation.

Keywords: adventitious rooting, auxin, receptors, jasmonic acid, cytokinin, nutrition, microRNAs, hormonal crosstalk

INTRODUCTION

If flowering is a key developmental process for sexual reproduction in plants, adventitious rooting (AR) occupies a central role in asexual propagation. Forestry, horticulture, and fruit crops depend to a large extent on the successful establishment of roots in cuttings and other propagules. Clonal propagation is of particular relevance to forestry, since genetic improvement in long lived species with large generation cycles is often limiting. Genetic gains from interspecific hybridization, mutations, and transgenic events can be captured and multiplied faster and more efficiently based on clonal propagation through AR of cuttings. Overall, the main application of AR is propagation by cuttings and its derived techniques adapted to clonal garden greenhouses and *in vitro* cultures, minicuttings and microcuttings, respectively (Assis et al., 2004). Therefore, rather than looking into the examples of developmentally programmed AR in intact plants, the focus of the present review is on AR of severed organs or in response to stressful conditions, such as flooding.

Most research on AR has been centered on the role of phytohormones, mainly auxins, and cutting physiological conditions. The role of stress responses associated with cutting severance and the relevance of mother plant status has often received less attention, although a shift in focus has been clearly taking place in the last two decades or so. Wound responses associated with cutting severance are integrated, and often necessary, in the steps leading to AR, and mother plant status is a key determinant of rooting propensity of cuttings derived from it. Therefore, the control of environmental variables of stock plants is rather relevant for the clonal propagation process. Clearly, a fundamental aspect governing AR responses to external and internal stimuli is cellular competence to respond. This developmental capacity to respond is responsible for many of the failures to obtain AR in mature cuttings, even upon careful manipulation of environmental variables and phytohormones that can modulate rooting.

The concept of adventitious root is based essentially on anatomical origin. Adventitious roots are formed in stems, leaves and

non-pericycle tissue in older roots, differing from primary roots, of embryonic origin, and lateral roots, which are derived from the pericycle layer (Li et al., 2009a). There are two main patterns of adventitious root development: direct and indirect. The tissues involved in the process of root development are most frequently the cambium and vascular tissues, which undergo the first mitotic divisions, leading directly to root primordia in the first pattern. In the indirect pattern of AR, albeit the same tissues often take part, the formation of a callus is observed prior to differentiation of root primordia. In both cases, before root primordia become distinguishable, clusters of usually isodiametric cells are formed (meristemoids; Altamura, 1996). In the indirect pattern of AR, a bottleneck is frequently observed, i.e., the establishment of an effective vascular connection between the newly formed root primordia and the stem. Poorly connected vasculature with the stem leads to non-functional roots, with negative consequences for cutting survival (Fleck et al., 2009).

Adventitious rooting is a complex process that can be affected by numerous variables, both internal and external. A large body of evidence has supported the existence of successive physiological phases in the process of adventitious root development, each with specific requirements that can even be antagonistic, but operate in complementary fashion. The most widely recognized AR phases are induction, initiation, and expression (Kevers et al., 1997).

The induction phase in cuttings or detached organs, such as leaves, is generally marked by the immediate consequences of the wounding response caused by severance. It encompasses the first hours after cutting removal, with a local increase in jasmonate, phenolic compounds and auxin at the cutting base, often associated with a transiently lower peroxidase (EC 1.11.1.7) activity, and the establishment of a sink for carbohydrates in the same zone (Schwambach et al., 2008; Ahkami et al., 2009). Peroxidases are heme-containing enzymes with catalytic action on diverse organic compounds, including indole-3-acetic acid (IAA), and their activity has been used as a biochemical marker of the rooting phases (Corrêa et al., 2012a). The induction phase is devoid of visible cell divisions and involves reprograming of target cells to the following establishment of meristemoids, which takes place in the initiation phase. Studying AR in apple, De Klerk et al. (1999) launched the concept of an early phase of dedifferentiation (0–24 h), taking place before the induction phase. In the concept of the present review, the dedifferentiation phase postulated by De Klerk et al. (1999) corresponds to the early steps of the induction phase. During initiation, besides cell divisions, meristemoids and development of root primordia, often a lower auxin and phenolic concentration and higher peroxidase activity are observed. The expression phase corresponds to the growth of root primordia through the stem tissues and the establishment of vascular connections between the newly formed root and the original stem cutting. For simplification purposes, it is not uncommon to join the initiation and expression phases under a single denomination of formation phase (Fett-Neto et al., 1992).

These overall changes in phytohormone balance, along with other less predominant but not unimportant changes to be discussed ahead, trigger a sequence of gene expression events that leads to proteomic changes, culminating with new root differentiation. Considering different systems and the fragmentary information available, these molecular events of gene expression and gene product accumulation can be putatively summarized in chronological sequence as follows: wounding and water balance stress-related, carbohydrate sink establishment, auxin transport systems, cell wall degradation and assembly, transcription factors involved in cell fate determination, replication machinery, transcription factors with roles in growth and differentiation (Brinker et al., 2004; Sorin et al., 2005; Ahkami et al., 2009).

At the molecular level, including the participation of various phytohormones, considerably more knowledge is available on lateral root development. Certainly, there is at least some overlap between the processes of lateral root development and AR. Most of the similarities include the requirement for an initial auxin increase, followed by a reduction, the participation of auxin transporters, cell wall dynamics, and the activity of specific transcription factors in these processes. Root growth responses to nutrient gradients, such as nitrate and phosphate (Desnos, 2008), seem to be another feature apparently shared between lateral and adventitious roots (Schwambach et al., 2005). Lateral root development depends at least partially on auxin activation of founder cells in the pericycle at the primary root differentiation zone, possibly mediated by an interaction of auxin with its receptor TIR1 (transport inhibitor response 1; Petricka et al., 2012). The role of root development inhibitors, such as cytokinins and strigolactones, which will be discussed ahead, also seems to be shared between lateral root development and AR. However, besides the usual histological origin, other very important differences exist between lateral and adventitious root development, most likely related to the often associated wound response and particular reorganization of auxin transport systems in the latter.

Although AR in intact plants may take place in certain conditions, such as flooding or programmed development, the typical AR features of stress signaling and major shifts in root–shoot correlative influences are usually present in excised plant parts, such as cuttings, hypocotyls and leaves. Rooting protocols based on pre-etiolated intact seedlings, commonly used to investigate AR in *Arabidopsis thaliana*, have roots formed mostly from the pericycle, which extends from primary roots into the hypocotyls of young seedlings, and do not face stresses capable of disrupting root–shoot correlative influences. A comparison of an intact seedling system with de-rooted older plants or with rooting of petioles of detached leaves showed significant differences, not only in root founding tissues, but also in auxin requirements, sensitivity, and rooting mutant phenotypes (Corrêa et al., 2012b). The fact that lateral and adventitious root developments have fundamental functional differences can be further highlighted by the opposite effects of ethylene on both processes, observed in studies with tomato (Negi et al., 2010). Perhaps the pathways leading to adventitious versus lateral root development could be viewed as different roads, which may intertwine in some portions, and end up leading to the same destination, i.e., new roots.

MOTHER PLANT STATUS – DEVELOPMENTAL COMPETENCE TO RESPOND AND THE RIGHT SUPPLIES FOR THE HURDLES AHEAD

In vegetative propagation, a mother plant provides cuttings with improved selected characteristics, and the formation of new

adventitious roots is essential for the restoration of the whole plant condition. Physiological and biochemical quality of mother plants, in addition to their genetic makeup, could limit rooting performance of cuttings derived therefrom (Osterc, 2009). The physiological condition of mother plants is directly affected by the environment in which they were raised or to which they were exposed, including light and temperature conditions, water and nutrient supplies (Moe and Andersen, 1988). Endogenous auxin, carbohydrate content, mineral nutrients, and other biochemical components, such as phenolics that could act as rooting co-factors or auxin transport modulators, may be affected by environmental factors and are transferred from the stock plants to the propagules when the cutting is severed. The content, metabolism, and interactions of these metabolites and components will influence early responses to wound and root induction of cuttings.

Auxin endogenous concentration varies over the course of rooting phases, and is needed at higher concentration during the induction phase for proper rooting (Kevers et al., 1997). In this context, high auxin content immediately after cutting severance originating from the mother plant, may result in improved rooting. As far as light treatments are concerned, shade conditions (low red:far-red ratios) induced auxin biosynthesis and increased IAA levels in *Arabidopsis* seedlings (Tao et al., 2008). Light availability and quality have been shown to affect auxin transport rate and its predominant anatomical path in the stem (Morelli and Ruberti, 2002). Sorin et al. (2005) described an interaction between light and auxin metabolism affecting *Arabidopsis* rooting. Mutants with low rooting capacity (*ago1*) had upregulated light responses and disturbed auxin homeostasis.

Mineral nutrition of stock plants is an important factor in determining AR capacity. The biosynthesis of one of the main auxin precursors, the amino acid tryptophan, requires zinc (Blazich, 1988; Marschner, 1995), which is also a structural component of the auxin receptor ABP1 (Auxin-Binding Protein 1; Tromas et al., 2010). Manganese and iron are co-factor and structural component of peroxidases, respectively. Therefore, these nutrients can affect this class of auxin catabolism enzymes (Campa, 1991; Fang and Kao, 2000). The appropriate management of light quality and fertilization schemes applied to mother plants, in a way to positive influence auxin biosynthesis, transport, and metabolism, could implicate in a better rooting response on subsequent cuttings produced by these stocks. The relevance of mineral nutrition for AR is highlighted by the fact that rooting phase-specific mineral nutrient compositions, optimized for cuttings themselves, have been shown to improve rooting and survival of *Eucalyptus globulus* plants (Schwambach et al., 2005). High nitrogen supply to stock plants and the resulting elevated N content in herbaceous cuttings have been shown to strongly promote AR (Druege et al., 2000, 2004; Zerche and Druege, 2009).

The initial content and composition of phenolic compounds are also transferred to cuttings from mother plants and the interaction of these metabolites with auxin and peroxidases may have effects on adventitious root formation (De Klerk et al., 1999). Flavonoids, a major class of phenolic compounds, can influence auxin transport (Peer and Murphy, 2007), mainly by interacting with efflux carrier PIN2 (PIN-FORMED 2) or affecting the distribution of other PIN proteins (Buer et al., 2010). Phenolics are also important in modulating peroxidase activity and could also act as antioxidants, preventing auxin degradation at cutting bases (De Klerk et al., 1999).

Several investigations have pointed out that the initial carbohydrate content of the cutting, should be enough to supply the energy reserves throughout the rooting period (Veierskov, 1988; Husen, 2008). On the other hand, there is evidence that carbohydrate allocation and distribution within the cutting could be more important than the content itself (Druege et al., 2000; Druege, 2009; Ruedell et al., 2013). Light and current photosynthesis of cuttings could play an important role in this scenario, influencing carbohydrate metabolism and reallocation (Hoad and Leakey, 1996; Rapaka et al., 2005). In the rooting recalcitrant *E. globulus*, donor plants grown in medium devoid of sugar and exposed to white irradiance promoted AR in cuttings, whereas presence of exogenous sugar in donor plant media favored rooting in the easy-to-root *E. saligna*, with no significant effects of irradiance (Corrêa et al., 2005). Appropriate light environment applied to mother plants may increase carbohydrate sink capacity at the root formation site in cuttings derived therefrom.

Maturation negatively affects the regenerative ability of plant material and, as a consequence, diminishes its AR potential. The content and profile of phenolic compounds, as well as the contents of carbohydrates and auxins, switch according to maturation state, correlating with rooting competence (Fernández-Lorenzo et al., 2005; Husen and Pal, 2007; Osterc et al., 2009). The use of juvenile-like material can help overcoming this limitation (Cameron et al., 2003; Kibbler et al., 2004). In vegetative propagation of trees, the use of minicutting technique, both in hydroponic or sand bed minihedges, affords a better environmental control of ministumps (mother plants), improving their physiological quality, and, consequently, the rooting propensity of the minicuttings obtained (Assis et al., 2004; Schwambach et al., 2008).

The molecular basis of rooting competence is an essential aspect of AR. In principle, even if all environmental variables are ideally manipulated so as to favor AR, unless developmental competence is present, responses to the root-promoting signals do not take place and rooting fails. Developmental responsiveness is likely dependent on presence and density of functional phytohormonal receptors and signaling pathways, particularly those for auxin. A detailed investigation on AR of hypocotyls (able to root proficuously upon exposure to auxin) and epicotyls (root poorly even in presence of auxin) of 50-day old seedlings of *Pinus taeda* showed that lack of rooting responsiveness in epicotyls was not related to auxin uptake, transport, distribution among cells, or metabolism. Localized fast cell division and root meristem organization were lacking in epicotyls (Diaz-Sala et al., 1996). Application of the auxin transport inhibitor *N*-(naphthyl)phthalamic acid (NPA) up to the first 3 days after cutting severance inhibited rooting without affecting auxin concentration or metabolic status at the rooting site, suggesting a role for auxin polarity in rooting capacity that would be different than simply moving auxin to the rooting zone.

Auxin capacity to trigger gene expression has been suggested as an early and critical point in AR competence of *Pinus taeda* stem cuttings, for example (Greenwood et al., 2001). In this system,

the inability to root in mature cuttings was apparently due to the lack of cells capacity to arrange themselves into root meristems in presence of auxin. Cell division and callus formation, however, occurred similarly, both in physiologically juvenile and mature cuttings, leading the authors to suggest the existence of an auxin transduction pathway specific to root meristem organization. Members of the expansin gene family are among the early auxin-induced genes during AR of pine cuttings, particularly in non-growing zones of the stem before cell divisions that result in root development (Hutchison et al., 1999). Some auxin-responsive transcription factors have been shown to play roles in the control of cell division leading to root primordia differentiation in cuttings of tree species (Sánchez et al., 2007; Solé et al., 2008; Vielba et al., 2011; Rigal et al., 2012) and are discussed in further detail in the Section "Cell Cycle and Division-New Meristems" below.

The loss of AR capacity at physiologically mature stages is often associated with the transition to flowering (phase shift from juvenile to adult stage). However, in specific organ parts or under specific culture conditions, loss of rooting capacity can take place and become easily noticeable at much earlier stages of development (e.g., seedling), providing interesting experimental systems to study this process in trees (Fett-Neto et al., 2001; Greenwood et al., 2001). Another useful model to study AR and the loss of rooting capacity is *Arabidopsis thaliana*. Using de-rooted hypocotyls of young (12 day old) and adult (26 day old) plants of the Landsberg ecotype, it was shown that AR was much slower in adult de-rooted plants and that endogenous polar auxin transport (evaluated with NPA application) was crucial for AR (Díaz-Sala et al., 2002). These authors also showed that rooting was not dependent on phase shift to reproductive phase, although a correlation was observed. The decline in rooting capacity was probably linked to age-related processes. A correlation between reduced AR capacity and flowering phase shift was also shown in detached leaves of *Arabidopsis* plants of the Columbia ecotype, but only in leaves harvested 2–3 weeks after bolting (Corrêa et al., 2012b). A possible link between flowering and AR of detached leaves was not observed by analyzing the AR kinetics in two early and two late flowering time mutants of each of two ecotypes, Antwerpen and Columbia (Corrêa et al., 2012b). Interestingly, Díaz-Sala et al. (2002) showed that AR in de-rooted hypocotyls of *Arabidopsis* adult plants depended on RGD (Arg-Gly-Asp) peptides (a family of peptides bearing this signature domain), although these were not sufficient for rooting to occur and had no effect on young plant hypocotyls. The RGD peptides may be important in causing changes to the plasma membrane of plant cells and their interaction with cell walls, perhaps affecting cytological events required for AR in adult plant hypocotyls. Taken together these data indicate that *Arabidopsis* and *in vitro* culture systems of tree species are useful tools to study developmental competence to AR.

FEATURES ASSOCIATED WITH EXOGENOUS AUXIN SUPPLY

In addition to the already mentioned effect of endogenous auxin in adventitious root formation, it is well-established that this phytohormone can also act when exogenously supplied, entering the stem via the cut surface of cuttings. In many rooting recalcitrant species, application of exogenous auxin is needed to achieve satisfactory rooting responses (Diaz-Sala et al., 1996; Fett-Neto et al., 2001). In these cases, endogenous auxin produced in the shoot apex and transported basipetally to the cut surface may be complemented by exogenously applied phytohormone aiming at improving the rooting response (Pop et al., 2011). The absence of a shoot apical meristem has not limited AR in *Eucalyptus* microcuttings exposed to exogenous auxin (Fogaça and Fett-Neto, 2005).

Uptake of exogenously provided auxin implicates in a new auxin transport route, which enters the cuttings mostly via the cut surface (Kenney et al., 1969; Guan and De Klerk, 2000) and may be taken up by cells both through a pH trapping mechanism (Rubery and Sheldrake, 1973) and through influx carriers (Delbarre et al., 1996). Most of the supplied auxin acts at the wound site, inducing cell dedifferentiation, leading to a new root meristem later on. A portion of the supplied auxin could also be redistributed along the cutting, mostly via the xylem transpiration route (Osterc and Spethmann, 2001). In this case, auxin influx and efflux carriers would not take significant part in the process, losing directionality of the polar auxin transport throughout the plant (auxin transport is discussed ahead in detail). In fact, auxin uptake may also occur through the phloem and a better rooting performance in *Prunus subhirtella* juvenile cuttings was related to this kind of absorption path (Osterc and Stampar, 2011). However, studies with auxin transport inhibitors provided evidence that rooting in *Pinus taeda* hypocotyls is improved when exogenous auxin is incorporated in the polar auxin transport system (Diaz-Sala et al., 1996). Much of the data from different reports on interactions of exogenous auxins with the polar auxin transport system is probably difficult to compare because of the use of different auxins in the various experiments, including synthetic forms, for which the transport systems are poorly known.

CARBOHYDRATE ALLOCATION

Carbohydrates contribute to the formation of adventitious roots by supplying energy and carbon necessary for cell divisions, establishment of the new root meristems and root formation itself. The efficient partitioning of carbohydrates between the new sink of developing roots at cutting base and the shoot meristem sink could be critical for AR (Druege, 2009). Ahkami et al. (2009) proposed that the early establishment of a carbohydrate sink at the rooting site is a key metabolic event in *Petunia hybrida* adventitious root formation. Pre-incubation of *Petunia* cuttings in the dark increased carbohydrate levels at their bases upon transfer to light, improving AR (Klopotek et al., 2010). Similarly, a higher content of soluble sugars and starch in the rooting zone were associated with higher rooting response in *Tectona grandis* cuttings (Husen and Pal, 2007). Higher accumulation of soluble carbohydrates and starch at the root formation zone in microcuttings was associated with improved rooting capacity of *E. globulus* without exogenous auxin. This condition was observed when cuttings were obtained from mother plants grown in medium devoid of sucrose and exposed for a few weeks to far-red irradiation-enriched environment (Ruedell et al., 2013). When mother plants were grown in sucrose containing medium, the positive effect of exposing stock plants to far-red enriched irradiance on microcutting rooting capacity was abolished. Inhibition of AR in carnation cuttings

by high carbohydrate content has also been proposed, although the importance of establishing an auxin-stimulated carbon sink was pointed out (Agulló-Antón et al., 2011).

Growth and differentiation of tissues can be modulated by carbohydrate signals through alterations in metabolic fluxes and carbohydrate concentrations during development, which may regulate gene expression (reviewed by Rolland et al., 2006). These carbohydrate signals are generated by photosynthesis and carbon metabolism in source and sink tissues and probably play a regulatory role in adventitious root induction (Druege, 2009). Interactions between phytohormones and carbohydrates are essential part of the sugar sensing and signaling network (Rolland et al., 2006): and a glucose and auxin signaling crosstalk was shown to be important for controlling root development and growth in *Arabidopsis thaliana* seedlings (Mishra et al., 2009). Auxin supply to *Dalbergia sissoo* cuttings enhanced the content of total soluble sugars and starch, promoting AR (Husen, 2008). Different carbon sources may affect the rooting capacity of eucalypt microcuttings in a rooting phase-dependent fashion, even in absence or with suboptimal supplied auxin concentrations, particularly in the difficult-to-root *E. globulus* (Corrêa et al., 2005). Taken together, available data suggest that low carbohydrate allocation to the root formation site may limit AR. Adequate supply of these compounds is a combined function of sink strength and the capacity of the source to meet sink demand (Druege, 2009). Carbohydrates play important roles, not only by providing energy and carbon chains for biosynthetic processes in new meristems and roots, but also by affecting gene expression, in co-action with auxin.

WOUND RESPONSE

Severance of a cutting from the donor plant has immediate consequences, including injury and the isolation from functional integrity of the whole plant condition, i.e., loss of root–shoot correlative influences (Druege, 2009). Excision of *Petunia* cuttings led to a fast and transient increase in the wound-phytohormone jasmonic acid (JA) and a continuous accumulation of soluble and insoluble carbohydrates during adventitious root formation (Ahkami et al., 2009). There is some evidence that AR is also influenced by ethylene production caused upon wounding during explant preparation, and a stimulatory role of endogenous ethylene would depend on achieving a relatively narrow concentration range (Mensuali-Sodi et al., 1995). In fact, for some *in vitro* studies, the use of anti-ethylene chemicals has resulted in improved rooting responses (De Klerk et al., 1999).

Adventitious rooting in cuttings may be compared to a stress-induced reprogramming of shoot cell fate. Acclimation to stress is often accompanied by metabolic re-adjustment. The alternative oxidase (AOX) plays a central role in determining reactive oxygen species equilibrium in plants and can be induced in response to diverse abiotic and biotic stress conditions (Santos-Macedo et al., 2012). Secondary metabolism during AR may be associated with AOX activity. Phenylpropanoid derivatives, especially phenolic acids and lignin, are known to be closely related to the regulation of cell division and differentiation. Enhanced accumulation of phenolic acids and some flavonoids was found to correlate with *in vitro* rooting (De Klerk et al., 1999). Moreover, a complex interaction between AOX and H_2O_2 signaling is apparent. Application of H_2O_2 could replace added auxin as a rooting agent in olive cuttings (Santos-Macedo et al., 2009) and the presence of an AOX inhibitor, salicylhydroxamic acid (SHAM), reduced rooting even in presence of exogenous auxin (Santos-Macedo et al., 2012).

Phenolic compounds are known to protect plants from oxidative stress (Jaleel et al., 2009) and allow the containment of excessive wound response that may inhibit subsequent regeneration processes (De Klerk et al., 2011). Phloroglucinol and ferulic acid displayed antioxidant action, protecting IAA from decarboxylation and the tissue from oxidative stress in *Malus* "Jork 9," thereby promoting AR. The decarboxylation was attributed to the wound response and did not occur to such an extent in non-wounded plant tissues. The action of the phenolic compounds suggests that, at least in part, rooting depends on the inhibition of IAA decarboxylation caused by wounding, so that more auxin is available to induce roots (De Klerk et al., 2011).

Hydrogen peroxide, a form of reactive oxygen, functions as a signaling molecule that mediates various physiological and biochemical processes, as well as controls responses to various stimuli in plants (Neil et al., 2002). Li et al. (2009b) showed that H_2O_2 might function as a signaling molecule involved in the formation and development of adventitious roots in mung bean seedlings. Production of H_2O_2 was markedly induced in indole-3-butyric acid (IBA)-treated seedlings suggesting that IBA induced overproduction of H_2O_2 and promoted AR via a pathway involving H_2O_2. In another study, Li et al. (2009c) suggested that the mechanism underlying the IBA and H_2O_2-mediated facilitation of adventitious root formation is the early decrease of peroxidase and ascorbate peroxidase activities in IBA and H_2O_2-treated seedlings. The decrease in activity of these enzymes would be relevant to generate the necessary high level of auxin and H_2O_2 required for adventitious root induction.

WATER RELATIONS

The availability of water is one of the most important factors favoring root development, as cuttings have to maintain a positive water balance while roots develop (Loach, 1988). Puri and Thompson (2003) carried out a study to examine the influence of three levels of initial water potential in stem cuttings of *Populus* (dried, soaked, and fresh) on plant water status and rooting capacity under controlled environmental conditions, in combination with planting in soils with different water potential. Results clearly showed that soil moisture had a major effect on rooting. Water-stressed cuttings took a longer time to root and formed fewer roots. Pre-soaking of cuttings had a positive effect on rooting, mainly under the drier soil moisture conditions. Although unrooted hardwood cuttings needed moister soil to initiate rooting, once roots were established, they could tolerate somewhat drier conditions. In good agreement, cutting survival and AR were highest in moister substrate for stem cuttings of juniper (*Juniperus horizontalis*), azalea (*Rhododendron*), and holly (*Ilex crenata*; Rein et al., 1991).

Gas exchange and water relations have also been simultaneously evaluated. Relative water content (RWC) of leaves and osmotic potential increased upon formation of root primordia in *Poinsettia* cuttings (Svenson et al., 1995). Following formation of root primordia, and concurrent with increasing RWC and osmotic

potential, stomatal conductance (g) increased. As roots initially emerged, net photosynthesis and g increased rapidly and continued to increase with further root primordia development and subsequent emergence of adventitious roots. Abscisic acid (ABA) often accumulates under water stress conditions and is a known inhibitor of cell cycle progression (Wolters and Jürgens, 2009). Hence, the level of water stress is a relevant factor for cutting establishment that should be minimized in order to avoid losses and slow establishment of plants.

PHYTOHORMONAL BALANCES: THE SEESAW OF PROMOTION VERSUS REPRESSION

Auxins have a rhizogenic action during the root induction phase (generally from cutting severance up to 96 h) and stimulate cells at the cutting base to engage in the establishment of meristemoids (Garrido et al., 2002). The same phytohormones become inhibitory after 96 h and may arrest or inhibit growth of root primordia (De Klerk et al., 1999). Diaz-Sala et al. (1996), using NPA treatments, showed that the initial 48 h were crucial for auxin-dependent root induction in pine. In addition, mRNA levels of transcription factors possibly related to root meristem fate, as well as cell wall remodeling genes, were increased in presence of exogenous auxin at 24 h (Hutchison et al., 1999; Sánchez et al., 2007; Solé et al., 2008; Vielba et al., 2011).

In general, free IAA endogenous levels have a transient increase during the induction phase, pass through a minimum at the initiation step and resume an increase in the expression phase (Bellamine et al., 1998). The importance of auxin at the induction and expression phases (first and last steps) of the rooting process was demonstrated through the use of anti-auxins, which prevent auxin from exerting its functions. In poplar cuttings, anti-auxins present at one of these phases caused significant inhibition of AR (Bellamine et al., 1998). Moreover, Negishi et al. (2011) compared easy and difficult-to-root lines of *E. globulus* and verified that IAA level was twofold higher in the easy rooting line, confirming the importance of IAA in AR.

A screen for chemicals that cause inhibition of cytochrome P-450 identified one chemical, MA65, which led to an increase in the number of roots of *Arabidopsis* seedlings and twofold higher IAA levels compared to the untreated *Arabidopsis* (Negishi et al., 2011). The observed phenotype was similar to the mutant *superrot2* (*sur2*) which contains high concentrations of free IAA (Delarue et al., 1998) due to a defect in the *SUR2* gene, which encodes the CYP83B1 protein, a cytochrome P450-dependent monooxygenase (Barlier et al., 2000). This increase in IAA production probably happens because cytochrome P450 inhibition blocks the synthesis of indole glucosinolates, providing more substrate (indole-3-acetaldoxime) for the biosynthesis of IAA (reviewed by Bak et al., 2001). The same chemical MA65 was effective for inducing AR in *E. globulus*, but the exact mechanism of action of the chemical in this species awaits further investigation.

The regulation of auxin levels can be done by conjugation of excessive auxin to inactive forms, preventing phytohormone accumulation in the tissue. Auxin degradation, e.g., by peroxidases, is another means of controlling the activity of these regulators. Auxins of different metabolic lability may be conjugated: high stability 1-naphthalene acetic acid (NAA), low stability IAA and moderate stability IBA (De Klerk et al., 1999). IAA can form conjugates with sugars, amino acids, and peptides and these forms are considered resistant to oxidases. IAA can be stored in higher plants as IAA conjugates which might be hydrolyzed depending on the plant demand for free auxin; IBA can also yield IAA by β-oxidation (Woodward and Bartel, 2005). Even if in some cases the conjugation process can be irreversibly inactivated by oxidation (Epstein and Ludwig-Müller, 1993), the most part of auxin conjugates are reversible (De Klerk et al., 1999). When IAA and IBA were exogenously applied to cuttings of *Pisum sativum* L. during adventitious root formation, conjugation of auxins with aspartic acid was the predominant route of metabolism, forming indole-3-acetylaspartic acid (IAAsp) and indole-3-butyrylaspartic acid (Nordström et al., 1991). The authors also verified that the levels of IBA remained high for longer time than those of IAA, indicating higher stability of IBA in rooting solution.

Some gene members of the *GH3* family are involved in the maintenance of auxin homeostasis, contributing to regulation of the auxin pool (Staswick et al., 2005; Chapman and Estelle, 2009). *GH3* genes encode IAA-amide synthetases, which act in the conjugation of physiologically active free IAA excess to amino acids (Staswick et al., 2005). In the moss *Physcomitrella patens*, knock-out of *GH3* genes increased the sensitivity to auxin causing growth inhibition (Ludwig-Müller et al., 2009). Altered auxin sensitivity was also observed in *Arabidopsis thaliana* by overexpression and insertional mutation of *GH3* genes (Staswick et al., 2005). Gutierrez et al. (2012) reported a crosstalk of IAA and JA in which AR-inhibitory JA levels are reduced by conjugation with amino acids through expression of *GH3.3*, *GH3.5*, and *GH3.6* auxin-induced genes, via the action of *ARF6* and *ARF8*, leading to increased number of adventitious roots. GH3 genes would be required for fine-tuning adventitious root initiation in the *Arabidopsis thaliana* hypocotyl, where JA homeostasis is under auxin control (Gutierrez et al., 2012). Curiously, JA accumulation at the cutting base has been shown to be an early, transient, and critical event for rooting of *Petunia* cuttings, and has been discussed to contribute to increasing cell wall invertases and sink strength at the cutting base (Ahkami et al., 2009). Brassinosteroids (BR) have been shown to exert a mild negative regulation of JA-induced inhibition of root growth (Huang et al., 2010). If this applies to AR as well, there could be an additional antagonist crosstalk between JA and BR, regulating the formation phase.

Cytokinins and ethylene have an overall inhibitory effect on induction, but can play a promotive effect during the first 24 h, when cytokinins start to drive cell cycle movement, culminating in mitotic processes (De Klerk et al., 1999; De Klerk, 2002), and ethylene may contribute to auxin transport regulation (Lewis et al., 2011) or to increase the number of auxin-responsive cells (De Klerk and Hanecakova, 2008). Corrêa et al. (2005) observed that kinetin inhibited AR if present during the induction phase in *E. globulus*. The cytokinin type-B response regulator PtRR13, a transcription factor that acts as positive regulator in the cytokinin signaling pathway, has been shown to negatively regulate AR in *Populus*; PtRR13 inactivation upon cutting severance due to the removal of root sources of cytokinin, would alleviate AR inhibition, allowing basipetally transported auxin to accumulate at

cutting base, promoting AR (Ramírez-Carvajal et al., 2009). Ethylene has been shown to promote adventitious root and inhibit lateral root development, predominantly by affecting auxin transport in distinct ways (Negi et al., 2010). Lateral root development inhibition by ethylene was linked to increased expression of PIN3 and PIN7 and auxin transport, preventing auxin accumulation maxima required for pericycle cell activation in roots; in contrast, adventitious root stimulation by ethylene in shoots was due to reduced auxin transport in these organs, favoring auxin accumulation and AR (Lewis et al., 2011). Stimulation of AR in flooded tomato plants was dependent on ethylene accumulation followed by auxin transport increase and allocation to flooded parts of the stem base. Local accumulation of auxin can cause further ethylene production, enhancing the process (Vidoz et al., 2010).

Strigolactones are also involved in adventitious root formation, mostly as repressors, by inhibiting the first divisions of founder cells independently of cytokinins, and perhaps negatively regulating basipetal auxin movement in *Arabidopsis thaliana* and pea (Rasmussen et al., 2012). Upon cutting severance, the content of strigolactones would reduce, since roots are a major source of these phytohormones.

Nitric oxide (NO) has also been proposed as a player in the control of AR. In cucumber, AR was favored by NO, acting downstream of auxin, possibly through different transduction pathways (Lanteri et al., 2009). Auxin-stimulated NO production would increase phosphate cyclic nucleotides cGMP (cyclic guanosine monophosphate) and cADPR (cyclic adenosine 5′-diphosphate ribose), triggering activation of Ca^{2+} channels in the plasmalemma. The release of phospholipids promoted by NO would provide substrates for phospholipases, whose activity and released products could further activate Ca^{2+} release to the cytosol and activate both calcium-dependent protein kinases (CADPKs) and mitogen-associated protein kinases (MAPK). These kinases would in turn lead to cell growth and differentiation associated with AR. NO-promoted AR was also reported for other species, including greenhouse–grown cypress (Lanteri et al., 2009) and *E. grandis* (Abu-Abied et al., 2012). In these studies a co-action of NO and auxin has often become apparent, with NO being induced by auxin. In sunflower, it was suggested that NO could participate with auxin in adventitious root initiation and expression (extension), whereas induction would depend only on auxin (Yadav et al., 2010). Studies on AR of *Tagetes erecta* (marigold; Liao et al., 2009), *Vigna radiata* (mung bean; Li and Xue, 2010), and *Chrysanthemum* (Liao et al., 2010) have suggested that H_2O_2 and NO may act together, possibly as parallel independent pathways dependent on Ca^{2+}, converging on the activation of MAPK cascades leading to AR. A novel interaction of NO and auxin has been shown at the level of NO dependent *S*-nitrosylation of TIR1 auxin receptor, enhancing TIR1-Aux/IAA binding and degradation of the latter, promoting auxin-mediated gene expression (Terrile et al., 2012). The extent of this interesting mechanism in the context of AR is a key research topic to be explored.

Gibberellins (GAs) are generally considered inhibitors of AR. This has been shown, for example, in poplar (Busov et al., 2006). Moreover, lateral root number and growth were promoted in plants with defects in GA production or perception, so that higher root mass and highly branched roots were produced. This inhibitory effect of GA on lateral root development has been partially attributed to changes in polar auxin transport (Gou et al., 2010). In contrast, initiation and elongation of adventitious roots was promoted by GA in deep water rice (Steffens et al., 2006). It is possible that GA may have an AR phase-dependent effect, being inhibitory to root induction and stimulatory to formation. ABA also acts as an inhibitor of lateral root development in *Arachis hypogaea* by blocking cell cycle progression (Guo et al., 2012). Inhibition of adventitious root formation step by ABA was also reported in deep water rice (Steffens et al., 2006).

Polyamines are nitrogen containing, polycationic, low molecular weight aliphatic compounds that can be found in meristematic and actively growing tissues. These metabolites (e.g., putrescine, spermidine, spermine) play various roles, mostly related to control of cell division, development, and stress responses. Because of their positive charges, polyamines are capable of binding to nucleic acids, proteins, and membranes, therefore potentially being able to interfere in processes such as gene expression, cell signaling, membrane stabilization, and modulation of some ion channels (Kusano et al., 2008). Polyamines have been treated as biochemical markers of AR because their concentration peak is consistently associated with the end of the induction phase, similar to auxins. In various unrelated species, AR or promptness to develop adventitious roots is often observed when polyamines peak at the end of adventitious root induction and are metabolized before or at the formation phase (Neves et al., 2002; Arena et al., 2003; Naija et al., 2008).

A tentative model summarizing some of the main data on phytohormonal control of AR is shown in **Figure 1**.

Given the importance of phytohormones, particularly auxins, to the control of AR, the next three sections will examine fundamental aspects of cell cycle control, root tissue differentiation, auxin transport, metabolism, and action. However, it must be emphasized that most of the knowledge presented in these sections is derived from investigations directed to general plant development or development of primary or lateral roots. Although it is clear that these processes are important in AR, their exact contribution in the specific context of the process is far from complete.

CELL CYCLE AND DIVISION – NEW MERISTEMS

Cell divisions in meristems depend on the cell cycle, which involves a mechanism governed by cyclin-dependent kinases (CDKs; Inzé and Veylder, 2006; De Veylder et al., 2007). The association of CDKs and cyclins is required for the induction of cell cycle progression, through phosphorylation of substrates at the transition points of some of its phases (Inzé and Veylder, 2006). G1–S transition is regulated by D-type cyclins (CYCD), which might also be involved in G2–M transition. A-type cyclins (CYCA) are present in S–M, whereas B-type cyclin (CYCB) act in G2–M transition and during M period (De Veylder et al., 2007). G1–S transition may be blocked by abscisic acid, causing inhibition of lateral root primordia initiation in peanut (Guo et al., 2012).

Cyclin-dependent kinases present in plants are A-type (CDKA) and B-type (CDKB), the latter being plant-specific. CDKB

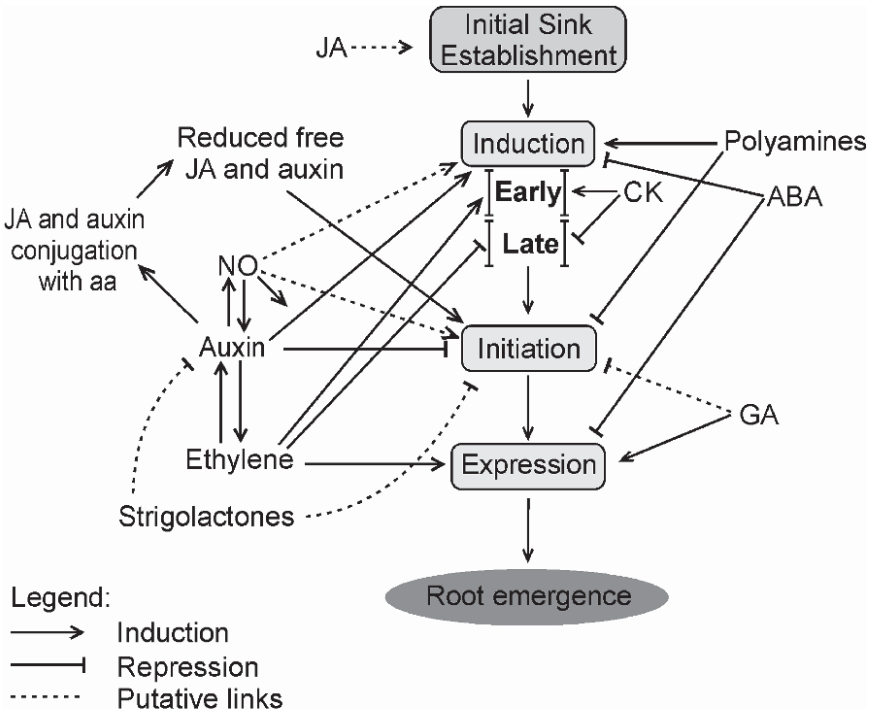

FIGURE 1 | Possible phytohormonal interactions during distinct phases of the adventitious rooting process. JA may promote initial carbohydrate sink establishment before induction or at its early moments. Induction phase is positively regulated by auxin, polyamines and, in early stage, by CK and ethylene. However, during late induction, cytokinin and ethylene act as negative regulators. ABA has a negative effect on AR induction. Initiation phase is inhibited by auxin, polyamines, and GA. JA and auxin are conjugated with aa, so the levels of these phytohormones decrease allowing the progress of the initiation phase. Strigolactones may repress auxin action by reducing its transport and accumulation, or may directly inhibit AR. In contrast, NO is regarded as a stimulator of AR, both during induction and initiation phases. Ethylene increases auxin transport, stimulates expression, but shows a direct repressor effect at induction phase, except perhaps at its early stage, as pointed out above. Auxin may also promote ethylene biosynthesis. Expression phase is induced by ethylene and GA, and suffers repression of ABA. Root emergence is the visible phenotype after the expression phase. Relative positions of phytohormone names within the scheme are not meant to represent differences in importance, but aim at better clarity of the layout. JA, Jasmonic acid; CK, cytokinin; ABA, abscisic acid; GA, gibberellin; NO, nitric oxide; aa, amino acids.

accumulation depends on the cell cycle period, specifically the B1 subclass in the S phase and after G2 until mid-M and B2 subclass, reaching a peak in G2 and M (Boudolf et al., 2006; De Veylder et al., 2007). Moreover, a plant homolog of the tumor suppressor Retinoblastoma (pRb), the RETINOBLASTOMA-RELATED (*RBR*) gene is considered a key cell cycle regulator downstream of the SCARECROW (*SCR*) patterning gene, a member of the GRAS family of transcription factors, acting in the control of cell division, differentiation and cell homeostasis (Wildwater et al., 2005; Borghi et al., 2010). The transcription factors *E2F* and *MYB3R* also take part in the cell cycle control, involved in activation/inactivation of the S-phase and M-phase genes, respectively (De Veylder et al., 2007). Cytokinin and auxin are the main hormones involved with cell proliferation and are indispensable for the progression of the cell cycle (Dewitte and Murray, 2003).

Plant development depends on meristem growth, which happens when cell division predominates over differentiation. The root meristem size is controlled by the balance between cell division and differentiation, where cytokinins and auxins act antagonistically and play important roles (Dello Ioio et al., 2007, 2008; Moubayidin et al., 2009, 2010). In *Arabidopsis*, the *short hypocotyl 2* (*SHY2*) gene acts as a negative regulator of auxin signaling (Tian et al., 2002) by forming heterodimers with *ARF* transcription factors and thus avoiding the activation of auxin-responsive genes. *SHY2* expression is activated by the presence of cytokinins via the route of AHK3 (*Arabidopsis* histidine kinase 3) receptor kinase/cytokinin-responsive *ARR1* transcription factor, and leads to negative regulation of *PIN* genes, involved in the efflux of auxin, which consequently causes a reduction in the root meristem size (Dello Ioio et al., 2008). Moreover, auxins

can cause *SHY2* degradation, and promote the expression of *PIN* genes (Dello Ioio et al., 2008). Furthermore, the transcription factor *ARR12* and GAs also seem to participate in this regulation, *ARR12* inducing a low level of *SHY2* expression and GAs repressing expression of *ARR1* during post-germination meristem growth (Moubayidin et al., 2010).

The root apical meristem is composed of sets of self-renewing and undifferentiated stem cells that allow continued root growth. The quiescent center (QC) takes part in maintaining this condition by supporting meristematic identity of the initial cells around it (Van den Berg et al., 1997; Osmont et al., 2007; Arnaud et al., 2010). The QC cells form part of a region that has a low rate of mitosis, and are histologically distinct from neighboring cells (Doerner, 1998). QC serves as a reservoir of cells for regeneration and ensures the persistence of the apex meristem, as they have self-renewal and self-maintenance capacities. Hormonal activity is important for the QC maintenance and organization (Sabatini et al., 1999; Ortega-Martínez et al., 2007). Reporter genes fused to promoters regulated by auxin were visualized with maximum expression in the position of the QC and root columella (Sabatini et al., 1999). Data obtained by Ortega-Martínez et al. (2007) suggest that ethylene promotes cell division in the QC, indicating that auxin alone would not be sufficient to carry out this function. Surrounding QC, initial cells perform stem cell-like divisions to generate a new initial and a daughter cell, so that the meristem gives rise to all different cell types (Van den Berg et al., 1997).

Some transcription factors, such as *SCR* and *SHORTROOT* (*SHR*), also belonging to the GRAS family of proteins, have crucial role in maintaining the meristematic cells pluripotent identity (Sabatini et al., 2003; Cui et al., 2007). *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) are also involved with meristem maintenance, are induced by auxin, and act in parallel with *SHR* and *SCR*, encoding transcription factors *AP2*-like (Aida et al., 2004). *SCR* expression appears to depend of the gene *PDR2* (Ticconi et al., 2009), acting indirectly on QC maintenance. The distribution of *PLT* mRNA is associated with the peak of auxin in stem cells and QC in root meristem (Sabatini et al., 1999). The homeobox transcription factor *WOX5* (*WUSCHEL-RELATED HOMEOBOX 5*), root homologue of the shoot *WUSCHEL* (*WUS*), also has a function in the stem cell maintenance and signaling (Sarkar et al., 2007; Miwa et al., 2009; Stahl et al., 2009).

The involvement of some of these transcription factors in AR in cuttings of tree species has been described. An approach based on cDNA subtractive libraries from rooting competent cuttings of *Pinus radiata* and *Castanea sativa* treated or not with exogenous auxin (Sánchez et al., 2007) yielded data supporting the involvement of clones with homology to *SCR* (*SCR*-like or *SCL*). The content of the corresponding mRNA of these genes increased in both species upon auxin exposure within the first 24 h of the rooting process, coinciding with cell reorganization preceding divisions and establishment of defined root primordia. In *Pinus radiata*, an *SHR*-related clone was identified with an expression pattern similar to that of *SCL*, except for the fact that it was auxin-independent, possibly playing a role in root meristem formation and maintenance, as well as in the cambium zone of hypocotyls (Solé et al., 2008). The expression of *SCL* in *C. sativa* cuttings of juvenile and mature stages was examined in detail (Vielba et al., 2011). A combination of quantitative real time polymerase chain reaction (PCR) and *in situ* hybridization showed that *CsSCL1* was upregulated by auxin, localizing more strongly in the cambium layer and derivative cells in rooting competent shoots, whereas for root incompetent shoots its signal was more diffuse and evenly distributed in the phloem and parenchyma (Vielba et al., 2011). The authors suggest that *CsSCL1* may determine which cells will engage in the root differentiation route, athough they observed that expression of this gene was also present in lateral roots and axillary buds.

Recently, *AINTEGUMENTA LIKE1* (*PtAIL1*), a member of the *AP2* family of transcription factors, has been shown to be associated with cell division and further establishment of adventitious root primordia in *Populus trichocarpa* (Rigal et al., 2012). Transgenic poplar overexpressing *PtAIL1* displayed higher number of adventitious roots, whereas RNA interference (RNAi) downregulation of the same gene transcript resulted in delayed AR. A number of genes were co-regulated with *PtAIL1* based on microarray and comparative analyses of modified poplar lines up or downregulated for the *AP2* transcription factor, included among these additional transcription factors, such as *AGAMOUS-Like6* and *MYB36* (Rigal et al., 2012).

THE CENTRAL ROLE OF AUXINS: TRANSPORT, CONTROL OF LOCAL CONCENTRATION, TIMING, AND METABOLIC DYNAMICS

Auxins are very important for determining pattern in plants. Their spatial distribution is determinant for proper formation of the axis along the plant body. Auxin transport has two main forms: (a) rapid (up to 10 cm per h), often referred to as non-polar, bidirectional transport in the phloem sieve elements, (b) slow (approximately 10 mm per h) or polar, mediated by transporters (Kerr and Bennett, 2007), mostly in vascular parenchyma. Rapid transport in the phloem conducting cells essentially obeys source–sink relations and involves both free IAA and inactive conjugates (Friml and Palme, 2002). Studies with radiolabeled IAA applied to pea leaves indicated that both transport pathways may communicate, at least from the non-polar to the polar system (Cambridge and Morris, 1996). There is also evidence that phloem-based transport may become relatively more important than polar transport, at least in roots, at later stages of seedling development (Ljung et al., 2005).

The polar transport of the major endogenous auxin IAA has specific carriers, which allow intercellular auxin flow and are well-known in *Arabidopsis*. In stems, the transport is active, polar, and basipetal. According to the chemiosmotic model (Raven, 1975), there is a pH gradient between the intra- and extracellular medium, generated by the action of proton pumps in the plasma membrane, which drive protons into the apoplast, making it acidic. In the apoplast, IAA can be found both in anionic and protonated forms, the latter being more lipophilic and capable of easily diffusing through the plasma membrane (Woodward and Bartel, 2005; Zazimalová et al., 2010). On the other hand, the anionic form lacks this capacity and, for it to enter the cell, the action of auxin influx carriers is required. These carriers are amino acid permease-like proteins of the AUX1/LAX family (reviewed in Vieten et al., 2007). These proteins act as H^+/IAA^-

symporters and may participate in lateral root emergence and root hair development (reviewed by Vanneste and Friml, 2009).

Members of the PIN Formed (PIN) protein family are involved with auxin efflux and their asymmetric distribution in the cells is fundamental to the characteristic polar basipetal transport along the stems. The correct localization of PIN proteins is determined by its phosphorylation status, defined by the balance between the kinase protein PID (PINOID) and the phosphatase PP2A. In the case of emerging primordia, the expression of PID is activated, turning PIN protein to a phosphorylated form, leading to its apical localization in the cell. On the other hand, in most situations, PP2A is more active than PID, leading to dephosphorylated PIN protein, resulting in a basal localization in the cell (Michniewicz et al., 2007). Furthermore, the NPA-binding protein and actin filaments of the cytoskeleton also function in the correct positioning of the PIN proteins (Muday and DeLong, 2001). This family of transmembrane proteins has eight members in *Arabidopsis* which are considerably homologous and functionally redundant, being involved in tropisms, embryo development, root meristem patterning, organogenesis, and vascular tissue differentiation (reviewed by Krogan and Berleth, 2007 and Vanneste and Friml, 2009). The Multidrug/P-glycoproteins of the ABCB (ATP-binding cassette B) transporter family (ABCB/MDR/PGP) also contribute to auxin transport, being more closely related to non-polar auxin efflux and maintenance of the main auxin fluxes (Geisler and Murphy, 2006). These transporters may also play a possible role in short-distance lateral auxin movement.

Basipetal auxin transport is also affected by the red/far-red (R:FR) light ratio (Morelli and Ruberti, 2002). In open daylight (high R:FR), auxin moves from the shoot to the root mainly through the central cylinder. However, in shade conditions (low R:FR), a new route, by the outer cell layers, is preferred. This alternative route is less effective and leads to increase in auxin levels in cell layers external to the central cylinder in the stem, enhancing cell elongation in this organ. Consequently, less auxin is transported through the vascular system, decreasing vascular differentiation and the auxin content reaching the root.

Recent findings revealed the function of a new family of putative auxin transporters, the PIN-LIKES (PILS; Barbez et al., 2012; Feraru et al., 2012). These proteins are considered evolutionarily older than PIN proteins and probably preceded the PIN-dependent auxin transport (Feraru et al., 2012), but are similar to PIN family members and also contain the auxin transport domain, predicted to carry out this function. The PILS proteins are localized in the endoplasmic reticulum (ER) and are involved in the intracellular transport of free IAA from cytosol to ER (Barbez et al., 2012; Feraru et al., 2012). According to these findings, PILS activity promotes auxin accumulation in the ER by increasing amide auxin conjugates, reducing free auxin levels. This action could be involved in a compartmentalized-type regulation of auxin metabolism (Barbez et al., 2012). The PIN family member PIN5, which is localized in the ER, is also suggested as an intracellular auxin carrier, stimulating the formation of auxin amino and ester conjugates and their transport to the ER (Barbez and Kleine-Vehn, 2012).

Auxin amino acid and glucose conjugates can also be stored in the vacuole (Ueda et al., 2011). The transport into this cellular compartment has been suggested as an action of the ABC transporter AtMRP5 (*Arabidopsis thaliana* multidrug resistance 5). *Atmrp5-1* mutants, defective in *MRP5* expression, have shown higher free auxin levels and inhibition of root elongation (Gaedeke et al., 2001). This could be due to increased levels of free auxin in the cytoplasm of root cells caused by a disruption in moving auxin conjugates away from the cytoplasm.

Considering other auxins, such as the endogenous IBA and the synthetic auxin (NAA), relatively little is known about transport and metabolism. IBA is more stable than IAA and persists for longer in plant tissues (De Klerk et al., 1999), being basipetally transported in seedling hypocotyls (Rashotte et al., 2003), similarly to IAA. However, IBA seems not to be transported in inflorescences, unlike IAA (Rashotte et al., 2003). Mutations affecting IAA transport did not cause significant effects in IBA transport. The differences between IBA and IAA transport suggest that IBA might use distinct transporters from those used to move IAA (Strader and Bartel, 2011). NAA is more stable than the above auxins and is probably transported by different carriers, as revealed by *aux1* loss-of-function mutants, which respond normally to NAA (Yang et al., 2006).

The formation of auxin gradients, originated by the combined processes of biosynthesis, conjugation, and degradation, as well as inter- and intracellular transport, independently of type, is relevant for both plant morphogenesis and determination of tissue patterns (Vanneste and Friml, 2009; Overvoorde et al., 2010; Simon and Petrasek, 2011). Previous studies of PIN expression and auxin distribution in *pin* mutants showed that PIN proteins are the major players in directional distribution networks that mediate auxin maxima and gradients during different developmental processes (reviewed by Vieten et al., 2007). In the developing embryo, the localization of PIN proteins assumes positions of auxin accumulation along the stages of development and form auxin convergence points, necessary for cotyledon initiation and positioning at the late globular stage (reviewed by Krogan and Berleth, 2007). In shoot apical meristems, auxin promotes *PIN1* expression, which generates auxin accumulation at the sites of leaf primordia formation. These, once established, promote a drain of auxin, which will accumulate at a certain distance from the early primordia, enabling the phyllotactic pattern to be established (reviewed by Berleth et al., 2007).

Recent evidence points to a possible role of APY (apyrases) in regulating auxin transport (Liu et al., 2012). Exogenous ATP is capable of inhibiting auxin transport and gravitropic response in *Arabidopsis*. Apyrases (triphosphate diphosphohydrolases) are enzymes that participate in limiting ATP content. Polar IAA transport in roots and hypocotyls was reduced in *apy2* null mutants when these were suppressed of *APY1* (apyrase 1) expression by an estradiol-induced RNAi. Basal portions of APY-suppressed hypocotyls accumulated less free IAA and morphological defects were seen in roots with the same genetic modification. Problems in gravitropic asymmetry of auxin content were detected by means of DR5::GFP constructs in APY reduced plants, either genetically or treated with APY chemical inhibitors. The relevance of apyrase participation in auxin transport during AR is presently unclear and should be object of further investigation.

Auxin gradients are also very important for root organogenesis and both primary and lateral root formation are issues that

had good advances in the last decades. Studying root outgrowth in *Arabidopsis*, Blilou et al. (2005) concluded that PIN-mediated modulation of auxin distribution controls both cell division and elongation, affecting meristem, elongation zone, and final cell sizes. Dubrovsky et al. (2008) revealed a spatial and temporal correlation of auxin maxima with developmental reprogramming, resulting in lateral root initiation (LRI). The sites and frequency of LRI are controlled by variations in auxin concentration in pericycle cells, which might be correlated with changes in PIN protein localization upon gravistimulation (Benkova and Bielach, 2010). These events will culminate in lateral root primordia formation.

Genetic studies revealed that *pils2pils5* double loss of function mutant had higher free auxin levels, increased hypocotyl growth and presence of lateral roots, which were longer and more abundant than in the PILS5 gain of function phenotype. This evidence suggests that PILS2 and PILS5 could have specific functions in the cellular regulation of root growth (Barbez et al., 2012).

However, relatively little is known about the effects of polar and non-polar auxin transport during adventitious root formation. Using inhibitors of polar auxin transport, various investigations in cuttings or de-rooted seedlings have provided evidence for a significant contribution of this type of transport to AR (e.g., Nordström and Eliasson, 1991; Liu and Reid, 1992; Koukourikou-Petridou and Bangerth, 1997; Guerrero et al., 1999; Garrido et al., 2002; Nicolás et al., 2004). Few studies analyzing the expression of genes encoding auxin carriers during adventitious rhizogenesis were conducted in de-rooted pine seedlings (Brinker et al., 2004), intact rice plants (Xu et al., 2005), carnation cuttings (Oliveros-Valenzuela et al., 2008; Acosta et al., 2009), and mango cotyledon segments (Li et al., 2012). The studies with carnation and mango showed the requirement of increased expression of auxin transporters and increase of polar auxin transport during the induction and formation phase of AR. However, in the case of pine seedling cuttings, increased expression was linked to root formation (Brinker et al., 2004). In rice, the expression of *OsPIN1* was also important during root formation (Xu et al., 2005). Taken together, these findings corroborate the role of auxin in controlling organogenesis, but more studies are necessary to clarify the effects of auxin carriers in AR, mainly in woody species. A summary of mechanisms and factors possibly contributing to transport and local concentration of auxin during AR is illustrated in **Figure 2**.

Considering cuttings used for vegetative propagation, the progressive accumulation and local concentration of auxin in the base of the cuttings seems to be important to generate the peak necessary for starting the rooting process (Acosta et al., 2009) and often this can be facilitated by exogenous application of auxins in recalcitrant species. Meanwhile, recent studies indicate that basipetal auxin transport and auxin accumulation in the rooting zone may be negatively regulated by strigolactones (Rasmussen et al., 2012). This phytohormone class could act reducing auxin levels in the pericycle, decreasing root initiation. This could be a direct effect or via regulation of the amount of local auxin levels, presumably involving impairment of the rooting zone (Rasmussen et al., 2012). Thus, although auxin is the main hormone involved in AR, it clearly does not act alone, since crosstalk between several phytohormones is necessary for the success of this process.

AUXIN RECEPTORS AND ACTION MECHANISMS

Even though auxin is known to play a central role in AR, the specific mechanisms of auxin action in this process are far from being understood. However, considering plant development in general, in the past decade a vast amount of data was reported regarding auxin perception (Mockaitis and Estelle, 2008). At the cellular level, auxin induces various rapid changes in cell physiology, such as membrane depolarization, apoplast acidification, cell wall loosening, activation of plasma membrane ATPases, and control of gene expression (Scherer, 2011). Although many of the signaling pathways leading to the responses mediated by auxin are still to be elucidated, significant knowledge on nuclear receptors for auxin is available. In the recent literature two different proteins are accepted as true auxin receptors, ABP1 and TIR1/AFB (auxin signaling F-box) proteins. The TIR1/AFB-family of F-Box protein members were the first authentic auxin receptors to be discovered (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). These proteins form nuclear regulatory complexes called SCF-E3-ubiquitin ligases and are responsible for the targeted degradation of a family of transcriptional repressors called AuxIAA proteins (Gray et al., 2001).

AuxIAA proteins are transcriptional repressors that act via dimerization with auxin-responsive transcription factors called ARFs (auxin-responsive factors). Upon binding of auxin to the F-Box (TIR1/AFB) subunit of the SCF TIR1/AFB complexes, their affinity toward the domain II of AuxIAA proteins is greatly enhanced with auxin acting as a "molecular glue" bringing the two proteins together; this binding triggers the ubiquitination of the AuxIAA by the SCF complex leading to its destruction by the 26S proteasome (Tan et al., 2007; Chapman and Estelle, 2009; Maraschin et al., 2009). The degradation of the transcriptional repressor releases the transcriptional activity of ARFs and auxin-responsive genes are expressed (**Figure 3**). The control of AR in intact seedlings of *Arabidopsis* by auxin, for example, involves activation of transcription factors *ARF6* and *ARF8* (Gutierrez et al., 2009). The TIR1/AFB family of auxin receptors is composed of 6 distinct members in *Arabidopsis* (namely TIR1, AFB1, AFB2, AFB3, AFB4, and AFB5), all of which are able to bind auxins specifically and show auxin-enhanced binding to AuxIAA proteins (Mockaitis and Estelle, 2008). Although much of the phenotypes of TIR1/AFB mutants indicate a large degree of redundancy, some specific features have already been identified. For example, TIR1 and AFB2 display a higher affinity for AuxIAA proteins compared to other members.

On the basis of the phenotype of single mutants, TIR1 appears to make the largest contribution followed by AFB2. Both AFB1 and AFB3 contribute to auxin response, but this contribution is only apparent in higher order mutant combinations. The *afb4* and *afb5* mutants are more resistant than *tir1* to picolinate auxins such as picloram, suggesting alternative substrate specificity (Parry et al., 2009). All of the defects observed in *afb4-2* mutant seedlings can be simulated in wild-type seedlings by treatment with auxin, indicating that AFB4 acts as a negative regulator of auxin-dependent processes. The *afb4-2* mutants have shorter roots

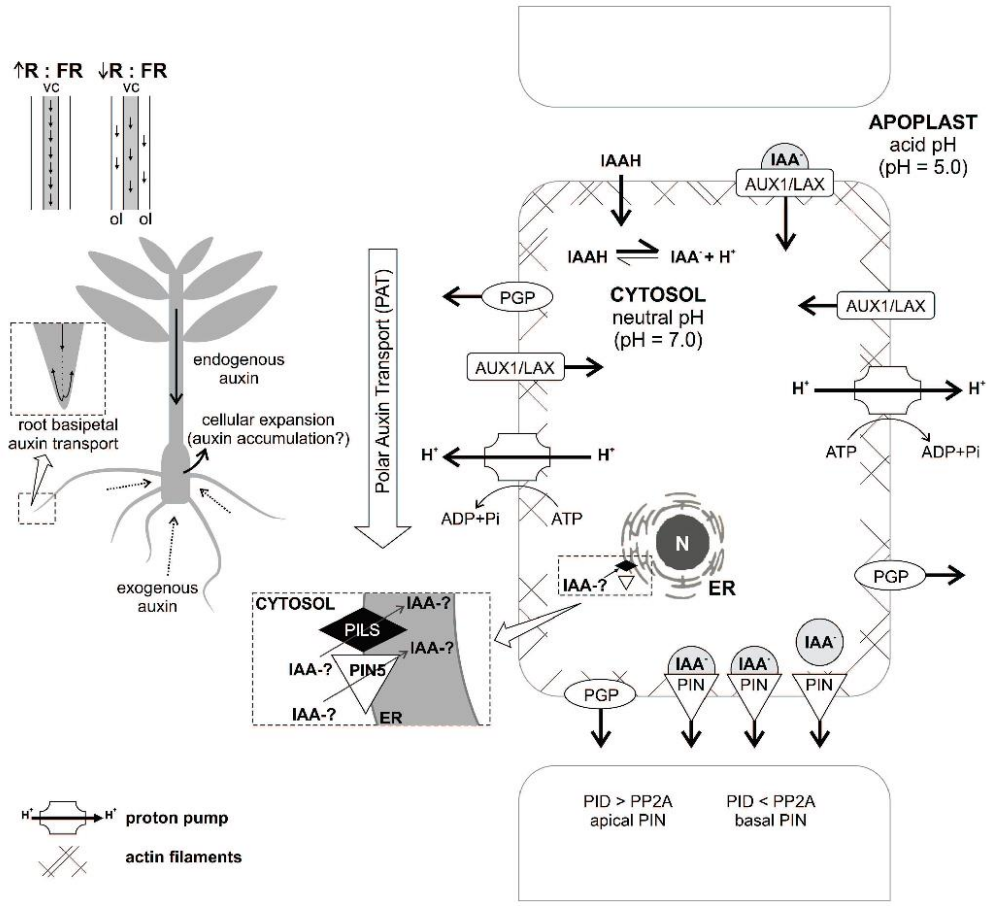

FIGURE 2 | Concept of auxin transport processes probably involved in AR. Active, polar, and basipetal auxin transport contributes to auxin accumulation in the rooting zone of a cutting. Endogenous auxin is transported through the stem in an active, polar, and basipetal way. In daylight, when there is high red:far-red ratio (R:FR), the transport is mainly through the central cylinder (vc). In shade conditions, with low R:FR ratio, a less efficient route by the outer cell layers (ol), is preferred. When exogenous auxin is applied to the medium, it is absorbed by diffusion, which can cause cellular expansion in the basal part of the plant, perhaps due to auxin accumulation. Once adventitious roots are established, they provide a follow up to stem basipetal transport, continuing through the stele as acropetal transport in roots and then basipetal through the subepidermal cell layers of the newly formed organs. Intercellular auxin transport involves specific carriers: AUX1/LAX proteins, related with auxin influx; PGP proteins, related with auxin efflux and lateral transport; and PIN proteins, which have an asymmetrical distribution and allow directed auxin efflux. The PIN correct localization in the cell and the route of the auxin flow is determined by the balance between the kinase protein PID and the phosphatase PP2A. Concerning intracellular transport, auxin can be transported into the endoplasmic reticulum (ER) by the action of PILS proteins and PIN5, which reduce free auxin levels and increase auxin conjugates. It remains to be elucidated if auxin conjugates can be formed into the ER or just in cytosol. For more details, see text. N – nucleus; IAA-? – auxin in free or conjugated form.

and display a higher lateral roots/primary root length ratio than wild-type seedlings, suggesting that AFB4 has a role in anchor or adventitious root production (Greenham et al., 2011).

The expression patterns of the TIR/AFB genes are highly overlapped and not auxin-responsive, with the most significant regulation so far described being due to post-translational repression of TIR1, AFB2, and AFB3 by miR393 upon pathogen attack (Navarro et al., 2006). The structural specificity of auxin binding to TIR1 has been investigated to atomic level via X-ray crystallography. The details of this interaction provided valuable information

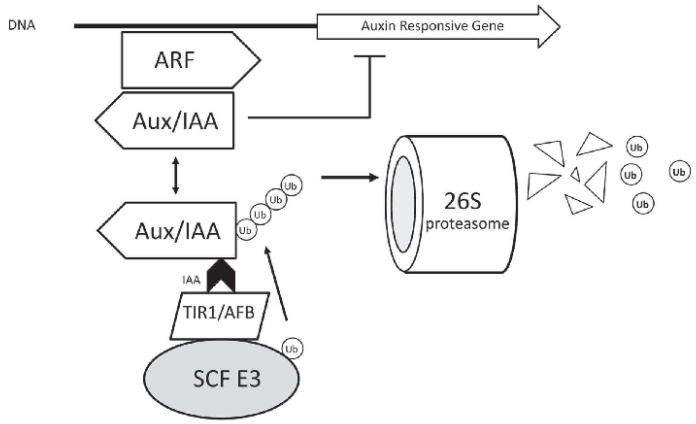

FIGURE 3 | Auxin action mechanism based on TIR1. Upon binding of auxin to the F-Box (TIR1/AFB) subunit of the SCF TIR1/AFB complexes, their affinity toward the domain II of AuxIAA proteins is greatly enhanced with auxin acting as a "molecular glue" bringing the two proteins together; this binding triggers the ubiquitination of the AuxIAA by the SCF complex leading to its destruction by the 26S proteasome. The degradation of the transcriptional repressor releases the transcriptional activity of ARFs and auxin-responsive genes are expressed. E3 – ubiquitin protein ligase.

to understand the mechanism of binding and structural details for active auxins (Tan et al., 2007). Recently, intensive efforts have been successful in designing TIR1-specific auxin antagonists, such as BH-IAA (tert-butoxycarbonylaminohexyl-IAA) and auxinole (Hayashi et al., 2008, 2012). These molecules specifically interact with the auxin-binding pocket on the TIR1 protein, blocking the access to the domain II of AuxIAAs. By testing the effects of blocking TIR1/AFB responses one is able to determine the contribution of TIR1/AFB-dependent transcriptional responses on whole plant phenotypes such as adventitious root formation. Although such inhibitors were designed based on the *Arabidopsis* TIR1 protein, the conservation of the TIR/AFB-AuxIAA mechanism goes all the way to mosses such as *Physcomitrella* sp., broadening the application of chemical tools to investigate physiological events in many unrelated plant species. A scheme on the TIR1 model of auxin action is shown in **Figure 3**.

Auxin-Binding Protein 1 was the first auxin-binding protein discovered, about 40 years ago (Hertel et al., 1972). ABP1 binding to auxin is highly specific and pH-dependent. Null *abp1* mutants are embryo-lethal and the functions of ABP1 on auxin signaling remained obscure since its discovery (Tromas et al., 2010). With the analysis of multiple TIR1/AFB mutants it became clear that nuclear perception of auxin and the degradation of AuxIAAs cannot account for all auxin-dependent cellular responses (Dharmasiri et al., 2005b). It is believed that plasma membrane localized ABP1 acts as an extracellular auxin receptor inducing rapid responses on the membrane and cytosol (Shi and Yang, 2011). The mechanism through which ABP1 is able to transduce the auxin signal to other molecules is still unknown. ABP1 has emerged as the receptor responsible for fast, protein synthesis independent, membrane and cytosolic responses to extracellular auxin concentrations. Many early auxin-dependent responses are attributed to ABP1 signaling: a fast (few milliseconds) drop on plasma membrane polarization, K^+ influxes (0.5 s), rise in cytosolic Ca^{2+} (30 s), phospholipase A activation (2 min), MAPK activation (5 min), among other rapid auxin-triggered responses (Tromas et al., 2010). Recently, it has been demonstrated that auxin binding to ABP1 is able to inhibit clathrin-dependent PIN protein endocytosis at the plasma membrane (Robert et al., 2010). It has been proposed that ABP1 would be the receptor to regulate auxin transport throughout the plant whereas the TIR1/AFB proteins would be the receptors responsible for intracellular auxin transcriptional responses.

The current scenario suggests that ABP1 and TIR1/AFB proteins are components of a two-receptor mechanism for auxin responses (Scherer, 2011) with ABP1 being an early sensor of apoplastic auxin concentrations regulating auxin transport and early, fast, transcriptional-independent, membrane and cytosolic responses, such as apoplast acidification and early elongation. TIR1/AFB would be the receptors responsible for the perception of nuclear and cytosolic auxin concentrations, involved in later, long term developmental responses, triggering transcriptional adaptive responses to the signal input generated by the ABP1-regulated auxin transport (Scherer, 2011). The relative participation of these auxin receptors in AR is currently unclear, but could putatively require sequential and conjunct activity in a rooting phase-dependent fashion. A putative model of ABP1 action and its interaction with TIR1 is shown in **Figure 4**.

miRNA CIRCUITRY

Several miRNAs were reported as involved in root development modulation, reinforcing the growing awareness that miRNAs play pivotal roles in many biochemical or biophysical processes

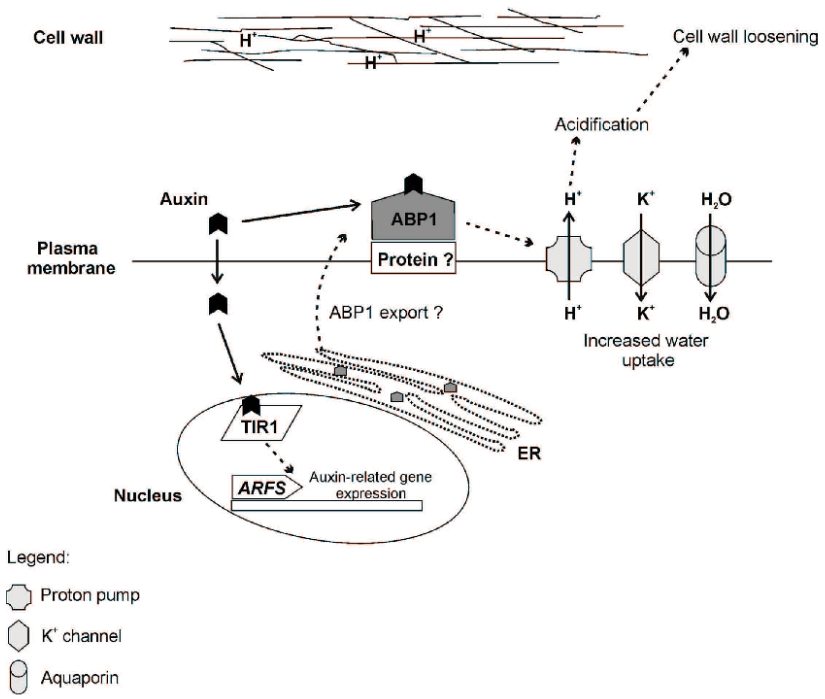

FIGURE 4 | Summary of the auxin perception by ABP1 and TIR1 receptors. Rapid auxin responses are thought to be mediated by ABP1. Auxin is perceived by ABP1 at the outer surface of the Plasma membrane. In this case, ABP1 is anchored by an unknown membrane-associated protein (Protein?). In flowering plants, ABP1 is mainly located at the endoplasmic reticulum (ER) due to an ER retention motif (KDEL). Currently, it is not known how ABP1 is exported to the plasma membrane. Binding of auxin to ABP1 induces several events including activation of proton pumps, which culminates in acidification of the outer space and contributes to cell wall loosening. There is also activation of potassium inward channels, which increase the intracellular K^+ and, consequently, lead to increased water uptake, allowing cell expansion. For slower responses, auxin is perceived by the F-box TIR1, which directs the auxin repressors Aux/IAAs for degradation and releases the auxin response factors (ARFS) to induce auxin-related gene expression.

in planta (Meng et al., 2010). Gutierrez et al. (2009) established that microRNAs miR160 and miR167 were implicated in adventitious root formation through auxin signal further transduced by their downstream ARF targets (Meng et al., 2010). ARF6 and ARF8 targeted by miR167 were shown to be positive regulators of shoot-borne root emergence, whereas ARF17, a target of miR160, was a negative regulator (Gutierrez et al., 2009). ARF17 affects both miR167-dependent and independent regulation of ARF6 and ARF8. Conversely ARF6 represses ARF17 by activating miR160, whereas ARF8 directly represses ARF17. Finally, miR167 and miR160 appear to have opposite roles in controlling the expression of the auxin homeostatic enzyme GH3, which are required for fine-tuning adventitious root initiation in the *Arabidopsis thaliana* hypocotyl, acting by modulating JA homeostasis (Gutierrez et al., 2012). Thus miR160 targets reduce active auxin and AR, whereas miR167 targets act in opposite way (Rubio-Somoza and Weigel, 2011).

ROOT GROWTH AND EMERGENCE THROUGH THE STEM

Adventitious root primordia, with apical meristem and differentiation of the basic root body, are formed and grow through the cortex toward the surface of the stem. Ethylene seems to be important to induce cell wall loosening and facilitate root passage through the stem tissues (Vidoz et al., 2010). Once newly formed roots reach the surface of the stem, a disruption of the epidermis and additional cell wall loosening take place, leading to root emergence. Afterward, the stem itself develops a periderm around the opening of each of the adventitious roots formed, important for protection against microorganism attack and drought (Hatzilazarou et al., 2006). The vascular reconnection between newly formed roots and the shoot is then fully established, allowing root nutrition, hydration, and growth (Hatzilazarou et al., 2006). In this process of vascularization and vascular connection, auxins and cytokinins are relevant for phloem and xylem tissue differentiation. In deepwater rice,

a model has been proposed for phytohormonal interactions regulating root emergence. In this model, ethylene would promote epidermal programmed cell death, root emergence and elongation, and these processes would be co-stimulated by GAs and inhibited by ABA (Steffens et al., 2006).

FINAL REMARKS AND PERSPECTIVES

In spite of a large volume of information on AR accumulated over the last few decades, a complete picture of this key developmental process is far from sight. Phytohormones are certainly at center stage in the conundrum of factors that influence AR. Not surprisingly, their actions involve significant degree of crosstalk, adding to the complexity of the process. In addition, a relevant participation of carbohydrate metabolism and mineral nutrition is evident, frequently modulating phytohormone-based controls. The wound response associated with the typical AR protocols add other players such as JA, H_2O_2, phenolics, and the action of enzymes on phytohormone content.

Faster advances of significant impact (both fundamental and practical) in the field of AR may depend on a number of strategies and scientific decisions for possible consideration by researchers. Although model species are a highly valuable tool for unveiling complex developmental processes, it is probably useful to somewhat diversify research objects, at least a couple of species for each general type of plant material (small herbaceous, monocots and dicots, horticulture/flower like crops, fruit crops, forest species, angiosperms, and gymnosperms) and within these seek for a few genotypes of easier or harder-to-root phenotype, in order to gain a better view of the process. A shift or at least a better balanced focus between research aiming at cuttings and at mother plant status and its implications on subsequent rooting may help achieve a more global understanding/predictable manipulation of AR. The recognition and identification of the main phases of AR should be taken into account in the various materials under investigation, for the process is quite dynamic and requisites and needs change along the process of re-establishing a root system.

From the experimental view point, solid associations must be established between structure and function, with a refinement of sampled cell types and tissues (cell/tissue-specific gene expression, proteomics, and metabolic profiling), always with a kinetic perspective of the successive phases. Another key association in the realm of methodologies is to maintain an open dialog between the basic and applied research with mutual benefits arising from exchanging operational strategies, investigation methods, and process modulation tools. Finally, a conjunct effort to establish clearer boundaries between lateral and adventitious root development and to seek an integrated look at these two processes within the various plant materials investigated may help clarify some of the contradictory data populating the rooting literature.

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ANEXO 2

The role of non-peptide factors and dimerization in the interaction of N-glycosylated Auxin Binding Protein 1 (ABP1) with different auxins as assessed by molecular dynamics simulations

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Artigo apresentado em sua formatação preliminar a ser submetido futuramente.

The role of non-peptide factors and dimerization in the interaction of N-glycosylated Auxin Binding Protein 1 (ABP1) with different auxins as assessed by molecular dynamics simulations

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Keywords: Auxin, ABP1, Maize, *Arabidopsis*, molecular dynamics, glycan

Introduction

Auxin is a critical phytohormone for plant growth, development and responses to changes in the environment, influencing several aspects of cell division, elongation and differentiation (Woodward and Bartel, 2005; Teale et al., 2006; Tromas et al., 2009). Auxin regulates different processes in plants, such as tropic responses to light and gravity (Luschnig et al., 1998; Friml et al., 2002; Friml, 2003), root (Aloni et al., 2005, Malamy, 2005) and shoot architecture (Bennett et al., 2006; Marlgorzata and Leyser, 2011), adventitious rooting (da Costa et al., 2013), organ patterning and vascular development (Blilou et al., 2005; Scarpella et al., 2006, Woodward and Bartel, 2005). Indole-3-acetic acid (IAA) is a natural form of auxin and is predominant in plants, whereas 1-naftalen acetic acid (NAA) is a synthetic form, with higher stability (Dunlap et al., 1986; De Klerk et al., 1997; Teale et al., 2006).

Currently, the TIR1/AFB family of F-box proteins and the Auxin Binding Protein 1 (ABP1) are widely accepted as true auxin receptors. The TIR1/AFB family promotes the activation or inhibition of auxin-related genes transcription (Santner and Estelle, 2009). Besides the nuclear TIR1/AFB auxin receptors, Auxin Binding Protein 1 (ABP1) has been identified as an extracellular auxin receptor. ABP1 is located both in the Endoplasmic Reticulum (RE) and in the apoplast close to the plasma membrane (Shimomura et al., 1999; Bauly et al., 2000; Tromas et al., 2010). Null mutations of ABP1 in *Arabidopsis* are embryo-lethal (Chen et al., 2001) indicating that ABP1 plays a crucial role in embryogenesis. However, the real role of ABP1 is still unclear, recent findings identified null alleles of ABP1 that develop exactly like the wild type plants under normal growth conditions (Gao et al., 2015). Several reports indicate that ABP1 is also involved in regulation of auxin responsive gene expression (Braun et al., 2008; Tromas et al., 2010; Shi and Yang, 2011). The signal cascade triggered by ABP1 includes activation of ATPase protons pumps, acidification of the extracellular space and activation of input K+ channels, which may eventually lead to changes in gene expression (Tromas et al., 2010; Scherer et al., 2011). ABP1 promotes clathrin-mediated endocytosis of PIN auxin efflux carriers, acting in the regulation of auxin transport. Binding of auxin inhibits ABP1 action, thereby stabilizing PINs at the plasma membrane, providing a mechanism for auxin to enhance its own efflux (Robert et al., 2010).

The structure of ABP1 from maize was previously established at 1.9 Å resolution (Woo et al., 2002). ABP1 is a dimer in crystal (Woo et al., 2002) and in solution, with one auxin binding site per dimer (Shimomura et al., 1986). The affinity to NAA is pH dependent, with an optimum binding at pH 5.0 - 5.5, and the binding protein becomes unstable below pH 6.0 (Löbler and Klämbt, 1985; Shimomura et al., 1986). The calculated free energy of binding of IAA and NAA were compared to experimental data and proved that NAA has a stronger interaction with ABP1 than IAA (Grandits and Oostenbrink, 2014). In each subunit of ABP1 in maize there is a glycosylation site at Aparagine95

(Asn95) and a Zinc ion (Zn^{+2}) coordinated with the histidines His57, His59 and His106, the glutamate Glu63 and with one water molecule (Woo et al., 2002). The residues that coordinate with Zn^{+2} together with Ile22, Leu25, Trp44, Gln46, Thr54, Pro55, Ile130, Phe149 and Trp151 are in the binding pocket of ABP1 (Woo et al., 2002; Grandits and Oostenbrink, 2014).

In the crystal structure, the carboxylate group of 1-NAA was in bidentate contact with the Zn^{+2} deep inside the binding pocket and replaced the Zn^{+2} -water interaction (Woo et al., 2002). Molecular dynamics (MD) simulations of ABP1 in complex with auxins showed water entrance in the Zn^{+2} coordination sphere simultaneously with auxin exit, suggesting that water assists the protonation and deprotonation of auxin molecules (Bertoša et al., 2008). The glycosylation is a high mannose-type glycan structure with 1865 Da of molecular mass and the total molecular mass of the protein ZmERabp1 is 20243 Da (Feckler et al., 2001). Dicot species have an additional glycosylation site at Asn11 (Woo et al., 2002), such as *Arabidopsis* (Palme et al., 1992).

There is some evidence that auxin binding to ABP1 causes changes in the C-terminal conformation of the protein (Thiel et al., 1993), with participation of the tryptophan 151 (Trp151) (Woo et al., 2002; Bertoša et al., 2008). Without auxin molecules in the binding site, the C-terminus keeps extended and Trp151 is pulled out of the binding site, whereas with the binding of an auxin related molecule, the C-terminus is not extended and Trp151 engages in π-π interactions with the planar aromatic group of the auxin related molecule (Fig. 1) (Woo et al., 2002; Bertoša et al., 2008). The KDEL sequence at the end of the C-terminus contributes to helix stability (Grandits and Oostenbrink, 2014). The C-terminal region of ABP1 presumably interacts with one transmembrane protein to transmit the auxin signal (Shi and Yang, 2011). One candidate encoding a glycosylphosphatidylinositol (GPI)-anchored protein named C-terminal peptide-binding protein 1 (CBP1) was found in maize seedlings (Shimomura et al., 2006), but the interaction between CBP1 and ABP1 has not been demonstrated (Tromas et al., 2010; Shi and Yang, 2011). Recently, it was shown that transmembrane kinase (TMK) members of the receptor-like kinase family can serve as docking proteins to ABP1 on the cell surface and that this interaction is auxin-dependent (Xu et al., 2014).

In this work, the Zn^{+2} coordination with the binding site of auxin, as well as the effects of different glycosylation patterns and the dimeric form of ABP1 from maize (*Zea mays*) and *Arabidopsis thaliana* were evaluated through computational techniques. Comparative modeling was employed using ABP1 protein of maize (PDB ID: 1LR5) as a template to obtain an ABP1 protein model for *Arabidopsis thaliana*, followed by MD simulations of both molecules. The impacts of IAA and NAA presence on ABP1 were also assessed.

Methods

Modelling and software

The crystal structure of ABP1 in maize was retrieved from RSCB Protein Data Bank, code 1LR5 to the protein with Zn^{+2} and 1LRH to the protein in complex with auxins (Woo et al., 2002). Monomer chains A and C were used in this work. The sequence from the crystal structure was submitted to alignment performed by Protein BLAST (BLASTp) to search for the most similar sequence in *A. thaliana* (64%). Modeller (9.1 version) (Sali and Blundell, 1993) was employed for the comparative modelling of the protein structure and Verify3D (Bowie et al., 1991; Lüthy et al., 1992) and Procheck (Laskowski et al., 1993) were applied for the assessment of the quality of the model. VMD (Humphrey et al., 1996) and PyMOL (Schrodinger, 2010) were used to visualize the trajectories and manipulate the structures. To perform the MD simulations and analysis, the GROMACS simulation suite version 4.5.4, and GROMOS 53a6 force field were employed. Carbohydrates and glycoproteins topologies construction was based on previous works (Pol-Fachin et al., 2010; Fernandes et al., 2010; Pol-Fachin & Verli, 2011; Pol-Fachin et al., 2012), employing the most populated geometries in solution of the glycosidic linkages that compose a disaccharide or a monosaccharide and an Asparagine residue. Auxin NAA structure topology was derived from PRODRG server and refined by using atomic charges from GROMOS96 53a6 force field, whereas auxin IAA topology was obtained by adapting existing parameters of a Tryptophan residue.

MD simulations protocol

Altogether fourteen systems were generated and simulated, seven for each ABP1: (1) monomeric without Zn^{+2} and non-glycosylated; (2) monomeric with Zn^{+2} in the binding site and non-glycosylated; (3) monomeric with Zn^{+2} in the binding site and glycosylated; (4) dimeric without Zn^{+2} and non-glycosylated; (5) dimeric with Zn^{+2} and glycosylated; (6) dimeric with Zn^{+2}, glycosylated and with IAA and (7) dimeric with Zn^{+2}, glycosylated and with NAA. All the systems were solvated in triclinic boxes using periodic boundary conditions and SPC water model (Berendsen et al. 1987). To neutralize the system, counter ions were added (Na^{+}) when necessary.

The systems were thermalized increasing the temperature gradually from 50 to 300 kelvin. Then, the temperature and pressure of the system were kept constant (300 K and 1 atm) by coupling of molecules and solvent to external temperature and pressure baths with coupling constants of τ= 0.1 and 0.5 ps, respectively. The Lincs method (Hess et al., 1997) was applied to constrain covalent bond lengths, allowing an integration step of 2 fs after an initial energy minimization using Steepest Descents algorithm. Electrostatic interactions were calculated with Particle Mesh Ewald method (Darden et al., 1993). The MDs simulations were conducted for 200ns in triplicates to enhance the sampling of each system, generating 8.4 μs of total simulation time.

Results and discussion

Zn^{+2} and glycosylation are important to provide protein stability.

The root mean square deviation (RMSD) was calculated for the monomer without Zn^{+2} and non-glycosylated, the monomer with the Zn^{+2} ion and non-glycosylated and the monomer in the N-glycosylated form with the Zn^{+2} ion to check for the influence of these elements on protein stability. The ABP1 of maize had more influence of non-peptide factors in its modulation than that of *Arabidopsis* (Fig. 2A and B). In both proteins, it is possible to observe that RMSD values decreased in presence of Zn^{+2} (Fig. 2A and B). However, when glycosylation is applied to the complex in maize, these values are intermediate between those of the non-glycosylated forms of the protein with or without Zn^{+2} (Fig. 2A). This suggests that Zn^{+2} is responsible for providing higher stability to the protein, especially in maize.

When we checked the root mean square fluctuation (RMSF) of the proteins we observed a higher flexibility in the N- and C-terminal regions in both species. These regions are coil structures and this conformation may explain the higher flexibility. When Zn^{+2} was added to the complex, it caused a decrease in the flexibility of these regions in maize (Fig. 3A). In *Arabidopsis*, both Zn^{+2} and glycosylation seem to play this role (Fig 2B), except for the C terminus, in which this effect was not observed (Fig 3B). To evaluate the influence of the N- and C-terminal regions in protein stability, the residues 1 to 15 and from 155 up to the end of the proteins were removed and the RMSD was recalculated. The values decreased in RMSD analysis, revealing that the instability observed when the protein is complete is caused mainly by fluctuation in the residues of these regions (Fig. 2C and D). Thus the ion and the glycan do not seem to play major roles in the stability of the central part of the protein of maize, although the glycan has a small effect in the protein rigidity (Fig. 2C). In *Arabidopsis*, however, the glycan seemed to play a major role, somehow changing the protein structure (Fig. 2D). For both proteins, Zn^{+2} and glycosylation caused residues His57, His59, His106 and Glu63 that coordinate with Zn^{+2}, to be more stable (Fig. 3). This indicates that the presences of the ion and N-glycosylation stabilize the residues of the binding pocket. Although the proteins of Maize and *Arabidopsis* share 64% of identity, molecular dynamics showed important differences between them.

C-terminus helix

During the MD simulations, most of the native structure was maintained on the different systems analyzed, keeping the parallel β helix structure. The main difference observed is in the C-terminus region of ABP1 that has a helix structure in the crystal. After some time of molecular

dynamics, the helix unfolded in most simulations and the C-terminus region became a more extended structure, showing that it is a labile and fragile region (Fig. 4). The helix unrolled even when the protein was simulated in the dimeric form or in complex with auxins (data not shown). The helix structure seems to be more stable in the predicted *Arabidopsis* ABP1 than in the maize protein. In *Arabidopsis* the helix is maintained in most of the monomer simulations (Fig. 4A) and unfolded when the protein is in the dimeric form. This conformational change is in agreement with previous observations (Bertoša et al., 2008). The KDEL sequence can help to stabilize the protein, especially the C-terminal region (David et al., 2001; Grandits and Oostenbrink, 2014) and, in the present simulation, the KDEL sequence is missing. In some cases, the unrolling happened close to or after 100ns of simulation. This observation points out to the importance of long simulations to allow the detection of some relevant phenomena that may go unnoticed in shorter time experiments.

Dimerization and auxin binding

In an attempt to check the effects of dimerization, the proteins were simulated as a dimer in two forms: only with the Zn^{+2} in the binding pocket and with the Zn^{+2} in the binding pocket and the N-glycosylation. The dimerization did not cause striking differences in the protein stability for both species with or without the glycosylation.

Simulations of the dimeric N-Glycosylated proteins with the Zn^{+2} ion in the binding pocket were performed in complex with the auxin types IAA and NAA. When the *Arabidopsis* ABP1 was bound with IAA or NAA the glycosylation structures arranged around the proteins along the simulation as can be seen after 200ns (Fig. 5). In some cases, it seems that the glycosylation is covering the putative site of entrance or egress of the ligand in the binding pocket. The presence of IAA or NAA also reduced the fluctuation in the C- and N-terminal regions of the protein.

The binding of IAA to maize ABP1 caused a higher flexibility in residues 77 to 87, mainly in the Lys82 of the Chain A and this pattern was repeated in the three replicates (Fig. 6). The flexibility also increased from residues 136 to 144, mainly in the His140. In the case of maize, the RMSD values with IAA or NAA did not change in relation to the dimeric form. However, in the *Arabidopsis* ABP1 the protein flexibility had a significant decrease with IAA in the binding pocket and this was even stronger with NAA (Fig. 7). When NAA was bound to the proteins, the RMSD values decreased in both species, indicating a higher stability with NAA than with IAA along the simulation (Fig. 7). This result might be correlated with the calculated and experimental free energies previously described, which revealed a higher binding affinity of ABP1 for NAA (Grandits and Oostenbrink, 2014). Moreover, it was shown that in maize NAA has stronger effects in some aspects of the plant

development, such as shoot growth, primary root growth and crown root formation in maize (Martínez-de la Cruz et al., 2015).

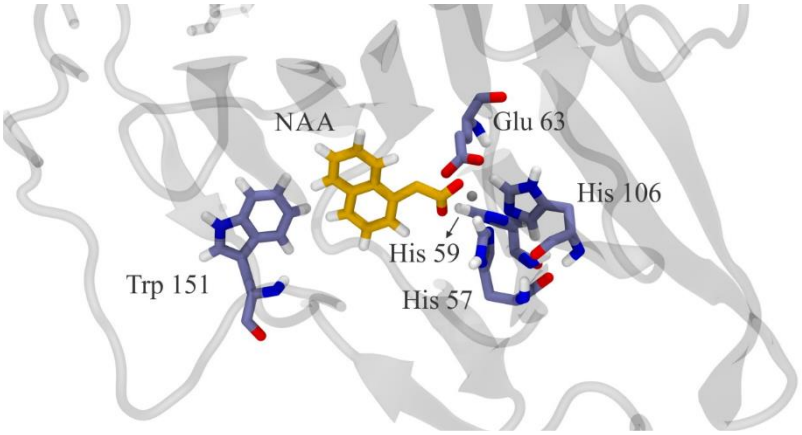

Figure 1. 3D view of the binding pocket of ABP1, with NAA, Zn^{+2} and the residues His57, His59, Glu63, His106 and Trp151 highlighted.

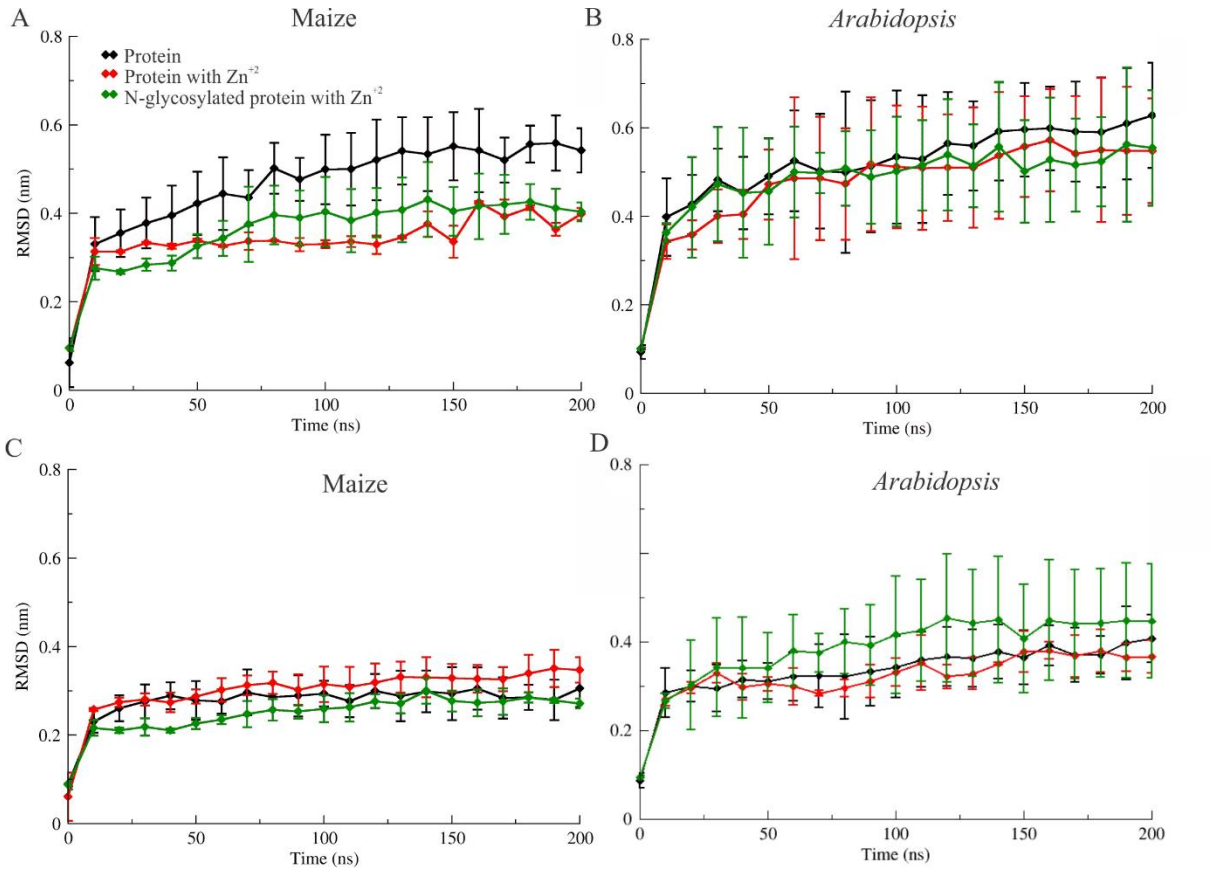

Figure 2. Root mean square deviation (RMSD) of the protein in relation to the corresponding crystal structure is shown for the monomer: without Zn^{+2} and non-glycosylated, with Zn^{+2} and non-glycosylated and N-glycosylated with Zn^{+2}. (A) Maize ABP1. (B) *Arabidopsis* predicted ABP1. (C and D) RMSD of the residues 15 to 155 (without C- and N- terminal regions) of the protein in relation to the corresponding crystal structure: (C) Maize ABP1. (D) *Arabidopsis* predicted ABP1. The bars represent the standard deviation values of three replicates.

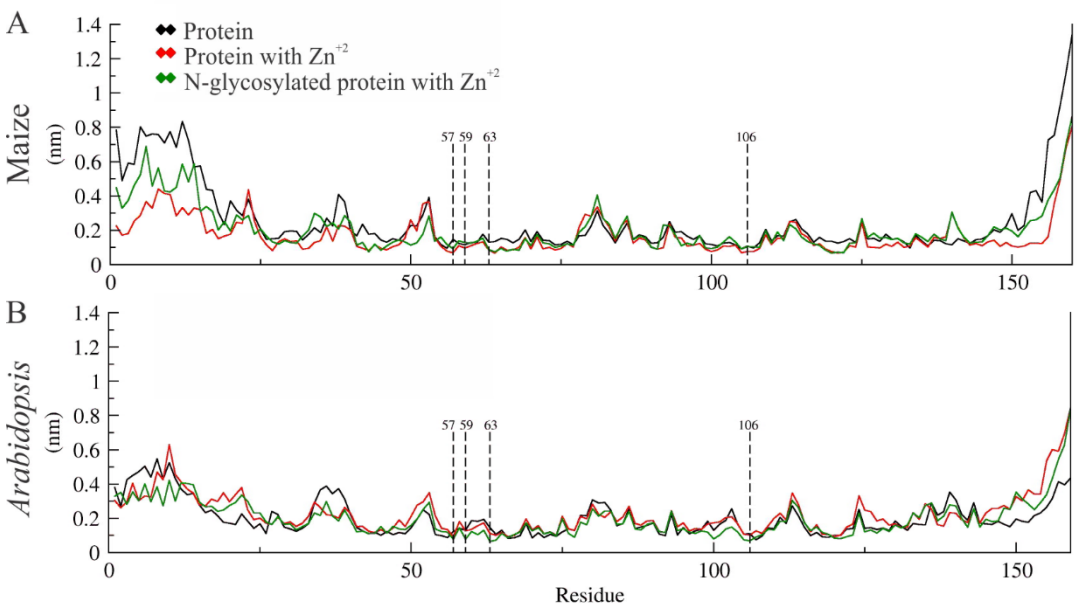

Figure 3. Maize and *Arabidopsis* ABP1 overall flexibility in three different forms: protein alone, protein with the Zn^{+2} ion and N-glycosylated protein with the Zn^{+2} ion. (A) Maize ABP1. (B) *Arabidopsis* predicted ABP1. 57, 59, 63 and 106 correspond to the residues His57, His59, His106 and Glu63 that coordinate with Zn^{+2}.

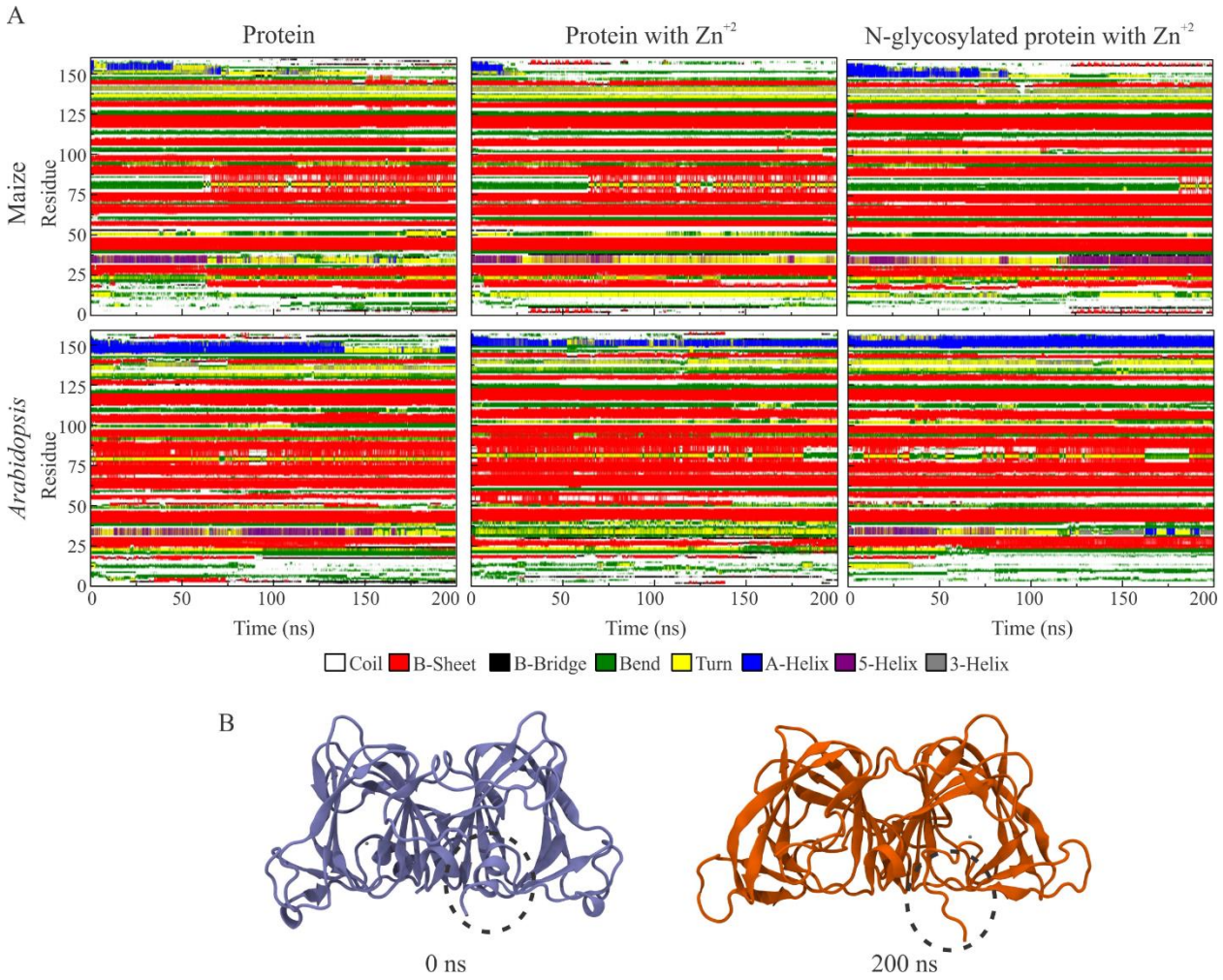

Figure 4. Secondary structure of monomeric protein ABP1. (A) Evolution of the secondary structure along the time of simulation by DSSP of the protein without Zn^{+2} and non-glycosylated, with Zn^{+2} and non-glycosylated and N-glycosylated with Zn^{+2}. (B) 3D view of the dimeric ABP1 protein of *Arabidopsis*. Dotted circles: the helix in the C-terminal region is highlighted in the crystal structure (0 ns) and at 200ns of simulation the highlighted region shows that the C-terminal portion is unrolled (200ns).

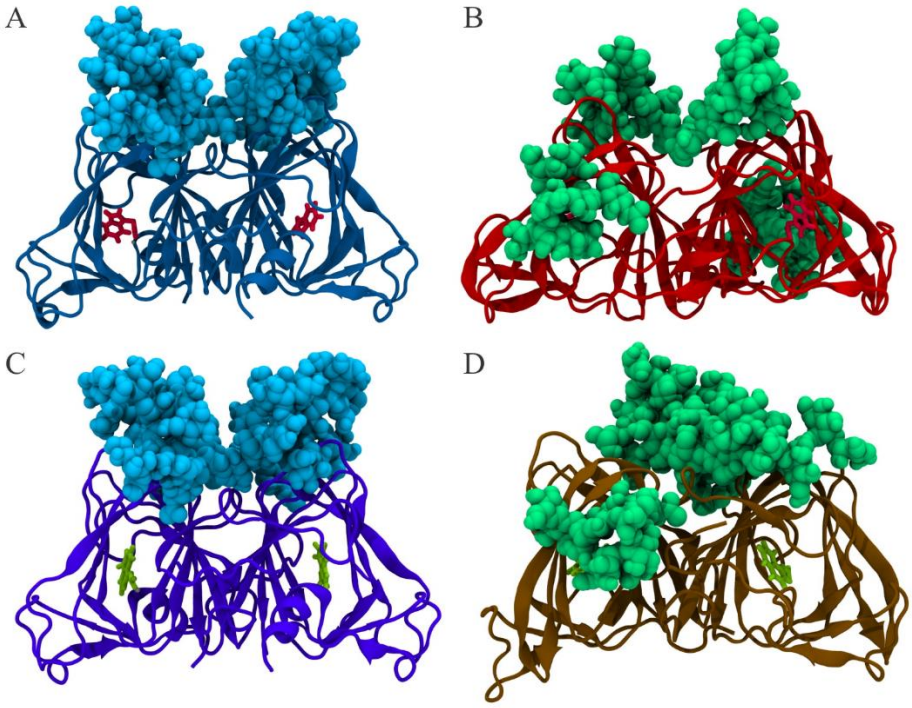

Figure 5. 3D view of the N-glycosylated and dimeric protein ABP1 of *Arabidopsis* (A) ABP1 in complex with IAA in the beginning of the simulation and (B) at 200ns of simulation. (C) ABP1 in complex with NAA in the beginning of the simulation and (D) at 200ns of simulation.

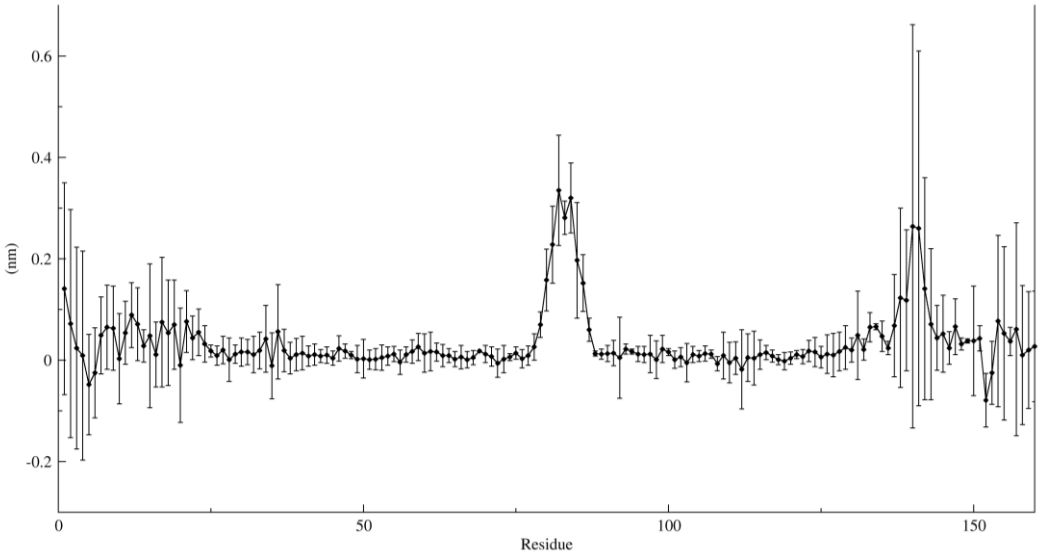

Figure 6. RMSF of dimeric N-glycosylated ABP1 of maize with Zn^{+2} and in complex with IAA. The graphic represents the values of Chain D minus the values of Chain A. The bars represent the standard deviation values of three replicates.

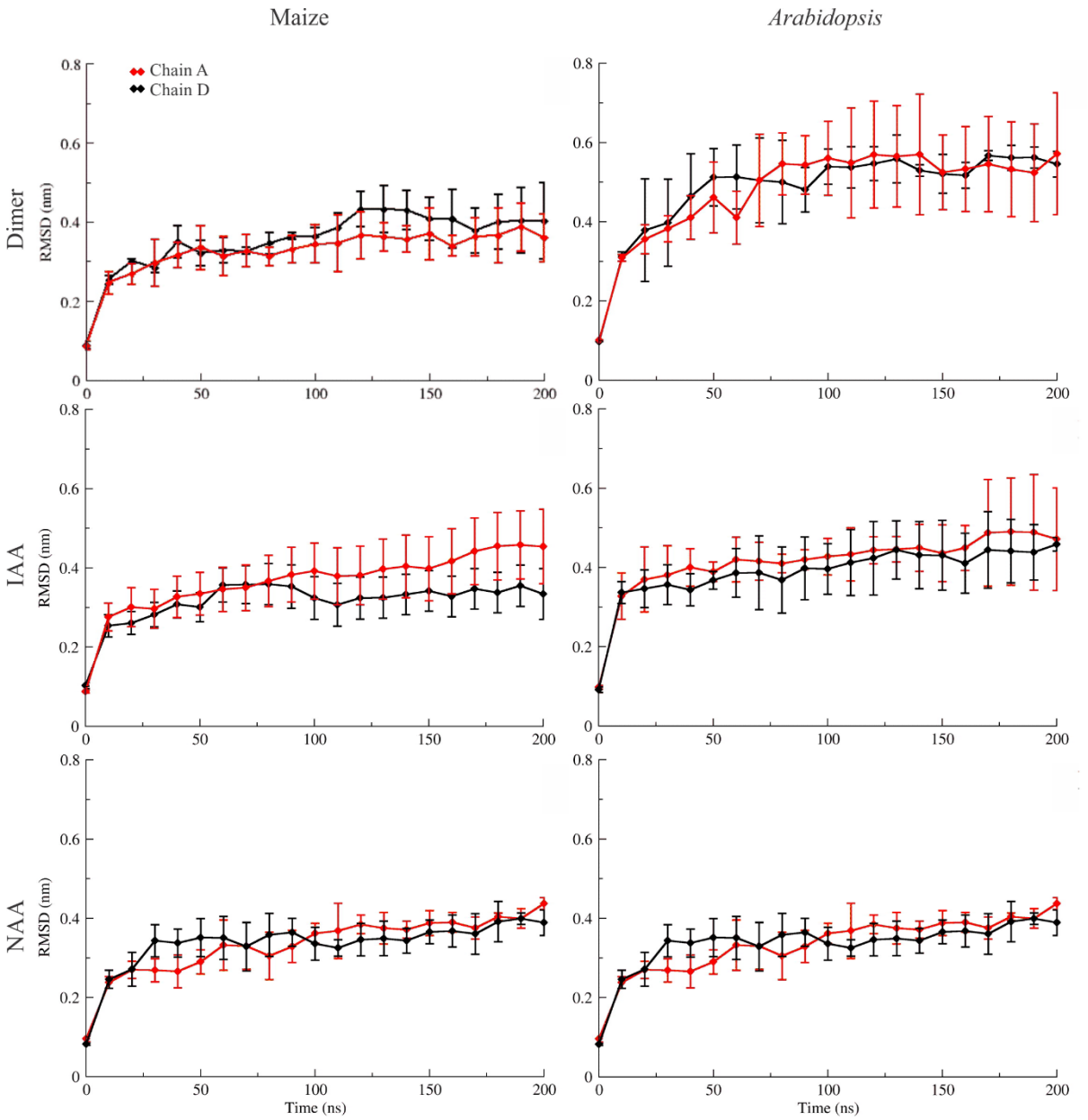

Figure 7. RMSD of maize and *Arabidopsis* ABP1 in the dimeric form with Zn^{+2} and in the dimeric form and N-glycosylated with Zn^{+2} in complex with IAA or NAA. The bars represent the standard deviation values of three replicates.

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ANEXO 3

Biosynthesis of Plant Triterpenoid Saponins: Genes, Enzymes and their Regulation

Anna Carolina A. Yendo, Fernanda De Costa, Cibele T. da Costa, Luana C. Colling, Greice Gosmann and Arthur G. Fett-Neto

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292 *Mini-Reviews in Organic Chemistry,* 2014, *11,* 292-306

Biosynthesis of Plant Triterpenoid Saponins: Genes, Enzymes and their Regulation

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Abstract: Saponins are ubiquitous plant natural products, essentially involved in plant defense against biotic stresses, with numerous pharmaceutical and agricultural applications. The common precursor for triterpenoid saponins is squalene (30 carbon molecule) which, *via* cationic intermediates, is oxidized to 2,3-oxidosqualene. After cyclization, the basic triterpenoid cyclic structure undergoes oxidation by monoxygenases and glycosylations of hydroxyl groups. Chemical synthesis of saponins essentially recapitulates the main biosynthetic steps. However, to date, plants are the most viable source of these molecules. Jasmonic or salicylic acid, as well as their respective methylated derivatives, are important signaling molecules in the responses culminating in triterpenoid saponin production. The current challenges to improve triterpenoid saponin production include a better understanding of the signal transduction pathways leading to their accumulation (with emphasis on late enzymes and "master" regulatory transcription factors), isolation and heterologous expression of biosynthetic genes, and structural and modeling studies of biosynthetic enzymes and their catalytic mechanisms.

Keywords: Biosynthesis, elicitor, enzymes, gene expression, signal transduction, triterpenoid saponins.

INTRODUCTION

Secondary metabolites (SMs), also known as natural products, are low molecular weight organic compounds highly relevant in plant defense against herbivore and pathogen attacks, as well as for coping with several abiotic stresses. The production and accumulation of these compounds are part of plant adaptation in response to environmental changes. During the course of evolution, secondary metabolism pathways were shaped in specific ways for different plant taxa, leading to great chemical diversity. Since SMs are not directly involved in plant development and growth, these metabolites often occur in relatively low yields [1]. Knowledge of biosynthetic pathways and of the corresponding enzymes and genes associated with them are key elements for improved production and higher yields of secondary metabolites of interest.

SMs derive from simple precursors of primary metabolism - amino acids, nitrogenous bases and intermediates of photosynthetic, respiratory, carbohydrate or fatty acid metabolism - through a sequence of reactions catalyzed by specific enzymes. There are over 200,000 known SMs, revealing the high diversity of these compounds. One reason for such diversity is the sequence of small modifications that occur on core compound structures, allowing a large variety of products to arise, particularly at later biosynthetic steps. A second reason is due to the frequently found mixed origin compounds, such as flavonoids, assembled from intermediates derived from the acetate-mevalonate and shikimate pathways. These hybrid-like pathways contribute to the complex regulation of metabolic fluxes leading to SMs [2, 3].

The activation of inducible SM biosynthesis occurs with the perception of an extracellular or intracellular signal, initiating a signal transduction network [1]. This network usually leads to the induction of stress-related genes, primarily at the level of transcription. Temporal and spatial regulation of expression patterns of specific stress genes is an important part of adaptive responses in plants. Transcription factors, sequence-specific DNA-binding proteins, play important roles in regulating gene expression in response to developmental programs and environmental changes in plants [4]. Efforts to engineer secondary metabolic pathways for better yields of products of interest have been more successful by targeting transcription factors that control the expression of various biosynthesis-related genes [3].

The availability of transcriptomic and genomic information for triterpenoid saponin metabolism is still limited. For *P. notoginseng*, one of the most economically important saponin-producing species, for example, only approximately 95 expressed sequence tag (ESTs) were available in the National Center for Biotechnology Information (NCBI) database (in March 2014). EST analysis is a useful tool in identifying putative biosynthetic genes, particularly in non-model plants for which no reference genome sequences are available. ESTs represent the expressed portion of a genome and can be used to characterize patterns of gene expression in different organs or tissues [5]. The discovery and predic-

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ANEXO 4

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2002-2006: Graduação em Ciências Biológicas – Universidade de Passo Fundo (UPF). Orientadora: Ana Christina Sagebin Albuquerque.

ESTÁGIOS REALIZADOS

2006 – 2006: Embrapa Recursos Genéticos e Biotecnologia
2005 – 2006: Embrapa Trigo
2004 – 2005: Laboratório de Citogenética Humana – UPF
2003 – 2003: Museu Zoobotânico Augusto Ruschi – UPF

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2007-2009: Bolsa de apoio técnico – Embrapa Trigo

ARTIGOS COMPLETOS PUBLICADOS EM PERIÓDICOS

Yendo, A.C.; De Costa, F; Costa, C.T.; Colling, L.C.; Gosman, G.; Fett-Neto, A.G. Biosynthesis of Plant Triterpenoid Saponins: Genes, Enzymes and their Regulation. Mini-Reviews in Organic Chemistry, v. 11, p. 292-306, 2014.

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