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EFEITOS DA SINALIZAÇÃO DEPENDENTE DE LUZ NO DESENVOLVIMENTO DE RAÍZES EM *ARABIDOPSIS THALIANA*

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Tese apresentada como requisito parcial para a obtenção do título de Doutor em Ciências ao Programa de Pós- Graduação em Genética e Biologia Molecular da Universidade Federal do Rio Grande do Sul.

Orientador: Prof. Dr. Felipe dos Santos Maraschin

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“Não estudo para saber mais, mas para ignorar menos”

(Sor Juana Inés de la Cruz, 1651-1695)

- Sumário -

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- Lista de abreviaturas -

ABCB/PGP: do inglês, *ATP-binding cassete-B/P-glycoprotein*

AIA/IAA: ácido indol-3-acético

amiR: do inglês, *artificial micro RNA*

AXR: do inglês, *auxin resistant*

bHLH: do inglês, *basic helix-loop-helix*

bZIP: do inglês, *basic leucine-zipper*

cDNA: DNA complementar

CHI: do inglês, *chalcone isomerase*

CHS: do inglês, *chalcone synthase*

CO: do inglês, *constans*

Col-0: Columbia-0

COP1: do inglês, *constitutive photomorphogenesis 1*

cry: do inglês, *cryptochrome*

CUL4-DDB1: do inglês, *cullin4/damage DNA binding 1/ring box 1*

DAG: do inglês, *days after germination*

DAO: do inglês, *dioxygenase for auxin oxidation 1*

DEG: do inglês, *differentially expressed gene*

DEX: do inglês, *dexamethasone*

DNA: ácido desoxirribonucleico

D-root: do inglês, *dark-grown root*

F3'H: do inglês, *flavonoid 3' hydroxilase*

F3H: do inglês, *flavone 3-hydroxilase*

FLS: do inglês, *flavonol synthase*

GFP: do inglês, *green fluorescent protein*

GO: do inglês, *gene ontology*

GR: do inglês, *glucocorticoid*

GUS: do inglês, *β-glucuronidase*

HFR1: do inglês, *long hypocotyl in far-red 1*

HY5: do inglês, *elongated hypocotyl 5*

HYH: do inglês, *HY5-homolog*

K: kaempferol

LAX: do inglês, *like AUX1*

LB: meio de cultivo Luria Bertani

LD: do inglês, *light-protect roots*

LR: do inglês, lateral root

MeOH: methanol

N: naringenina

NAA: do inglês, *1-Naphthaleneacetic acid*

NPA: do inglês, *naphthymlphthalaymic acid*

PAT: do inglês, *polar auxin transport*

phot: do inglês, *phototropin*

phy: do inglês, *phytochrome*

PID: do inglês, *pinoid*

PIF: do inglês, *phytochrome interacting factors*

PIN: do inglês, *pin-formed*

PM: do inglês, *plasma membrane*

Q: quercetina

RbcS1A: do inglês, *ribulose carboxylase small subunit 1A*

RNA: ácido ribonucleico

ROS: do inglês, *reactive oxygen species*

RT-qPCR: do inglês, *reverse transcription - quantitative polymerase chain reaction*

SE: do inglês, *standard error*

SPA: do inglês, *supressor of phyA-105*

TAA1: do inglês, *tryptophan aminotransferase of Arabidopsis*

T-DNA: do inglês, *transferred*-DNA

TIBA: do inglês, *2,3,5-triiodobenzoicv acid*

TWD1: do inglês, *twisted dwarf 1*

UVR8: do inglês, *UV-B resistance 8*

WT: do inglês, *wild-type*

YUC: do inglês, *yucca*

- Resumo -

Além da função de fixação das plantas ao solo, as raízes atuam na absorção de nutrientes e água necessários para o desenvolvimento vegetal. Seu crescimento e desenvolvimento, assim como os demais órgãos, se dá por uma complexa rede de sinalização. O crescimento das raízes, em condições naturais, ocorre abaixo do solo na ausência de luz. Análises prévias demostram que, embora as raízes cresçam abaixo do solo na escuridão, a iluminação da parte aérea é essencial para que as raízes se desenvolvam normalmente. O trabalho apresentado nesta tese teve como objetivo investigar o efeito da iluminação da parte aérea no crescimento de raízes mantidas no escuro na planta modelo *Arabidopsis thaliana*. Os resultados apresentados nos capítulos II à VI mostram que à luz desempenha um papel chave na indução do crescimento da raiz primária. Derivados fotossintéticos atuam sinergisticamente à luz e não como os únicos sinais de longa distância como descrito previamente. A presença de luz na parte aérea leva a alterações significativas no transcriptoma de raízes, envolvendo genes relacionados a diversas classes metabólicas. Utilizando mutantes de perda de função, observamos que o fator de transcrição HY5 possui um papel chave no crescimento de raízes e que sua estabilização em raízes depende da funcionalidade das quinases da família AGC3. Além disso, análises de mutantes de perda de função em genes que apresentaram expressão alterada em resposta à luz demostraram que variações na intensidade luminosa as quais as partes aéreas são expostas influenciam diretamente no crescimento da raiz principal e emissão de raízes laterais. A indução da rota de biossíntese de flavonoides em raízes sugere esses metabólitos como sinais importantes para o crescimento destes órgãos.

- Abstract -

Besides their role in anchoring plants in the soil, roots are necessary for nutrient uptake and water absorption. Root growth and development, just as other organs, are regulated by a complex signaling network. In natural conditions, root development mostly happens underground, in darkness. Previous reports have shown that even though roots grow place in darkness under the soil, shoot-illumination is essential for root development. This thesis aimed to investigate the effect of root illumination on dark-grown roots in the model plant *Arabidopsis thaliana.* The results presented on chapters II to VI show that light has a major effect on primary root elongation. Photosynthetic sugars act synergistically with light and not solely as a long-distance signal. Shoot illumination leads to changes in root transcriptome, influencing different metabolic pathways. Employing loss-of-function mutant lines, the transcription factor HY5 showed a key role in the control of root photomorphogenesis. Its stabilization in dark-grown roots is dependent of AGC3 kinases. Moreover, we show that changes in the light intensity to which shoots are exposed lead to changes in primary and lateral root development. The high induction of flavonol biosynthesis in roots in response to shoot illumination suggests that these metabolites act as a signal in dark-grown roots.

Capítulo I

- Introdução -

1. Morfogênese vegetal

Plantas desenvolveram ao longo do processo evolutivo um complexo e intricado sistema de percepção das condições ambientais. Por serem organismos sésseis, respostas rápidas e altamente coordenadas são essenciais para assegurar sua sobrevivência. Quando germinadas no escuro, plântulas apresentam um padrão de desenvolvimento estiolado ou escotomorfogênico, alocando as reservas de energia contidas nas sementes para o alongamento do hipocótilo em contrapartida ao desenvolvimento dos cotilédones e do sistema radicular. Em dicotiledôneas, como *Arabidopsis thaliana*, o desenvolvimento no escuro é caracterizado pela repressão da abertura dos cotilédones e à formação do gancho plumular. A estrutura de gancho facilita a passagem pela barreira do solo e garante proteção mecânica aos frágeis cotilédones e ao meristema apical caulinar. Essa estratégia garante que as limitadas reservas contidas na semente sejam investidas na busca pela luz, pré-requisito para sobrevivência fotoautotrófica das plantas. Após ultrapassar a barreira do solo, as plântulas expostas à luz passam a apresentar respostas fotomorfogênicas que incluem a abertura do gancho plumular, desenvolvimento das folhas e, consequentemente, o estabelecimento dos processos fotossintéticos que permitem a alocação dos açúcares recém-formados para o desenvolvimento das raízes, antes negligenciado (Lee et al., 2017).

Os processos fotomorfogênicos são desencadeados pela percepção da luz pelos fotorreceptores. Plantas possuem um complexo e eficiente sistema de fotorreceptores capazes de perceber sutis variações de intensidade e qualidade luminosa (Kami et al., 2010; Galvão and Fankhauser, 2015; van Gelderen et al., 2017). Fitocromos são sensores de luz vermelha e vermelho-extremo que sofrem alterações conformacionais reversíveis em resposta à razão vermelho/vermelho-extremo (phyA-phyE em *Arabidopsis thaliana*; Franklin and Quail, 2010). Três classes de proteínas são conhecidas pelas respostas a UV-A/luz azul: criptocromos (cry1 e cry2; Chaves et al., 2011), fototropinas (phot1 e phot2) e ZEITLUPE (ZTL, FKF1 e LKP2; Demarsy and Fankhauser, 2009). A luz UV-B é percebida pelo receptor UVR8 (Jenkins, 2014). Embora sejam mais abundantes nos tecidos aéreos, já foi demonstrado que os fotorreceptores também são expressos nas raízes (van Gelderen et al., 2017). Os efeitos fotomorfogênicos desencadeados pelos fotorreceptores nas partes aéreas têm sido extensamente estudados (revisado em Kami et al., 2010). Entretanto, as cascatas de sinalização que induzem o desenvolvimento do sistema radicular foram por

muito tempo negligenciadas. Evidências mais recentes revelam que o sistema radicular, mesmo no escuro abaixo do solo, passa por mudanças drásticas de morfologia e desenvolvimento em resposta à luz (Yokawa et al., 2013a; van Gelderen et al., 2018; Zhang et al., 2019).

2. HY5 – o elo da sinalização fotomorfogênica

A percepção de luz desencadeia cascatas de sinalização que se baseiam na interação dos fotorreceptores com diversas classes de proteínas, como os PHYTOCHROME INTERACTING FACTORS (PIFs; Leivar and Monte, 2014), e a ubiquitina E3 ligase CONSTITUTIVE PHOTOMORPHOGENESIS 1 (COP1; Huang et al., 2014). COP1 é uma proteína RING *finger* presente em plantas e animais que, em plantas, juntamente com as proteínas da família SUPRESSOR OF PHYA-105 (SPAs), controla a abundância de diversas proteínas, incluindo os fatores de transcrição ELONGATED HYPOCOTYL 5 (HY5; Ang et al., 1998), HY5-HOMOLOG (HYH; Holm et al., 2002) e LONG HYPOCOTYL IN FAR-RED1 (HFR1; Yang et al., 2005); o ativador do florescimento CONSTANS (CO); bem como phyA, phyB e cry2 (Hoecker, 2017; Podolec and Ulm, 2018). *COP1* é um gene de cópia única em Arabidopsis, enquanto a família SPA é composta por quatro membros (SPA1-4). Mutantes de perda de função *cop1* e *spa1234* apresentam crescimento fotomorfogênico mesmo no escuro, caracterizando uma resposta constitutiva à luz (Menon et al., 2016). Mutantes *spa1234* apresentam um nanismo severo, já plântulas *cop1* possuem o crescimento interrompido ainda nos estágios iniciais de desenvolvimento (Deng et al., 1991). COP1/SPA atuam como receptores do complexo E3 ligase CULLIN 4/DAMAGED DNA BINDING 1/RING BOX 1 (CUL4-DDB1$^{COP1/SPA}$; Lau and Deng, 2012).

Na ausência de luz, o complexo CUL4-DDB1$^{COP1/SPA}$ adiciona ubiquitina aos fatores de transcrição HY5 e HYH, direcionando-os para degradação mediada pelo proteassomo 26S (Ang et al., 1998; Osterlund et al., 2000; Holm et al., 2002). Por sua vez, na presença de luz, o complexo é desativado pela interação com os fotorreceptores (revisado em Podolec and Ulm, 2018), permitindo o acúmulo de HY5/HYH e desencadeando as respostas fotomorfogênicas dependentes desse *hub* de sinalização. Recentemente, um modelo adicional de regulação da fotomorfogênese desencadeada por luz UV-B e mediada pelo módulo COP1/SPA-HY5 foi descrito (Huang et al., 2014). Em resposta à luz UV-B, COP1

e SPA acumulam em complexos associados a UVR8, promovendo a expressão e estabilização de HY5 (Huang et al., 2013).

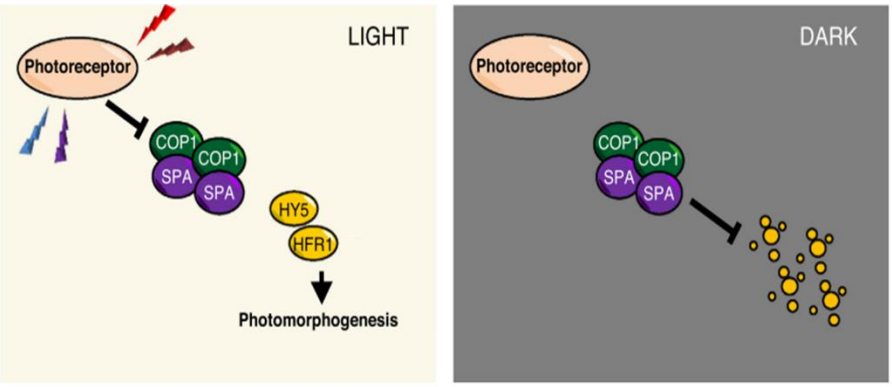

Figura 1. Regulação das respostas fotomorfogênicas mediadas pelo módulo COP1-HY5 (Modificado de Podolec and Ulm, 2018).

A identificação dos mutantes *hy* nos anos 1980 possibilitou que abordagens genéticas e moleculares fossem utilizadas para uma maior compreensão dos mecanismos envolvidos na regulação do crescimento vegetal em resposta à luz. Essa classe foi denominada *hy* devido ao fenótipo de maior alongamento do hipocótilo (*long hypocotyl* em inglês), apresentado por esses mutantes (Koornneef et al., 1980). Dentre os cinco mutantes *hy*, *hy5* foi o último a ser caracterizado, sendo o único que não está diretamente relacionado a fotorreceptores de luz (Oyama et al., 1997). O mutante *hy5* apresenta um alongamento exagerado do hipocótilo quando crescido na luz, fenótipo semelhante ao observado em plântulas estioladas (Oyama et al., 1997; Sibout et al., 2006). Em contrapartida ao alongamento do hipocótilo, essa mutação leva a raízes principais mais curtas (Sibout et al., 2006; Chen et al., 2016; Zhang et al., 2019). Ambos fenótipos sugerem que a mutação *hy5* reduz as respostas à presença de luz, sugerindo que HY5 desempenha um papel-chave na modulação dessa resposta. Outro fenótipo bastante interessante apresentado por esse mutante é o aumento do número de raízes laterais (Ang et al., 1998), bem como do ângulo dessas raízes (Oyama et al., 1997), tornando o sistema radicular dessas plantas bastante agravitrópico, sugerindo que além das respostas à luz, a mutação *hy5* leva a alterações nas respostas mediadas pelo hormônio auxina.

O *locus hy5* codifica uma proteína do tipo bZIP (*basic leucine-zipper*), com localização nuclear (Oyama et al., 1997; Ang et al., 1998). A expressão de *HY5* é altamente induzida pela luz e ocorre principalmente nos estágios iniciais de desenvolvimento (Hardtke

et al., 2000; Osterlund et al., 2000; Zhang et al., 2017). Análises genômicas e de imunoprecipitação da cromatina mostraram que esse fator de transcrição é capaz de ligar-se à região promotora de mais de 11000 genes em Arabidopsis (Lee et al., 2007b; Zhang et al., 2011), demostrando a importância de HY5 como um regulador-chave de diversas cascatas de transdução de sinal (Eckardt, 2007). HY5 controla a expressão de genes que possuem sequências-consenso "elementos-ACGT", como os elementos G-box (CACGTG), C-box (GACGTC), Z-box (ATACGGT) e A-box (TACGTA) em sua região promotora (Song et al., 2008). Surpreendentemente, dentre os alvos, foram observados genes relacionados não apenas à luz, mas também resposta a estresse, hormônios, florescimento e ritmo circadiano (Lee et al., 2007; Zhang et al., 2011). Dentre os muitos alvos de HY5, já foi demostrado que este liga-se à região promotora e é capaz de induzir genes responsivos à luz, como *Chalcone synthase* (*CHS*) e *Ribulose bisphosphate carboxylase small subunit 1A* (*RbcS1A*; Lee et al., 2007; Zhang et al, 2011). Embora a interação entre HY5 e os genes da rota de biossíntese de flavonoides/antocianinas tenha sido demostrada há mais de 20 anos (Ang et al., 1998; Chattopadhyay et al., 1998), os efeitos resultantes desse *crosstalk* ainda são pouco compreendidos.

A formação do sistema radicular é altamente dependente de sinais externos e internos. Os principais fenótipos observados no mutante *hy5*, como o crescimento acelerado de raízes laterais, pelos radiculares mais longos e baixa resposta gravitrópica, são fenótipos semelhantes aos observados em resposta a hormônios (Enders and Strader, 2015). Genes de sinalização de auxina, como AUXIN RESISTANT 2 (AXR2)/IAA7 e SOLITARYROOT (SLR)/IAA14, possuem expressão reduzida no mutante *hy5*. Além disso, raízes de plântulas *hy5* mostraram-se pouco responsivas à aplicação exógena de auxina e citocinina (Cluis et al., 2004). Já foi demostrado que HY5 desempenha um papel importante na manutenção da distribuição intracelular do transportador de auxina PIN-FORMED2 (PIN2; Laxmi et al., 2008). O enriquecimento de fatores de transcrição mediadores de respostas hormonais relatados como alvos de HY5 (Lee et al., 2007; Zhang et al., 2011), bem como os fenótipos resultantes desta mutação, indicam que HY5 atua como um integrador das vias de sinalização hormonais e luminosas (Cluis et al., 2004). Neste contexto, o fator de transcrição HY5 tem um papel essencial, não apenas na resposta fotomorfogênica, mas também regulando direta e indiretamente as respostas hormonais.

3. Sinais que desencadeiam respostas fotomorfogênicas em raízes

Na natureza, o sistema radicular da maioria das plantas se desenvolve abaixo do solo, na escuridão, sugerindo que moléculas sinalizadoras enviadas pela parte aérea iluminada, são os responsáveis pela indução do crescimento das raízes. Três vias de transdução de sinal são aceitas como promotoras da fotomorfogênese radicular: percepção direta de luz pelas raízes; passagem de luz pelo sistema vascular com indução local das respostas fotomorfogênicas; e sinais móveis enviados pela parte aérea (revisado em Lee et al., 2017). A passagem de luz pelo sistema vascular da parte aérea para as raízes é capaz de ativar nas raízes os módulos de resposta dependentes de phyB e HY5 (Lee et al., 2016). Embora essa possibilidade tenha sido demostrada em espécies herbáceas e lenhosas, a transmissão é bastante afetada em longas distâncias (van Gelderen et al., 2018b). Trabalhos recentes sugerem que a iluminação da parte aérea tem efeito principal no desenvolvimento de raízes (Chen et al., 2016; Sakaguchi and Watanabe, 2017). Fitormônios, açúcares, RNAs móveis e proteínas já foram considerados sinais móveis produzidos pela parte aérea e mobilizados até as raízes, onde promovem o desenvolvimento do sistema radicular (van Gelderen et al., 2018; Lee et al., 2017).

Já foi demostrado que os açúcares produzidos durante os processos fotossintéticos são transportados via floema até as raízes, onde estimulam seu crescimento (Kircher and Schopfer, 2012). Além da sacarose, a proteína HY5 é capaz de migrar da parte aérea para as raízes via floema, onde induz sua própria expressão e de genes promotores da captação de nitrogênio (Chen et al., 2016). O hormônio auxina tem grande influência no desenvolvimento do sistema radicular, sendo que a auxina derivada da parte aérea é essencial para a emergência de primórdios de raízes laterais (Bhalerao et al., 2002). O característico transporte polar desse hormônio, juntamente com a estabilização dependente de luz de seus transportadores (Laxmi et al., 2008; Sassi et al., 2012), sugerem que auxina seja um importante candidato a sinalizador móvel que regula o crescimento das raízes em resposta à luz. Grande parte dos estudos de desenvolvimento fotomorfogênico das raízes foram realizados com raízes completamente iluminadas, condição diferente da encontrada na natureza. Embora esses estudos tenham tido grande importância na elucidação dos mecanismos genéticos e moleculares envolvidos nas respostas luminosas, pouco ainda se

conhece das rotas de transdução de sinal que levam ao desenvolvimento de raízes em ausência de luz.

4. Auxina – regulador-chave do desenvolvimento radicular

Auxina foi o primeiro regulador do crescimento vegetal identificado, estando envolvido em praticamente todos os aspectos do desenvolvimento vegetal, incluindo embriogênese, organogênese, tropismos e padronização dos tecidos vegetais. Sintetizado preferencialmente em regiões jovens da parte aérea e transportado de maneira polarizada para as raízes, auxina é considerado o principal regulador do desenvolvimento do sistema radicular (Saini et al., 2013). O ácido indol-3-acético (AIA) é a principal auxina presente nas plantas, sintetizada de forma dependente ou independente do triptofano (Zhao, 2014). A via de síntese dependente de triptofano ocorre nos plastídeos e é catalisada pela enzima TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA1), juntamente com as mono-oxigenases da família YUCCA (YUC; Mashiguchi et al., 2011). Nosso entendimento das rotas de biossíntese de AIA ainda é bastante fragmentada; entretanto, diversos trabalhos demostram que esta pode ser modulada por sinais internos, como outros hormônios, assim como por sinais ambientais, como luz ou gravidade (Zhao, 2014; Ikeuchi et al., 2019).

Além da biossíntese local de auxina, seu transporte define a ocorrência de gradientes de concentração que são essenciais para as respostas por ela mediadas (Sabatini et al., 1999; Benková et al., 2003; Friml et al., 2003). O transporte direcional é coordenado por gradientes quimiostáticos com a ajuda dos carreadores de influxo AUXIN RESISTANT 1/LIKE AUX1 (AUX1/LAX), carreadores de efluxo da família PIN-FORMED (PIN; Gälweiler et al., 1998) e pelos transportadores da família ATP-BINDING CASSETTE-B/P-GLYCOPROTEIN (ABCB/PGP; Vanneste and Friml, 2009). A polarização dos transportadores PIN na membrana plasmática é regulada pelo balanço da atividade da proteíno-fosfatase 2A (PP2A; Michniewicz et al., 2007) e das proteíno-quinases da família AGC3 (Friml et al., 2004). As quinases AGC3 PINOID (PID), WAG1 e WAG2 são capazes de fosforilar resíduos de serina em três motivos TPRXS(N/S) conservados, encontrados na alça citoplasmática das proteínas PIN (Dhonukshe et al., 2010). A fonte de auxina assim como a intensidade de transporte desse hormônio para as raízes está relacionada com o estágio de desenvolvimento vegetal.

Sabe-se que entre o quinto e o sétimo dia após a germinação as raízes experimentam um aumento dos níveis endógenos de auxina, somando-se a auxina oriunda da parte aérea àquela proveniente dos primórdios de raízes laterais em desenvolvimento, gradativamente tornando as raízes menos dependentes da auxina sintetizada nos tecidos aéreos (Bhalerao et al., 2002).

A distribuição de PINs não é unicamente regulada pelas quinases AGC3, mas também pela sinalização dependente de luz. Em plântulas crescidas no escuro, as proteínas PINs são direcionadas para o vacúolo lítico para degradação, enquanto plântulas crescidas na luz apresentam uma localização estável de PINs na membrana plasmática (Kleine-Vehn et al., 2008; Laxmi et al., 2008; Sassi et al., 2012). O direcionamento de PINs para o vacúolo no escuro reduz o fluxo de auxina da parte aérea para a raiz (Laxmi et al., 2008), explicando o menor desenvolvimento das raízes de plântulas mantidas no escuro. Entretanto, em plântulas crescidas sob condições naturais, com a parte aérea exposta à luz, o sistema radicular desenvolve-se normalmente, mesmo abaixo do solo na escuridão. Isso sugere que sinais enviados pela parte aérea desencadeiam cascatas de resposta que levam à estabilização das proteínas PINs na raiz, permitindo a formação de gradientes de auxina e consequentemente o desenvolvimento das raízes.

O entendimento de como à luz influencia a estabilização das proteínas PIN e os efeitos dessa estabilização ainda são muito limitados. Embora ainda pouco elucidada, sabe-se que é dependente de COP1 e HY5 (Laxmi et al., 2008; Sassi et al., 2012). Dados recentes do nosso grupo sugerem que as quinases da família AGC3 poderiam ser o elo entre COP1-HY5/HYH e a estabilização dos transportadores de auxina. Mais especificamente, foi demostrado em plântulas estioladas que PID interage com e fosforila COP1 especificamente no resíduo Ser20 (Lin et al., 2017). Dados adicionais (van Gelderen e Offringa, dados não publicados) demonstram que outro membro dessa família, a quinase AGC3-4, assim como PID, é capaz de fosforilar COP1. Portanto, a fosforilação de COP1 mediada pelas quinases AGC3 possivelmente leva à desestabilização do complexo COP1/SPA, inibindo a degradação dos promotores da fotomorfogênese HY5/HYH e possibilitando a indução das rotas fotomorfogênicas nas raízes.

5. Flavonoides e o desenvolvimento das raízes

Além do já descrito efeito na regulação do transporte de auxina, a luz também tem efeitos sobre o metabolismo secundário, incluindo a indução da produção de flavonoides (Buer and Muday, 2004). Flavonoides são compostos fenólicos de baixo peso molecular com função não-essencial para a sobrevivência vegetal (Buer et al., 2010). Embora estruturalmente semelhantes, flavonóis, flavonas, isoflavonas e antocianinas possuem ramificações na sua fórmula básica, C_6-C_3-C_6, que levam à formação de diferentes estruturas com diferentes funções *in planta* (Taylor and Grotewold, 2005; Saito et al., 2013). Devido às diversas possibilidades de ramificações na cadeia principal, mais de 9000 estruturas já foram descritas, estando relacionadas com diversos processos, incluindo pigmentação, proteção contra luz UV, interação com microrganismos, homeostase de espécies reativas de oxigênio (ROS), bem como inibição do transporte de auxina (revisado em Falcone Ferreyra et al., 2012).

A identificação da série de mutantes *transparent testa* (*tt*) em Arabidopsis gerou uma ótima ferramenta para estudos bioquímicos e genéticos envolvendo essa rota de biossíntese (Koornneef, 1990). A ausência de pigmentação nas sementes definiu marcadores genéticos facilmente reconhecíveis, permitindo o estabelecimento das principais etapas da via de biossíntese de flavonoides (Winkel-Shirley, 2001). O fluxo de carbono dessa via de biossíntese é controlado pelas enzimas CHS (*tt4*), CHALCONE ISOMERASE (CHI, *tt5*), FLAVONONE 3-HIDROXYLASE (F3H, *tt6*), FLAVONOL SYNTHASE (FLS) e FLAVONOID 3' HYDROXYLASE (F3′H, *tt7*). Kaempferol e quercetina são as principais agliconas produzidas; entretanto, agliconas são raramente detectadas em plantas, sugerindo que derivados glicosilados são os responsáveis pelos fenótipos observados *in vivo* (Saito et al., 2013).

Devido ao seu potencial antioxidante, flavonoides foram propostos como tampões inespecíficos, que atuam limitando os níveis celulares de ROS (Gayomba et al., 2017). Seu acúmulo em regiões de alta concentração de auxina sugere que flavonoides podem ser essenciais para a redução de radicais gerados durante o catabolismo de auxina (Brunetti et al., 2018), corroborando sua atividade inibitória sobre a enzima DIOXIGENASE FOR AUXIN OXIDATION1 (DAO1; Zhang et al., 2016). Além disso, o mutante *anthocyanin reduced* (*are)* em tomate, em comparação com o genótipo selvagem, apresenta uma elevação

no transporte de auxina assim como altos níveis de ROS. Esta observação reforça que a ausência de flavonoides está relacionada com alterações nos máximos de auxina e, consequentemente, com um maior acúmulo de ROS (Maloney et al., 2014; Gayomba et al., 2017).

Apesar de documentados no final dos anos 1980 como inibidores naturais do transporte de auxina (Jacobs and Rubery, 1988), os mecanismos pelos quais flavonoides controlam negativamente o transporte de auxina *in vivo* permanecem pouco elucidados. Sabe-se que o flavonol quercetina possui o maior potencial inibitório, sendo capaz de inibir a interação entre a imunofilina TWISTED DWARF1 (TWD1) e o transportador de efluxo ABCB1 (Bailly et al., 2008), além de reprimir a atividade da quinase PID (Kuhn et al., 2017), responsável pela ciclagem de PIN1 (Friml et al., 2004). Experimentos usando a linhagem mutante *tt4*, que não produz flavonoides devido à ausência da enzima CHS, demonstraram que a ausência de flavonoides leva ao alongamento da raiz principal, bem como um aumento no número de raízes laterais e adventícias (Brown et al., 2001). O aumento no transporte de auxina observado em plântulas *tt4* foi revertido pela aplicação exógena do intermediário naringenina, consistente com a hipótese de que os flavonoides endógenos atuam como inibidores desse processo (Murphy et al., 2000; Brown et al., 2001). Recentemente, trabalhos envolvendo raízes iluminadas e não iluminadas propuseram que os flavonoides podem atuar como sinais locais, integrando respostas hormonais e ROS em resposta à presença de luz (Silva-Navas et al., 2016). Embora a relação entre flavonoides e auxina tenha sido extensivamente estudada nos últimos 20 anos, os resultados obtidos até o momento sugerem que a interação entre eles é muito mais complexa do que o simples modelo de que flavonoides atuam como inibidores naturais do transporte de auxina.

6. Desenvolvimento de raízes iluminadas *vs.* no escuro

A possibilidade de cultivo de Arabidopsis em um ambiente estéril e controlado como as placas de Petri possibilitou um imenso salto no conhecimento acerca dos sistemas de percepção de luz em plantas. Em 1971, apenas um fotorreceptor era conhecido; hoje, são conhecidas quatorze diferentes proteínas responsáveis pela percepção de luz em Arabidopsis (Briggs and Lin, 2012). A maioria dos experimentos que levaram à caracterização das respostas fotomorfogênicas foram realizados em placas de Petri e, consequentemente, com

as raízes expostas à luz. Nos últimos anos, diversos trabalhos têm demonstrado que a iluminação das raízes leva a mudanças drásticas de morfologia e desenvolvimento e pode ser considerada uma situação de estresse (Xu et al., 2013; Yokawa et al., 2013b; Silva-Navas et al., 2015; Chen et al., 2016; Zhang et al., 2019). Diferentes sistemas estão sendo desenvolvidos buscando mimetizar as condições naturais de crescimento das raízes, alguns mais complexos, como o sistema D-Root (Silva-Navas et al., 2015) e o *improved agar-plate culture* (Xu et al., 2013) e outros mais simples, como o usado pelo nosso grupo (Miotto et al., 2019).

Apesar de ainda pouco documentado, o efeito da sinalização luminosa em raízes escuras parece ser um ponto-chave para um melhor entendimento do desenvolvimento do sistema radicular em condições mais similares aquelas experimentadas na natureza. Nossa hipótese é que a sinalização dependente de luz na parte aérea preserva o transporte de auxina em raízes mantidas no escuro através de uma função alternativa das quinases AGC3. A fosforilação de COP1 por AGC3-4 nas raízes levaria à estabilização dos fatores de transcrição promotores da fotomorfogênese HY5/HYH nas raízes abaixo do solo. A maior abundância destes fatores de transcrição levaria à expressão de um conjunto de genes responsivos à luz e necessários à estabilização das proteínas PIN na membrana plasmática, mantendo a sinalização de auxina advinda da parte aérea para induzir o desenvolvimento do sistema radicular (Figura 1).

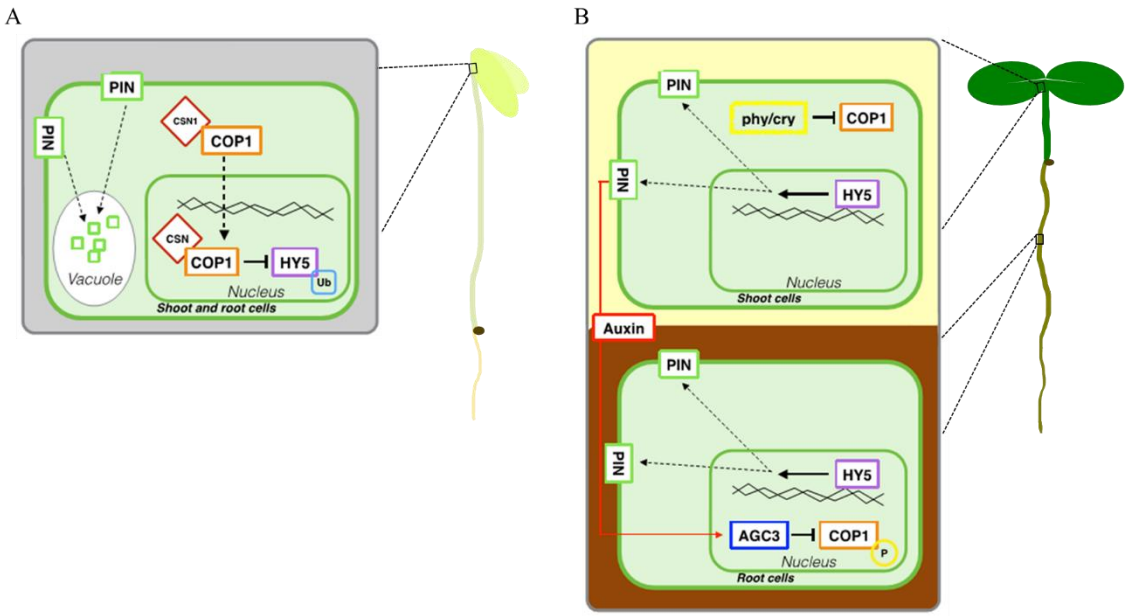

Figura 1. Modelo de crescimento das raízes em resposta à luz. (A) No escuro, o complexo COP1 marca para degradação as proteínas promotoras da fotomorfogênese HY5/HYH impedindo a expressão de genes responsivos à luz. Em resposta a ausência de luz, transportadores de auxina são direcionados para o vacúolo para degradação, diminuindo o aporte de auxina para as raízes e diretamente reprimindo o desenvolvimento do sistema radicular. (B) Na presença de luz, o complexo COP1 é desfeito pela interação com os fotorreceptores, permitindo o acúmulo de HY5/HYH e a indução de genes fotomorfogênicos. A auxina sintetizada nos tecidos jovens é transportada polarmente para as raízes, funcionando como um sinal de longa distância. A presença de auxina nas raízes induz a fosforilação de COP1 pelas quinases da família AGC3, descomplexando a proteína e liberando o fator de transcrição HY5 para induzir genes fotomorfogênicos que, possivelmente, atuam na estabilização de PIN1 na membrana plasmática. A permanência de PIN1 na membrana preserva o transporte de auxina oriundo da parte aérea e possibilita o desenvolvimento das raízes.

- Objetivos -

1. Objetivo geral

Tendo em vista a escassez de dados na literatura a respeito do efeito da iluminação da parte aérea no crescimento e desenvolvimento de raízes mantidas no escuro e buscando um melhor entendimento das rotas moleculares envolvidas na sinalização entre parte aérea e raízes, a presente tese de doutorado se propôs a investigar como a sinalização dependente de luz na parte aérea afeta o desenvolvimento de raízes mantidas no escuro na planta modelo *Arabidopsis thaliana*.

2. Objetivos específicos

2.1 Comparar o padrão de transcritos de raízes de plântulas crescidas no escuro ou com apenas suas partes aéreas expostas à luz por meio de sequenciamento;

2.2 Avaliar fenotipicamente mutantes perda de função de genes com expressão alterada entre as condições acima citadas, bem como caracterizar o efeito da intensidade luminosa no crescimento de raízes mantidas no escuro;

2.3 Analisar o efeito da iluminação da parte aérea na sinalização e na manutenção dos máximos de auxina nas raízes por meio de linhagens repórter;

2.4 Abordar a relação de flavonoides com a manutenção do transporte de auxina e o efeito dessa interação no desenvolvimento de raízes;

2.5 Verificar o envolvimento das quinases AGC3 na estabilização do fator de transcrição HY5 em raízes através da análise de mutantes de perda de função e linhagens repórter;

Capítulo II

- Identification of root transcriptional responses to shoot illumination in *Arabidopsis thaliana* -

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Identification of root transcriptional responses to shoot illumination in *Arabidopsis thaliana*

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Abstract

Light is a key environmental factor regulating plant growth and development. *Arabidopsis thaliana* seedlings grown under light, display a photomorphogenic development pattern, showing short hypocotyl and long roots. On the other hand, when grown in darkness, they display skotomorphogenic development, with long hypocotyls and short roots. Although many signals from shoots might be important for triggering root growth, the early transcriptional responses that stimulate primary root elongation are still unknown. Here, we aimed to investigate which genes are involved in the early photomorphogenic root development of dark grown roots. We found that 1616 genes in 4 DAG, and 3920 genes after 7 DAG were differently expressed in roots when the shoot was exposed to light. Of these genes, 979 were up regulated in 4 days and 2784 at 7 DAG. We compared the functional categorization of differentially regulated process by two methods: GO term enrichment and transcriptogram analysis. The expression level of nine selected candidate genes in roots confirm the data observed in the RNAseq analysis. Loss-of-function mutants of selected differentially expressed genes suggests the involvement of these genes in root development in response to shoot illumination. Our findings are consistent with the hypothesis that the roots respond to the aboveground light environment even growing in the darkness.

Introduction

Light is an important signal to plant growth and development, providing energy for photosynthesis and enabling seed germination, seedling photomorphogenesis, phototropic movements and photoperiodic responses (Jiao et al., 2007; Li et al., 2012). Plants have developed a complex photoreceptor sensory system to adapt to different light conditions (Mo et al., 2015; Su et al., 2017). During photomorphogenic development, the exposure of seedlings to light enables the activation of the photosynthetic process (Wu, 2014). Photosynthetic-derived sugars are essential to induce and maintain root development (Kircher and Schopfer, 2012). Besides sucrose, polar auxin transport (PAT) plays a key role in root growth (Bhalerao et al., 2002; Chen et al., 2014; van Gelderen et al., 2018). Roots grown in darkness have less rootward auxin transport, due to relocation of the PIN-FORMED (PIN) auxin efflux carriers from the plasma membrane to vacuoles (Laxmi et al., 2008; Sassi et al., 2012). Although auxin and sugars play important roles in root growth and development, evidence indicate that other molecules affect root development by modulating auxin activity and signaling or by auxin-independent pathways (Fukaki and Tasaka, 2009).

In natural conditions, the root systems of most terrestrial plants grow underground and in darkness, nevertheless, the roots are able to respond to illumination of the shoot (Galen et al., 2007; Xu et al., 2013; Lee et al., 2016; Sakaguchi and Watanabe, 2017). It has been reported that the light perceived by the photoreceptors in the shoot tissues can affect root development via long-distance signaling pathways (Salisbury et al., 2007; Sassi et al., 2012; Lee et al., 2016; van Gelderen et al., 2018). The key photomorphogenic regulator HYPOCOTYL ELONGATED 5 (HY5) was recently shown to be transported from shoots to roots (Chen et al., 2016). When in the root system, HY5 activates its own expression and target genes involved in N uptake, acting as a shoot-to-root mobile signal (Chen et al., 2016; Zhang et al., 2017). However, the mechanism by which HY5 regulates root photomorphogenesis is not fully understood.

One of the key mechanisms that enable plants to respond to external conditions is a fine regulation of gene expression. In recent years, next-generation sequencing (NGS) technologies have made possible gene expression profiling in different cells and tissues, different physiological conditions, in response to external stimuli and others (Zhang et al., 2014; Conesa et al., 2016). RNA sequencing (RNA-Seq) has been considered the

experimental standard for transcriptomic investigations (Kukurba and Montgomery, 2015; Hrdlickova et al., 2017). Even with the large number of methodologies available, still there is no optimal pipeline for transcript quantification, normalization and differential expression analysis (Costa-Silva et al., 2017). The performance of the applied methodology changes according to the different studies. In order to obtain more successful results, the use of more than one tool is recommended (Soneson and Delorenzi, 2013). The transcriptogram is a tool that provides a hierarchical view of gene expression, starting from a global view of the main metabolic pathways, followed by the indication of the most responsive pathways, allowing the identification of the main genes responsible for this alteration (Rybarczyk-Filho et al., 2011; da Silva et al., 2014). The starting point of a trancriptogram is the generation of an ordered gene list based on previously described protein-protein interactions (Rybarczyk-Filho et al., 2011). The ordered gene list is the basis for the identification of gene groups significantly altered in response to the treatment. The transcriptogram methodology has successfully been used in human (da Silva et al., 2014; De Almeida et al., 2016), *Saccharomyces* (Rybarczyk-Filho et al., 2011) and microorganism (Ferrareze et al., 2017) data-sets. Until now, no work has reported its use to analyze plant data-sets.

Here, in an attempt to address the genes involved in the early photomorphogenic development of dark-grown roots in response to shoot-illumination, we performed a transcriptomic analysis of *Arabidopsis thaliana* roots from plants grown with shoots exposed to light and roots in the dark, as well as roots from seedlings grown in complete darkness. We demonstrate that the shoot-illumination significantly changes the transcriptome of dark-grown roots altering several metabolic pathways that reprogramed roots to induce their growth.

Results

Shoot illumination largely affects root transcriptomic profiles

To understand the effects of the shoot illumination on dark-grown roots in Arabidopsis, we adapted the D-root system (Silva-Navas et al., 2015) to grow roots in darkness and shoots in light to mimic the natural root growth environment. Seedlings grown with the shoots exposed to light and roots in dark (LD condition) showed longer roots than seedlings grown in complete darkness (D condition; Figure 1a) in all time-points. Based on

this observation, we attempted to analyze the transcriptome profile of roots at 4 and 7 days-old in both D and LD conditions. When we compared the transcriptome data of roots grown on LD with D, we found 1616 DEGs at 4 days-old, and 3920 DEGs at 7 days-old (Figure 1b, Supplementary Table 2) indicating a striking effect of the shoot illumination on the root transcript profiles. Among all light-influenced genes, 515 were down-regulated whereas 847 were up-regulated in both time points (Figure 1c). We found 360 DEGs between 4 and 7 days-old in D condition and 36 between 4 and 7 days-old in LD condition (Figure 1b; Supplementary Table 2). The low number of DEGs between the 4 and 7 days-old datasets in the same growing conditions (Figure 1b) indicates that there is very little variation on gene expression over time in the seedlings, although there is a significant phenotypic difference in root growth (Figure 1a) over time. This observation suggests that by 4 days-old there is already a fully established transcriptional response in roots that will lead to the growth differences observed in later stages as 7 and 10 days-old.

Gene ontology analysis showed that photosynthesis, cell wall organization and response to karrikin were the main categories up-regulated in roots by shoot illumination. In addition, other significantly up-regulated categories were related to secondary metabolic process, response to organic substance and hormonal signaling as well as flanonoid biosynthesis (Figure 1d). Among the down-regulated processes, the main categories affected were response to drug, response to stimulus and response to wounding with an interesting appearance of jasmonate-responsive genes among the top down-regulated genes in 4DL condition (Supplementary Table 2). These results suggest that roots from dark grown seedlings induce defense-related pathways that repress root elongation in the absence of light signals, whereas light signals highly induce cell-wall reorganization and hormonal signaling to promote root elongation.

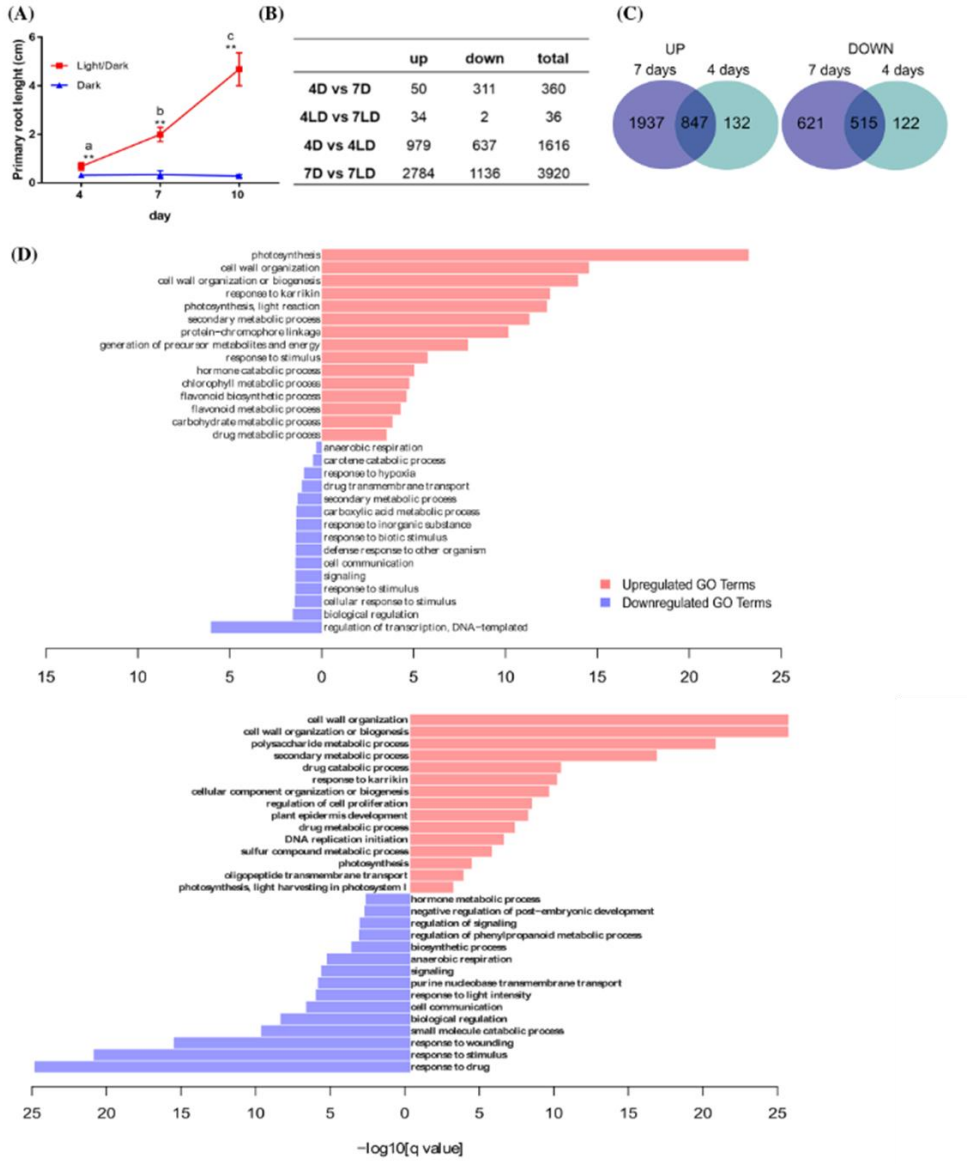

Figure 1 Shoot-illumination affects growth and gene expression in dark-grown roots. (A) Primary root length of wild-type Arabidopsis seedlings grown in LD and D at 4, 7 and 10 days after germination (n≥ 10). Statistical significance was determined by Student's t-test (* p≤ 0.05; ** p<0.01). Different letters represent a significance difference (p≤ 0.05) determined by one-way analysis of variance (ANOVA) with post hoc Tukey test. Error bars indicate SD. (B) Root RNA-seq analysis comparison between the 4 and 7 days after germination timepoints in D or LD conditions. Number of transcripts showing significantly altered expression levels between two libraries. Genes with p-value ≤0.05 and a 2-fold change were considered differentially expressed between every two libraries (C) Venn diagram summarizing the overlap between up- and down- regulated genes between 4LD and 7LD libraries. (D) Functional enrichment analysis of up- and down-regulated genes at 4LD (top panel) and 7LD (bottom panel) compared to the dark (4D and 7D respectively) libraries. The top 15 enriched terms were listed were red bars represent up regulated and blue bars represent down regulated GO terms respectively.

Arabidopsis thaliana transcriptogram: window selection

RNAseq analysis is based on the quantitative gene expression pattern in different conditions. The performance of the applied methodology changes according to the study. To complement our expression analysis we used the transcriptogram methodology (Rybarczyk-Filho et al., 2011). We were able to retrieve from the STRING v10.5 *Arabidopsis thaliana* database (Szklarczyk et al., 2017) information for 15.000 proteins and 525.212 interactions (interaction score $\geq$ 0.800). Using this information, we developed the first *Arabidopsis thaliana* trascriptogram described (Figure 2a).

In order to determine the expression profile of our data-set, we tested seven possible windows (30, 45, 60, 90, 120, 150 and 300 neighboring genes) on the gene list. Decreasing the window size increases the peaks' height and decreases width (Supplementary Figure 2) leading to a very high number of peaks. On the other hand, increasing the window size decreases the peaks' height and increases width. This can lead to merge neighboring pathways, which may decrease the signal and introduce false positive results. By comparing the different window profiles, we chose to use window size 90 as the final window to obtain the expression profiles of our data-set.

Transcriptogram analysis identifies a large number of gene sets in roots affected by shoot-illumination

As we aimed to elucidate the shoot-light influence in the dark-grown root, we focused our comparison between LD and D conditions. When we compared LD condition with D condition, the same peak distribution is seen among 4 and 7 days-old (Figure 2b) with mostly the height of the peaks (significance) being increased over time. The similar transcriptogram peaks distributions observed between 4 and 7 days-old timepoints suggest that there is an increased quantitative response to the light condition over time in roots, but qualitatively, the regulated processes remained the same. We observed 20 significantly induced regions in 4 days-old and 14 regions in 7 days-old libraries. Three regions were repressed in 4 days-old and 19 in 7 days-old samples. The main categories changed on samples were photosynthesis, ion transport, hormone signaling pathways and RNA processing (Figure 2c). The significant regions comprise 2916 genes in 4 days-old and 4004 genes in 7 days-old (Supplementary Table 3).

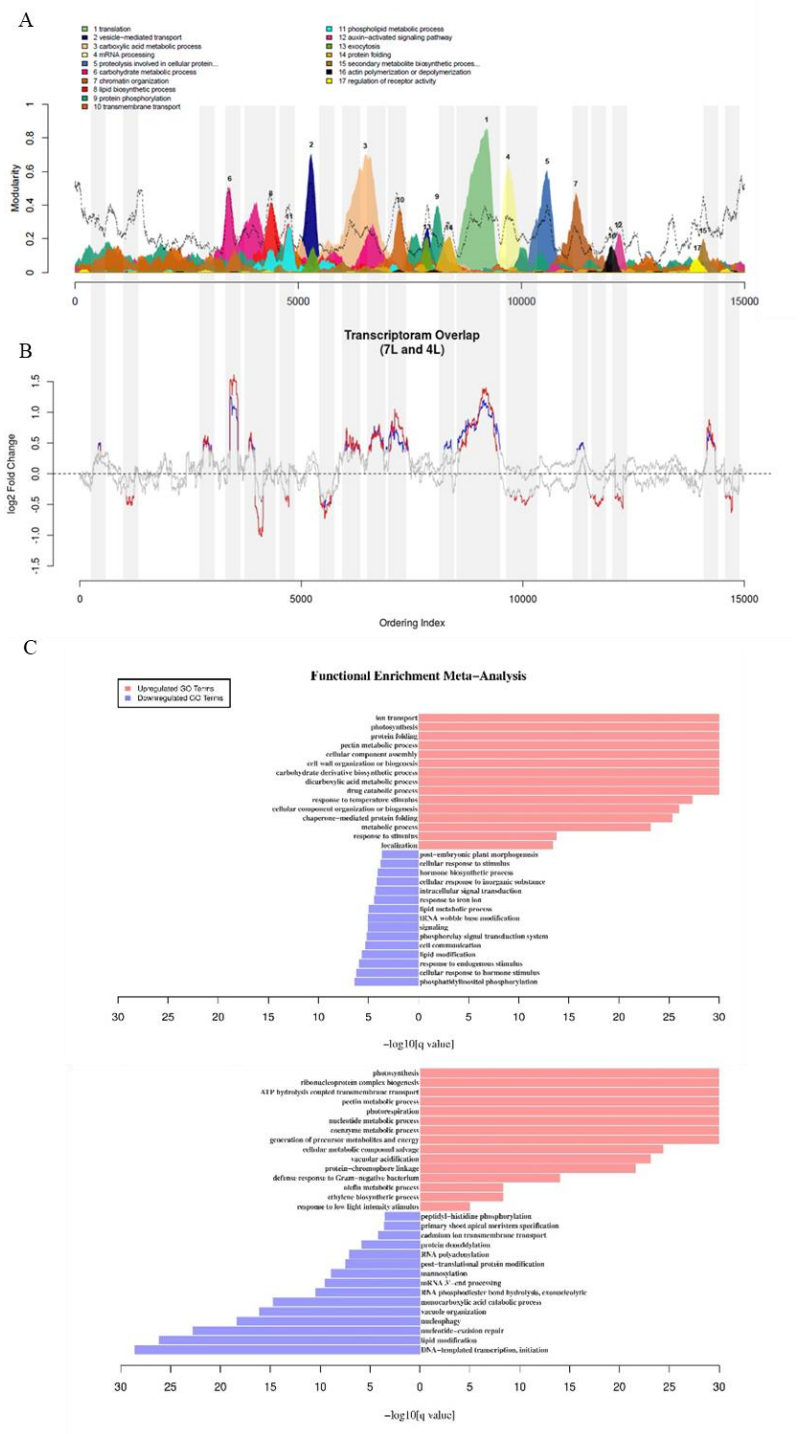

Figure 2 *Arabidopsis thaliana* transcriptogram. (A) The x-axis relative to gene position have been divided by the total number of proteins retrieved from STRING. Projection of Gene Ontology terms is color-coded. (B) Average transcriptograms for r=90, light x dark significant peaks (up-regulation) or valleys (down-regulation) are represented by blue and red lines in 4LD and 7LD, respectively. Grey lines show non-significant regions. (C) Functional enrichment analysis of peaks or valleys from B. The top 15 enriched terms were listed. Red bars represent up regulated and blue bars represent down regulated GO terms respectively.

Root genes affected by shoot-illumination

Both methods used (DEseq and Transcriptogram) resulted in a large set of DEGs between LD and D conditions. In order to filter the total number of genes, we combined the final result of both methodologies. When overlapped the results, about 10% of the genes altered in response to light were common in both adopted methodologies (201 genes at 4 days-old and 609 genes at 7days-old; Figure 3a; Supplementary Table 4). A detailed look of the genes presents at the intersection showed that most of these genes are related to primary metabolic processes of plant development, such as photosynthesis, synthesis and catabolism of organic compounds, amino acids and translation (Figure 3b). Remarkably, the applied methodologies resulted in two very distinct set of genes/processes altered in dark-roots in response to shoot-illumination, opening up new possibilities that have not been explored by the GO term enrichment (DeSeq).

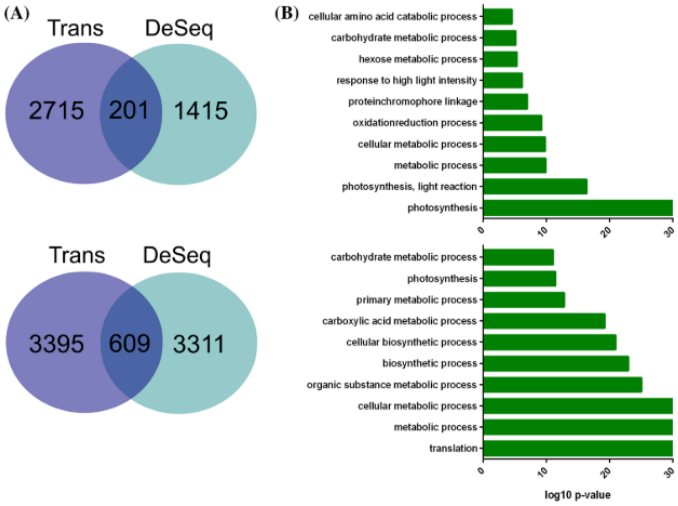

Figure 3 (A) Venn diagram of DEG from DeSeq and transcriptomic analysis (Trans) from 4 and 7 DAG of light modulated genes. (B) Summary of the functional enrichment analysis of the genes present in the overlapping of A, 4 days-old (top panel) and 7 days-old (bottom panel).

To validate our expression data, we selected 10 genes which have already been described as related with: light responses, root development, auxin and the positive photomorphogenic regulator HY5 (Figure 4a and b, Supplementary Table 4). The expression of *Flavonol Synthase 1* (*FLS1*) in the RNAseq data was around 8-fold higher in roots under shoot-illumination, when compared to the dark condition in both time points. The same expression pattern was observed in the qPCR with a higher intensity (around 45-fold). The

Indole-3-Acetic Acid 17 (*IAA17*), *Rotamase CYP 1* (*ROC1*), *ROC5*, SCAR1, *Short Hypocotyl 2* (*SHY2*), *WAG1*, *YUCCA3* and *YUCCA9* were up-regulated in the RNA-seq analysis and showed the same expression pattern in the qPCR. The qPCR results were fairly consistent with that of the RNA-seq analysis, this finding verified the accuracy of the RNA-seq results.

Mutants for root light-responsive genes display diverse primary root growth phenotypes

To investigate whether these identified genes are involved in root development, we examined the primary root growth in loss-of-function mutants. It is noteworthy that the phenotyping experiments (Fig. 4B and 4C) were performed in a different laboratory and, although the absolute lengths of the roots look different than Figure 1, the overall relative growth of the plants is comparable. In general, root length was altered in all mutants analyzed as shown in Figure 4c. The *fkbp-like* loss-of-function mutant showed shorter roots than Col-0 in both times, *fls1* and *yuc9* mutants were shorter than the wildtype only in the dark condition. The *iaa17* and *wag1* displayed increased primary root length when the shoots were illuminated and shorter root when growth in the darkness. The *roc1-2*, *roc5* and *yuc3* mutants showed longer roots than Col-0 when the shoot was illuminated. The *shy2-2* seedlings showed changes in the root development in both times and conditions, whereas *scar1-t1* seedlings were pretty similar to the wildtype plants. The *fkbp-like* mutant exhibited shorter roots than the wild-type in 7 days-old in the D condition, as well as in LD in 10 days-old. On day 7, *fls1* and *iaa17* seedlings showed shorter roots at D condition, as opposed to *yuc9*, which showed shorter roots at day 10. The *roc1-2* and *roc5* mutants showed longer roots than wild type in 7 and 10 days-old only in the LD condition. Interestingly, *shy2-24* seedlings were the only ones that presented difference in both time and light conditions, with shorter roots in D and longer roots in LD. The *wag1* seedlings had less root development in the D condition at 7 days-old while it had longer roots than the WT at 10 days-old in the LD condition. The results presented above suggest that these genes are somehow involved in the photomorphogenic development of dark-grown roots, however further studies are necessary to investigate their role in this process.

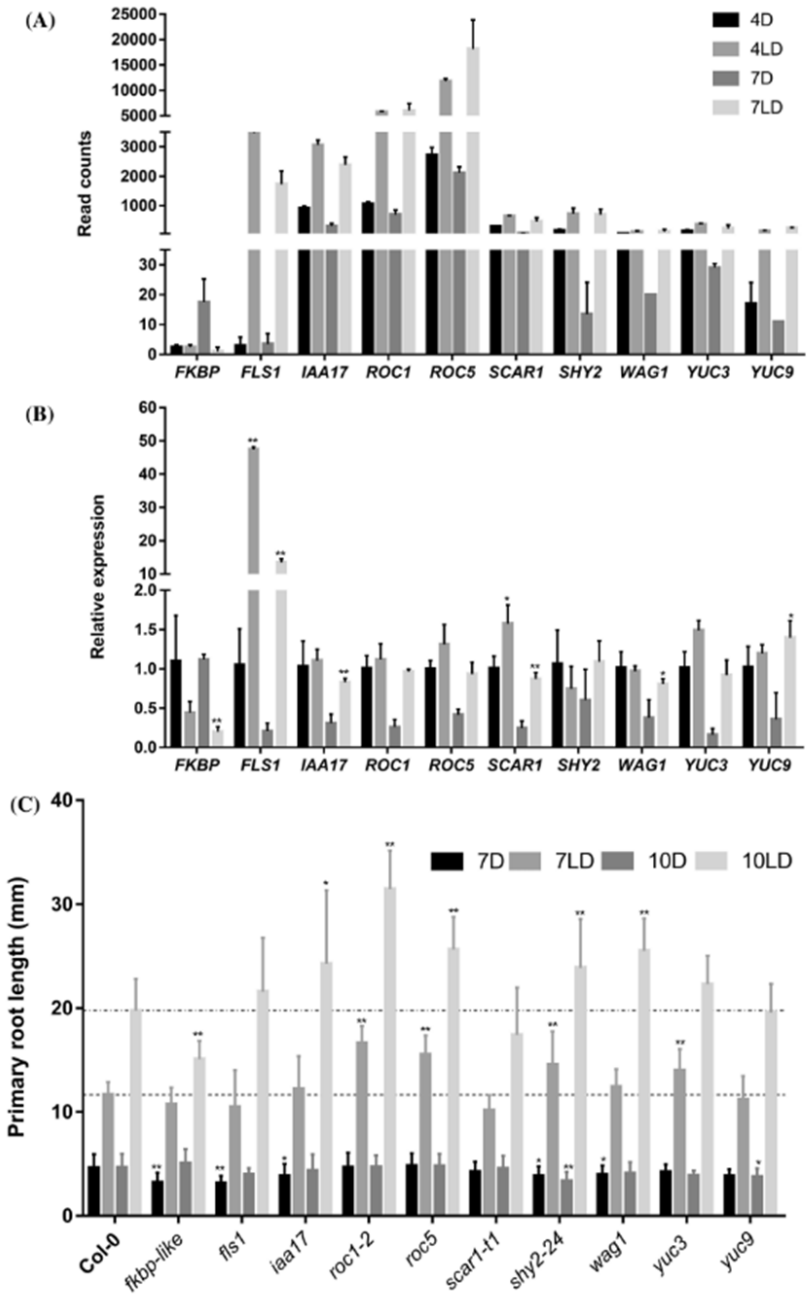

Figure 4 Expression patterns of ten root-related genes in Arabidopsis dark-grown roots. (A) (B) Number of read counts obtained in the RNA-seq for the selected genes.Validation of the transcriptomic analysis by qRT-PCR analysis of gene expression in roots. Gene expression was normalized using an internal control (AT3G18860 and AT2G42500) for each reaction. The expression levels of 4D condition were set as 1. The LD samples were compared with D samples at the same time. Statistical significance was determined by Mann-Whitney test when necessary or by the Student's t-test (* $p \leq 0.05$; ** $p<0.01$). (C) Primary root growth lengths of single knockout mutants ($n \geq 15$). Statistical significance was determined by Kruskal-Wallis test with Dunn's post-test (* $p \leq 0.05$; ** $p<0.01$). Error bars indicate SD.

Discussion

In this work, we provided a transcriptome survey to understand the main genes and processes that are regulated in dark-grown roots in response to shoot illumination. In nature, roots are plant organs that typically grow belowground in the darkness. Several reports have suggested that *Arabidopsis* growing under traditional agar-plate culture system (with shoots and roots exposed to light) is not under a natural environment for the roots and may cause artifact responses (Xu et al., 2013; Qu et al., 2017). With this concern in mind, we used a modified D-root system (Silva-Navas et al., 2015) in order to emulate the natural growth conditions in an *in vitro* setting, with roots in the dark and shoots exposed to light. Our modified D-Root system prevented light illumination from the sides and the bottom, whereas not completely prevented the light illumination from the top. Even though our system does not completely block the top-light, it allows roots to grown in a light gradient. The data presented here are in agreement with previous studies that primary dark-grown roots grow longer when cultivated with the shoot exposed to light (Xu et al., 2013; Silva-Navas et al., 2015; Qu et al., 2017; Sakaguchi and Watanabe, 2017). It has been shown that roots exposed to light show changes in the gene expression profile when compared to dark-grown roots (Silva-Navas et al., 2015). Our data shows that shoot illumination leads to changes in the transcriptional profile in dark-grown roots when compared roots of seedlings grown in darkness. When we compared or DEGs with a previous data-set from roots using the D-Root system (Silva-Navas et al., 2015), we found that only 443 genes were differentially regulated in both experiments (Supplementary Figure 4). This shows that both datasets describe very distinct processes and that shoot illumination has potentially a more drastic effect on gene expression than root illumination. Also, our findings are consistent with the hypothesis that the roots are affected by the aboveground light environment and reprogram the transcriptional responses in roots to optimize growth. To further elucidate this hypothesis, we addressed the shoot-illumination effect on dark-grown roots of several mutant lines. All the mutant lines tested showed longer roots when shoots were exposed to light. The overall root length of the mutants was longer than wild-type plants, even though the respective knocked out genes were induced by light. This indicates that some early light responsive genes might act as repressors of photomorphogenic growth.

It is notable that the transcriptional responses in the roots to shoot-illumination led to changes in metabolic and catabolic processes in the dark-grown roots, reinforcing the idea that signaling molecules derived from the illuminated-shoots induce the development of dark-grown roots (Chen et al., 2016; Lee et al., 2016; van Gelderen et al., 2018). As expected, changes in the shoot light condition are responsible for the regulation of some key process in roots, as photosynthesis, cell wall organization, response to oxygen and wounding, ion transport and translation. Interestingly, induction of genes related to photosynthesis and cell wall organization have already been observed in roots not exposed to light directly (Lee et al., 2016). The induction of photosynthetic responses in dark-grown roots could be related to the fact that our system does not completely block the top-light. Roots of 4 days-old were probably more affected by the top-light gradient, as these roots are shorter when compared to 7 days-old roots. Beyond these, the biosynthesis of flavonoids has been linked to light responses in the roots (Lee et al., 2016; Silva-Navas et al., 2016; Qu et al., 2017). In our data-set, processes related to the biosynthesis of flavonoids were observed as differentially expressed in 4 days-old, although genes from the biosynthetic route were induced at both times. An induction in the pathways related to biosynthesis flavonols have already been reported (Lee et al., 2016; Qu et al., 2017). Silva-Navas et al. (2016) suggested that flavonols may act as positional signals to control the light-responses in root tropism. Taking this information into account, flavonols could also act regulating the primary root growth in response to shoot-illumination. Our transcriptogram analysis showed an up regulation in translation processes that was not identified by the DeSeq analysis, reinforcing that different approaches on analyzing the transcriptome data can lead to different sets of differentially expressed genes.

Auxin has been reported several times to play an important role in root development (Bhalerao et al., 2002). In darkness, the expression and localization of PIN1 is shifted, thus reducing auxin transport to the root system (Sassi et al., 2012), suggesting that light directly influences the auxin transport. The AGC3 kinase WAG1 was found to be a negative regulator of root waving (Santner and Watson, 2006) and act as well as PINOID in the control of PIN1 and PIN2 polarity establishment in roots (Huang et al., 2010; Dhonukshe et al., 2015). The expression level of *WAG1* was increased in shoot-illuminated samples, moreover, *wag1* mutant line showed shorter primary roots in darkness. The closely related AGC3 kinase WAG2 has been shown to regulate the maintenance or formation of a local

auxin maximum in the apical hook by phosphorylation of the central intracellular loop of all PIN proteins (Willige et al., 2012). The expression pattern showed by *WAG1* together with the altered root phenotype suggests that the AGC3 kinase WAG1 possibly can play a similar role in root cells.

In higher plants, the FKBP and cyclophilin families are involved in specific developmental functions. Recently, the plant immunophilins were identified as regulators of polar auxin transport (Ivanchenko et al., 2015; Geisler et al., 2016). Whereas the ROC1/AtCYP18-3 protein has been considered a link between photoreceptors signaling and hormone sensitivity in Arabidopsis (Trupkin et al., 2012), as well as a shoot-to-root long-distance signaling (Spiegelman et al., 2015), the immunophilins can be good candidates to regulate dark-grown roots. In this study, *ROC1* and *ROC5* expression was induced in roots of light-grown seedlings and their respective loss-of-function mutants showed longer roots than WT, in contrast of *FKBP*-like, which was repressed in light-grown seedlings and *fkbp1-like* showed a shorter main root than the WT. These observations indicate that the cyclophilin genes could possibly act as integrators between light and the auxin transport in the control of dark-grown roots to maintain normal development. Besides shoot-to-root auxin gradient, the local auxin biosynthesis contributes for normal root development and root gravitropic responses (Bhalerao et al., 2002; Petersson et al., 2009; Chen et al., 2014). The auxin biosynthetic *YUC3* and *YUC9* genes were up-regulated in roots in response to shoot illumination. Previous studies have also reported a higher expression of these genes in root cells (Chen et al., 2014), which contributes to the normal root development. Future studies will be necessary to address accurately the function of these genes in dark-grown roots.

Although the auxin perception and signaling mediated by the auxin/indole-3-acetic acid (Aux/IAA) is well established, its contribution to the light-mediated root growth has not yet been clarified. Gain-of-function mutations in several Aux/IAA genes, such as *shy2-2*/*IAA3* and *axr3-3/IAA17* mutants, result in altered photomorphogenic phenotype (Nagpal et al., 2002; Tian et al., 2002) which reinforces the biological relevance of the link between auxin- and light- regulated genes. In this study, we observed an induction of *SHY2* and *IAA17* gene expression in response to shoot-illumination, fairly consisted with previous reports for *SHY2* (Tian et al., 2002). We found that loss-of-function mutants showed longer roots than WT when light-grown. It is noteworthy that we found *shy2-24* and *iaa17* to have shorter

roots in dark-grown condition. This is consistent with the suggestion that the turnover of Aux/IAAs may be an important factor in modulating dark-grown root.

High-throughput sequencing has become the main choice for analyzing global transcript levels. In this work, we show that shoot-illumination affects about four thousand transcripts in roots. The overall expression profiles in roots do not change over time and are much more affected by light condition than seedling age. Although the total number of genes altered in the transcriptogram were similar to that observed in the DeSeq analysis, the overlap pattern of the two methodologies (DeSeq vs Transcriptogram) was reduced to a small number of common genes. By combining the two approaches, we believe that it was possible to select relevant genes more robustly, as the majority of the mutants we tested for root growth displayed significant phenotypes. Based on the shared genes, we revealed a new repertoire of genes involved in dark-grown roots development. It would be of special interest to explore the functions of these genes in the early dark-grown root photomorphogenesis in Arabidopsis.

Here, we aimed to investigate the mechanism by which light influences dark-root growth. Transcriptomic analysis showed that shoot-illumination triggers broad alterations of gene expression in dark-grown roots. Combining two differential expression methodologies, we were able to define a robust gene-set differentially expressed in dark-grown roots. In addition, transcriptogram analyses showed to be a good approach to investigate global gene expression.

Materials and methods

Plant materials

Arabidopsis Columbia (Col-0) was used as wild-type (WT), and the mutants *fkbp-like* (SALK_047305), *fls1* (SALK_009992), *iaa17* (SALK_065697C), *roc1-2* (SALK_121820C), roc5 (GK-177D02), *scar1-t1* (SALK_017554), *shy2-24* (Tian and Reed, 1999), *wag1* (SALK_002056), *yuc3* (SALK_030785) and *yuc9* (SALK_066251) are in Col-0 ecotype background and were obtained from the The European Arabidopsis Stock Centre (NASC, http://arabidopsis.info/).

Growth conditions for the RNA-seq experiment

Arabidopsis thaliana Col-0 seeds were surface sterilized and cold-stratified at 4°C for two days in complete darkness to synchronize germination. Plants were grown on half-strength sucrose-free Murashige and Skoog (Sigma-Aldrich, M5519) media supplemented with 1.5% agar (w/v; Merck Millipore,107881) and 0.05% MES hydrate (w/v; Sigma-Aldrich, M8250), pH 5.7, on vertically oriented 12-cm-square plates. Seedlings were grown at 21°C ± 2°C under long days photoperiod (16-hour light and 8-hour dark) with white light illumination (130µmol m−2 s−1) in a modified D-Root system (Silva-Navas et al., 2015) which consisted of a black paper surrounding ¾ of the petri dish length. Seeds where plated at the limit of the black cover so that the roots would grow into the darkened side of the plate (Supplementary Figure 1). For dark grown plants, the plates were completely covered with aluminum foil. Roots of 4 and 7 days-old seedlings were used for RNA isolation. The experiment was performed in the Plant Physiology laboratory at UFRGS, Porto Alegre, Brazil.

RNA isolation and RNA-seq analysis

Four and seven day-old roots from both light (4LD and 7LD, respectively) and dark grown plants (4D and 7D, respectively) were immediately frozen in liquid N_2 (two independent biological samples, composed of approximately 70 seedling each) and total RNA was isolated using NucleoSpin® RNA (Macherey-Nagel) according to the manufacturer's instructions. RNA concentrations were verified using the Nanodrop spectrophotometer (Thermo Scientific). Samples with concentrations ranging from 250 ng to 1 µg of RNA were precipitated with 3M NaOAc, 5mg/mL glycogen and ethanol and the pellet was kept in 70% ethanol for shipping. RNAseq sequencing was performed by Fasteris SA, Switzerland. Library preparation followed RiboZero treatment and sequenced in Illumina HiSeq 2500, generating High-Output Single-reads, 1x 125 bp.

Data filtering and mapping of reads

The raw reads were filtered before data analysis, the presence of adapters and quality of reads were determined using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Based on these data, the Trim Galore! software (http://www.bioinformatics.babraham.ac.uk/ projects/trim_galore/)

was used to eliminate sequences of reads with a quality below 30, as well as the adapters. The cleaned reads were anchored with TopHat2 (Kim et al., 2013) to the reference genome of *Arabidopsis thaliana* (http://plants.ensembl.org/Arabidopsis_thaliana/Info/Index). During mapping, zero mismatches and unique mapped reads were allowed. The counting tables of the reads mapped to each gene were generated by the Subread software (Liao et al., 2013). The raw data for the sequencing libraries are deposited on NCBI GEO GSE132249.

Differentially expressed genes (DEGs) analysis

Quantification of DEGs was performed with the R package DESeq2 (Love et al., 2014). This method represents the widely accepted and accurate analysis approaches of RNA-Seq data. Those genes with p-value ≤0.05 were considered as significantly differentially expressed, and a 2-fold change was used to identify the genes differentially expressed between every two libraries.

Growth conditions for the qRT-PCR and mutant phenotyping experiments

Seeds were surface sterilized and cold-stratified at 4°C for two days in complete darkness to synchronize germination. Plants were grown on half-strength sucrose-free Murashige and Skoog (Duchefa, M0222) media supplemented with 1.5% agar (w/v; Duchefa, D1004) and 0.05% MES hydrate (w/v; Sigma-Aldrich, M8250), pH 5.7, on vertically oriented 12-cm-square plates. Seedlings were grown at 21°C ± 2°C under long days photoperiod (16-hour light and 8-hour dark) with white light illumination (120μmol m−2 s−1) in a modified D-Root system (Silva-Navas et al., 2015) as described above. For dark grown plants, the plates were completely covered with aluminum foil. Roots of 4 and 7 days-old seedlings were used for RNA isolation. The experiment was performed in the Institute of Biology Leiden at Leiden University in The Netherlands.

Validation of RNA-Seq analysis by qRT-PCR

Total RNA (three independent biological samples) was isolated from 4 and 7 days-old *Arabidopsis* roots as previously described. About 100ng of total RNA was used to synthesize first-strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fischer) following the manufacturer's instructions. Primers for each gene were designed using the QuantPrime (Arvidsson et al., 2008; Supplementary Table 1). qRT-PCR was

performed in a BioRad CFX96™ Real-time System C1000 Thermal Cycler using SYBR® Premix Ex Taq™ (TaKaRa, Japan) according to the manufacturer's protocol. Three independent technical replicates from three individual cDNA templates were used. The transcript levels were normalized against the reference genes AT3G18860 and AT2G42500, defined using the geNorm software (Supplementary Figure 1; Vandesompele et al., 2002).

Arabidopsis thaliana transcriptogram

Protein-protein interaction data for *Arabidopsis thaliana* was downloaded from STRING v10.5 database (Szklarczyk et al., 2017), with a cutoff combined score of 0.800. The protein ordering process was performed by the Transcriptogramer v1.0 software (Rybarczyk-Filho et al., 2011; da Silva et al., 2014). The transcriptograms were generated using the R package Trancriptogramer (Morais et al., 2019) for both 4 and 7 days treatments and plotted as: For each position i of the ordering indexes in the transcriptograms, the attributed relative expression value for such position was determined by

$$Log_2\left(\frac{T_i^{LIGHT}}{T_i^{DARK}}\right)$$,where

T_i is the mean expression of the genes encoding for the proteins belonging to the i^{th} windowed interval. The individual gene expression values were determined by the read counts mapped to each gene.

Statistically significant peaks and valleys on the transcriptograms were spotted through a Monte Carlo sampling process, where random sets of ordering indexes permutations were drawn and used to generate *random transcriptograms*, from which null distributions of peaks/valleys lengths were inferred. Critic values for statistically significant peaks and valleys ($p < 0.05$) were then determined based on such null distributions. The number of permutations for each test was set after the convergence of critic values was observed. This statistical analysis was performed through in-house scripting on the R programming environment. All of the functional enrichment analysis were done using the topGO package (classic Fisher test with Benjamini-Hochberg correction; Alexa and Rahnenfuhrer, 2016) and the GO term annotations for the *Arabidopsis thaliana* genes were obtained from the ENSEMBL Plant database through the biomaRt package (Durinck et al., 2009). The software REVIGO was used to remove redundant terms (http://revigo.irb.hr/).

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Capítulo III

- Effects of light intensity on root development in dark-grown roots -

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Frontiers in Plant Science – Technical Advances in Plant Science

Research Topic – Highlights of the 2nd D (dark-grown)-root meeting

Effects of light intensity on root development in dark-grown roots

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Abstract

Plant development is known to be affected by light quality, quantity and intensity. The effect of light on root growth suggests there is a light dependent signaling pathway from illuminated shoots to the root system. Here, we addressed the potential role of sucrose and auxin as long-distance signals and we also explore the transcriptome data provided in Miotto et al. (2019) looking for key genes involved in the early photomorphogenic root development of D-roots. We observed that the presence of sucrose increases root length to a much lower extent than the effect of an illuminated shoot. Auxin transport proteins accumulated in roots grown with shoots exposed to light, however synthetic auxin response reporters and auxin quantification suggest that it does not prompt changes the auxin maxima in roots. We selected genes that had been related to light/auxin or the photomorphogenic regulator HY5 and confirmed the RNA-seq expression patterns for 84% of the candidate genes using qPCR. Primary and lateral root development of wild-type and single T-DNA lines seedlings were highly sensitive to the light intensity to which shoots are exposed. These results provide a background evidence that changes in light intensity in the aerial tissue highly influence the development of roots in the dark. Further studies are necessary to identify the role of each candidate gene in the root adaptation to shoot-illumination.

Introduction

Communication between adjacent cells is essential to fine tune the coordination of cell proliferation and differentiation. In animals, cell-to-cell communication is facilitated by the neuronal and circulatory systems. In plants, the vascular system provides the major route for long distance intercellular communication (Ko and Helariutta, 2017). A broad range of substances including RNA, proteins, peptides and phytohormones have been detected in plant vasculature (Notaguchi and Okamoto, 2015), acting in long distance communication to adapt to the environment by balancing growth and resource allocation (Chaiwanon et al., 2016). Long-distance signals enable plants to coordinate shoot/root growth and development in response to internal and external oscillations (Notaguchi and Okamoto, 2015). In roots, grafting studies have provided evidence that shoot derived signals are required for nutrient uptake and root development (Lee et al., 2017; van Gelderen et al., 2018b). Root development takes place underground in the darkness. However, the phenotypes observed between skoto- and photomorphogenic seedlings clearly suggest that even in the dark, root morphology is modulated by long-distance signals, derived from the aerial tissues. Plant hormones, photosynthetically-derived sugars, and more recently, proteins have been directly linked to the long-distance signaling between shoots and roots (Lee et al., 2017; van Gelderen et al., 2018b).

The cross-talk between shoots and roots have been extensively studied, the plant hormone auxin (indol-3-acetic acid, IAA) was the first described candidate as a shoot-to-root integrator. Young leaves are the main source of auxin synthesis, which is mainly transported to the root via phloem, where it regulates root development (Bhalerao et al., 2002). Cellular localization of the PIN-FORMED (PIN) auxin efflux carriers was shown to be mediated by shoot signals. When grown in darkness, PIN1 is de-localized from the plasma membrane, reducing auxin delivery to the root system (Laxmi et al., 2008; Sassi et al., 2012), suggesting that auxin is a good candidate to mediate shoot-to-root communication in response to light. Photosynthetic derivates have also been proposed as long-distance signals between shoots and roots. Root growth has been shown to be inhibited when photosynthesis is blocked and restored by adding sucrose to the medium (Kircher and Schopfer, 2012). Photosynthetic-derived sugars are essential to induce and maintain root development as they

can transmit information from the shoot to the root system (Kircher and Schopfer 2012).

Mobile proteins and RNAs are also described as long-distance signals. In Arabidopsis, the transcription factor HY5 moves from the shoot to the roots via phloem, where it induces its own expression. The positive feedback loop created by HY5 promotes root development and nitrate uptake in response shoot illumination (Chen et al., 2016). By grafting experiments in tomato, a cyclophilin was shown to be necessary to lateral root initiation in tomato. The translocation of SlCyp1 from the shoots in response to changing light intensities readjust auxin responses and the transcriptome status in roots (Spiegelman et al., 2015). More than 2000 genes were shown to produce mobile RNAs in Arabidopsis whereas a high number of these seems to move in the shoot-to-root direction (Thieme et al., 2015), suggesting that a large number of mobile RNAs mediate root development in response to a signal delivered by the shoots.

It has been reported that plants can adjust root architecture using long-distance signaling pathways in response to the quality and intensity of the light stimulus (Chen et al., 2016; van Gelderen et al., 2018a). The increase in light intensity was shown to promote root growth, NO_3 uptake and to increase biomass accumulation (Nagel et al., 2006; Chen et al., 2016b; Kumari et al., 2019). On the other hand, the lack of a functional HY5 protein suppresses the main root elongation as well as shoot and root biomass accumulation (Chen et al., 2016). Roots exposed to various light intensities (38 to 150 μmol m^{-2} s^{-1}) showed a large increase in lateral and adventitious roots, together with changes in the expression of genes related to catalytic activity, hormone and light signaling, and clock-regulated pathways (Kumari et al., 2019).

Here, we addressed the effect of sucrose on root development and how root auxin homeostasis is affected by shoot-illumination. Next, we revisited the RNA-seq data from Miotto et al., (2019) and selected 18 additional candidate genes related to auxin and the transcription factor HY5. Root phenotype of T-DNA lines showed that light intensity affects root photomorphogenesis in a dose-dependent manner which might mask phenotyping of relevant mutations controlling the process.

Results

Sucrose does not overcome the light requirement for root morphogenesis

To determine the involvement of auxin and sucrose as possible mobile signals we used an adapted D-Root system (Silva-Navas et al., 2015) to grow Arabidopsis seedlings in a square Petri dish with shoots in light and roots in dark (Miotto et al., 2019), mimicking the natural root growth environment. We therefore evaluated the effect of sucrose in fully illuminated seedlings (L condition), illuminated shoots and light-protect roots (LD condition) and seedlings grown in darkness (D condition). As shown in Figure 1A, 7 days after germination (DAG) seedlings grown without sucrose in L or LD showed a similar main root length, whereas D seedlings showed a shorter main root when compared with both illuminated conditions. The addition of sucrose in the culture media induced root development in all conditions. Even though sucrose could induce the root growth of D seedlings, it was not enough to reach the light effect observed in L and LD seedlings, suggesting that light and sucrose act synergistically to induce root growth.

To further test this hypothesis, we checked the effect of light-dark and dark-light transitions in the primary root length, *i*) 4 DAG seedlings grown in LD with or without sucrose were transferred to D for additional 6 days and; *ii*) 4 DAG seedlings grown in D with or without sucrose were exposed to 8h light in the LD condition. In both cases, the lack of light produced short roots that could not be converted to long roots by sucrose supplementation (Figure 1B). Thus, these experiments combined suggest that light is essential to promote root growth, also that a short (8h) light treatment is not enough to trigger root growth if plants are transferred back to darkness, whereas sucrose can induce root development but it is not sufficient to complement the light absence. It has been shown that darkness reduces meristem size and inhibits cell proliferation (Sassi et al., 2012), we then decided to check how it is affected in our system. Seedlings grown in D condition showed a smaller root meristem (Figure 1C) with lower mitotic activity according to the CYCB1;1:GUS reporter (Figure 1D) than LD seedlings, suggesting that the short root observed in D seedlings (Figure 1A) reflects a reduction of meristem size and cell proliferation.

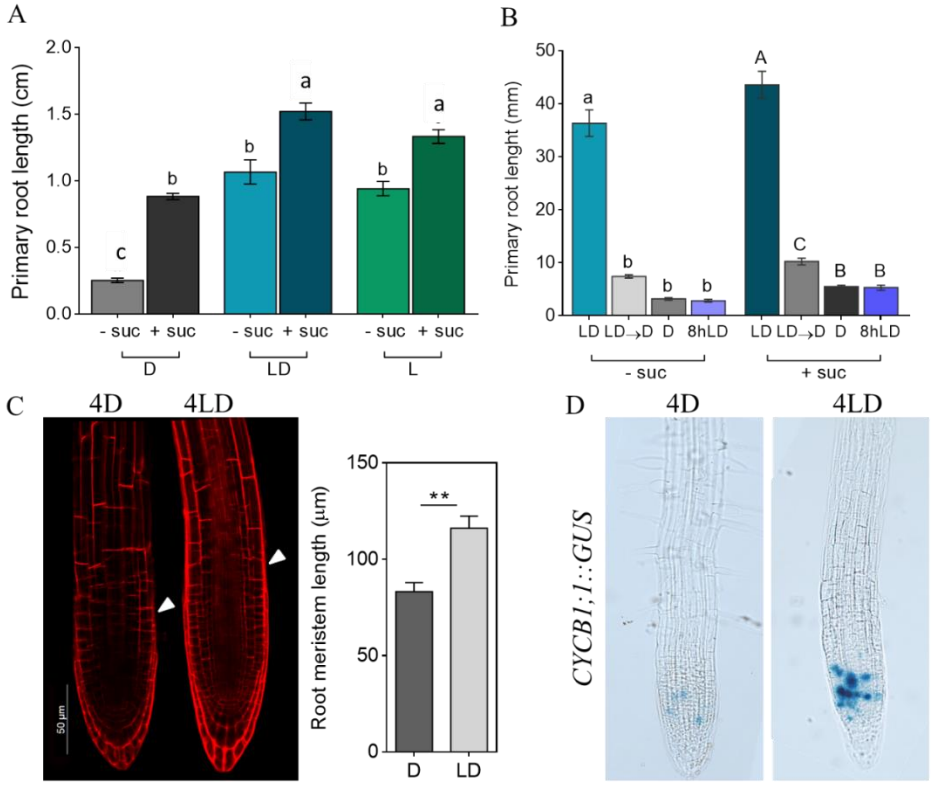

Figure 1. Sucrose and light act together in root development. (A) Primary root length of 7 DAG Col-0 seedlings grown without or with 1% sucrose in D, LD or L light conditions (n≥19). (B) Primary root length of 10 DAG Col-0. Wild-type seedlings were grown with or without sucrose for 4 days in LD and transferred to dark (LD →D) or kept in LD for additional 6 days (LD); alternatively, seedlings were grown for 4 days in darkness and kept in darkness for additional 6 days (D) or shoots were exposed to light for 8h (8hLD) (n≥10). (C) Root apical meristem length and (D) CYCB1;1:GUS expression patterns of 4-day-old D and LD grown seedlings. Root meristem is depicted as the distance between the quiescent center and the uppermost first cortical cell that is twice as long as it is wide indicated by the white arrowheads. Letters denote different significant classes (p≤0.05) by Anova test with Tukey post-test. Asterisks denote significant difference against the Control by Student's t test (**p≤0.001). Error bars indicate SE.

Systemic PAT inhibition represses primary root growth

Auxin has been suggested to be a long-distance signal integrating light and root development (Sassi et al., 2012). We therefore decided to test how local application of the auxin transport inhibitors naphthylphthalamic acid (NPA) and 2,3,5-Triiodobenzoic acid (TIBA) affect the main root growth. Local applications of a paste containing 10µM NPA or TIBA at the shoot-to-root junction of 6 DAG seedlings were unable to repress primary root growth (Figure 2A and B), suggesting that shoot-to-root polar auxin transport is not essential for primary root growth. However, supplementation of NPA or either TIBA in the growth

medium caused a strong inhibition of the main root growth (Figure 2C), resembling the seedling root phenotype when grown in the dark, supporting the idea that the root development of dark-grown roots may be caused by an inhibition in auxin transport as long as PAT is suppressed systemically. Furthermore, we grew seedlings in the D condition for 2 days in medium supplemented with 10nM of 2,4D or NAA. Both treatments showed no effect on primary root growth in D condition, whereas in both cases primary root growth was repressed in LD (Figure 2D). These results suggest that systemic repression of polar auxin transport suppresses the primary root growth, however local inhibition seems to have no effect.

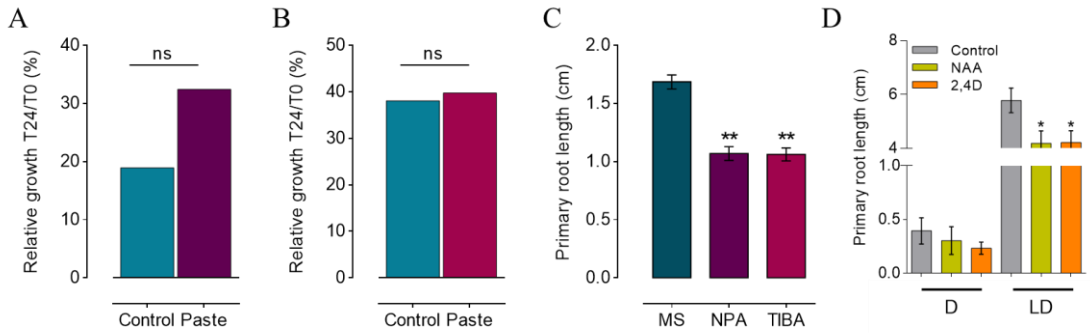

Figure 2. The effect of auxin transport-inhibitors and synthetic auxin on Arabidopsis primary root growth. (A) 10µM NPA and (B) 10µM TIBA were applied in the shoot-to-root junction in 6 DAG seedlings grown in LD. Primary root lengths were measured before the treatment (T0) and 24h after the application (T24). The data were analyzed by Student's t test for each sample compared T24 with T0 (*p≤0.05; **p≤0.01). (C) Primary root length of 7 DAG Col-0 seedlings grown without or with 10µM NPA or TIBA in LD light condition. Seedlings were grown in MS for 4 days and then transferred to a new medium supplemented with NPA or TIBA for additional 3 days. (D) Root length of seedlings grown for 5 days in MS and then transferred to a medium supplemented with 10nM NAA or 2,4D for 48 hours. Asterisks denote significant difference against the Control by Student's t test (**p≤0.001). Error bars indicate SE (n≥15).

Enhance in PIN polarity does not enhance auxin maxima in LD roots

Shoot-to-root auxin transport have been shown to be regulated by the photomorphogenesis repressor COP1 (Sassi et al., 2012), leading to changes in root elongation and lateral root initiation (Sassi et al., 2012; van Gelderen et al., 2018a). We showed previously that systemic chemical inhibition of polar auxin transporters strongly represses primary root development (Figure 2C). To gain insights into how LD root morphogenesis is controlled by auxin transport, we compared the PINs and ABCBs proteins cellular distribution by accessing GFP-fusion reporter lines in LD and D light conditions.

When shoots are exposed to light (LD condition), we observed a clear plasma membrane (PM) localization of PIN1 and ABCB19, the two main rootward auxin transporters (Figure 3A and B), whereas the PM signal was much reduced when seedlings were grown in D condition. A similar result was observed to PIN2 (Figure 3C) and ABCB1 (Figure 3D), suggesting that shoot illumination triggers additional signals acting to stabilizing PINs and ABCBs at PM.

To further elucidate the role of auxin in the early root photomorphogenesis, we checked how the enhancement of PINs and ABCBs stability at PM influences auxin maxima and minima in roots. We used the R2D2 (Liao et al., 2015) and DR5::GFP auxin sensors combined with endogenous auxin quantification. We observed an increase in the D2 signal in LD and L roots when compared to D (Figure 3E), indicating lower nuclear auxin signaling in the root apical meristem. Whereas the DR5 fluorescence was similar in D and L condition, LD root showed an increase in fluorescence signal and expansion of the DR5::GFP domain in the meristematic region (Figure 3F). In addition, endogenous auxin levels were similar in LD and D shoots and roots (Figure 3G). Although shoot illumination seems to enhance shoot-to-root auxin transport, the auxin response reporters suggest that shoot derived IAA has a minor effect on the root apical auxin accumulation.

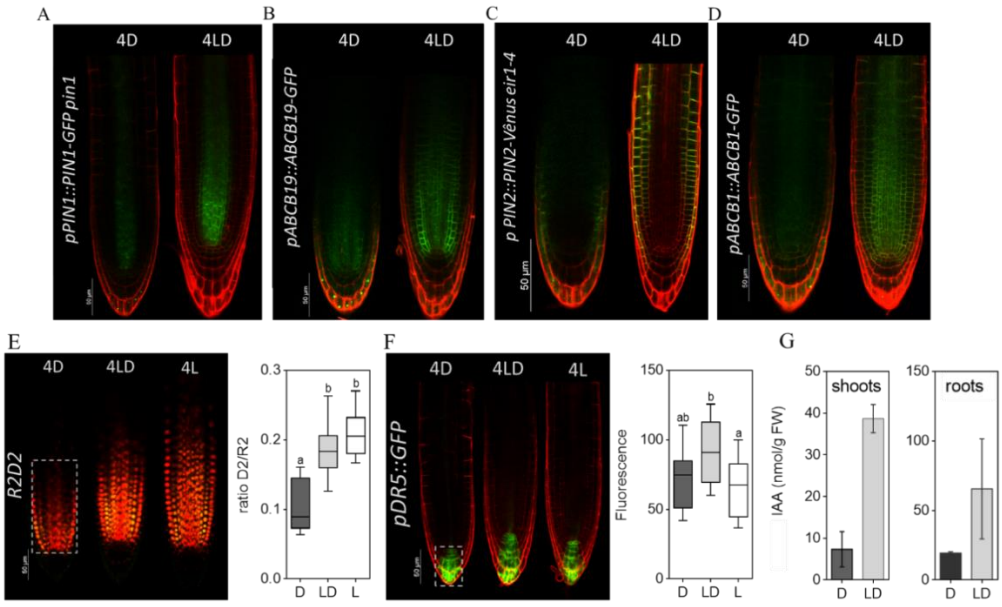

Figure 3. Light regulates the cellular localization of auxin transporters and auxin maxima in roots. (A) PIN1-GFP, (B) ABCB19-GFP, (C) PIN2-GFP and (D) ABCB1-GFP signals in 4-day-old seedlings grown on LD and D. (E) Merged confocal images of R2 (red) and D2 (green) expressing R2D2 marker and quantification of D2/R2 ratio in 4-day-old seedlings grown on D, LD and L condition (n≥6). (F) confocal images and

fluorescence intensity quantification of DR5::GFP signals in 4-day-old seedlings grown on D, LD and L condition (n≥9). (G) IAA levels in shoots and roots of 4 DAG seedlings (n≥2). Letters denote different significant classes (p≤0.05) by Anova test with Tukey post-test. Error bars indicate SE.

Shoot illumination effects on gene expression of dark-grown roots

Aiming to establish a set of genes involved in the early photomorphogenic root development of dark-grown roots we further evaluated candidate genes in our RNA-seq data (Chapter 2). We selected genes influenced by both auxin and the positive photomorphogenic regulator HY5 (Supplementary Table 1), by combining transcriptome data (Nemhauser et al., 2004) and Chip-seq data (Zhang et al., 2011) from published works. From this list, we identified a subset of light-responsive transcripts that are commonly auxin- and HY5-regulated (Supplementary Figure 1). Around 20% of the identified light-induced genes were HY5 putative targets, whereas only 5% of transcripts that are light-regulated were also auxin-related genes. Examining this further, we selected 18 additional genes which have already been described as involved in light responses, root development and signal transduction to validate the RNA-seq results by RT-qPCR. From the differentially expressed genes identified, genes such as *AIAMT1*, *PILS4*, *PIN5*, *PIN6* and *YUC8* were selected to assess the effect of shoot-illumination on root auxin homeostasis, while *ROPGEF4*, *LBD29*, *NRT2.1* and *Shaven2* were selected to for their putative roles on root development. Genes involved in response to stimulus and signal transduction such as of *BTB-POZ*, *MAKR6*, *NPH3*, *ROP9*, *RIC1*, *KAI2*, *NPY4*, *PP2A-B* and *Vid-27* were selected to investigate the downstream light signaling components.

We confirmed, by RT-qPCR analysis, that *KAI2*, *NPY4*, *PP2A-B*, *ROPGEF4*, *Shaven2* and *Vid-27* expression recapitulate their RNA-seq observed patterns (Figure 4A and B). *KAI2* showed more read counts at 4 LD whereas, in the qPCR data, this gene was more expressed at 7 LD seedlings. *NPY4*, *PP2A-B*, *ROPGEF4*, *Shaven2* and *Vid-27* showed the same expression pattern in the RNA-seq data as the one observed in the qPCR. Expression levels of *AIAMT1*, *BTB-POZ*, *LBD29*, *MAKR6*, *NPH3*, *NRT2.1*, *PILS4*, *PIN5*, *PIN6*, *ROP9*, *YUC8* and *RIC1* showed similar expression patter as the RNA-seq, whereas not similar statistics (Figure 5A and B). Most of the genes showed the same expression pattern in both techniques, with larger abundance of transcripts when shoots were exposed to light. Unlike most of the tested genes, *YUCCA8* was identified as repressed in the RNA-seq data when in the presence of light, whereas in the qPCR data the gene was up-regulated

in both time points. Overall, the qPCR result was fairly consistent with that of the RNA-seq analysis. This finding verified the accuracy of the RNA-seq results.

To further investigate whether these selected genes are involved in root development, we examined primary root growth in single T-DNA insertion knockout lines at 7 and 10 days in the same experimental conditions as the expression analysis. Most of the lines had shorter roots than the wild-type (Figure 4C and 5C), suggesting that the selected genes are required as positive regulators of primary root development in Arabidopsis. The *nph3* and *yuc8* lines were the only which produced longer roots than the wild-type, indicating that these genes can be involved in the negative regulation of primary root growth in response to shoot illumination in Arabidopsis. The *kai2* and *shaven2* mutants showed significantly shorter roots than Col-0 in 7- and 10-day-old seedlings as well in the LD and D condition, suggesting that these genes are involved in root development regardless of shoot illumination. The *pin6* mutant showed shorter roots in the D condition at both times, as opposed to *nph3* lines which showed longer roots than Col-0 when shoots were illuminated. This suggests both genes are involved in root development in a light-dependent manner. The *pp2-a*, *nrt2.1-1*, *pils4* and *rop9* lines were similar to wild-type plants, while *aiamt1*, *btb-poz*, *lbd29*, *makr6*, *npy4*, *pin5*, *ric1*, *ropgef4* and *vid-27* seedlings displayed changes in root development at least at one time or condition. The phenotype presented by the T-DNA lines indicates that these genes are somehow involved in primary root growth in the dark, although their role in this process remains to be elucidated.

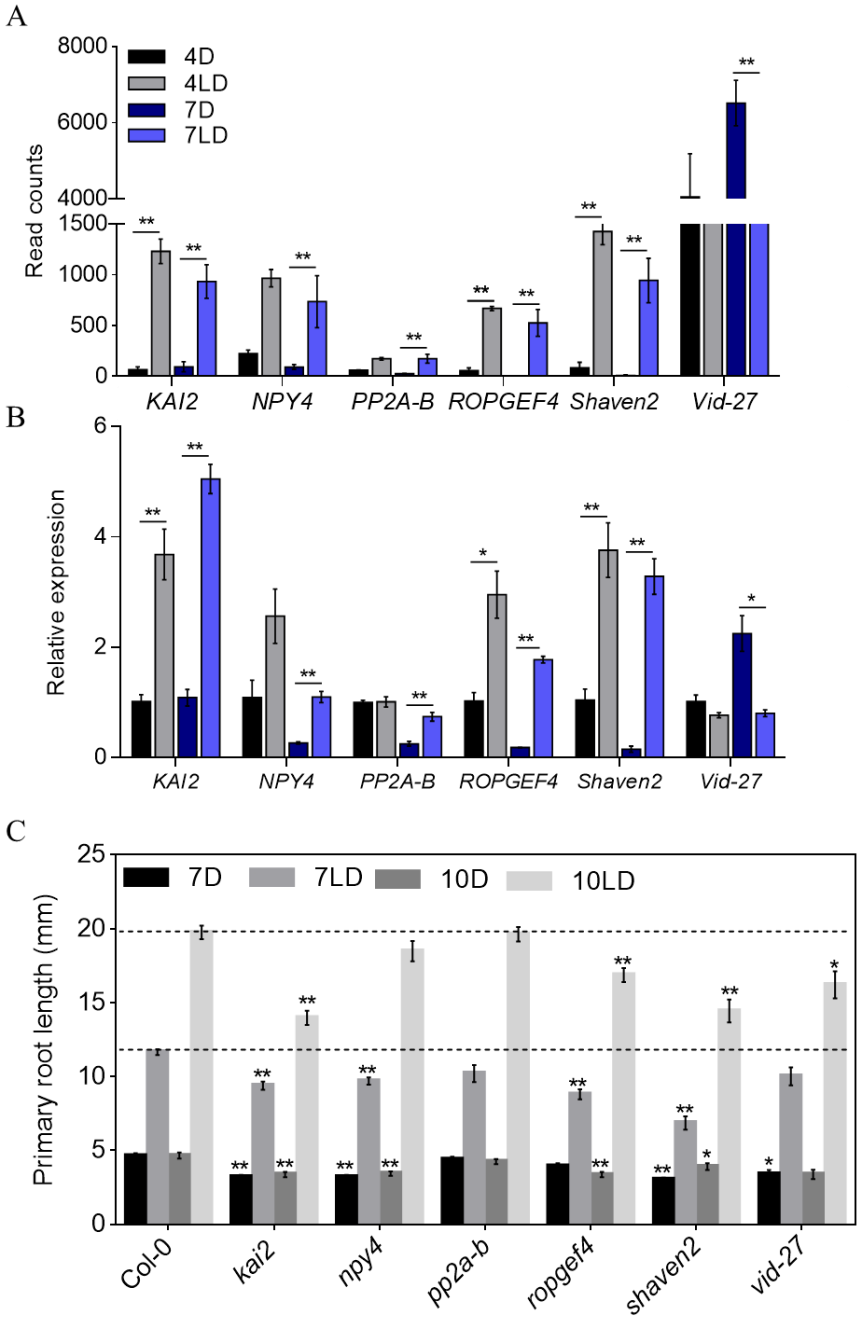

Figure 4. Overlapped significantly genes in the RNA-seq and RT-qPCR. (A) Number of read counts of the selected genes. Asterisks denote differentially expressed genes with p-value ≤ 0.05 (padj) and a ≥ 2-fold change. (B) RT-qPCR analysis of gene expression in roots. Gene expression was normalized using an internal control (AT3G18860 and AT2G42500; Miotto et al., 2019) for each reaction. The expression levels of 4DAG D condition were set as 1. Statistical significance was determined by Mann–Whitney test when necessary or by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) between D and LD condition to every time. (C) Primary root growth lengths of single T-DNA insertion lines (n≥ 30) were average. Seedlings were grown under 120 μmol m^{-2} s^{-1}. Error bars indicate SE. The means were compared by Kruskal–Wallis test with Dunn's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against the wild-type genotype.

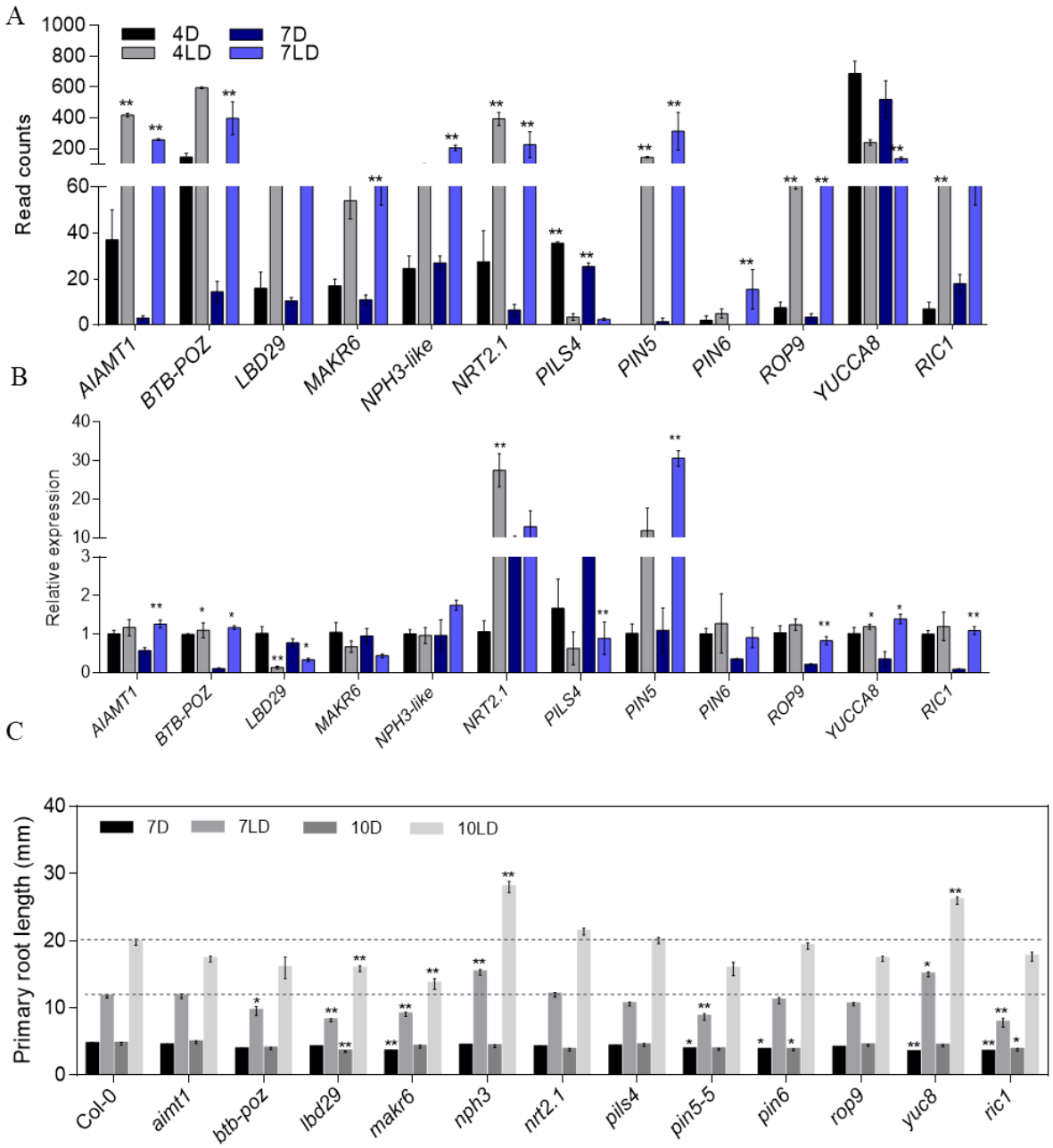

Figure 5. Candidate genes expression and primary root phenotype. (A) Number of read counts of the selected genes. Asterisks denote differentially expressed genes with p-value ≤ 0.05 (padj) and a ≥ 2-fold change. (B) RT-qPCR analysis of gene expression in roots. Gene expression was normalized using an internal control (AT3G18860 and AT2G42500; Miotto et al., 2019) for each reaction. The expression levels of 4DAG D condition were set as 1. Statistical significance was determined by Mann–Whitney test when necessary or by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) between D and LD condition to every time. (C) Primary root growth lengths of single T-DNA insertion lines (n≥ 30) were average. Seedlings were grown under 120 μmol m^{-2} s^{-1}. Error bars indicate SE. The means were compared by Kruskal–Wallis test with Dunn's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against the wild-type genotype.

Light intensity regulates Arabidopsis root growth

Although we have investigated the effect of shoot illumination in dark-grown roots, the effect of variations in light intensity was not initially considered. It has been reported that changes in light intensity leads to alterations in plant phenotype as well as in hormone homeostasis (Kumari et al., 2019). To gain insight into how root growth is controlled by light, we analyzed Col-0 seedlings in dark- and light- exposed root development for several days. Wild-type seedlings showed an increase in root growth in response to the light intensity, reaching maximum growth at 80 μmol m^{-2} s^{-1} (Figure 6A), whereas higher light intensity (105 μmol m^{-2} s^{-1}) inhibited primary root growth. Primary root length of light-exposed roots at 40 and 60 μmol m^{-2} s^{-1} was found to be slightly shorter than dark-grown roots at all the evaluated timepoints. The opposite pattern was found at 80 and 105 μmol m^{-2} s^{-1} at 6 and 10 days, where dark-grown roots were shorter than light-exposed roots. Interestingly, at 14 days, dark-grown roots showed longer roots than light-exposed roots at 80 μmol m^{-2} s^{-1}. Lateral root (LR) density showed a similar response as primary root growth, increasing the LR density with light intensity (Figure 6B). Surprisingly, at 80 μmol m^{-2} s^{-1}, light-exposed roots displayed much higher LR density than dark grown roots, suggesting that direct light exposure of roots promotes LR formation in a narrow irradiance dependent fashion, as this effect was absent in higher or lower light intensities. Low light intensity strongly reduced lateral root development and no lateral roots were observed at 40 μmol m^{-2} s^{-1} at all the evaluated timepoints.

The shorter root development observed at 40 μmol m^{-2} s^{-1} as well as the absence of lateral roots may be related to a lower photosynthetic output from the shoots. Because sucrose can stimulate primary root growth in dark-grown seedlings (Kircher and Schopfer, 2012), we wondered whether sucrose supplementation could rescue root growth. To test this, we grew wild-type seedlings on agar plates containing sucrose (Figure 6C and D) and low light intensity. The addition of sucrose to the medium did not affect significantly primary root growth or lateral root density at 40 and 80 μmol m^{-2} s^{-1}. Taken together, these data support the idea that root development is irradiance dependent and not essentially dependent on sucrose availability.

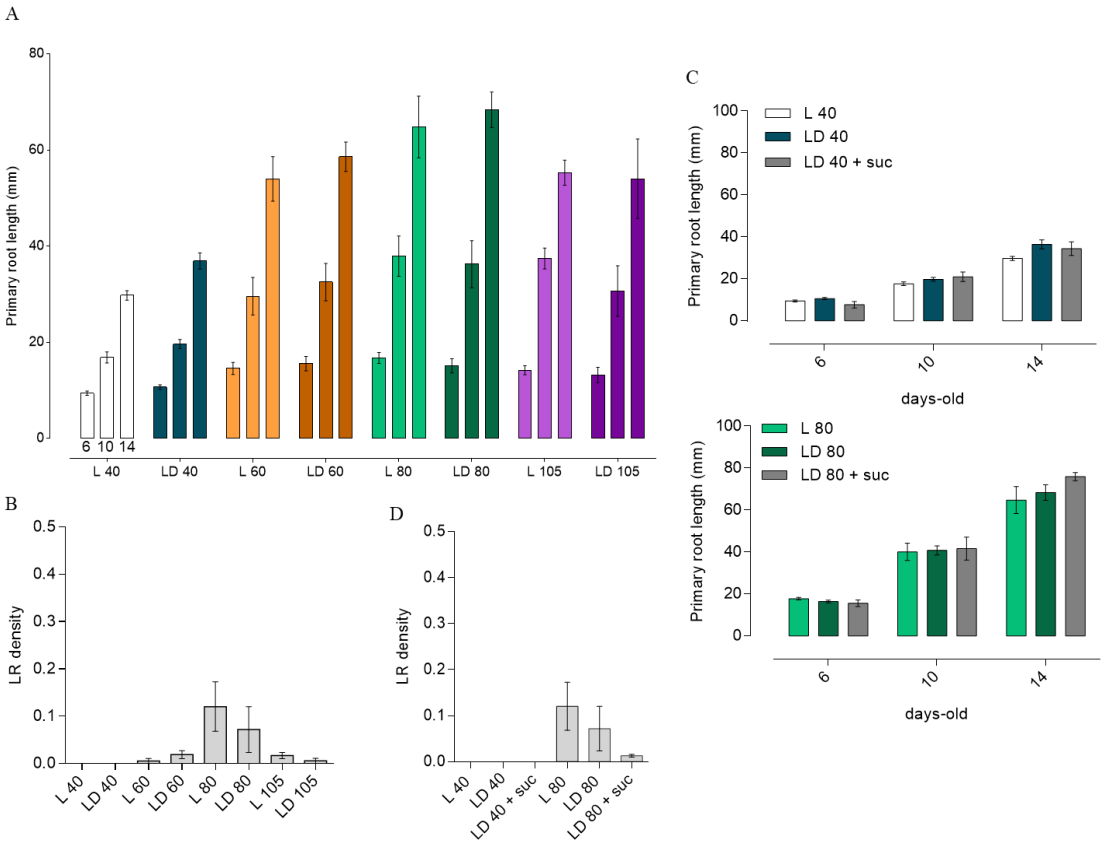

Figure 6. Light intensity dependent root growth in wild-type seedlings. (A) Primary root length of wild-type seedlings grown in L and LD at 6, 10 and 14 DAG (n≥5) under four different light intensities of 40, 60, 80 and 105 µmol m^{-2} s^{-1}. (B) Lateral root density of A seedlings. (C) Primary root length of wild-type seedlings without or with 1% sucrose. (D) Lateral root density of C seedlings.

Mutants for root light-responsive genes display changes in root development under different light intensities

To identify the mechanisms involved in the phenotype observed in wild-type seedlings, we measured primary root length and lateral root density of the T-DNA insertion lines described earlier in low (40 µmol m^{-2} s^{-1}) and medium light intensity (80 µmol m^{-2} s^{-1}), where primary root growth reaches a maximum. We found no significant differences in primary root growth for most of the T-DNA lines when compared to wild-type Col-0 at both light intensities (Figure 7A). However, we observed that most genotypes displayed significant differences between 40 µmol m^{-2} s^{-1} and 80 µmol m^{-2} s^{-1} light intensities. The *lbd29* and *yuc3* mutants only showed shorter roots than Col-0 at 7 days at 80 µmol m^{-2} s^{-1} only, whereas *nrt2.1-1* and *shy2-2* showed shorter roots in both light intensities. The *kai2* mutant had shorter primary roots only at 40 µmol m^{-2} s^{-1}. The differences observed at 7 days

to *lbd29*, *nrt2.1-1* and *kai2* lines were no longer observed in later time points. At 10 days, only *shy2-2* presented shorter roots than wild-type. The *yuc3*, *shy2-2*, *pin5* and *pils4* mutants showed shorter roots than Col-0 in 14-days at 80 μmol m^{-2} s^{-1} only. Conversely, for *pin5-5*, *vid-27* and *yuc3* mutants, 80 μmol m^{-2} s^{-1} had the same root lenght as 40 μmol m^{-2} s^{-1}, suggesting that these mutations impact the light dose responsivity of primary roots.

Lateral root development was shown to be modulated by differences in light intensity (Kumari et al., 2019). In our experiments, lateral root density was strikingly affected by the light intensity to which the shoot was exposed (Figure 7B) and many mutants displayed increased LR formation in response to light intensity. At 14 days, *gef4*, *nrt2-1*, *ric-1*, *makr6*, *pp2a-b*, *roc1*, *yuc8*, *yuc9* and *aimt1* all produced more LR than WT grown at 80 μmol m^{-2} s^{-1}, suggesting that these genes act as repressors of LR formation in response to light intensity. Overall, low light intensity strongly impaired lateral root development for most genotypes. Only *vid-27* and *pils4* lines showed higher lateral root density at 40 μmol m^{-2} s^{-1} than at 80 μmol m^{-2} s^{-1}. The *lbd29*, *scar1*, *shaven2*, *btb-poz*, *kai2* and *pin5* lines did not produce lateral roots at low light intensities even after 14 days. The auxin-related mutants *yuc3* and *shy2-2* presented no lateral root development in any light condition, possibly due to changes in auxin homeostasis in these lines. The *gef4* and *nrt2.1-1* lines showed an increase in lateral root density in both timepoints at 80 μmol m^{-2} s^{-1}. Overall, most of the evaluated mutants showed a similar response as the wild-type Col-0. Regardless, our results indicate that shoot irradiance strongly controls the root growth responses in Arabidopsis seedlings, and that some phenotypes are highly dependent on the light intensity.

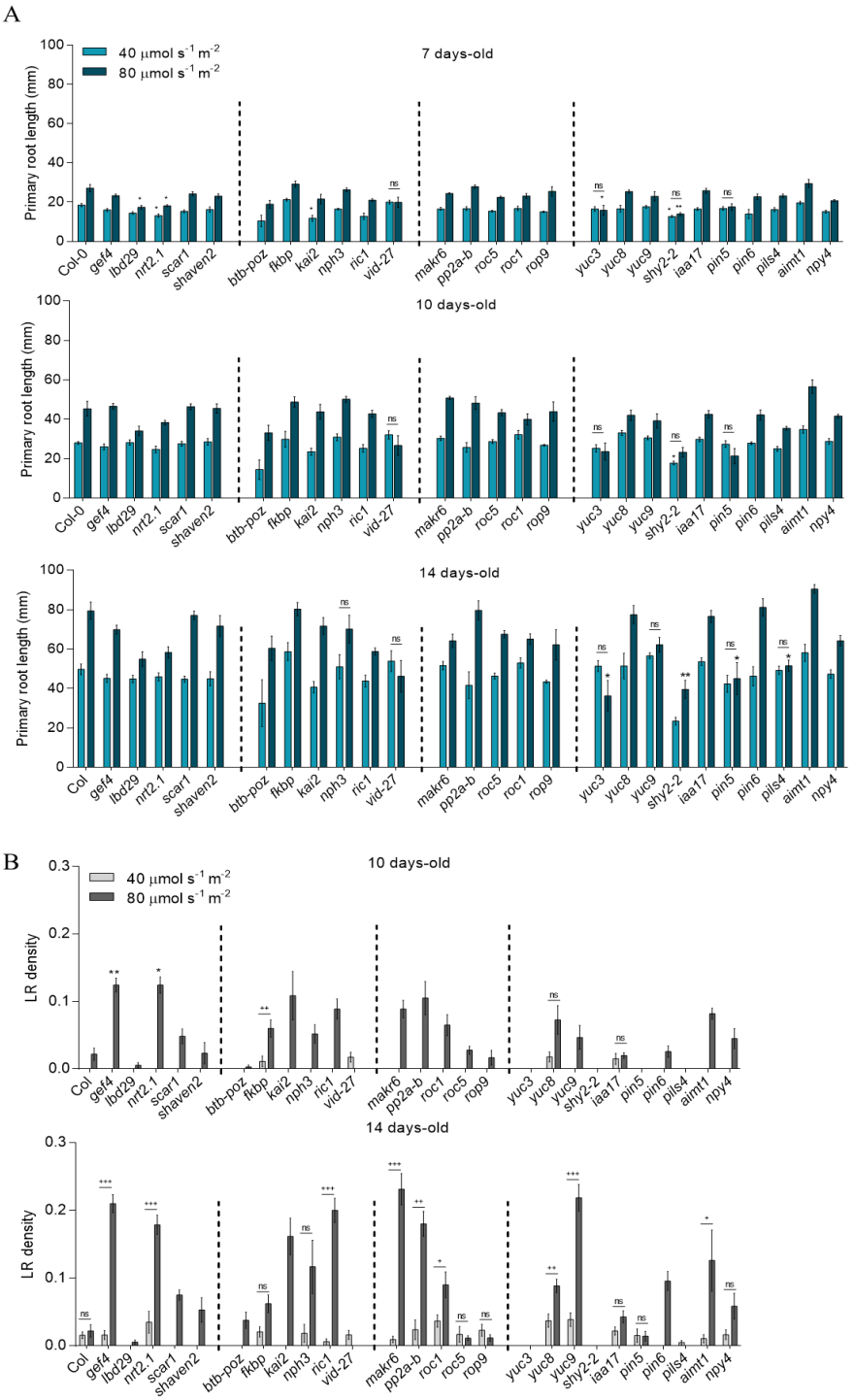

Figure 7. Dark-grown root of candidate genes under different light intensities in the shoots. (A) Primary root length of single T-DNA insertion lines (n≥ 10). Root measurement are done in 7, 10 and 14 days after germination. (B) Lateral root density of A seedlings. Error bars indicate SE. Asterisks denote significance difference by Kruskal–Wallis test with Dunn's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against the wild-type genotype. Genotypes were individually compared between 40 and 80 µmol m^{-2} s^{-1}, non-significant comparisons are denoted by ns.

Discussion

The effects triggered by light in plant morphogenesis were mostly studied in the aerial tissues. Nevertheless, a substantial part of the plant grows protected from light, and it can adjust its development to many different above ground factors. Here, employing a modified D-root system (Silva-Navas et al., 2015) we addressed how light perception in the shoot influences root development and the potential messengers delivered by shoots to roots. Using our modified D-root system we showed that exogenous sucrose supply induces root growth when roots are kept in darkness, however, it cannot fully restore the light induced growth. When roots are grown exposed to light, as normally happen in Petri dishes, they show a reduction in primary root length when compared to roots grown in the LD condition (Silva-Navas et al., 2015). We observed a slight increase in primary root growth in LD condition, whereas lateral roots were more abundant in the L condition. On the other hand, root illumination highly induced lateral root development when compared to LD seedlings.

We observed that an increase in light intensity increases the primary root growth as well as lateral root development, while the higher intensity (105 μmol m^{-2} s^{-1}) had an inhibitory effect on primary root elongation. It has been reported that higher intensities (280 μmol m^{-2} s^{-1}) have an inhibitory effect on root elongation (Silva-Navas et al., 2015), reinforcing the idea that roots sense direct illumination as a stress. The biggest differences were observed under 80 μmol m^{-2} s^{-1} in agreement what was previously observed at 75 μmol m^{-2} s^{-1} (Silva-Navas et al., 2015). Plants produce sucrose through photosynthesis in the presence of light. However, externally applied glucose, which is eventually originated from sucrose hydrolysis, was shown to be involved in seedling root growth direction, which could be mimicked by high light intensity (Singh et al., 2014). In our experiments, light-induced changes in dark-grown roots was not significantly altered by externally added sucrose. This finding indicates that light intensity and dark-grown root development are more dependent on photoreceptor signaling than sucrose availability.

Cell proliferation at the root meristem is known to be regulated by auxin gradients (Sabatini et al., 1999). Mutations in *pin* proteins result in defects in the meristem size due to the absence of PIN-mediated recirculation of shoot-derived auxin in the root apex (Blilou et al. 2005). The inhibition of cell proliferation in the root meristem showed by the D seedlings, lead us to check the stability of auxin carriers and the possible effects in auxin accumulation

in the apex zone in response to light. As previously reported (Laxmi et al., 2008; Sassi et al., 2012), root PIN1 and PIN2 PM localization is stronger in LD seedlings compared to D seedlings. Light is also able to induce PINs expression (van Gelderen et al., 2018). As showed before (Laxmi et al., 2008; Sassi et al., 2012, this work), the depletion of PIN auxin efflux from the PM is supposed to lead to very low levels of auxin in the root apex. Using the transcriptional reporter DR5 we observed that auxin maxima are similar in the root apex of D and LD seedlings. Moreover, auxin nuclear response in the meristematic zone showed to be higher in D root, suggesting that the reduction of PM PIN1 and ABCB19 in D roots do not lead to inefficient auxin transport from the meristem zone to the root apex.

We have shown in Chapter 2 that shoot illumination has a drastic effect on dark-grown root gene expression, reinforcing the idea that, even growing in the dark, roots are able to reprogram the transcriptional status to optimize growth in response to the shoot light condition. To further elucidate this process, we investigated 18 additional candidate genes. In general, the expression profile by qPCR recapitulated the RNA-seq data. Very few genes explored in this work such as *VID-27*, *PILS4*, *PIN5* and *YUC8* showed higher expression in dark. In addition, most of the evaluated mutants, except for *nph3* and *yuc8*, showed shorter roots than the wild-type when shoots were exposed to light, suggesting a positive role in primary root growth. Future studies will be necessary to address accurately the required function of these genes for primary root development. Control of root structure and development is regulated by phytohormone synthesis and distribution. Phytohormones such as auxins have been shown to influence root development (Jung and McCouch, 2013; Qin et al., 2019). Therefore, a few genes involved in auxin responses were analyzed in this current work. Remarkably, primary root development of *yuc3* and *pin5* lines was not affected by light in all evaluated timepoints both in low (40 μmol m^{-2} s^{-1}) or medium (80 μmol m^{-2} s^{-1}) intensities. This suggests that these genes are probable good candidates for transducing, in a quantitative manner, a signal sent by the illuminated shoot to the dark-roots. Nevertheless, it shows that both light and auxin-regulated pathways interact to regulate dark-grown root development. However, a more detailed study of these genes must be carried out to address they role in the control of root development in response to alterations in light intensity. From all the candidate genes analyzed in this work, it was observed that the vacuolar import/degradation Vid27-related protein, was the only line which presented an increase in lateral root density at low light intensity. In addition, primary root development

was unaltered under all evaluated timepoints both in 40 μmol m^{-2} s^{-1} and 80 μmol m^{-2} s^{-1} irradiances. Moreover, the transcriptome analysis shows that Vid27 is repressed in roots by shoot illumination. These results suggest that this gene may work as a repressor of lateral root development in seedlings exposed to light.

Here, we show that root growth responses are drastically affected by shoot irradiance. The light intensity affects primary and lateral root development in Arabidopsis in a dose dependent manner. Our results indicate that some mutants only display root growth related phenotypes at certain light intensities and that light perception modulates growth responses more strongly through photoreceptor activation than on photosynthate availability. Elucidating the possible long-distance signaling from illuminated shoot to trigger early root photomorphogenesis will need further experiments but our findings suggest that sucrose and auxin act in a positive way to coordinate root development in response to light, whereas sucrose and auxin cannot be considered the early long-distance signal to induce root development in response to light. Further investigation of the specific role of candidate genes in this response may help us to better understand how plants adapt root development in response to external stimuli.

Materials and methods

Plant material and growth conditions

Arabidopsis Columbia (Col-0) was used as wild-type (WT), and the mutants *aiamt1* (SALK_072125), *kai2* (SALK_128254), *pp2a-b* (SALK_027044), *ropgef4* (SAIL_184_C08), *btb-poz* (SALK_021843), *makr6* (SALK_082476), *nph3* (SALK_070901), *rop9* (SALK_019272), *ric1-1* (SAIL_210_E12), *kai2* (SALK_128254), *npy4* (SALK_046452), *pp2a-b* (SALK_027044), *cobl9-1* (SALK_099933) and *vid-27* (SALK_070099) are in Col-0 ecotype background and were obtained from the The European Arabidopsis Stock Centre (NASC, http://arabidopsis.info/). Genotyping on T-DNA insertions was performed following the SALK (http://signal.salk.edu/index.html) instructions. The primers used for genotyping can be found in Supplementary Table 2. All experiments were done using homozygous lines for the T-DNA insertion. Seeds were sterilized, germinated, and grown as described previously (Chapter 2). The plates were kept vertically and grown under different white light intensities (21 °C ± 3 °C, 16 h- photoperiod)

in the range of 40 to 120 μmol m^{-2} s^{-1}. Root phenotyping and RNA extraction were performed with 7, 10 and 14-days old and 4 and 7-days old seedlings respectively. Sample harvesting was approximately done at ZT=7. Primary root length was measured with ImageJ (Fiji) and plotted into graphs in GraphPad Prism 6.

Auxin transport inhibitors treatments

NPA and TIBA applications on hypocotyls were performed in 6 DAG seedlings grown in LD light condition. The stock solution of the chemicals (in DMSO) were dissolved in a pre-heated lanolin paste with 2,5% paraffin. The paste was manually administered in the hypocotyl with pipette tips. Roots were measured before the treatment (T0) and 24h after the treatment (T24).

Auxin isolation and HPLC analysis

For the auxin quantification, 4 DAG D and LD roots were used, the isolation and quantification were carried out as described (Vilasboa et al., 2019). IAA analytical standard was used for the calibration curve.

GUS staining and microscopy analysis

Seedlings were fixed in 80% acetone for 20 minutes at -20ºC, washed 3 times in water and incubated overnight in GUS staining buffer [10 mM EDTA, 100 mM sodium phosphate (pH 7.0), 0.1% (v/v) Triton X-100, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-glucuronide] at 37 °C. Subsequently, samples were washed in water once and cleared in 70% (v/v) ethanol at room temperature before imaging with ZEISS Axio Vert microscope.

Confocal Imaging and Quantification

Imaging was performed by using a Leica TCS SP5 confocal microscope, equipped with HyD in addition to the standard photomultiplier tubes (PMT). The fluorescence signal intensity of their presented markers was quantified by using the quantify tool of the Leica software (LAS AF Lite). For all markers, we defined a ROI in the region that showed the most representative signal distribution. We used the same ROI (size and shape) to analyze all images of the respective experiment.

RNA isolation and RT-qPCR

RNA extraction and RT-qPCR were performed as described previously (Miotto et al., 2019). All RT-qPCR values represent three biological replicates, each containing at least two technical replicates. Primer sequences used can be found in the Supplementary Table 2.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., CA, US). Data were tested for normal distribution by Shapiro-Wilk test and then applied the respectively statistic test and when significant ($p \leq 0.05$) were showed in the graphs. Statistical details of each experiment (test used, replicates, etc) can be found in the Results section and Figure-Figure Legend sections.

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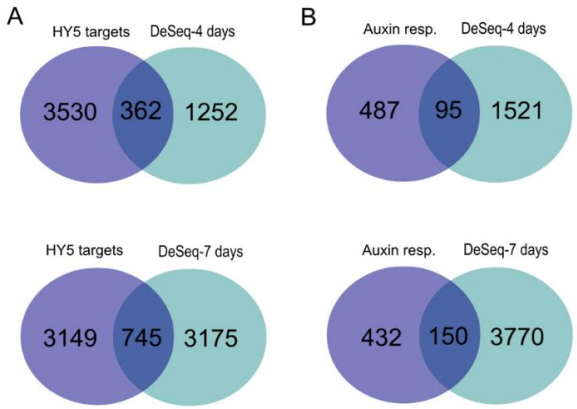
Supplementary data

Supplementary tables are available at:

https://drive.google.com/drive/folders/1jeLQ04CZDnFZuyD0jejfAG1SOheYoDgu?usp=sharing



Supplementary Figure 1. Comparison of our data-set with the HY5 Chip-seq data and auxin- responsive genes (common genes are listed in Supplementary Table 1).

Capítulo IV

- Light-dependent flavonoid metabolism effects on photomorphogenic root development -

Light-dependent flavonoid metabolism effects on photomorphogenic root development

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Abstract

Flavonols, are a class of specialized plant compounds that have been suggested to work as auxin transport inhibitors. In Chapter 2 we observed a high induction of genes related to flavonoid biosynthesis pathway in roots in response to shoot illumination. Aiming to determine their roles in root growth, we evaluated single loss of function mutants for different flavonoid biosynthesis genes. Because flavonoids have been reported to play a role in the regulation of polar auxin transport, we accessed auxin distribution in these mutant backgrounds. Using reporter lines we accessed the accumulation of auxin transport proteins in response to flavonol chemical treatment. Our results support the hypothesis that shoot-illumination induces localized biosynthesis of specific flavonoids in the roots, which helps to rearrange auxin gradients and, consequently, promote lateral root development.

Introduction

Flavonoids are a large group of specialized plant metabolites with a C_6-C_3-C_6 carbon structure, produced via the phenylpropanoid pathway (Falcone Ferreyra et al., 2012), which include flavonols, flavones, isoflavones and anthocyanins. The more than 9000 different flavonoids reported in plants are the result of carbohydrate side chains and chemical modifications in the basic skeleton (Taylor and Grotewold, 2005; Saito et al., 2013). Flavonoids have been described to have multiple roles in plant development, such as pigmentation, UV-B protection, plant–microbe interactions and scavengers of reactive oxygen species (Winkel-Shirley, 2001; Schulz et al., 2016). Flavonoids have also been implicated as modulators of auxin transport (Brown et al., 2001; Peer and Murphy, 2007), seed germination (Hernández and Munné-Bosch, 2012), and root and shoot development (Buer and Muday, 2004b; Silva-Navas et al., 2016). Due to their relevance to plant development, the flavonoid biosynthesis pathway has been extensively studied in the model plant Arabidopsis (reviewed in Buer et al., 2010). The *transparent testa* (*tt*) mutants, which are affected in seed coat pigmentation due to the lack of flavonoid biosynthesis, provided a tool to establish the crucial steps in the biosynthesis of these metabolites as well as genetic studies (Koornneef, 1990).

Most of the enzymes responsible for flavonoid biosynthesis in Arabidopsis are encoded by single-copy genes. The first step of flavonoid synthesis is catalyzed by CHALCONE SYNTHASE (CHS/*tt4*; Feinbaum and Ausubel, 1992), which converts 4-coumaryl-CoA and malonyl-CoA into naringenin chalcone. Next, CHALCONE ISOMERASE (CHI/*tt5*; Shirley et al., 1992) acts on naringenin chalcone to produce naringenin. Consequently, the *tt4* and *tt5* mutants fail to synthesize naringenin, or any of the downstream metabolites. Naringenin is used as a substrate for the production of dihydrokaempferol by FLAVONE 3-HYDROXYLASE (F3H/*tt6*; Owens et al., 2008a; Owens et al., 2008b). Dihydrokaempferol represents a branch-point in the pathway that can lead to the production of either kaempferol by FLAVONOL SYNTHASE 1 (FLS1) or quercetin by FLAVONOID 3′,5′-HYDROXYLASE (F3′H/*tt7*) followed by FLS1. Quercetin and kaempferol have been linked to a range of processes in plant development, whereas not much is known about the biological function of the intermediate naringenin.

Flavonoid biosynthesis is highly regulated by environmental signals, such as temperature, UV-B and blue light (Mierziak et al., 2014; Wan et al., 2018). At molecular level, the transcription of flavonoid biosynthesis genes is regulated in an organ-specific way via the R2R3-MYB, MYC-like basic helix-loop-helix (bHLH) and WD40-repeat transcription factors (Quattrocchio et al., 2006; Stracke et al., 2007; Gonzalez et al., 2008; Hichri et al., 2011). The key flavonols biosynthetic genes have been shown to be regulated by PFG1/MYB12, PFG2/MYB11 and PFG3/MYB111 transcription factors (Stracke et al., 2007). MYB12 is predominantly expressed in the roots whereas MYB111 is more abundant in shoots (Stracke et al., 2007). The photomorphogenic bZIP transcription factor ELONGATED HYPOCOTYL 5 (HY5) has also been linked as a positive regulator of MYB12 and some of the core flavonoid biosynthetic genes in response to light (Lee et al., 2007; Stracke et al., 2010). In addition, Arabidopsis seedlings grown in the darkness do not accumulate flavonoids (Buer et al., 2010). Altogether, these findings suggest a cross-talk between light and flavonoids signaling in the control of plant development, however, the regulatory mechanisms involved are still not clear.

Blockage of the flavonoid biosynthesis, by CHS mutation in the *tt4* mutant, leads to changes in PIN1 cycling (Peer et al., 2004) and an increase in auxin transport, which can be rescued by exogenous treatment with naringenin (Murphy et al., 2000). On the other hand, the over accumulation of flavonols in the *tt7* mutant shows an inhibition of auxin transport (Buer and Muday, 2004b; Peer et al., 2004). Besides inhibition of rootward auxin transport, endogenous flavonols are also able to affect PIN2-dependent auxin reflux in the root meristematic zone (Buer and Muday, 2004b), either by changing PIN localization or affecting PIN efficiency (Peer et al., 2004; Bailly et al., 2008; Kuhn et al., 2017). Auxin transport proteins are known to show varying sensitivity to flavonols. The interaction between TWD1 and ABCB19 is completely inhibited by the presence of quercetin (Bailly et al., 2008), while PIN2 seems to be less sensitive to inhibition by flavonols than PIN1 (Peer et al., 2004). The regulation of auxin transport by flavonols availability has been extensively explored, however their overall significance in the control of root development still remains unclear.

In this chapter, we aimed to determine the effect of flavonoids in the control of root development in Arabidopsis. To better clarify the involvement of flavonoids on the control of root development, we used a combined approach of mutant lines and chemical treatments.

We show that naringenin, a precursor of flavonols, inhibits early primary root growth and induces lateral root formation. In agreement with the pharmacological treatment, the naringenin over accumulating mutant, *tt6-3*, showed an increase in lateral root density. By using R2D2 and DR5 reporter lines, we also show that a lack of the core flavonoid biosynthetic enzymes leads to changes in auxin homeostasis. These results are consistent with a role for flavonoids as regulators of root development as a consequence of changes in auxin homeostasis.

Results

Flavonoid biosynthesis pathway is highly upregulated locally in LD roots

Root development in response to shoot illumination is not a fully understood process. In Chapter 2 we show that shoot illumination highly induces the core enzymes of the flavonol biosynthesis pathway in LD roots of Col-0 seedlings (Miotto et al., 2019). To confirm this result, we evaluated the *CHS::CHS-GFP tt4* (Lewis et al., 2011) and *FLS1::FLS1-GFP fls1* (Kuhn et al., 2011) lines in LD and D seedlings. In agreement with the transcriptional data (Supplementary Figure 1A) CHS-GFP and FLS1-GFP were more abundant in LD roots (Supplementary Figure 1B) showing a higher signal in the elongation zone of root cortex cells. Flavonoid biosynthesis is a known light dependent process, roots of Arabidopsis seedlings growing in complete dark do not accumulate flavonoids (Buer et al., 2010). However, it has been shown that flavonoids (Buer et al. 2008) and the RNA of core biosynthetic enzymes, such as CHS are phloem mobile (Thieme et al., 2015). Therefore, we decided to examine if the high expression observed in LD roots were locally induced or if it was delivered by the illuminated shoot. In order to investigate this option, we addressed the relative expression of some of the flavonoid biosynthesis genes (Figure 1A) in 4-day-old seedlings grown in dark after shoot illumination for 6, 12 and 48 hours. The qRT-PCR results demonstrated that shoot illumination lead to an increase in the expression of flavonoid biosynthesis pathway both in shoots and roots (Figure 1B). Upon light stimulus, *CHS* and *F3H* expression was much strongly induced in roots when compared to shoots within 12h, reaching 200- and 50-fold in 48h, respectively. Contrarily, *FLS1* levels were not altered in roots in the evaluated timepoints. In the roots, *CHI* induction happened at a slower rate, being significantly induced only after 48h. These results indicated that the flavonoid pathway

is probably induced in the roots as an effect of shoot illumination.

To examine the effect of flavonoids in LD roots, we evaluated the effect of flavonols treatments on primary root growth as well as on lateral root density. Early primary root development was significantly repressed by naringenin treatment at 6 DAG in a concentration dependent manner (Figure 1C and Supplementary Figure 2). Whereas both naringenin and quercetin were able to increase lateral root density in 14 DAG seedlings (Figure 1D), primary root length significantly increased in all flavonoid treatments (Figure 1E). To summarize, our results suggest that the flavonols are involved in the control of LD roots. However, the specific role of each compound in this process remains unclear.

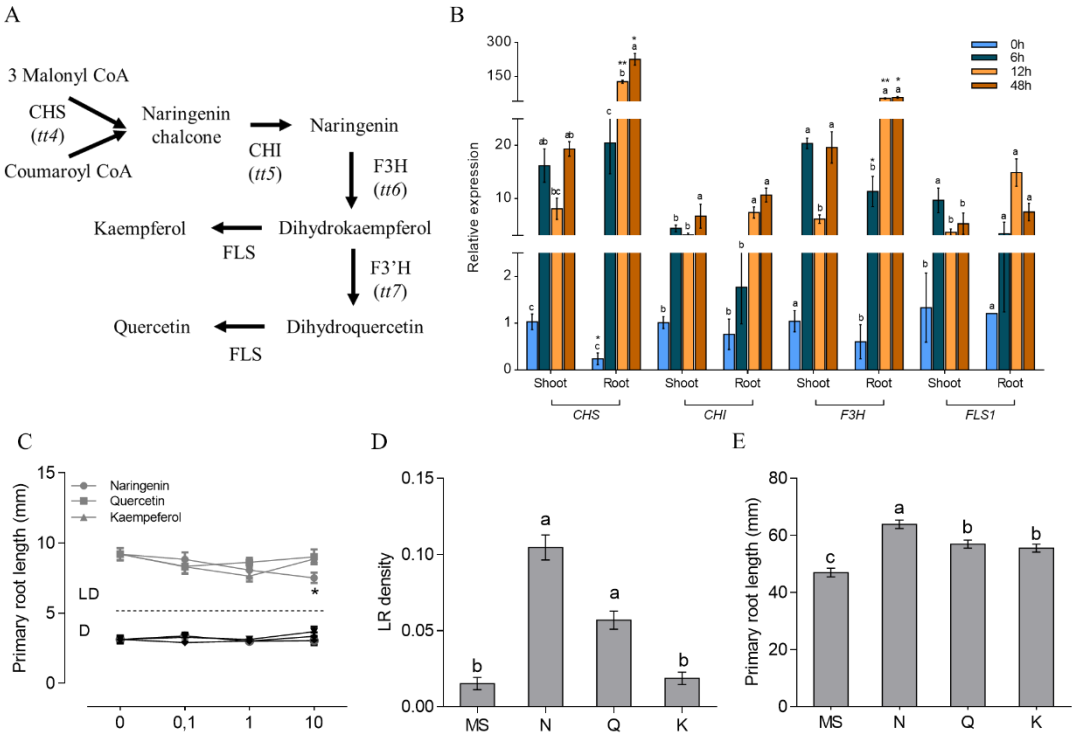

Figure 1. Flavonols effect root growth of wild-type seedlings. (A) The biosynthetic pathway leading to flavonols. (B) Root and shoot expression of the core biosynthetic genes in 4-day old seedlings after a long-term shoot illumination. Letters denote different significant classes, ($p \leq 0.05$) by Anova test with Tukey post-test and asterisks indicate a significant difference with the same gene and condition in shoots by t-test (*$p \leq 0.05$; **$p \leq 0.01$). (C) Naringenin repress primary root elongation in 6 DAG LD seedlings ($n \geq 10$). Wild-type seedlings were grown in MS supplied with 0, 0.1, 1 or 10µM of naringenin, quercetin or kaempferol. Asterisks indicate a significant difference by Anova test with Dunnett post-test (*$p \leq 0.05$) in the same treatment against the control. (D-E) Flavonols effect on 14 DAG in lateral root density (D) and main root growth (E; $n \geq 20$). Letters denote different significant classes, ($p \leq 0.05$) by Anova test with Tukey post-test (*$p \leq 0.05$). Wild-type seedlings were grown in MS medium as a control or MS with 10µM of naringenin (N), quercetin (Q) or

kaempferol (K). Error bars indicate SE.

Involvement of flavonoids in dark-grown root development

To explore the role of the flavonoid genes in regulating dark-grown roots, we selected single T-DNA insertions lines. We used previously described loss-of-function mutants on Col-0 background: *tt4-15* (Appelhagen et al., 2014), *tt5-2* (Appelhagen et al., 2014), *tt6-3*, *tt7-7* (Appelhagen et al., 2014) and *fls1*. In the *tt4-15*, *tt6-3* and *fls1* T-DNA lines (Figure 2A), primary root length was similar to the wild-type Col-0 when shoots were illuminated. However, *tt4-15* and *fls1* lines exhibited shorter roots compared to the wild-type when grown in the dark. In addition, the primary root development of *tt5-2*, *tt7-7* and *chil* lines was significantly different than those of wild-type. *tt5-2* and *chil* seedlings showed an increase in primary root length when compared to the wild-type, whereas *tt7-7* had the opposite phenotype. Root elongation has been correlated with the root meristem size (Sassi et al., 2012), we therefore analyzed the meristem size in these mutants. In agreement with its reduced primary root length, *tt7-7* had significantly smaller meristems than the wild-type Col-0. On the other hand, *tt4-15* showed larger meristem size (Figure 2B). Notably, *tt5-2* and *chil* lines showed similar meristem size than the wild-type. These results suggest that, in opposition to *tt4-15* and *tt7-7*, the primary root growth phenotypes of *tt5-2* and *chil* are not directly correlated to root meristem size.

Most of the mutants showed similar lateral root densities as the wild type (Figure 2C). The CHI (*tt5*) enzyme, catalyzes the second step in the flavonoid pathway, by converting the tetrahydroxychalcone substrate to naringenin (Figure 1A). Consequently, in the *tt5-2* mutant, naringenin or any of the downstream products are not accumulated. On the other hand, the *tt6-3* mutant fails to convert naringenin to dihydrokaempferol, resulting in an accumulation of naringenin or its glycosylated products. Interestingly, we observed a significant increase in the primary root growth in the *tt5-2* seedlings (Figure 2A) and a significant reduction in the lateral root development, whereas the *tt6-3* seedlings showed an increase in lateral root development (Figure 2D) was observed. To unravel the role of naringenin on lateral root density, we tested whether naringenin treatment was able to induce lateral root development in the *tt5-2* lines. Naringenin supplementation caused an increase in the lateral root density in Col-0 and *tt5-2*. Naringenin supplementation in the *tt5-2* seedlings lead to an increase in lateral density reaching the same extent as *tt6-3* seedlings

(Figure 2D). In conclusion, these results indicate that the intermediary naringenin or some derivative upstream of F3H (*tt6*) acts positively in LR development.

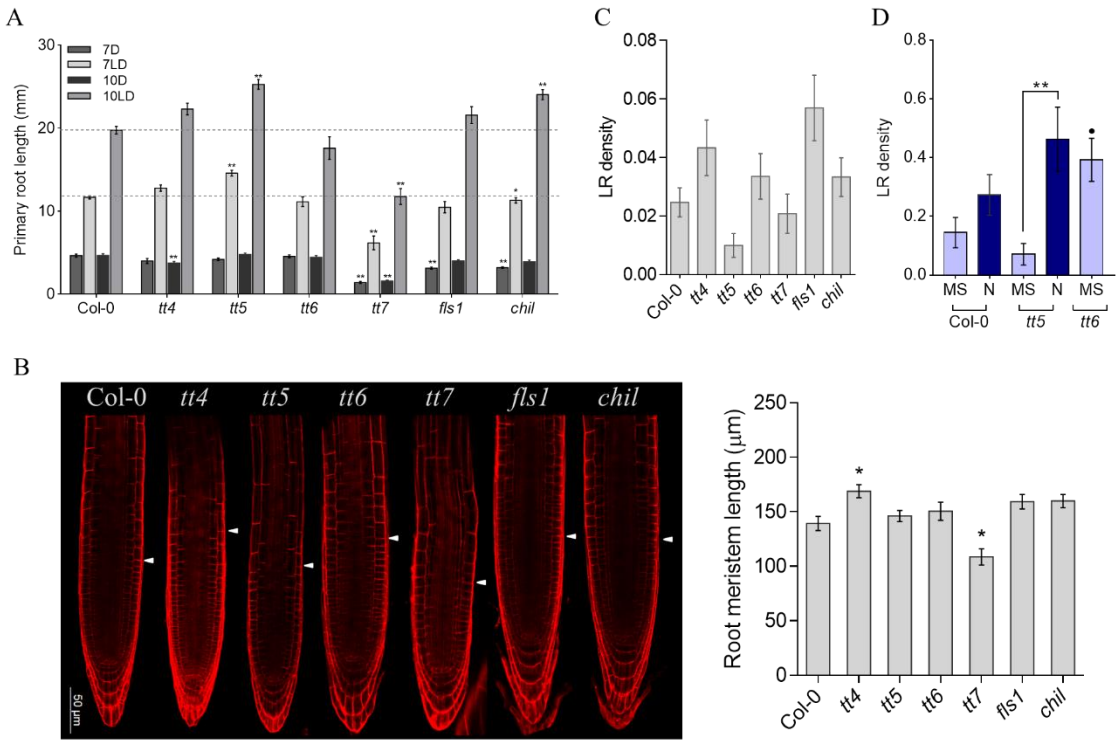

Figure 2. Effects of shoot-illumination on rot development of flavonols mutant lines. (A) Primary root length at 7 DAG (n≥17) seedlings grown in LD or D. (B) Root apical meristem size is altered in *tt* mutants grown in LD. Root meristem is depicted as the distance between the quiescent center and the uppermost first cortical cell that is twice as long as it is wide indicated by the white arrowheads. Statistical significance was determined by Anova test with Dunnett's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against Col-0 genotype. (C) Lateral root density in 14-day old of (A) seedlings. (D) 50µM naringenin (N) induces lateral root development in 14-day old seedlings grown in LD. • indicates significance by Anova test with Dunnett's post-test (•p ≤ 0.05) compared to wild-type and * indicates significance by Student's t test compared with control (**p ≤ 0.01). Error bars indicate SE.

Flavonols have a dual effect on the abundance of auxin transport proteins

Flavonoids are known as auxin transport regulators (Jacobs and Rubery, 1988; Murphy et al., 2000; Brown et al., 2001; Buer and Muday, 2004b). Among the flavonoids, only quercetin and naringenin have been reported as auxin transport inhibitors (Murphy et al., 2000; Brown et al., 2001; Peer et al., 2004). To investigate the effect of flavonols on auxin transport, we tested whether naringenin modulates the abundance of auxin transport proteins. To investigate the inhibitory potential of naringenin on auxin transport, we

quantified the fluorescence of FP-fusion reporter lines for the main mediators of rootward and shootward auxin transport in roots (Peer et al., 2011) in naringenin supplemented medium. In Chapter 3 we showed that ABCB1 and ABCB19 were more abundant in LD roots than in D, suggesting that root auxin transport mediated by ABCBs is regulated by shoot illumination. Naringenin application reduced ABCB1-GFP (Figure 3A) and ABCB-4-GFP abundance in LD roots (Figure 3B), but not in ABCB19-GFP (Supplementary Figure 3). On the other hand, quercetin treatment repressed ABCB1 but had no effect on ABCB4 and 19. Quercetin treatment reduced PIN1-GFP (Figure 3C) and PIN2-mCherry (Figure 3D) abundance. Naringenin treatment had no effect on neither of the evaluated PINs abundance, suggesting that naringenin does not affect their expression and/or cycling. Taken together, this suggests that the interaction between flavonoids and auxin transporters proteins appears to be protein-specific. Quercetin reduces the accumulation of PIN carriers, whereas naringenin has a major effect in ABCBs protein stability.

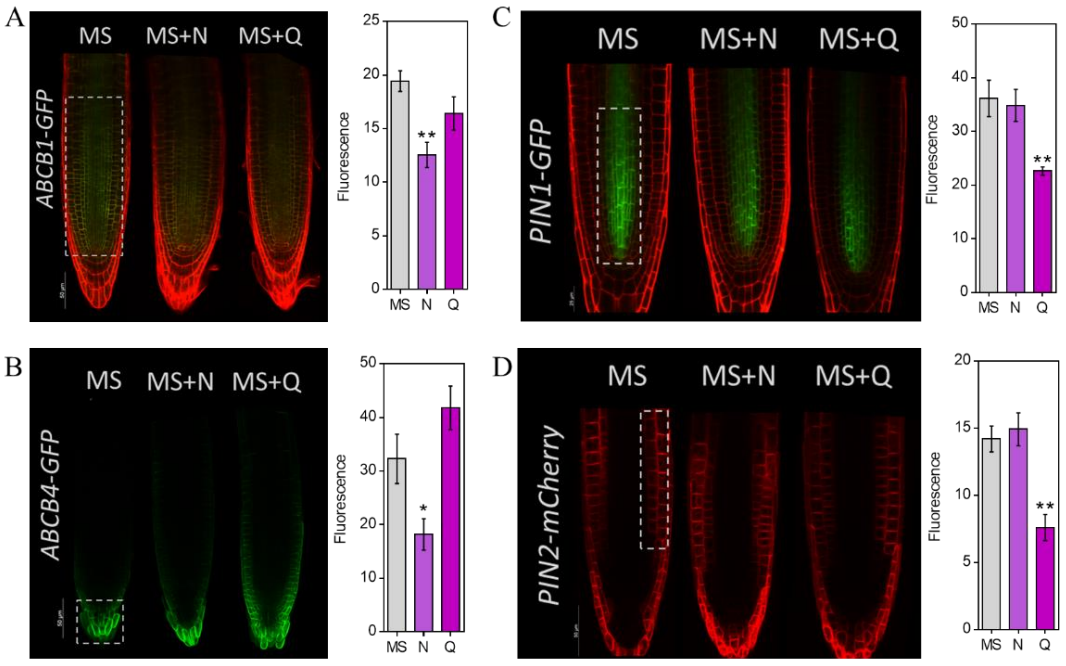

Figure 3. Flavonols treatments lead to changes in auxin transport proteins accumulation. (A) ABCB1-GFP, (B) ABCB4-GFP, (C) PIN1-GFP and (D) PIN2-mCherry signals in 4-day-old seedlings grown on LD in MS as a control or in MS supplemented with 10µM naringenin (N) or 10µM quercetin (Q). Asterisks indicates significance by Anova test with Dunnett's post-test (*$p \leq 0.05$; ** $p \leq 0.01$) compared with control (MS). The withe dashed boxes represent the ROIs used to quantify GFP and mCherry signal intensity.

Flavonoids lead to changes in auxin homeostasis during root photomorphogenic development

To provide further evidence for the role of flavonoids in root auxin responses, we analyzed the semi-quantitative auxin reporter R2D2 (Liao et al., 2015) in the *tt5, tt6* and *tt7* mutant backgrounds. No difference was observed in the D2 relative signal between LD and D grown wild-type seedlings (Figure 4A). This observation may imply that additional processes beyond auxin signaling regulate LD root development. On the other hand, *tt5-2* seedlings displayed a significant reduction in D2 fluorescence in LD seedlings, indicating higher auxin responses in 4-day-old roots. The increase in auxin response was also observed in 6-day-old roots (Supplementary Figure 4). Notably, LD *tt6-3* seedlings showed similar levels as wild-type, *tt6-3* lines are supposed to accumulate naringenin, but no impact on the levels of nuclear auxin signaling was observed. Furthermore, we observed no alteration in auxin responses in the *tt7-7* flavonol over-accumulating mutant.

We have demonstrated that the presence of naringenin and quercetin affects lateral root development (Figure 1D). The root phenotype of the mutant lines in our light conditions, suggest that flavonols are important integrators of environmental cues, especially light. To further investigate the developmental roles of naringenin and quercetin, we addressed the effect of these compounds on auxin homeostasis. Naringenin treatment was able to partially restore wild-type D2 levels in the *tt5-2* seedlings (Figure 4B), in agreement with these observations, *tt6-3* seedlings treated with naringenin showed even lower nuclear auxin response. The reduction in the nuclear auxin signaling also correlates with the reduction of *DR5::GUS* and *DR5::GFP* after naringenin treatment (Figure 4C and 4D). On the other hand, quercetin treatment induced auxin nuclear signaling (lower D2 levels) in both mutant backgrounds, whereas no effect was observed in the wild-type. Next, we assessed the effect of the lack of flavonoids in auxin signaling by using the auxin responsive promoter *pDR5::GFP* in the *tt4* background. *tt4 pDR5::GFP* lines showed no difference in the auxin responsive expression in D and LD roots (Figure 4E). In agreement with Figure 4C and D, naringenin treatments lead to a decrease in auxin DR5:GFP expression in the root apex (Figure 4F), whereas no alteration in auxin levels was observed in the quercetin treatment. This observation implies that specific flavonoids, and/or naringenin derivatives, act to modulate the nuclear auxin signaling and distribution in roots. Altogether, we conclude that the presence of naringenin has a negative effect on auxin signals. Notably, *tt5-2* and *tt6-3*

mutations displayed a very strong effect on root development, also the presence of naringenin impacts primary and lateral root growth, however the *tt6-3* showed a weaker alteration in auxin nuclear response by using the R2D2 marker. We conclude that other compounds, such as naringenin-glycosides products, may modulate the auxin homeostasis in roots.

To determine how flavonoid distribution was affected by the lack of the core biosynthetic enzymes, we investigated endogenous flavonoid derivatives present in wild-type, *tt5-2*, *tt6-3* and *chil* roots by UPHLC-ESI-QqTof. The root extracts showed altered accumulation of specific flavonol glycosides (Supplementary Table 2). In dark-grown roots, a predominance of quercetin-glycosides was observed, which suggests that quercetin levels may have a primordial effect of the root development in response to light. Surprisingly, in the *tt6-3* samples, we detected the presence of quercetin 3-O-b-D-glucopyranosyl-7-O-a-L-rhamnopyranoside, this indicates that somehow the activity of F3H can be partially complemented by a different enzyme in Arabidopsis. These data suggest that those compounds whose accumulation is affected in *tt5-2* and *tt6-3*, may be functionally important for the regulation of auxin levels in LD roots.

Lack of *tt5* and *tt6* does not impair NPA and IAA root response

The establishment and maintenance of auxin gradients in roots is controlled by the PIN and ABCB auxin carries (Zazímalová et al., 2010). In Figure 3 we showed that flavonols lead to changes in PINs and ABCBs proteins abundance. Based on that, we decided to check the effect of the synthetic auxin transport inhibitor NPA and IAA in the main root lenght of the flavonoid pathway mutants *tt5* and *tt6*. Both, NPA and IAA were able to repress primary root growth in WT and the mutants (Figure 5A and 5B). While, the inhibition of root growth was more severe in IAA than that was observed in NPA.

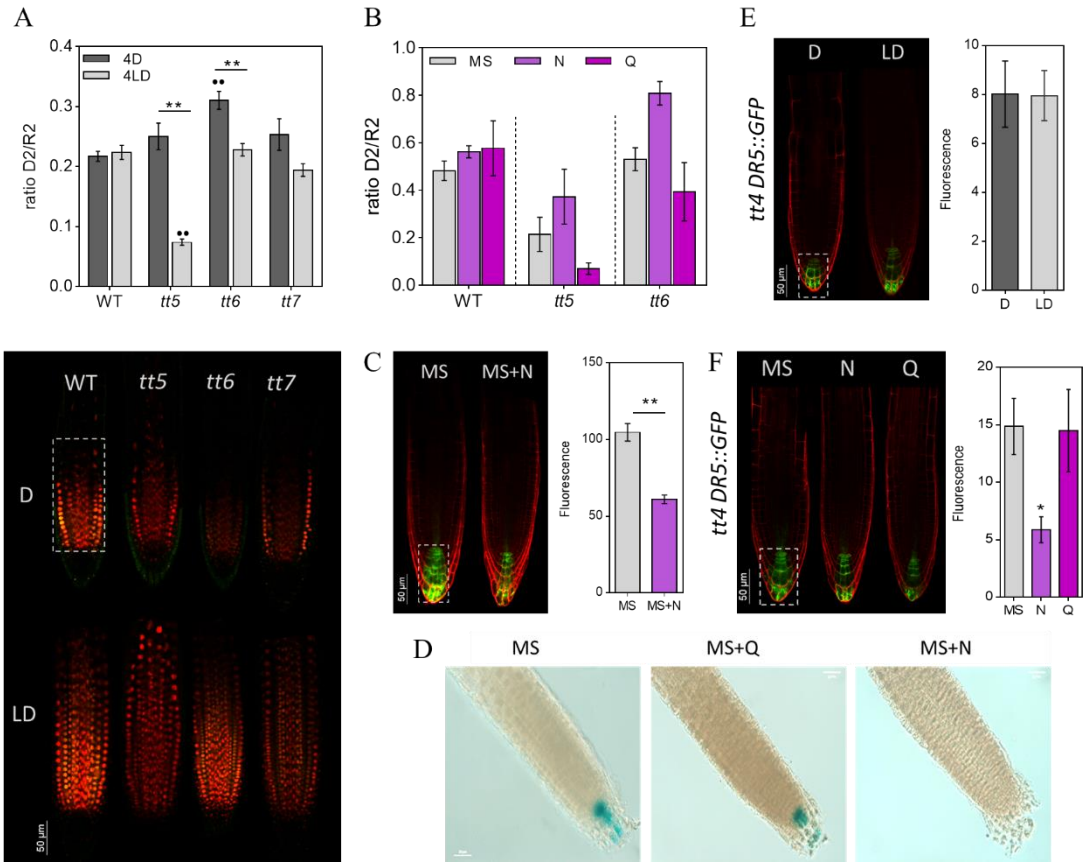

Figure 4. Naringenin affects auxin homeostasis. (A-B) Merged confocal images of R2 (red) and D2 (green) expressing R2D2 marker and quantification of D2/R2 ratio in *tt* backgrounds. (A) 4 DAG seedlings were grown in LD and D light condition (n≥5). (B) 4-day-old seedlings grown on LD in MS as a control or in MS supplemented with 10µM naringenin (N) or 10µM quercetin (n≥4). • indicates significance by Anova test with Dunnett's post-test (••p ≤ 0.01) compared to wild-type and * indicates significance by Student's t test between LD and D light conditions (**p ≤ 0.01). (C) 10µM naringenin represses *DR5::GFP* expression at the root meristem in 4-day old LD seedlings (n≥10). * indicates significance by Student's t test compared with control (**p ≤ 0.01). (D) *DR5::GUS* expression of 4-day old seedlings grown on LD in MS as a control or in MS supplemented with 10µM naringenin (N) or 10µM quercetin (Q). (E-F) 4-day old *tt4 DR5::GFP* expression in (E) LD and D roots (n≥8) (F) MS supplemented with N or Q (n≥6). Asterisks indicates significance by Anova test with Dunnett's post-test (* p ≤ 0.05) compared with control (MS). Error bars indicate SE. The withe dashed boxes represent the ROIs used to quantify GFP, R2 and D2 signal intensity.

Sucrose is known to be a positive regulator of flavonol biosynthesis (Solfanelli et al., 2006). As discussed in Chapter 3, sucrose was also reported a long-distance signal from illuminated shoots that induce root growth (Kircher and Schopfer, 2012). To explore this possibility, we tested the effect of sucrose treatment in *tt5* and *tt6* primary root growth. *tt6* seedlings showed shorter roots than the wild-type in LD condition, which was recovered by

sucrose supply (Figure 5C). These results indicate that light activated signals prime roots for sugar uptake. This process seems to be blocked in *tt6* mutants which fail to activate the root growth in response to shoot illumination possibly due to carbohydrate starvation in the roots.

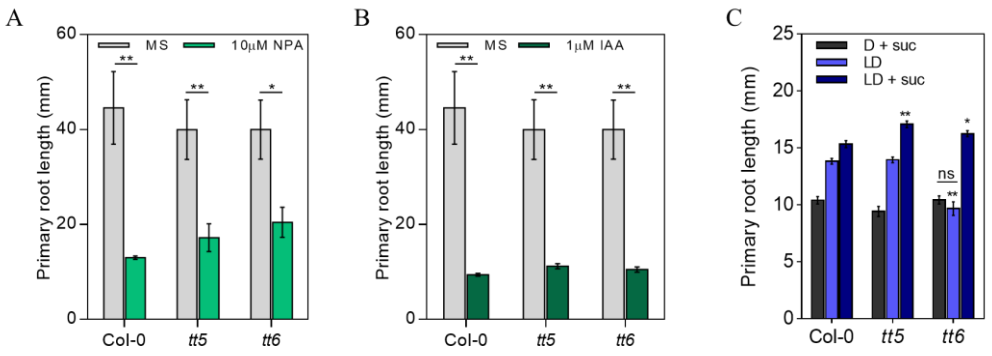

Figure 5. F3H is involved in sucrose-dependent root growth. (A-B) Main root length in 14 DAG grown in MS medium or supplemented with 10μM NPA (A), 1μM IAA (B) after day 4. Asterisk indicate statistical significance by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) between control and treatment for each line (n=5). (C) Primary root length in 7 DAG LD and D seedlings without or with 1% sucrose. Statistical significance was determined by Anova test with Dunnett post-test (*p ≤ 0.05; **p ≤ 0.01) in the same timepoint against the wild-type genotype (n≥25). Error bars indicate SE.

Discussion

Flavonols are known as modulators of auxin transport by direct and indirect regulation of auxin transporters (Peer and Murphy, 2007). Here, we addressed the effect of shoot illumination in root flavonol biosynthesis and its effects in auxin accumulation and root development. Flavonols are known to be involved in a range of physiological process during plant development acting as molecular buffers during stresses or as endogenous regulators (Peer and Murphy, 2006). These compounds were shown to be accumulated in response to high light, sucrose and auxin. Here, we show that shoot illumination highly increases the expression of flavonol biosynthetic enzymes in dark roots. Roots of 4-day old seedlings showed higher expression levels of the flavonols biosynthesis genes than 7-day old seedlings, matching the previously described profile of flavonol accumulation in young seedlings (Peer et al., 2001). As a possibility, the mRNA of the some of these genes could work as a long-distance signal delivered by the shoot to act locally in roots. We checked this possibility by addressing gene expression in shoots and roots in a shoot-illumination time

course. Our results showed that even being expressed overall in the plant (Supplementary Figure 5), shoot-illumination induces root expression of these genes. The root induction suggests a complex mechanism involving long-distance signals and lay this pathway as an interesting target for future studies aiming the exploration for possible long-distance signals between shoots and roots.

Modulation of root architecture is a complex process which involves several hormones, such as auxin, cytokinin and ethylene (Benková and Hejátko, 2009). Besides hormonal control, nutrient status and endogenous metabolites are also involved in the control of this process (Ristova et al., 2018). Flavonols have been implicated in the control of root development as in lateral root and gravitropism responses (Brown et al., 2001). In this work, we show that single loss of function mutants in different flavonoid biosynthesis genes affect primary root development in covered-roots. Experiments using different *tt4* alleles linked this gene with an increase in lateral and adventitious roots (Brown et al., 2001). We did not find consistent differences in lateral root development among the lines we used, whereas chemical treatment and chemical complementation showed a positive role for quercetin and naringenin in promoting lateral root development.

Previous studies have shown that flavonols can inhibit auxin transport through PINs and ABCBs proteins (Buer and Muday, 2004; Peer et al., 2004; Peer and Murphy, 2007; Kuhn et al., 2017) or affecting regulatory enzymes (Peer and Murphy, 2006; Bailly et al., 2008). The lack of CHS/*TT4* significantly reduces PIN1 transcript abundance and affects PIN1 protein accumulation/cycling (Peer et al., 2004). We did not find differences in PIN1 and PIN2 protein levels after naringenin treatment, whereas their abundance was reduced when quercetin was supplemented to the medium. Quercetin was shown to bind to the ATP-binding site of ABCB proteins and inhibit their activity (Geisler et al., 2005), however in our case, quercetin had no effect on ABCB1, ABCB4 or ABCB19 abundance. On the other hand, ABCB1 and ABCB4 protein levels were reduced in naringenin treatments. Flavonols are routinely used as phosphatases and kinases inhibitors in animal cell lines (Peer and Murphy, 2006), suggesting that in plants, kinases and phosphatases associated with auxin transport can be reasonable targets of these compounds (Peer and Murphy, 2007). The observed changes in protein accumulation observed in this work can be either the result of direct interaction between flavonols and auxin carriers; or indirectly by modulation of key enzymes responsible for PINs and ABCBs cycling and regulation.

The *transparent testa* mutants provide useful tools for studying the roles of flavonoids in plant development. The *tt4* and *tt7* loss of function mutants were found to have altered auxin transport (Peer et al., 2001; Peer et al., 2004). By addressing the effect of individual flavonoid biosynthesis genes on auxin accumulation in roots, we showed that *tt5* mutation has a significant increase in root auxin signaling in response to shoot-illumination. In the *tt5* mutant, naringenin or any of the downstream products are not accumulated. Naringenin treatment partially restored wild-type auxin responses in *tt5* background. Combining with the repression of DR5 signal in naringenin supplemented medium, this suggests that naringenin act as a negative auxin modulator. The role of flavonols in light-induced root development seems to be a combination of factors that include modulation of auxin transporters, nutrient status, ROS scavenger activity and interaction of regulatory enzymes (Peer and Murphy, 2007). In conclusion, we propose that flavonols act as light-responsive regulators contributing to the root development through rearrangements in auxin gradients.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana seeds were sterilized, germinated, and grown as described previously (Chapter 2). T-DNA insertion lines used are: *tt4-15* (GK-545D04), *tt5-2* (GK-176H03), *tt6-3* (SALK_113904C), *tt7-7* (GK-629C11), *fls1* (SALK_009992) and *chil1-1* (SALK_096551C) are in Columbia (Col-0) ecotype. Genotyping on T-DNA insertions was performed following the SALK (http://signal.salk.edu/index.html) instructions, the primers used for genotyping can be found in the Supplementary Table 2. All the experiments were done using homozygous lines for the T-DNA insertion. The *tt5-2 R2D2*, *tt6-3 R2D2* and *tt7-7 R2D2* lines were obtained by crossing the previously described *R2D2* (Liao et al., 2015) with the single mutant lines.

Molecular cloning and plant transformation

To generate the *Promoter::turboGFP:GUS* fusions, approximately 1.5 kb of the *CHI*, *F3H* and *F3'H* promoter regions were amplified from Col-0 genomic DNA by polymerase chain reaction (PCR) using specific primers listed in Supplementary Table 2.

The purified PCR product was then cloned into the pENTR/D-TOPO (#K240020, Thermo Fischer), the resulted fragment were subsequently recombined with the pMDC163(gateway)-TurboGFP:GUS (Xiao and Offringa, 2019, #11791020, Thermo Fischer). The *Promoter-TurboGFP:GUS* constructions were transformed into wild-type plants (Col-0) using *Agrobacterium tumefaciens* (strain Eha105) floral dip method (Clough and Bent, 1998; Zhang et al., 2006).

RNA isolation and qPCR

For Supplementary Figure 1 RNA extraction and RT-qPCR were performed as described in Miotto et al. (2019). For Figure 1B RNA extraction and RT-qPCR were performed as described in Chapter 2. All RT-qPCR values represent three biological replicates, each containing at least two technical replicates. Primer sequences used can be found in the Supplementary Table 2.

GUS staining, confocal imaging and microscopy analysis

GUS staining and confocal imaging and quantification were performed as described Chapter 3.

Metabolite isolation and analysis

Seedlings were incubated overnight in darkness at 4°C in 400µL of 80% (v/v) methanol (MeOH) and centrifuged at 16,000g for 15 min at 4°C. The supernatant was concentrated in speed-vac and resuspended in 50µL of 80% MeOH. Aliquots were analyzed by a reverse-phase HPLC (Shimadzu) using a Shim-Pak C18 column (4.8 x 150mm, Waters) using acetonitrile solvent-A, water as solvent-B and 20mM phosphoric acid as solvent-C. The HPLC program was started with B/C (6/80, v/v) for 4 min, 6%-30% B and 80%-0% C for 25 min, then B/C (30/0, v/v) for 2 min, followed by equilibrating for 3 min. The flow rate was 1 mL min^{-1}, and the injection volume for standards and samples was 20µL. Quercetin and kaempferol were detected at 365 nm and naringenin at 290 nm. Flavonoid standards including quercetin (Sigma-Aldrich, cod), kaempferol (Sigma-Aldrich, cod), and naringenin (Sigma-Aldrich, cod) were used for the calibration curve.

For the LC/MS elucidation, 6-days old dark-grown roots were used. HPLC-MS analyses were performed on a Shimadzu UHPLC system (UFLCXR). Chromatographic

conditions were Shim-pack CLC-ODS C18 (250 × 4.6 mm i.d., 5 µm, Shimadzu); flow rate 0.6 ml min^{-1}, gradient (step, time, %B over A) 1, 10 min, 10–100%; 2, 10-14 min, 100%; 3, 14-14.2 min, 100-10%; 14.2-25 min, 10%). Solvent A was formic acid 0.1% (v/v) and solvent B was formic acid 0.1% (v/v) in methanol. The UHPLC was connected to a Bruker micrOTOF-QIII. The UHPLC output was directly interfaced to the ESI ion source. The MS conditions were: nebulizer gas (N_2) 4 Bar, dry temperature 300 °C, HV capillary 4000 V, transfer time 55 us and collision energy 2 eV. The MS acquisitions were performed in the negative electro- spray ionization mode.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., CA, US). Data were tested for normal distribution by Shapiro-Wilk test and then applied the respectively statistic test and when significant ($p \leq 0.05$) were showed in the graphs. Statistical details of each experiment (test used, replicates, etc) can be found in the Results section and Figure-Figure Legend sections.

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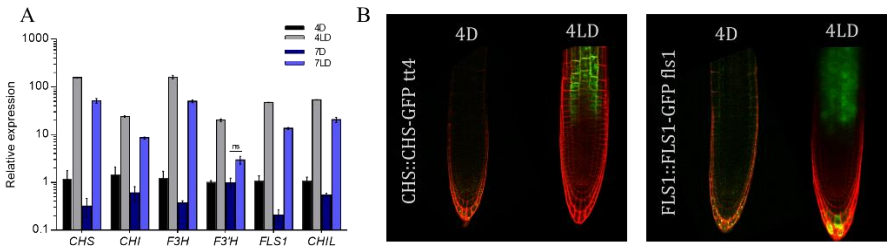
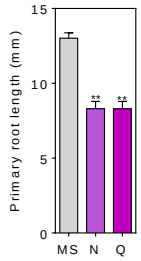
Supplementary information

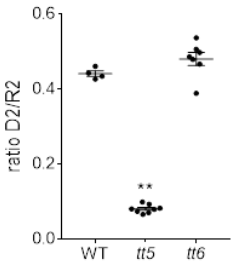
Supplementary tables are available at:
https ://drive.google.com/drive/folders/1jeLQ04CZDnFZuyD0jejfAG1SOheYoDgu?usp=sharing



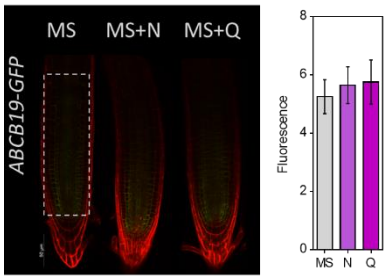
Supplementary Figure 1. (A) RT-qPCR analysis of flavonols gene expression in LD and D roots. Gene expression was normalized using an internal control (AT3G18860 and AT2G42500; Miotto et al., 2019) for each reaction. The expression levels of 4DAG D condition were set as 1. Statistical significance was determined Student's t-test between D and LD condition to every time. Ns represent no significant comparison. (B) CHS-GFP and FLS1-GFP expression in 4 DAG LD and D roots.



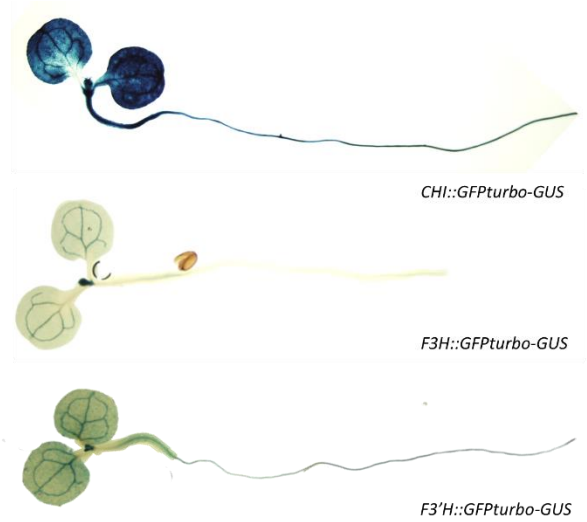
Supplementary Figure 2. Flavonols effect on 7 DAG in primary root growth (n≥10). Asterisks denote different significant classes by Anova test with Dunnett post-test (**p ≤ 0.01). Wild-type seedlings were grown in MS medium as a control or MS with 100µM of naringenin (N) or quercetin (Q). Error bars indicate SE.



Supplementary Figure 3. R2D2 marker and quantification of D2/R2 ratio in *tt5* and *tt6* backgrounds. 6 DAG seedlings were grown in LD (n≥5). * indicates significance by Anova test with Dunnett's post-test (**p ≤ 0.01) compared to wild-type.



Supplementary Figure 4. ABCB19-GFP signal in 4-day-old seedlings grown on LD in MS as a control or in MS supplemented with 10µM naringenin (N) or 10µM quercetin (Q). The withe dashed boxes represent the ROIs used to quantify GFP signal intensity.



Supplementary Figure 5. CHI, F3H and F3'H promoter GUS expression in 4-day old LD seedlings.

Capítulo V

- HY5/HYH and AGC3 kinases regulate root development in the dark -

HY5/HYH and AGC3 kinases regulate root development in the dark

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Abstract

Light perception by plant photoreceptors triggers photomorphogenic seedling development, resulting in green and open cotyledons and a well-developed root system. A key regulator of photomorphogenesis is the E3 ubiquitin ligase subunit COP1, which mediates proteasomal degradation of the photomorphogenesis promoting bHLH/bZIP transcription factor HY5 and its close homolog HYH in the dark. Recently, the PID AGC3 kinase was shown to interact with and phosphorylate COP1. Although the mechanistic switch from skoto- to photomorphogenesis is well elucidated in the aerial tissues, less is known about how it is regulated in roots. In Chapter 3 we discussed the implications of auxin as a mobile signal delivered from illuminated shoot to promote dark-root growth. Here, we show that photoreceptors positively regulate root development. Mutant phenotype and confocal microscopy analysis show that HY5 has a major role in shoot and root photomorphogenesis. Furthermore, we show that AGC3 kinases promote lateral root development. The genetic repression of COP1, specifically in roots, does not correlate with an increase in primary root growth. Overall, these data provide molecular and genetic insights for the control of root photomorphogenesis by HY5/HYH and AGC3 kinases.

Introduction

During the evolution process plants have developed a complex system to monitor multiple signals of the environment. By monitoring light intensity, quality, duration and direction, plants get environmental information to optimize their growth and development. Light regulates a broad range of developmental processes which includes seedling photomorphogenesis. Such complex processes involve signaling and hormonal pathways that often result in dramatic changes in plant morphology (Lee et al., 2017). Plant development in response to light begins with light perception by several classes of photoreceptors. Far-red/red light is perceived by phytochromes (PHYA-PHYE in Arabidopsis), blue light by cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2) and ZEITLUPE, and UV-B light by UVR8 (Kami et al., 2010; Galvão and Fankhauser, 2015). The photomorphogenic process is repressed in the absence of light by the CONSTITUTIVE PHOTOMORPHOGENIC/DE-ETIOLATED/FUSCA (COP/DET/FUS) genes, and members of the SUPPRESSOR OF PHYA (SPA) family (Yi and Deng, 2005; Lau and Deng, 2012; Podolec and Ulm, 2018). In darkness, COP1/SPA complexes target positive regulators of photomorphogenesis for ubiquitination and degradation through the 26S proteasome pathway (Osterlund et al., 2000; Holm et al., 2002). By controlling the levels of photomorphogenesis-promoting transcription factors, the COP1/SPA complex regulates a wide range of light-dependent developmental processes, which includes de-etiolation (Wang et al., 2019).

One of the targets of the COP1/SPA complex is the photomorphogenic promoting transcription factor ELONGATED HYPOCOTYL 5 (HY5). In the dark, the nuclear localized COP1 targets HY5 and HY5 HOMOLOG (HYH) to proteasome degradation (Ang et al., 1998; Osterlund et al., 1999; Holm et al., 2002). Loss-of-function *hy5* seedlings have an increase in lateral root development when grown under constant light, also in the absence of *hy5* seedlings are more agravitropic, implicating a cross-talk with hormone signaling (Sibout et al., 2006). Interestingly, *hyh* loss-of-function seedlings show no obvious aberrant phenotype (Holm et al., 2002; Sibout et al., 2006). The expression levels of HY5 and HYH have been shown to be induced by light (Holm et al., 2002; Sibout et al., 2006; Singh et al., 2012), and more specifically, in a root-covered system (Zhang et al., 2019). In addition, HY5 acts as a mobile shoot-to-root signal that promotes nitrate uptake in response to light (Chen

et al., 2016). Many auxin-related genes were found to be differently expressed in *hy5* loss-of-function mutant (Sibout et al., 2006). Besides, HY5 has been shown to regulated auxin accumulation in the roots by modulating the intracellular distribution of the auxin transporter PIN-FORMED2 (PIN2; Laxmi et al., 2008). Moreover, *DR5::GUS* expression was strongly induced in lateral roots in *hy5* mutant background (Zhang et al., 2019). It suggests that the modulation of hormone signaling could be one of the mechanisms by which HY5 controls root growth.

The plant hormone auxin plays a central role in many processes of plant development. Directional cell-to-cell auxin transport generates accumulation of this hormone in specific sites that are responsible for controlling gravitropic responses, lateral root development, and tissue pattering (Motte et al., 2019). The asymmetric subcellular localization of PIN-FORMED (PIN) proteins at the plasma membrane determines the direction of auxin flow (Wisniewska et al., 2006). Phosphorylation of PINs at conserved serines in TPRXS motifs by the AGC3 kinases PINOID (PID), WAG1 and WAG2 switch PIN polarity from basal to apical in root meristem cells (Friml et al., 2004; Kleine-Vehn et al., 2009; Dhonukshe et al., 2010). Recently, based on etiolated seedlings, an alternative role was proposed to the AGC3 kinase PID. Besides its role in the control of auxin transport, PID interacts with and phosphorylates COP1 at Ser20, repressing COP1 activity and promoting photomorphogenic responses such as apical hook opening and repression of hypocotyl elongation (Lin et al., 2017). The PID close homolog, AGC3-4, was found to also interact and phosphorylate COP1 (van Gelderen, 2017). However, the mechanism by which light regulates COP1-AGC3 kinases-HY5 in roots has not been fully elucidated.

Here we explore the possible role of AGC3 kinases as positive regulators of root photomorphogenesis by stabilization of HY5/HYH transcription factors. We focused on comparing single and higher order loss-of-function mutants effects on primary and lateral root development, and the expression pattern of AGC3-4 and HY5 reporter lines. Our findings suggest that AGC3-4, WAG1 and WAG2 act opposite to HY5 in lateral root growth and that HY5 has an AGC3-dependent role in the control of root photomorphogenesis.

Results

Photoreceptors positively regulate root photomorphogenesis

Photomorphogenesis is triggered by light perception through the plants'

photoreceptors. To investigate whether light perception by the shoots would affect dark-grown root development, we evaluated phytochrome and cryptochrome mutants cultivated with an illuminated shoot and darkened root (light/dark condition, Chapter 2). As an indicator of root photomorphogenesis in response to shoot illumination, we measured the primary root length over time and lateral root density. *phyB*, *phyE*, *cry1*, *cry2* and the double mutant *cry1cry2* showed a significant decrease in primary root length at 7 DAG (Figure 1A), the same was observed at 14 DAG for all the lines except for *phyE* and *phyA*. Notably, *phyA* phenotype resembled wild-type seedlings in both timepoints. The lateral root density was significantly lower than Col-0 in *cry2* and *cry1cry2* (Figure 1B), suggesting a major effect of *cry2* in lateral root formation. These results indicate that photoreceptor activation in shoots is needed to trigger proper root development and that phyB and the cryptochromes CRY1 and CRY2 are the major photoreceptors involved in the activation of main root growth. Interestingly, phyE seems to be more relevant in early root growth and becomes less essential in later stages.

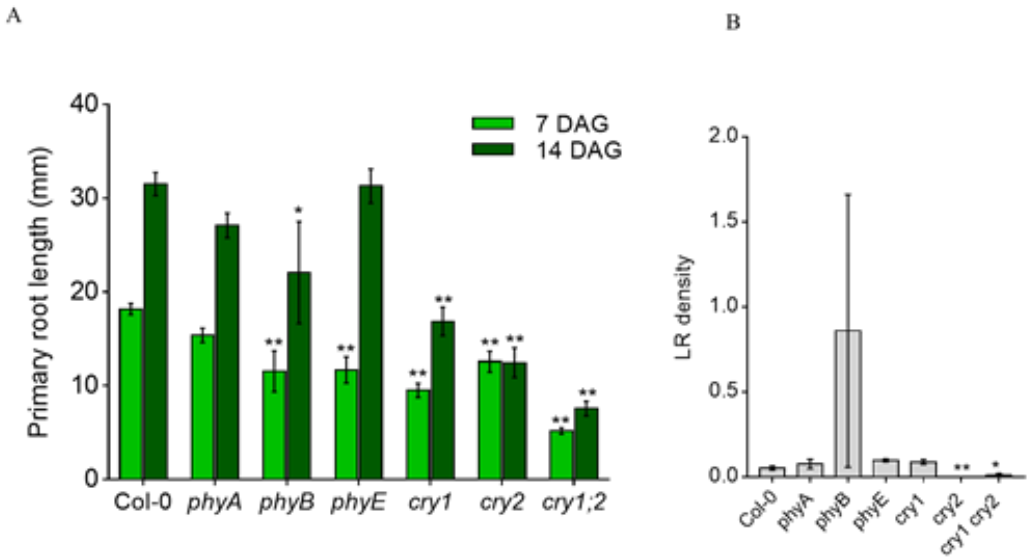

Figure 1. Shoot-illumination affects root growth in photoreceptor mutants. (A) Primary root lengths of single knock-out mutants at 7 and 14 DAG ($n \geq 15$). (B) Lateral root density. Error bars indicate SE. Statistical significance was determined by Anova test with Dunnett post-test ($*p \leq 0.05$; $**p \leq 0.01$) in the same timepoint against the wild-type genotype.

AGC3 kinases promote lateral formation but do not affect primary root growth in response to light

Shoot development mediated by light is a much better understood process than light-promoted root development. The switch from skoto- to photomorphogenesis in shoots involves several components of the CSN complex (COP9), the E3 ubiquitin ligase COP1, the downstream transcription factors, HY5 and HYH and the more recently reported AGC3 kinases PID and AGC3-4 (Lau and Deng, 2012; Lin et al., 2017; Wang et al., 2019). In Chapter 1 we hypothesized that AGC3-4 and it is homologs WAG1 and WAG2 play a key role in photomorphogenic root development. To investigate whether these AGC3 kinases are involved in the control of light responsive root growth we examined their expression in LD and D growth conditions (Chapter 2) and evaluated shoot and root phenotypes of single loss-of-function lines. No difference was observed in *AGC3-4* and *WAG2* expression when we compared LD and D at 4 and 7 DAG roots (Figure 2A), whereas *WAG1* showed higher expression in roots at 7 DAG. Consistently, the GFP signal of *AGC3-4::AGC3-4-GFP agc3-4-1* was similar in both conditions (Figure 2B). We further examined the expression pattern of WAG1 and WAG2 using promoter::GUS reporter lines. *WAG1* was predominately detected in the elongation zone in both in LD and D conditions (Figure 2C), matching with the qPCR data, whereas the GUS signal increased on the LD conditions at 7 DAG. Although *WAG2* showed similar expression in LD and D in the qPCR (Figure 2A), the reporter line, *WAG2p::GUS*, was induced in LD roots at 7 DAG (Figure 2C). However, both in D or LD conditions, *agc3-4, wag1* and *wag2* mutant lines did not differ from wild-type (Figure 2D) in hypocotyl and primary root growth, suggesting that these genes are not directly involved in the light responsive growth. Yet, none of the mutants showed emerged lateral roots in 14-days-old seedlings, suggesting they are needed for lateral root development (Figure 2 E). In summary, our result suggests that AGC3-4, WAG1 and WAG2 kinases have a role in the lateral root control instead of the main root.

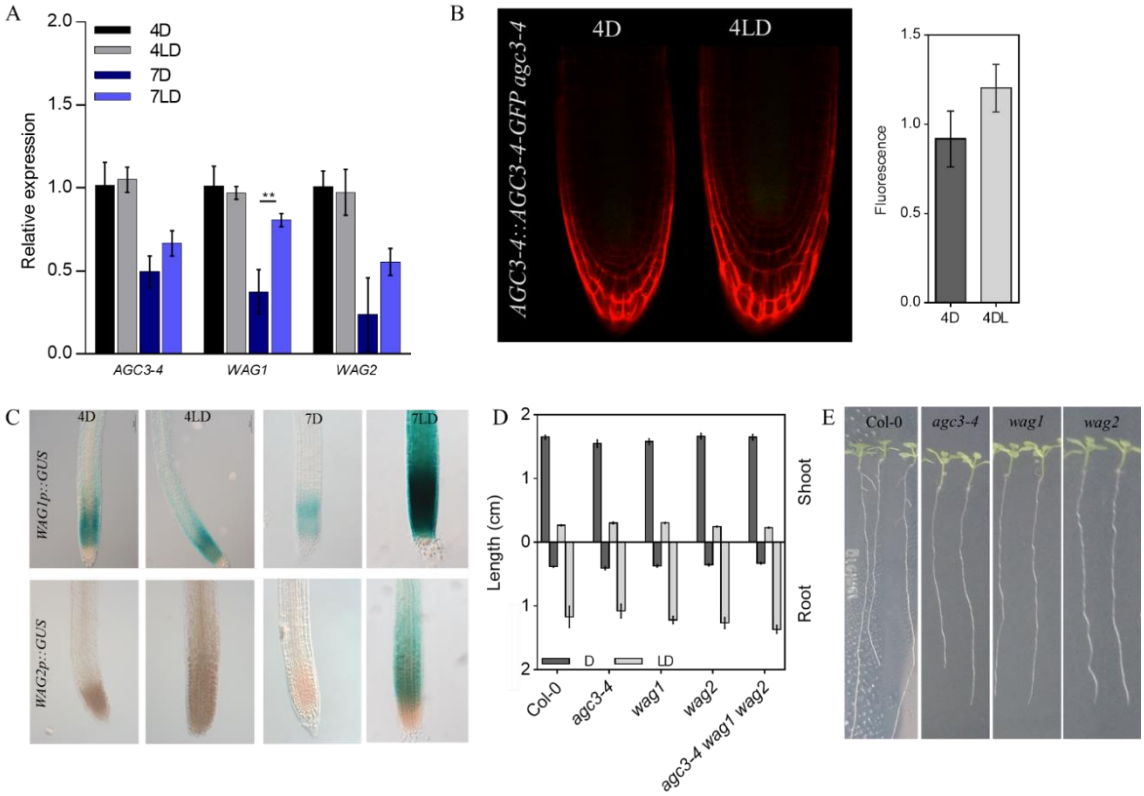

Figure 2. Effects of shoot-illumination on AGC3 kinases expression in roots. (A) RT-qPCR analysis of gene expression in roots. The expression levels of 4DAG D condition were set as 1. Statistical significance was determined by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) between D and LD condition to every time. (B) Confocal images of 4-day old *agc3-4-1 AGC3-4::AGC3-4-YFP* seedlings showing AGC3-4-YFP expression at the root meristem. (C) GUS expression of 4- and 7-day old *WAG1p::GUS* and *WAG2p::GUS* lines. (D) Shoot and primary root length at 7 DAG (n≥17). Error bars indicate SE. (E) AGC3 kinases affect lateral root development, representative images of 14 DAG seedlings grown in LD.

HY5 promotes root growth in response to shoot illumination

The transcription factor HY5 and its homolog HYH are known key regulators of root photomorphogenesis (Sibout et al., 2006; Chen et al., 2016; Zhang et al., 2017; Zhang et al., 2019). To investigate whether these genes are involved in the induction of root photomorphogenic growth, we examined single and double loss-of-function mutants grown in D and LD conditions. The *hy5* mutant displayed longer shoots than WT in LD and shorter roots both in LD and D condition (Figure 3A). In contrast to the pronounced phenotype of *hy5*, the *hyh* mutant showed no difference in root and hypocotyl lengths compared to wild-type. Nonetheless, in the *hy5hyh* double mutant, the root and shoot phenotypes observed in *hy5* were enhanced, suggesting an additive effect between both genes. Additionally, *hy5*

showed an increase in LR density that was not observed in *hyh* lines, whereas *hy5hyh* showed a similar increase in the LR density as the single mutant *hy5* (Figure 3B) suggesting that HY5 acts alone in suppressing LRs. The observed difference in phenotype suggests that HY5 has a major role in the control of root photomorphogenesis. To test this hypothesis, we measured the expression levels of HY5 and HYH roots after 4 and 7 DAG grown on LD or D conditions. Both genes showed the same expression pattern, with larger abundance of transcripts when shoots were exposed to light (Figure 3C). *HYH* showed a higher light induction than *HY5* in LD. *HYH* induction in roots under shoot-illumination was six to eight-fold higher, when compared to the dark condition in 4 and 7 days, respectively. On the other hand, *HY5* expression increased around two-fold in roots in the same comparison.

To evaluate how fast shoot illumination is able to induce HY5 and HYH in roots we examinated *HY5* and *HYH* root transcripts in dark grown etiolated plants transferred to shoot light. In 4 DAG seedlings, root expression of both genes was induced within 2 hours after shoot illumination (Figure 3D), reaching maximum levels in 4 hours. Consistently, the GFP signal of *HY5::HY5-GFP hy5* seedlings was strongly increased in roots after 3 hours of shoot illumination (Figure 3E). To further determine whether light-exposure affects their expression, we quantified endogenous *HY5* and *HYH* transcript abundance in 4 DAG shoots and roots after shoot illumination. The expression of both genes was highly induced by shoot light (Figure 3F). In the dark, both *HY5* and *HYH* transcripts are present at very low levels in roots, while they are significantly more abundant in shoots. Upon light stimulus, *HYH* expression was strongly induced around 12-fold within 6h in shoots, reaching 50-fold in 48h. Contrarily, *HY5* levels increased about 3-fold in 6h, not exceeding 5-fold in 48h time-point. In the roots, *HYH* induction happened at a slower rate than the shoots, being significantly induced, about 20-fold, only after 48h. The delay in HYH induction in roots might suggest that a shoot signal is necessary to trigger *HYH* expression in dark-grown roots. The expression of *HY5* in roots was significantly induced, about 4-fold, as early as 6h, reaching roughly 20-fold after 48h. Interestingly, the kinetics of HY5 induction happened faster in roots than in shoots, suggesting that HY5 induction in roots might respond to upstream signals of HY5 itself. These results suggest that the transcriptional regulation of HY5 and its close homolog HYH in roots is highly controlled by shoot illumination.

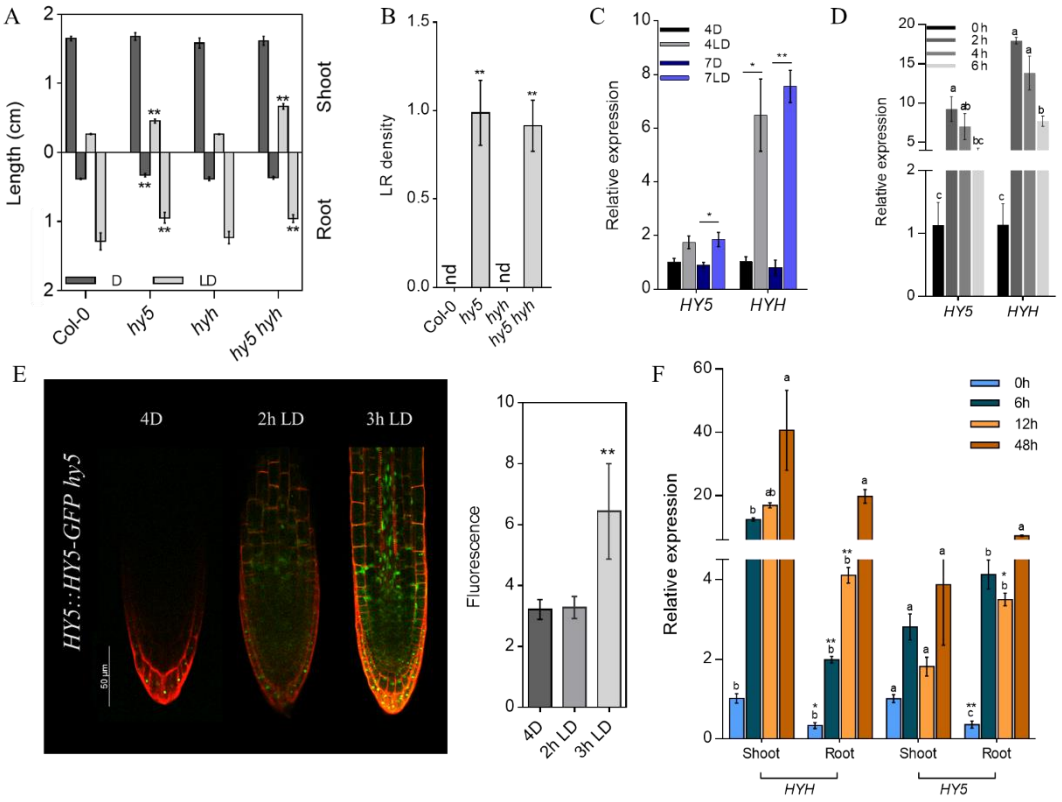

Figure 3. *hy5* mutation affects shoot and root development. (A) Shoot and primary root length of 7-day old seedlings (n≥15). (B) Lateral root density increases in *hy5* background in 14-day old seedlings. Statistical significance was determined by Anova test with Dunnett's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against Col-0 genotype. (C) RT-qPCR analysis of HY5 and HYH expression in roots. The expression levels of 4D condition were set as 1. Statistical significance was determined by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) between D and LD condition to every time. (D) Root expression of HY5 and HYH in 4-day old seedlings 0, 2, 4 and 6h after shoot illumination. Statistical significance was determined by the Anova test with Tukey post-test (Letters denote different significant classes, p ≤ 0.05). (E) Confocal images of 4-day old *HY5::HY5-GFP hy5* seedlings showing HY5 root expression. Statistical significance was determined by the Student's t-test (*p ≤ 0.05; **p ≤ 0.01) against 4D samples. (F) Root expression of HY5 and HYH in 4-day old seedlings after a long-term shoot illumination. Letters denote different significant classes, (p ≤ 0.05) by Anova test with Tukey post-test and asterisks indicate a significant difference with the same gene and condition in shoots by t-test (*p ≤ 0.05; **p ≤ 0.01). Error bars indicate SE.

AGC3 kinases and HY5/HYH putative role in the control of dark-root photomorphogenesis

In order to evaluate if AGC3 kinases influence the HY5/HYH photomorphogenic signaling cascade, we decided to check their possible role in HY5/HYH stability in the root. In an effort to examine a potential genetic interaction between HY5/HYH and AGC3

kinases, we combined higher order mutants. We compared the seedling phenotypes in 7 and 14 DAG (Figure 4A and 4B). After 7 days in the dark (D) *agc3-4 wag1 wag2 hy5* seedlings had longer hypocotyls than Col-0. Surprisingly, the long hypocotyl and short root phenotype of *hy5* in LD was still present in the *agc3-4 wag1 wag2 hy5 hyh* quintuple mutant but abolished in the *agc3-4 wag1 wag2 hy5* line. This suggests that the *hy5* phenotype is dependent of these AGC3 kinases (WAG1, WAG2 and AGC3-4) and that somehow these AGC3 kinases act redundantly to repress root length in a HY5-dependent way.

Roots of *hyh agc3-4* grown in LD were longer than the wild-type, whereas *agc3-4 hy5* had shorter roots, resembling the *hy5* single mutant and *hy5hyh* seedlings (Figure 3A). These contrasting phenotypes of *hyh agc3-4* and *agc3-4 hy5* suggest that HYH acts as a repressor of root growth in an AGC3-4 dependent manner. When we analyzed LR density at 14 DAG, *agc3-4 hy5* and *hy5 hyh agc3-4 wag1 wag2* seedlings had an increase in the lateral root development (Figure 4B). Interestingly, in agreement with the root length results, the *agc3-4 wag1 wag2 hy5* line suppressed the increase in LR observed in *hy5* and *hy5hyh* mutants (Figure 3B) and this suppression was absent in the *agc3-4 wag1 wag2 hy5hyh* quintuple mutant. These results point to an AGC3 kinase dependent regulation of HY5 and HYH in the roots where HYH is able to partially take over some HY5 functions in the absence of WAG1, WAG2 and AGC3-4.

Since previous data shows that AGC3-4 is able to interact with and phosphorylate COP1, we decided to evaluate the phenotype of AGC3-4 overexpression lines. The *35S::AGC3-4* and *35S::AGC3-4 hy5* seedlings grown in the dark for 7 days had shorter primary root length than Col-0 (Figure 4C). *35S::AGC3-4* repressed root elongation in the dark but had no effect in LD conditions. The presence of *hy5* mutation in the *35S::AGC3-4* had the same phenotype as the single *hy5* lines, with shorter main roots and longer hypocotyl. The same effect was observed in LR development (Figure 4D). This result suggests that AGC3 kinases act upstream to HY5 in the regulation of photomorphogenesis and that AGC3-4 overexpression does not affect directly light dependent root growth. To further elucidate the role of the HY5-HYH/AGC3-4 module in root photomorphogenesis, we examined the impact of *hy5*, *hyh* and *agc3-4* mutations in *HY5::HY5-GFP* and *AGC3-4::ACG3-4-GFP* expression. The expression pattern and signal abundance of *AGC3-4::ACG3-4-GFP* was unchanged in the *hy5* and *hy5hyh* backgrounds (Figure 4E), suggesting these transcription factors are not involved in the transcriptional control of AGC3-4

expression. Similarly, *HY5::HY5-GFP* was unchanged in the *agc3-4* and *hyh* backgrounds (Figure 4F).

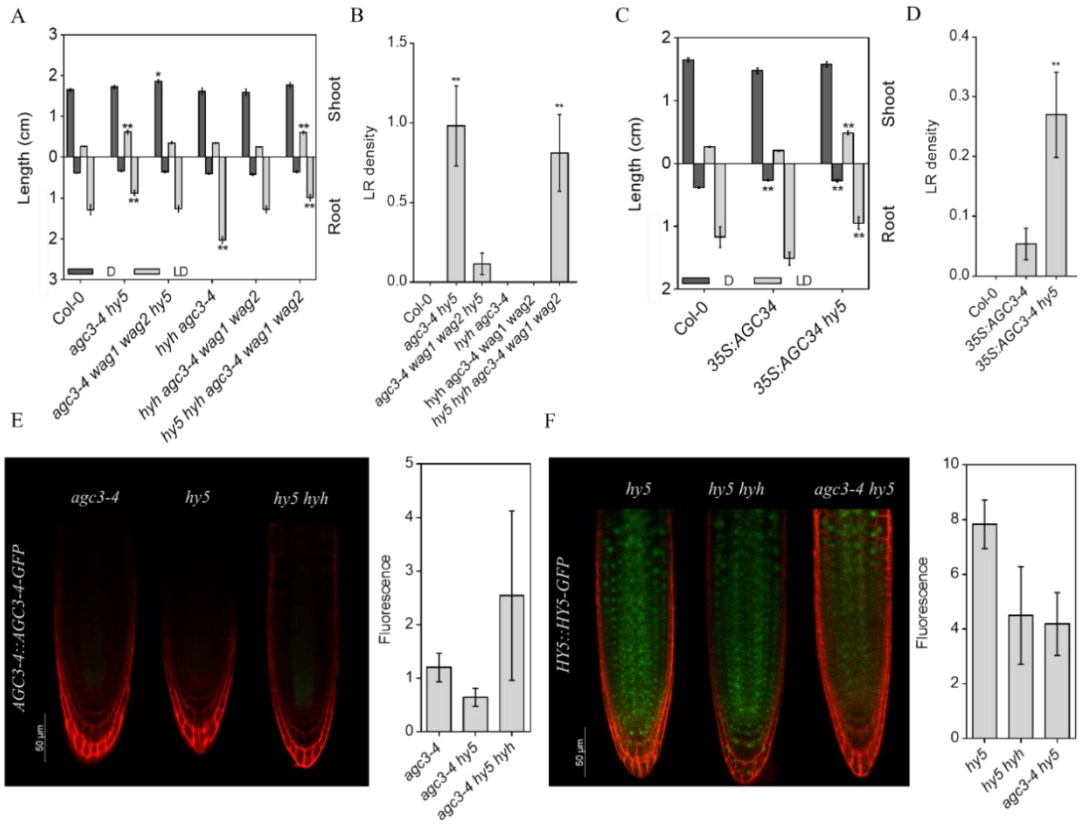

Figure 4. Seedling phenotypes of higher order mutants. (A and C) Shoot and primary root length of 7-day old seedlings (n≥17). (B and D) Lateral root density increases in *hy5* background in 14-day old seedlings. Statistical significance was determined by Anova test with Dunnett's post-test (*p ≤ 0.05; **p ≤ 0.01) in the same light condition against Col-0 genotype. (E and F) Confocal images of 4-day old (E) *AGC3-4::AGC3-4-GFP agc3-4-1* and (F) *HY5::HY5-GFP hy5* seedlings.

AGC3-4 is required for NPA response in dark-grown roots

The establishment and maintenance of auxin gradients in roots is controlled partially by the PIN efflux carriers (Benková et al., 2003). PIN stabilization in the plasma membrane is mediated through protein phosphorylation by AGC3 kinases (Huang et al., 2010; Weller et al., 2017). The shorter primary root, increase in lateral root density and long hypocotyl phenotypes observed in *hy5hyh* and *agc3-4 hy5* double mutants, are typical phenotypes of auxin-related mutants (Sibout et al., 2006; van Gelderen, 2017). To explore this possibility, we tested the effect of the synthetic auxin transport inhibitor NPA and IAA in the main root.

NPA treatment inhibited primary root growth in Col-0 and the double mutant *hy5hyh* (Figure 5A), however, this inhibition was not observed in *agc3-4 hy5* seedlings. When grown in the presence of 0.5 µM IAA, the inhibition of root growth was more severe than that observed with NPA (Figure 5B). This observation suggests that auxin transport inhibition by NPA is AGC3-4 dependent.

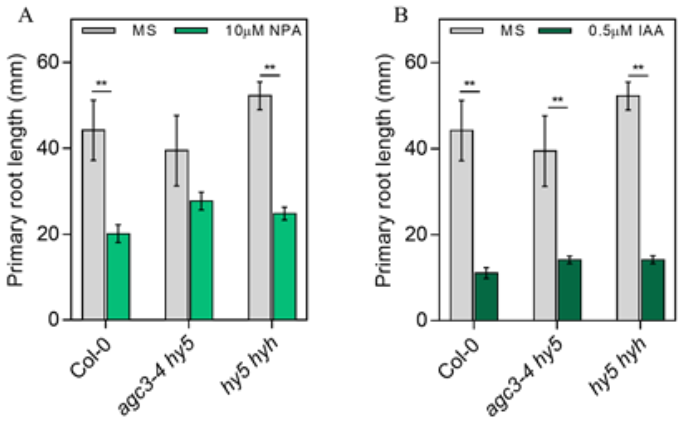

Figure 5. AGC3-4 is involved in NPA-repression root growth. (A-B) Main root length in 14 DAG grown in MS medium or supplemented with 10µM NPA (A), 0.5µM IAA (B) after day 4. Statistical significance was determined by the Student's t-test (**$p \leq 0.01$) between control and treatment for each line (n=5). Error bars indicate SE.

Root-specific repression of COP1 does not induce primary root growth

To gain insight into the role of COP1 in control root photomorphogenesis, we constructed an artificial microRNA specific to COP1 under the control of a glucocorticoid (GR)-inducible promoter. The GR promoter was driven by pTobRB7, a previously characterized root-specific promoter (Yamamoto et al., 1991). Among the lines we generated, pTobRb7-amiRCOP1-GR #4 and #6 lines were the most responsive to dexamethasone treatment (DEX; data not shown). Line #4 showed stronger COP1 repression levels, reaching 40% repression (Figure 6A). Interestingly, dark grown seedlings treated with DEX showed no effect on primary root length, suggesting that COP1 repression in roots is not sufficient to promote root elongation and that, indeed, a light dependent signal derived from the shoots is necessary to trigger this response.

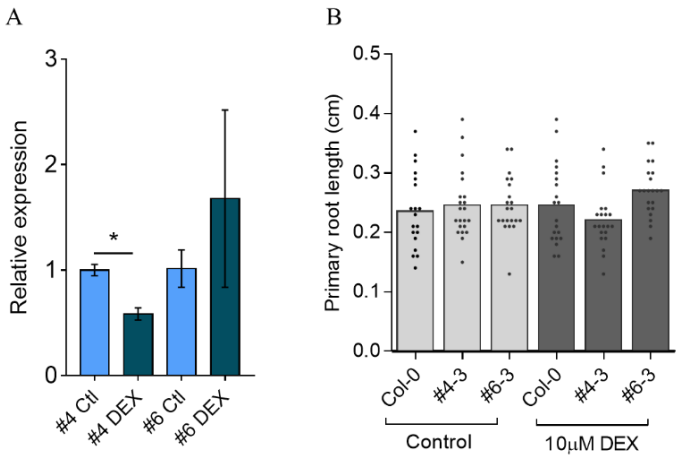

Figure 6. COP1 knock down does not trigger root development. (A) RT-qPCR analysis of COP1 expression in 7 DAG roots before and after 4h of 10μM DEX treatment. The expression levels of the control condition were set as 1. Statistical significance was determined by the Student's t-test (*$p \leq 0.05$) between control and DEX treatment for each line. (B) Primary root length of 7 DAG wild-type seedlings and amiR #4 and #6 lines without or with 10μM DEX grown in darkness ($n \geq 19$).

Discussion

Previously, it has been shown that the PID and AGC3-4 kinase positively regulate photomorphogenesis by COP1 phosphorylation (van Gelderen, 2017; Lin et al., 2017). However, no evidence was provided to support the importance of this regulation in the stabilization of the key photomorphogenic promoter HY5 and its close homolog HYH. Here, we showed that HY5 has a major role in control root morphogenesis in response to shoot illumination, that is somehow dependent of AGC3 kinases.

AGC3-4 is a homolog of PID, and together with WAG1 and WAG2 it forms the AGC3 subclade of AGCVIII kinases in Arabidopsis (Galván-Ampudia and Offringa, 2007). Recently, it has been shown that even though *agc3-4* loss-of-function showed normal early root growth, its overexpression resulted in auxin transport related phenotypes, supporting a role for this kinase in PIN recycling (van Gelderen, 2017). The single mutant *agc3-4*, *wag1* and *wag2* showed similar hypocotyl and main root length as wild-type in both light conditions. Additionally, the triple mutant *agc3-4 wag1 wag2* did not lead to a different phenotype. PINs localization in roots is dependent of its phosphorylation by the AGC3 kinases (Dhonukshe et al., 2010; Huang et al., 2010), in the single and triple loss-of-function seedlings we observed a lack of lateral root development, that can be related to changes in

auxin homeostasis due to alterations in PIN polarity. AGC3-4 and WAG1 were previously reported to be expressed in the lateral root primordia (van Gelderen, 2017), suggesting that both genes act as positive regulators of lateral root development. WAG1 was shown to be a direct target of HY5 (Zhang et al., 2011) what correlates with its transcriptional induction in LD roots showed by qPCR and promoter:GUS reporter line. The evidence that WAG1 can partially complement PID phenotype (van Gelderen, 2017), together with its expression pattern in roots, leads to consider this kinase, and possibly the others, as regulators of root development by maintaining auxin gradients in response to shoot illumination.

HY5 is a known key regulator of plant growth and development in response to environmental stimulus integrating light and hormone signaling (Gangappa and Botto, 2016). Recently, HY5 was shown to be transported from the illuminated shoot to the roots, where it triggers its own expression (Chen et al., 2016). Here, we examined HY5 and HYH contribution to root development in response to shoot-illumination. Both genes were induced in roots upon shoot dark-to-light transition, unlike *HYH*, *HY5* showed to be induced first in roots, suggesting that additional signals, besides HY5 itself (Chen et al., 2016), are responsible for HY5 expression in roots. The *HYH* late response observed in roots agree with previous data which shows a HY5-dependent regulation (Zhang et al., 2017). Interestingly, HY5 and HYH had distinct expression patterns in LD and D roots, although both are induced in response to light, *HYH* showed a greater induction than *HY5*. Both, HY5 and HYH were reported to modulate root development (Sibout et al., 2006; Zhang et al., 2019), in our system we observed a repression of primary root elongation and an increase in lateral root density in *hy5* backgrounds when grown in LD, indicating that HY5 plays a major role in light-regulated root development.

We hypothesize that AGC3-4 acts upstream of HY5, controlling COP1 phosphorylation status and therefore leading to HY5 stabilization. The root phenotype observed in *35S::AGC3-4* and *35S::AGC3-4 hy5* suggests that AGC3-4 overexpression does not directly affect light-dependent root growth. AGC3-4 have been proposed to have an opposite effect on PIN stabilization than the other AGC3 kinases (PID, WAG1 and WAG2; van Gelderen, 2017), suggesting that the repression observed in D root may be related to changes in auxin maxima and minima in the root tip. No differences were observed in AGC3-4 expression in *hy5* and *hy5hyh* backgrounds, however *hyh* seemed to have a positive effect on AGC3-4 expression (data not shown), suggesting that HY5 and HYH possibly have

distinct roles in light-dependent root development. HY5-GFP expression was not changed in the *agc3-4* background. Both AGC3-4 and PID kinases had been proposed as positive photomorphogenic regulators (Lin et al., 2017; van Gelderen, 2017). Our data suggests that in the lack of a functional AGC3-4, PID or WAGs might take over its function to stabilize HY5, however more evidence is needed to support this possibility. In shoots, HY5 and HYH stability is controlled by the COP1/SPA complex (Deng et al., 2000; Hardtke et al., 2000; Holm et al., 2002). It would be interesting to investigate how this interaction in roots contributes to root growth in our system. We generated amiRCOP1 inducible lines driven by a root-specific promoter, which showed that the COP1 suppression is not enough to induce root growth in darkness, reinforcing the idea that light dependent shoot-derived signals are necessary to induce physiological changes in roots. More detailed analysis of the effects of suppress COP1 in roots will give us insight on the molecular mechanism involved in root photomorphogenesis.

Our data, together with the fact that AGC3-4 and PID proteins promote photomorphogenesis by disassembling the COP1 complex (van Gelderen, 2017; Lin et al., 2017), as well as the demonstrated organ-specific HY5 control of root growth (Zhang et al., 2017), suggests that phosphorylation of COP1 by AGC3 kinases in roots play an important role in HY5-local stabilization and root growth. However, the effect of HY5 stabilization in roots and how it promotes root morphogenesis still remains unclear.

Materials and methods

Plant material and growth conditions

Arabidopsis Columbia (Col-0) was used as wild-type (WT), and the mutants *agc3-4-2* (SAIL_1262_B11), *hy5* (SALK_056405), *hyh* (WiscDsLox253D10), *wag1* (SALK_002056) and *wag2* (SALK_070240) are in Col-0 ecotype background. The *AGC3-4::AGC3-4-YFP agc3-4-1*, *35S::AGC3-4* and *35S::AGC3-4 hy5* (van Gelderen, 2017) and *HY5::HY5-GFP hy5* (Chen et al., 2016) lines were described previously. *phyA*, *phyB* and *phyE* seeds were kindly provided by Remko Offringa. *cry1*, *cry2* and *cry1 cry2* seeds were kindly provided by Chentao Lin. For the multiple order mutant lines, simple or double lines were crossed. The primers used for genotyping can be found in Supplementary Table 1. Seeds were sterilized, germinated, and grown as described in Miotto et al. (2019). The plates

were kept vertically and grown under white light (21 °C ± 3 °C, 16 h- photoperiod). Primary root length was measured with ImageJ (Fiji) and plotted into graphs in GraphPad Prism 6.

RNA isolation and RT-qPCR

For Figure 2A, 3C and 3D RNA extraction and RT-qPCR were performed as described in Miotto et al. (2019). For Figure 3F total RNA was isolated using TRIzol™ Reagent (Thermo Fischer, #15596026). The cDNA was synthesized using M-MLV reverse transcriptase (Promega, # M5313) following the manufacturer's instructions. qRT-PCR was performed in a StepOne™ Real-Time PCR System using Platinum Taq DNA Polymerase (Thermo Fischer, # 10966) according to the manufacturer's protocol. All RT-qPCR values represent three biological replicates, each containing at least two technical replicates. Primer sequences used can be found in the Supplementary Table 1.

GUS staining and microscopy analysis

Fresh seedlings and plant organs were washed two time with washing-buffer [10 mM EDTA, 100 mM sodium phosphate (pH 7.0), 0.1% (v/v) Triton X-100, 2 mM $K_3Fe(CN)_6$] under vacuum for 10 min. After, seedlings were transferred into GUS staining buffer [10 mM EDTA, 100 mM sodium phosphate (pH 7.0), 0.1% (v/v) Triton X-100, 1 mM $K_3Fe(CN)_6$, 1 mM $K_4Fe(CN)_6$, 1 mg/ml 5-bromo-4-chloro-3-indolyl-D-glucuronide] under vacuum for 30 min and incubated at 37 ℃ for overnight. Subsequently, samples were cleared in 70% (v/v) ethanol at room temperature before imaging with Leica DM-R.

Confocal Imaging and Quantification

Confocal imaging and quantification were performed as described Chapter 3.

Molecular cloning and plant transformation

All oligonucleotides used for cloning procedures are described in Supplementary Table 1. For construction of the pToBRb7 line driven the amiRCOP1 constructs the pINDEX4 based destination vector was used (Ouwerkerk et al., 2001). The promoter region of TobRb7 were amplified from *Nicotiana tabacum* genomic DNA using specific primers that can be find in the Supplementary Table 1 (An et al., 2004). The WMD3-Web app (http://wmd3.weigelworld.org/cgi-bin/webapp.cgi) was used to design the artificial

amiRNA sequence. The plasmid pRS300 was used as a template for cloning the amiRCOP1 (Schwab et al., 2006). Bacteria were grown on LB medium (Sigma, L3022) supplemented with 50 mg/L kanamycin for *E. coli* strain Top10 or Omnimax. The presence of the insert and the sequence was confirmed by sequencing. Binary plasmids were introduced into *Agrobacterium tumefaciens* strain Eha105 by electroporation and selection on LB medium containing 100 mg/L rifampicin and 50 mg/L kanamycin. Arabidopsis miRCOP1 transgenic lines were obtained by Agrobacterium-mediated transformation of the Col-0 ecotype allele by floral dipping (Zhang et al., 2006) using Agrobacterium strain Eha105 containing the pTobRb7-miRCOP1. T1 generation were selected on plates with carbenicillin (100 mg/L) and hygromycin (50 mg/L), T2 were selected on plates with 25 mg/L hygromycin.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, Inc., CA, US). Data were tested for normal distribution by Shapiro-Wilk test and then applied the respectively statistic test and when significant ($p \leq 0.05$) were showed in the graphs. Statistical details of each experiment (test used, replicates, etc) can be found in the Results section and Figure-Figure Legend sections.

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Supplementary data

Supplementary tables are available at:

https://drive.google.com/drive/folders/1jeLQ04CZDnFZuyD0jejfAG1SOheYoDgu?usp=sharing

- Considerações finais -

Embora pouco documentada, a percepção de luz pelos tecidos aéreos é necessária para o desenvolvimento do sistema radicular abaixo do solo. Ao longo do processo evolutivo, as plantas desenvolveram mecanismos complexos para proteção de eventuais condições adversas impostas pelo meio ambiente. Assim, estes organismos contam com vias complexas de sinalização entre órgãos distantes, os quais são responsáveis por otimizar o uso dos recursos disponíveis para crescimento e desenvolvimento. O desenvolvimento do sistema radicular é um exemplo de processo desencadeado por um sinal de longa distância.

Os resultados descritos no Capítulo II e III desta tese relacionam o efeito da iluminação da parte aérea na indução e/ou repressão de genes em raízes protegidas da luz. Estes resultados confirmam dados de outros grupos (Lee et al., 2016; Silva-Navas et al., 2016; Zhang et al., 2019) de que a presença de luz na parte aérea é capaz de desencadear mudanças significativas no trascritoma de raízes, assim como alterar diversas rotas metabólicas. Visando melhor aproveitar o expressivo número de genes diferencialmente expressos nas raízes, optamos pelo uso integrado de diferentes metodologias na análise dos genes diferencialmente expressos. O uso conjunto de duas metodologias com abordagem distinta possibilitou a identificação de dois conjuntos de genes com baixa sobreposição entre eles. Embora as estratégias sigam princípios diferentes na escolha de genes diferencialmente expressos, a sobreposição de um pequeno conjunto de genes entre ambas gerou um repositório robusto de genes possivelmente envolvidos no crescimento de raízes protegidas da luz. Devido ao expressivo número de genes obtidos, os dados não foram totalmente explorados. A combinação dos dados obtidos por esse sequenciamento com dados de bancos de dados públicos, pode refinar ainda mais esse conjunto de genes, possibilitando uma escolha mais assertiva dos genes responsáveis pela transmissão do sinal recebido da parte aérea.

Mostramos no Capítulo III que as raízes são capazes de responder a variações não só de presença e ausência de luz, mas também da intensidade de luz à que a parte aérea é exposta. Os genes alvos de HY5 potencialmente estão envolvidos na estabilização do transporte de auxina ou ainda no crescimento das raízes em resposta à luz. Uma grande quantidade de dados de genes alvos de HY5 é conhecida (Lee et al., 2007b; Zhang et al., 2011). Os previamente descritos alvos de HY5, juntamente com genes responsivos à auxina

foram sobrepostos aos genes diferencialmente expressos obtidos em nossas análises, possibilitando a definição de conjuntos de genes responsivos à luz, auxina e HY5. Sabe-se que além das respostas fotomorfogênicas, HY5 faz parte de rotas de transdução de sinal de escape a sombra (van Gelderen et al., 2018a). De modo geral, estudos envolvendo Arabidopsis são realizados em meio de cultura sob condições de luz, intensidade, duração e qualidade, controladas. O conjunto de informações obtidas no Capítulo III, de que intensidade luminosas maiores levam à repressão do crescimento das raízes, vai de encontro com o discutido anteriormente, que a presença de luz é essencial para a dissipação de sinais que promovem o desenvolvimento da raiz mesmo esta estando na escuridão.

Açúcares fotossintetizados e o fitohormônio auxina eram os principais candidatos a sinais de longa distância disparados pela parte aérea iluminada para as raízes, abaixo do solo. O papel de ambos como mensageiros foi avaliado no Capítulo III desta tese. Nossa análise mostra que embora a presença de açúcar seja capaz de induzir o crescimento de raízes mantidas no escuro, à luz tem papel principal neste processo, em contrapartida ao que foi descrito previamente (Kircher and Schopfer, 2012). Mostramos também que a auxina produzida nos tecido aéreos é capaz de induzir o desenvolvimento de raízes mantidas no escuro, entretanto, ensaios químicos onde o transporte polar de auxina foi bloqueado localmente, não geraram evidências suficientes para que este hormônio seja considerado o sinal inicial necessário para desencadear a resposta fotomorfogênica nas raízes. Parte deste efeito pode estar relacionado à inibição sistêmica do transporte polar de auxina causado por esses inibidores, que mesmo aplicados de forma pontual, espalham-se rapidamente gerando efeitos sistêmicos difíceis de interpretar. Estudo recentes (Chen et al., 2016) sugerem que o principal indutor da fotomorfogênese, HY5, após estabilizado na parte aérea, é capaz de migrar da parte aérea para as raízes via floema, onde induz sua própria expressão e de genes promotores da captação de nitrogênio. Embora sua mobilidade tenha sido demostrada de forma muito elegante por Chen e colaboradores (2016), outros trabalhos sugerem a possibilidade de uma indução local e autônoma desse fator de transcrição nas raízes (Lee et al., 2016; Zhang et al., 2017). Estas observações permitem a especulação da existência de sinais adicionais, ainda não identificados. Novas análises envolvendo bancos de dados de proteínas, metabólitos e RNAs móveis podem lançar novos candidatos a atuarem nesta via de sinalização.

A disponibilidade de mutantes de perda-de-função permite avaliar geneticamente o papel de genes de interesse. Baseado no Capítulo III, definimos um conjunto de genes candidatos a desempenhar um papel chave na manutenção das respostas fotomorfogênicas em raízes crescidas no escuro. Dentre os genes diferencialmente expressos, os genes codificadores das enzimas da rota de biossíntese de flavonoides foram altamente induzidos em resposta à luz. A avaliação fenotípica de mutantes de perda de função para esses genes juntamente com análises de complementação química, geraram um conjunto de evidências do papel desses metabólitos do desenvolvimento de raízes. A interação entre flavonoides e auxina é conhecida de longa data (Murphy et al., 2000; Peer and Murphy, 2007). Nossos resultados sugerem que o precursor naringenina desempenha um papel chave no desenvolvimento do sistema radicular, reprimindo o crescimento da raiz principal e induzindo a formação de raízes laterais. Observamos ainda que a ausência de flavonoides endógenos assim como a presença de homólogos sintéticos levam a mudanças na homeostase de auxina. Esse conjunto de informações nos leva a sugerir que os flavonoides podem estar atuando como bloqueadores localizados do transporte polar de auxina na raiz, separando as diversas fontes de auxina no início do desenvolvimento radicular e orquestrando a distribuição dos gradientes de auxina. Assim, os fenótipos contrastantes observados em raízes em resposta à presença de luz na parte aérea podem ser resultantes da interação entre auxina e flavonoides.

HY5 é considerado um integrador das vias de sinalização hormonais e luminosas. O conjunto de observações que postula HY5 como um sinalizador master nas respostas à luz em raízes foi obtido, em sua grande maioria, em trabalhos onde as raízes, assim como as partes aéreas, estavam iluminadas. A exposição das raízes à luz acaba por gerar respostas incongruentes com as observadas em condições mais similares às ambientais. A degradação deste fator de transcrição, assim como seu homologo próximo HYH, é relativamente bem estabelecida na parte aérea. Recentemente, o grupo de pesquisa do nosso colaborador concomitante ao grupo de Lin e colaboradores (2017) sugeriu um mecanismo adicional de repressão do complexo COP1-SPA dependente das quinases da família AGC3. PINOID e AGC3-4 são capazes de interagir e fosforilar COP1, desativando esse complexo e liberando as respostas mediadas por HY5/HYH. No capítulo V analisamos o efeito dessa interação no fenótipo de raízes crescidas protegidas da luz, assim como na estabilização da proteína HY5 e AGC3-4. Confirmamos a importância da funcionalidade da proteína AGC3-4 na

estabilização de HY5 nas raízes, entretanto os mecanismos adjacentes a essa sinalização não foram abordados. Plantas com menor expressão de COP1 nas raízes não apresentaram um alongamento da raiz principal. Em conjunto com os resultados apresentados no Capítulo III, esses dados reforçam a complexidade das respostas à luz e a importância da luz como um sinal permanente e constante para a indução dessa resposta. Diversas questões a respeito dos efeitos da desestabilização de COP1 permanecem não respondidas. Neste contexto, estamos analisando o proteoma de plantas amiRCOP1 e plantas não transformadas buscando identificar as proteínas alvo de degradação desse complexo em raízes crescidas no escuro. Esperamos que, com essa nova abordagem, possamos identificar genes chaves reprimidos na presença de COP1 que são necessários para o desenvolvimento das raízes.

Baseado nos dados apresentados nesta tese fomos capazes de corroborar alguns aspectos da nossa hipótese inicial, assim como inserir novas possibilidades nesse modelo de desenvolvimento (Figura 2). Diversas questões a respeito dos mecanismos que coordenam o desenvolvimento do sistema radicular permanecem não respondidas. Qual o ou quais os sinais enviados pela parte aérea que desencadeiam o crescimento das raízes mantidas na escuridão? Qual a importância fisiológica da interação entre COP1 e as quinases AGC3 nas raízes? Como essa interação está relacionada à estabilidade de HY5 e o desenvolvimento das raízes? Como o módulo AGC3-COP1-HY5 impacta na manutenção do transporte polar de auxina e no desenvolvimento do sistema radicular? Quais vias de sinalização são afetadas por diferentes intensidades luminosas nas raízes? Estas são algumas das perguntas que gostaríamos de responder.

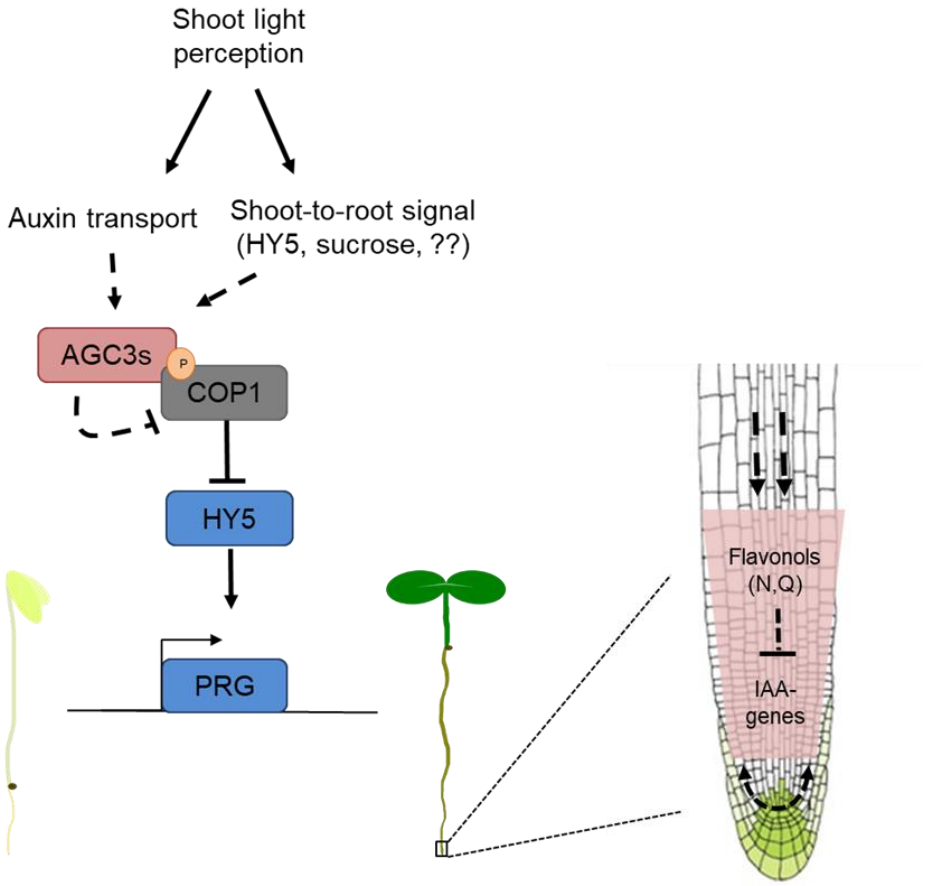

Figura 2. Modelo de crescimento das raízes em resposta à luz. A percepção de luz nos tecidos aéreos desencadeia a emissão de sinais de longa distância ainda não totalmente identificados que ao serem percebidos nas raízes induzem a interação entre quinases da família AGC3 e o complexo COP1-SPA. A desestabilização do complexo COP1, permite o acúmulo dos fatores de transcrição promotores da fotomorfogênese HY5 e HYH, responsáveis pela indução de genes responsivos à luz, os quais são importantes para a manutenção do transporte de auxina dos tecidos aéreos. A estabilização de PIN1 na membrana preserva o transporte de auxina oriundo da parte aérea. Concomitantemente, a indução da biossíntese de flavonoides leva ao acúmulo desses metabólitos na zona meristemática das raízes, que por sua vez atuam controlando a re-distribuição da auxina presente nesta região. O rearranjo definido pela presença de flavonoides determina o investimento em crescimento da raiz principal ou a emissão de raízes laterais, contribuindo com a estrutura do sistema radicular.

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