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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)

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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).

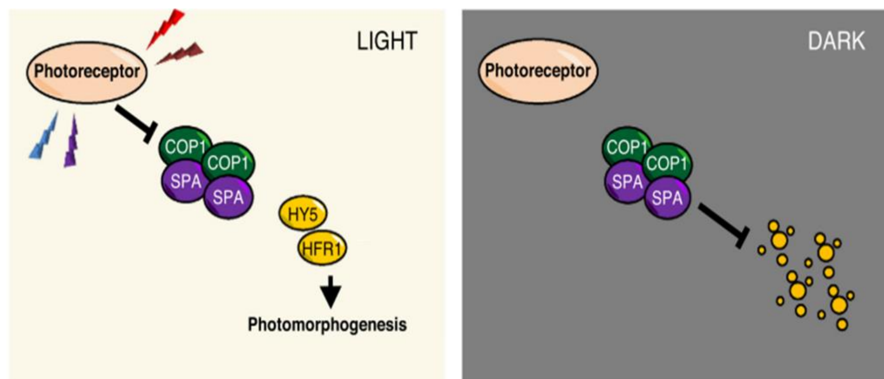

Figura 1. Regulação das respostas fotomorfogênicas mediadas pelo módulo COP1-HY5 (Modificado de Podolec and Ulm, 2018).

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).

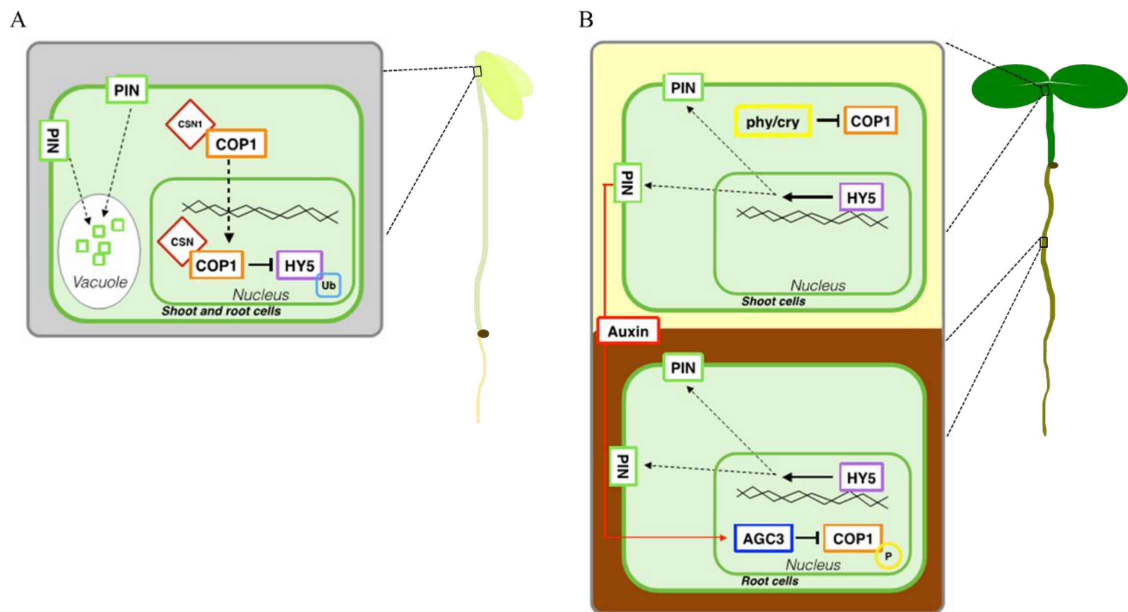

Figura 1. Modelo de crescimento das raízes em resposta à luz. (A) No escuro, o complexo COP1 marca para degradação as proteínas promotoras da fotomorfogênese HY5/HYH impedindo a expressão de genes responsivos à luz. Em resposta a ausência de luz, transportadores de auxina são direcionados para o vacúolo 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.

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.

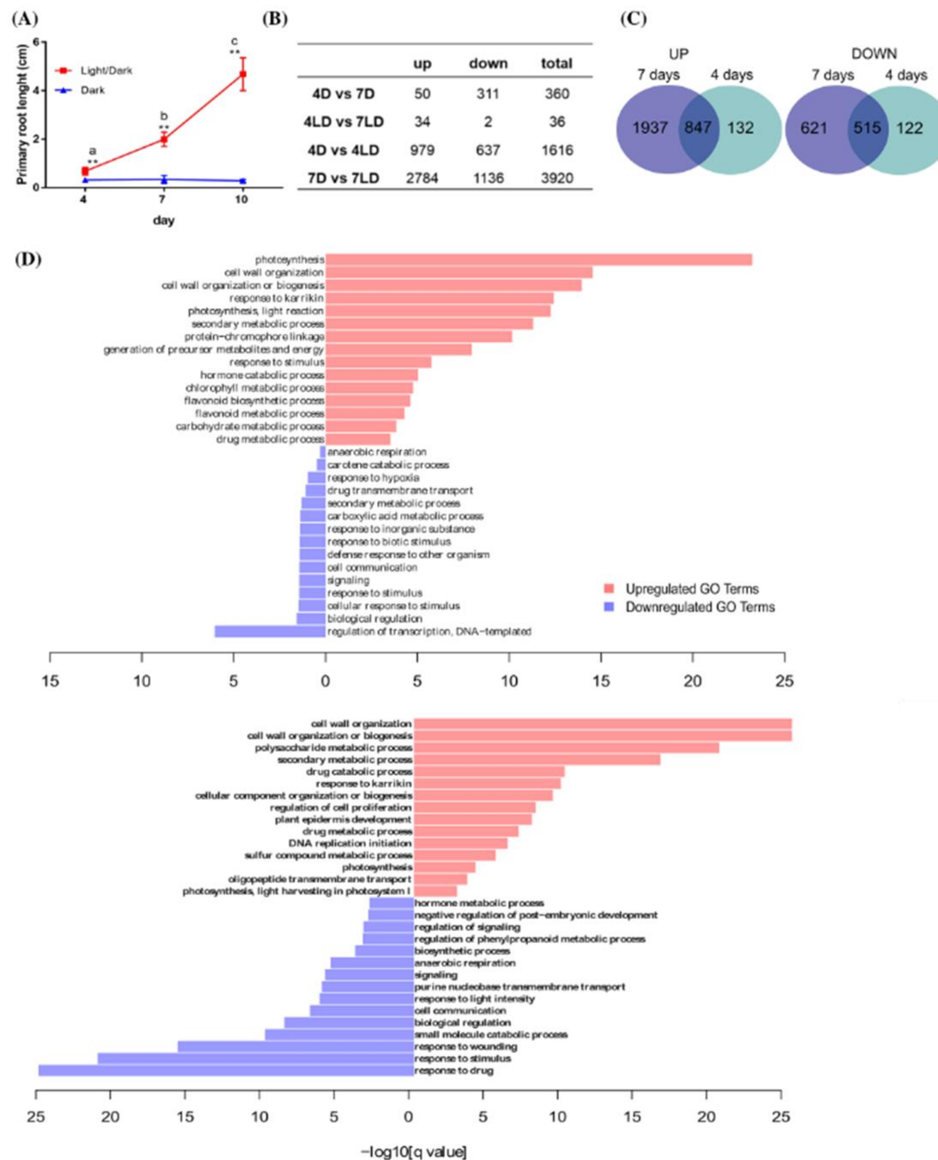

Figure 1 Shoot-illumination affects growth and gene expression in dark-grown roots. (A) Primary root length of wild-type Arabidopsis seedlings grown in LD and D at 4, 7 and 10 days after germination (n≥ 10). Statistical significance was determined by Student's t-test (* p≤ 0.05; ** p<0.01). Different 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 ≥ 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).

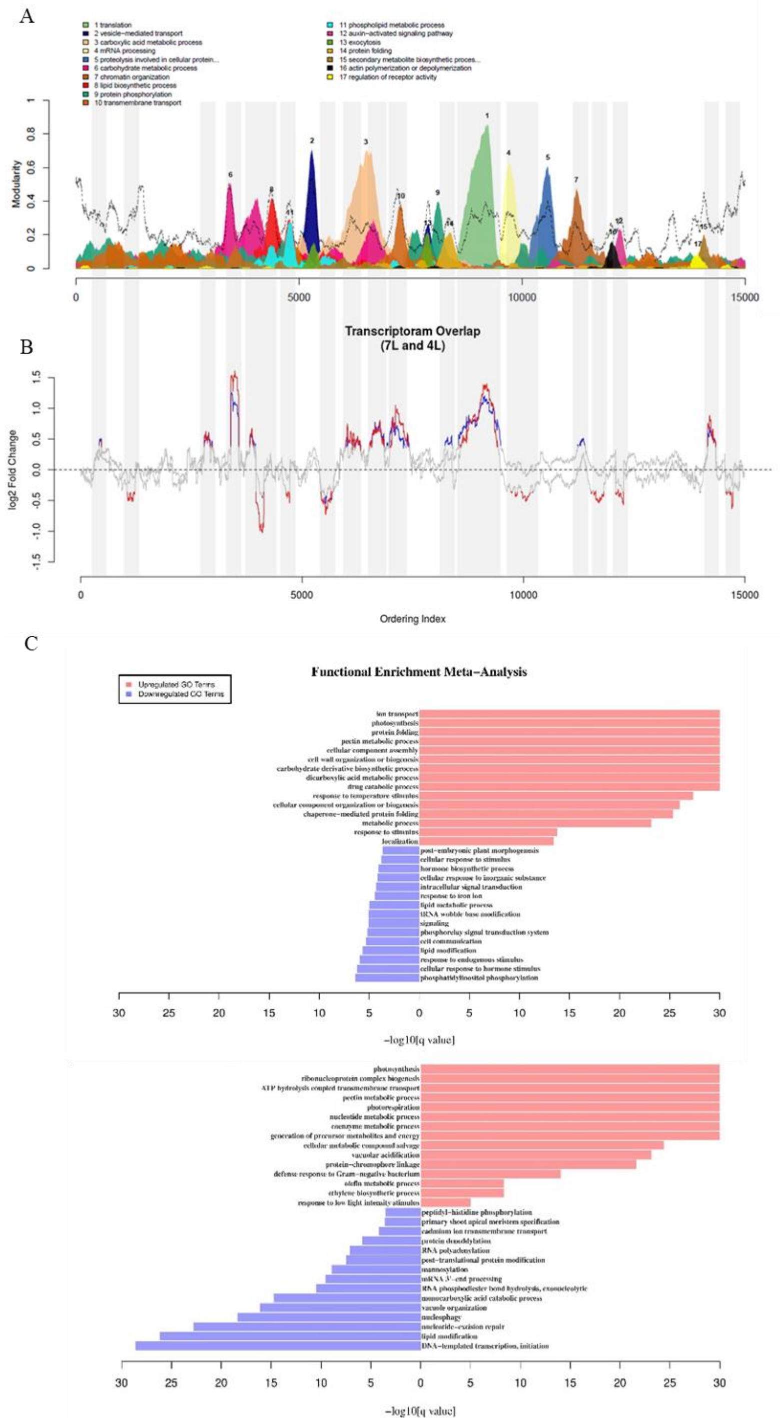

Figure 2 *Arabidopsis thaliana* transcriptogram. (A) The x-axis relative to gene position have been divided by the total number of proteins retrieved from STRING. Projection of Gene Ontology terms is color-coded. (B) Average transcriptograms for r=90, light x dark significant peaks (up-regulation) or 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).

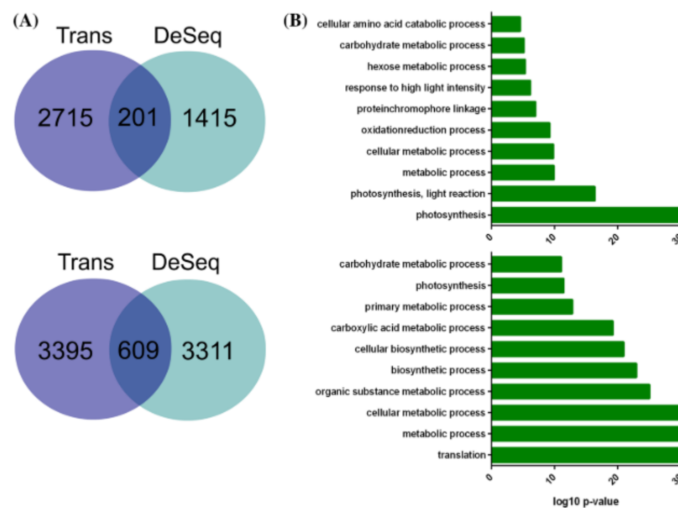

Figure 3 (A) Venn diagram of DEG from DeSeq and transcriptomic analysis (Trans) from 4 and 7 DAG of light modulated genes. (B) Summary of the functional enrichment analysis of the genes present in the overlapping of A, 4 days-old (top panel) and 7 days-old (bottom panel).

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.

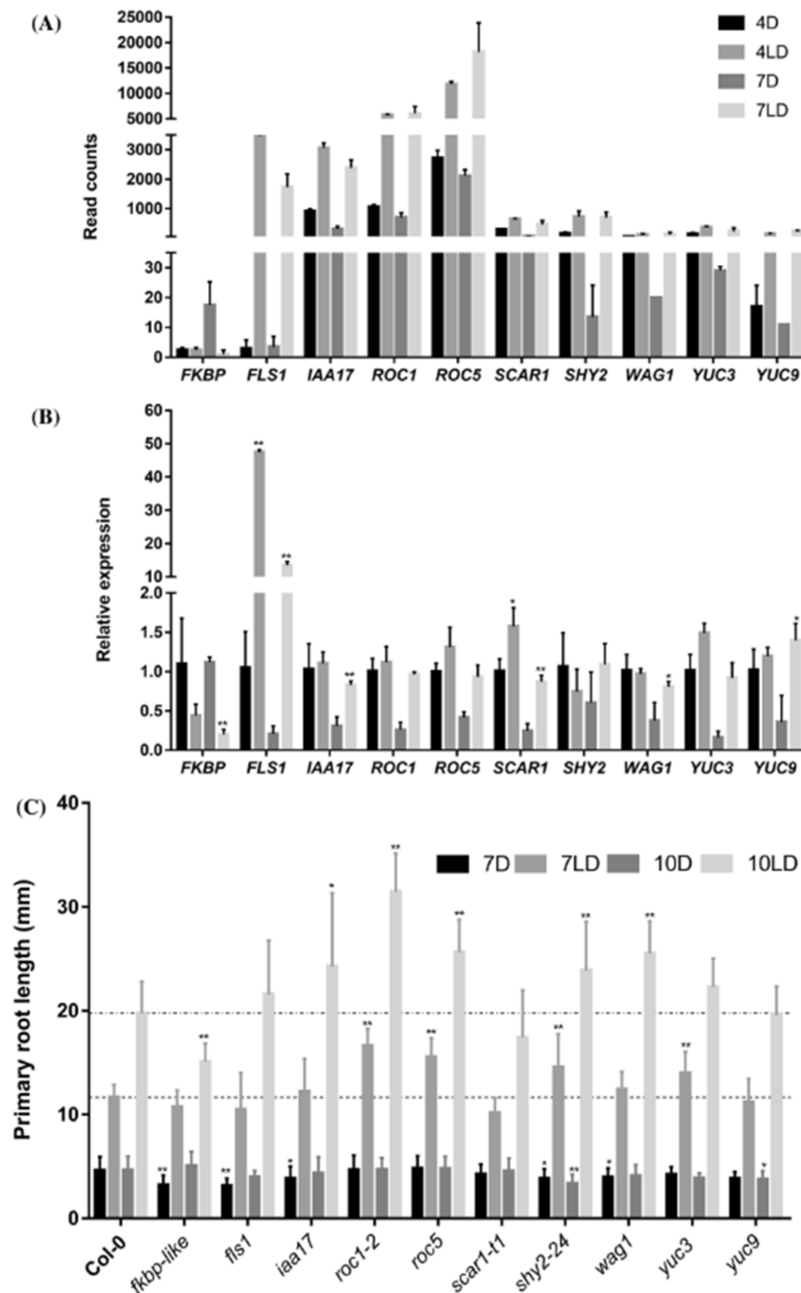

Figure 4 Expression patterns of ten root-related genes in Arabidopsis dark-grown roots. (A) (B) Number of read counts obtained in the RNA-seq for the selected genes.Validation of the transcriptomic analysis by qRT-PCR analysis of gene expression in roots. Gene expression was normalized using an internal 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

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

T_i is the mean expression of the genes encoding for the proteins belonging to the ith 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 $\mu mol\ m^{-2}\ s^{-1}$) 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.

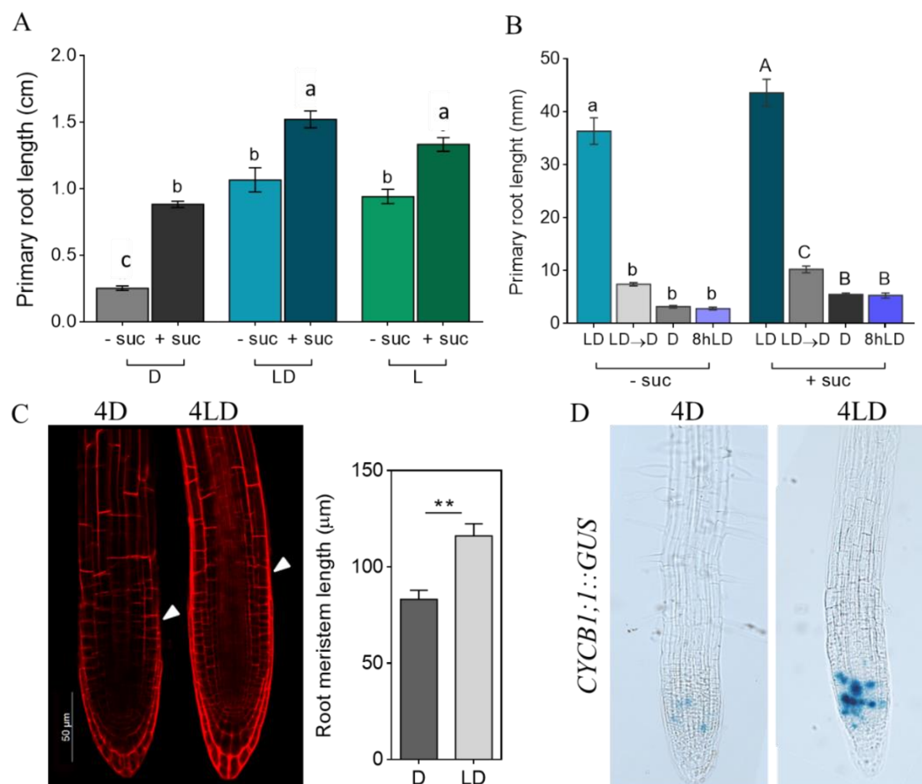

Figure 1. Sucrose and light act together in root development. (A) Primary root length of 7 DAG Col-0 seedlings grown without or with 1% sucrose in D, LD or L light conditions (n≥19). (B) Primary root length of 10 DAG Col-0. Wild-type seedlings were grown with or without sucrose for 4 days in LD and transferred 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.

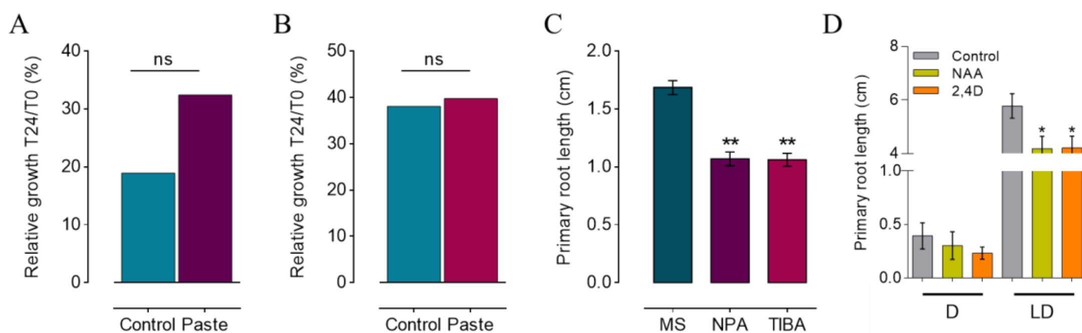

Figure 2. The effect of auxin transport-inhibitors and synthetic auxin on Arabidopsis primary root growth. (A) 10µM NPA and (B) 10µM TIBA were applied in the shoot-to-root junction in 6 DAG seedlings grown in LD. Primary root lengths were measured before the treatment (T0) and 24h after the application (T24). The data were analyzed by Student's t test for each sample compared T24 with T0 (* $p \leq 0.05$; ** $p \leq 0.01$). (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 (** $p \leq 0.001$). Error bars indicate SE ($n \geq 15$).

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.

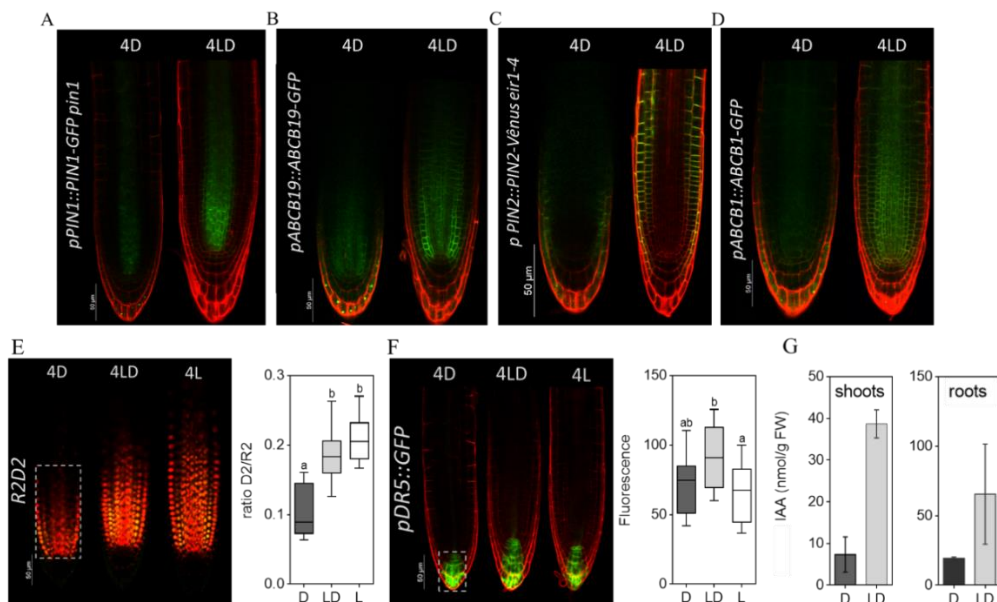

Figure 3. Light regulates the cellular localization of auxin transporters and auxin maxima in roots. (A) PIN1-GFP, (B) ABCB19-GFP, (C) PIN2-GFP and (D) ABCB1-GFP signals in 4-day-old seedlings grown on LD and D. (E) Merged confocal images of R2 (red) and D2 (green) expressing R2D2 marker and quantification 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 ($n \geq 9$). (G) IAA levels in shoots and roots of 4 DAG seedlings ($n \geq 2$). Letters denote different significant classes ($p \leq 0.05$) 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.

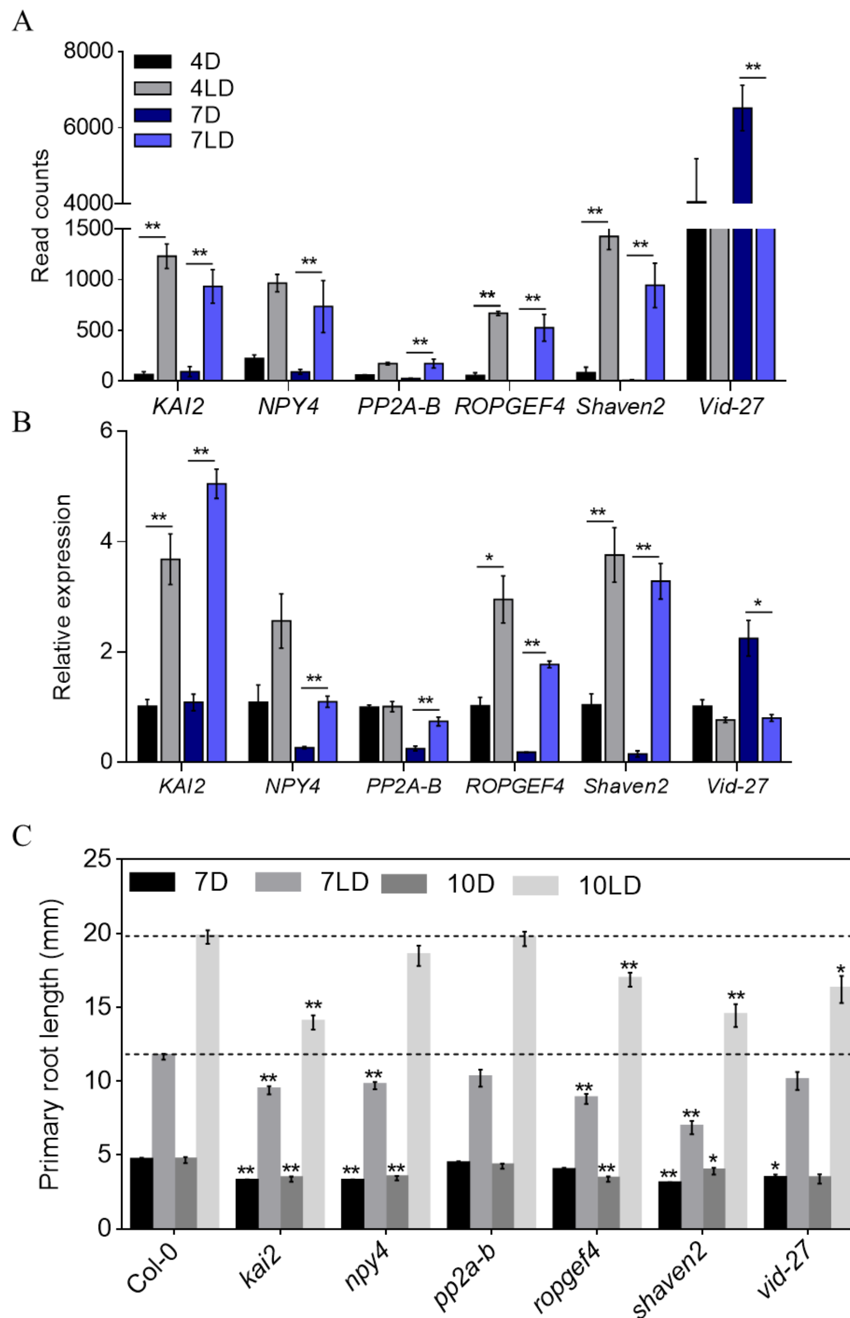

Figure 4. Overlapped significantly genes in the RNA-seq and RT-qPCR. (A) Number of read counts of the selected genes. Asterisks denote differentially expressed genes with p-value ≤ 0.05 (padj) and a ≥ 2-fold change. (B) RT-qPCR analysis of gene expression in roots. Gene expression was normalized using 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.

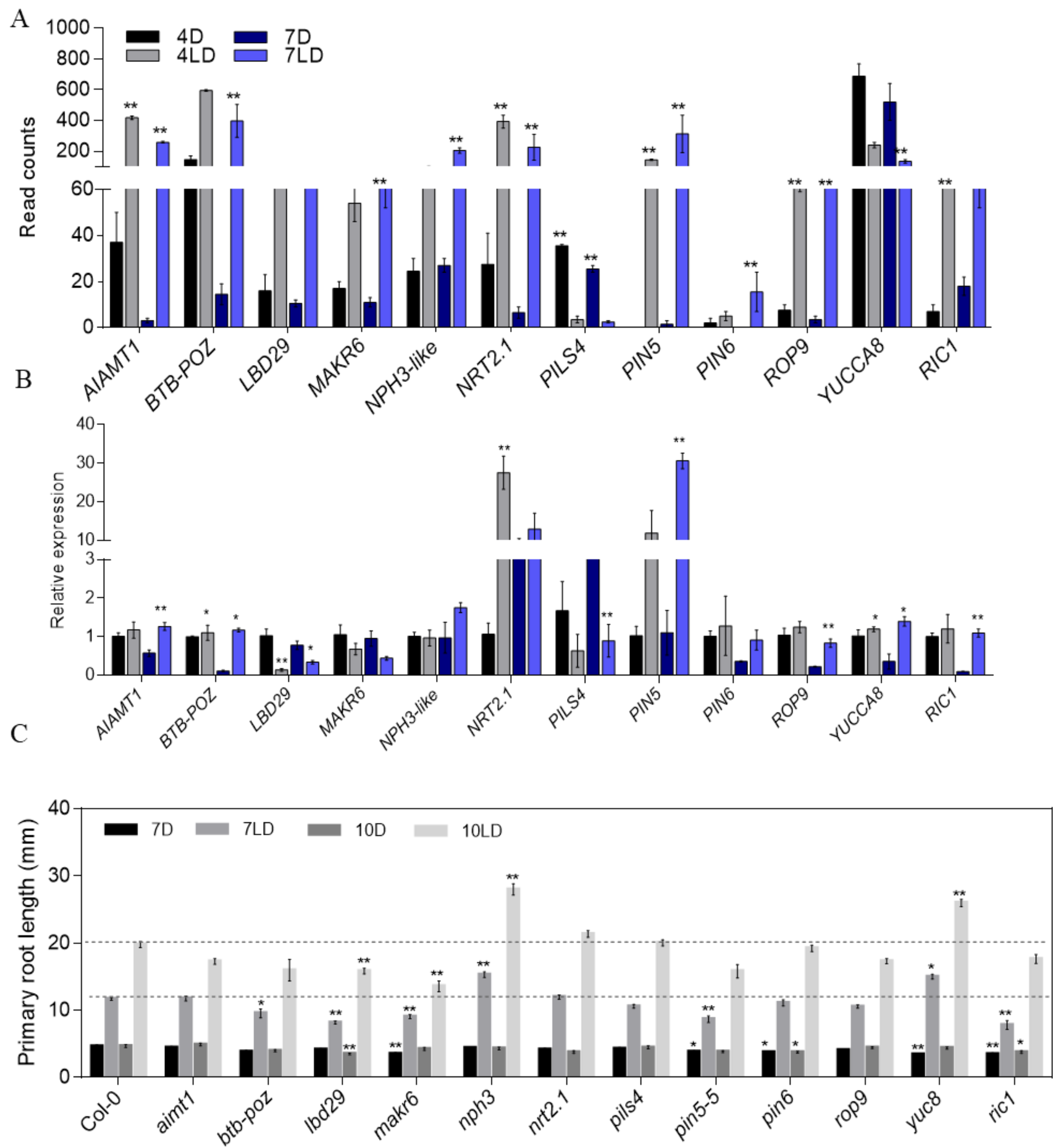

Figure 5. Candidate genes expression and primary root phenotype. (A) Number of read counts of the selected genes. Asterisks denote differentially expressed genes with p-value ≤ 0.05 (padj) and a ≥ 2-fold change. (B) RT-qPCR analysis of gene expression in roots. Gene expression was normalized using an 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.

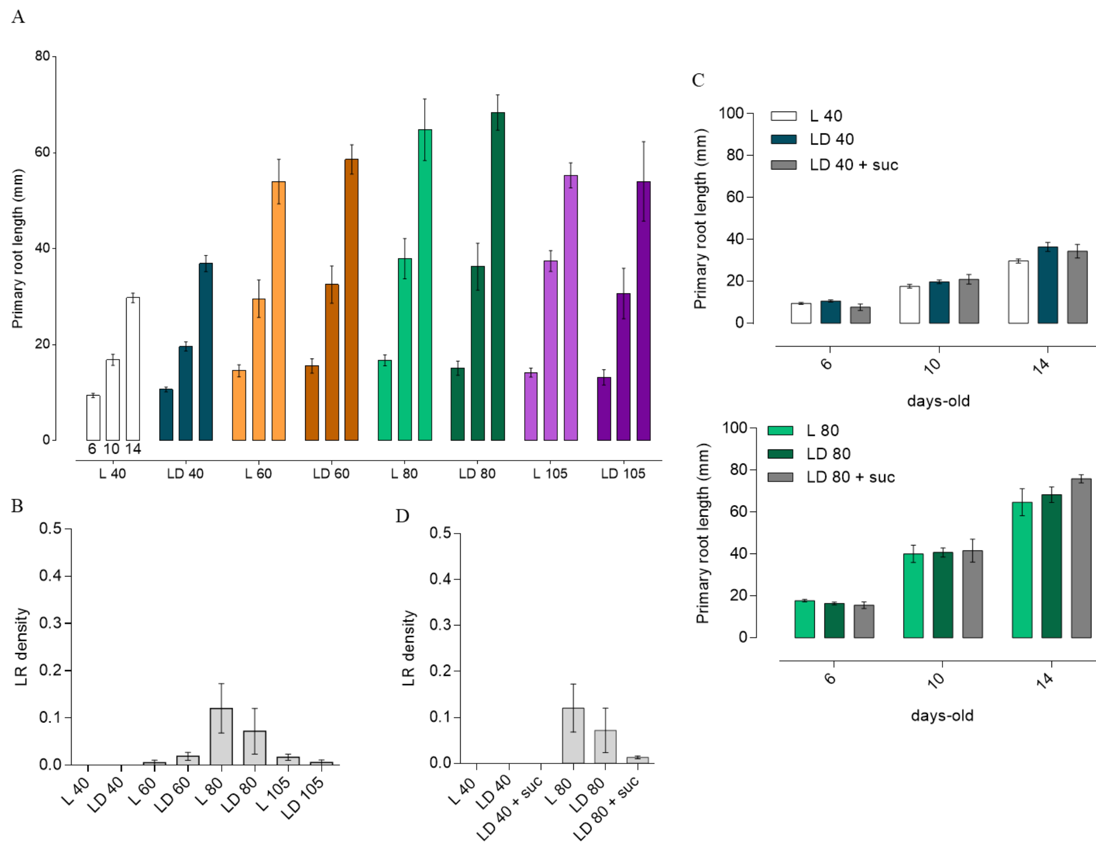

Figure 6. Light intensity dependent root growth in wild-type seedlings. (A) Primary root length of wild-type seedlings grown in L and LD at 6, 10 and 14 DAG (n≥5) under four different light intensities of 40, 60, 80 and 105 µmol m^{-2} s^{-1}. (B) Lateral root density of A seedlings. (C) Primary root 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.

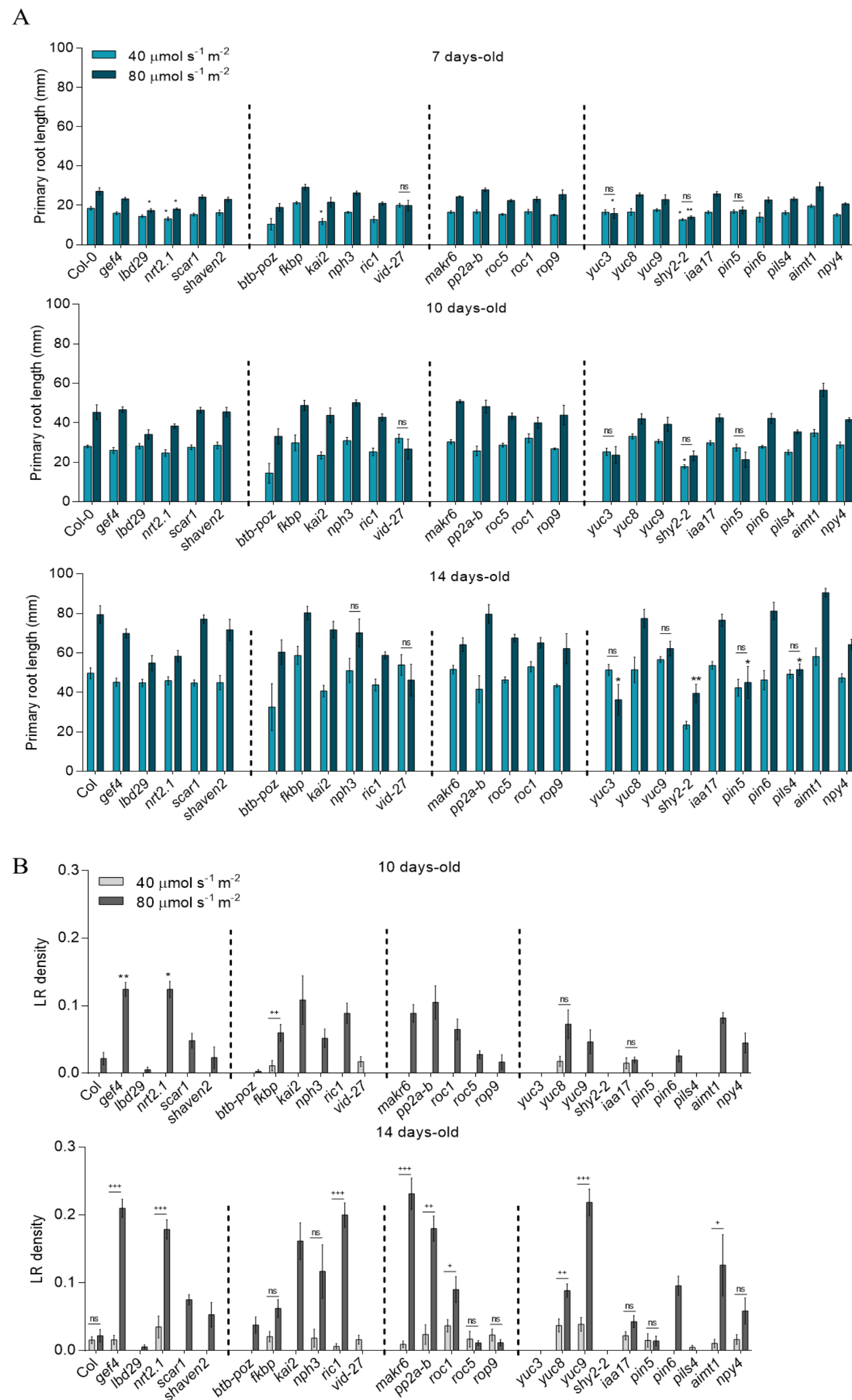

Figure 7. Dark-grown root of candidate genes under different light intensities in the shoots. (A) Primary root length of single T-DNA insertion lines (n≥ 10). Root measurement are done in 7, 10 and 14 days after germination. (B) Lateral root density of A seedlings. Error bars indicate SE. Asterisks denote 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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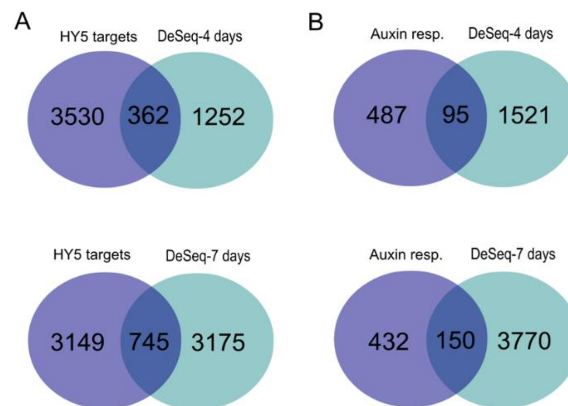
Supplementary data

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).

- 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.

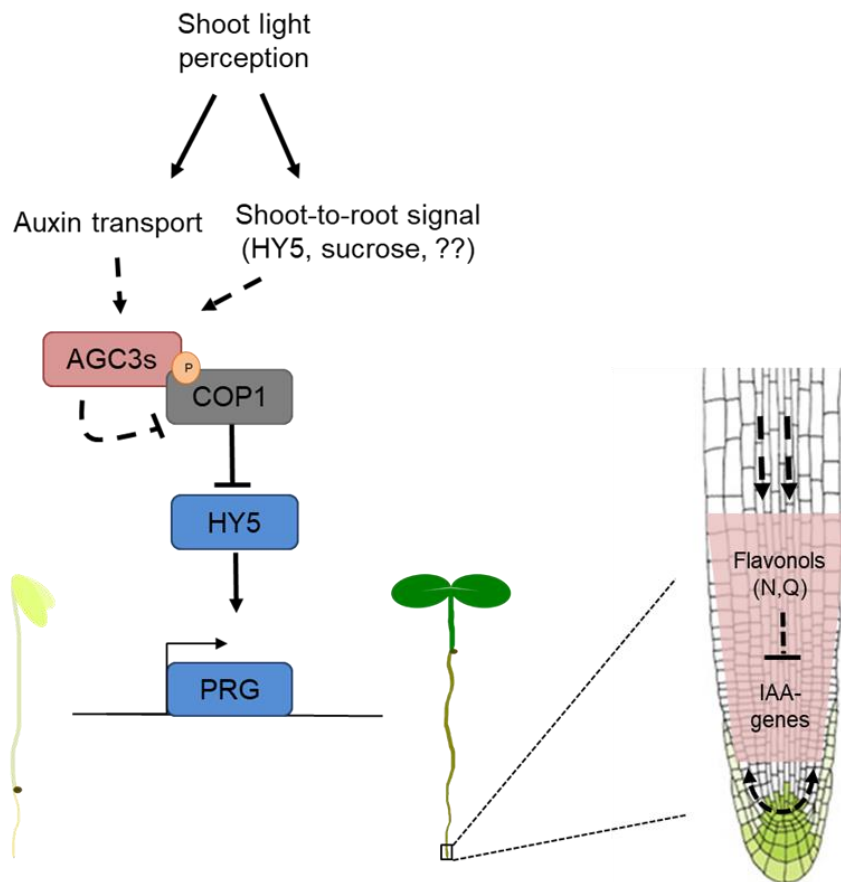


Figura 2. Modelo de crescimento das raízes em resposta à luz. A percepção de luz nos tecidos aéreos desencadeia a emissão de sinais de longa distância ainda não totalmente identificados que ao serem percebidos nas raízes induzem a interação entre quinases da família AGC3 e o complexo COP1-SPA. A desestabilização 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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