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AVALIAÇÃO DO PAPEL DA SÍNTESE DE FLAVONÓIDES NO DESENVOLVIMENTO FOTOMORFOGÊNICO DE RAÍZES EM *ARABIDOPSIS THALIANA*

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“Enquanto eu estiver vivo, as chances são infinitas”

-Monkey D. Luffy

Índice

Instituições e fontes financiadoras

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Resumo

A percepção de luz pela parte aérea da planta ativa o desenvolvimento das raízes abaixo do solo. Genes da via de biossíntese dos flavonóides têm sua expressão induzida nas raízes pela iluminação da parte aérea. Trabalhos anteriores em *Arabidopsis thaliana* já sugeriram várias potenciais moléculas envolvidas na sinalização de luz durante o desenvolvimento das raízes, como açúcares, auxina e o fator de transcrição ELONGATED HYPOCOTYL5 (HY5). HY5 é um regulador positivo da fotomorfogênese, controlando a transcrição de genes responsivos a luz, o que inclui os genes da via de biossíntese de flavonóides, os quais têm um papel importante na inibição do transporte polar de auxina. Portanto, HY5 funciona comunicando às raízes sob a situação luminosa percebida pela parte aérea e induzindo a biossíntese de flavonóides nas raízes, aumentando a expressão dos genes de biossíntese. Além disso, sabe-se que plantas cultivadas na sombra produzem menos raízes laterais (LR) e essa resposta é regulada por HY5, que reprime indiretamente dois transportadores de auxina (PIN3 e LAX3). Assim, o nosso objetivo é avaliar se a formação de LR depende do acúmulo de flavonóides promovido por HY5. Nossos resultados indicam que HY5 é necessário para a correta expressão da síntese dos flavonóides nas raízes e que a via metabólica deve estar íntegra para o efeito de HY5 nas LR ser expresso. Além disso, o intermediário naringenina (ou seus derivados), parece ter um papel positivo na formação de LR, atuando *downstream* a HY5. Além disso, os cruzamentos com mutantes dos transportadores de auxina revelaram que PIN3 e LAX3 são importantes para a formação de LR, atuando *downstream* aos flavonóides. Também identificamos uma rede molecular através de UHPLC-HRMS, para avaliar o efeito metabólico da naringenina. Essa rede indica que o perfil completo de flavonóides deve ser importante para o desenvolvimento da raiz e as alterações observadas especialmente no mutante *tt6-3* podem resultar de um desvio metabólico da via dos flavonóides, longe da subclasse dos flavonóis. Além disso, também avaliamos o possível papel dos flavonóis no zoneamento do meristema radicular. Sabe-se que esses compostos contribuem para o efeito de citocinina (CK) na zona de transição das raízes (zona que divide a zona meristemática da zona de alongamento), que é essencial para estabelecer um mínimo de auxina importante para o tamanho do meristema radicular. Observamos um acúmulo de flavonóis no meristema em resposta à CK que possivelmente afetam o tamanho do meristema. Em conclusão, nossos resultados apontam um papel cooperativo da luz, auxina, CK e flavonóis na organização do meristema radicular e indução do crescimento da raiz dependente de luz.

Abstract

Light perception by shoots activates the primary root development underground. Genes from the flavonol biosynthesis pathway have their expression in roots increased by shoot illumination. Previous works in Arabidopsis thaliana have shown several potential light signalling components involved in root development, such as sugars, auxin and the transcription factor ELONGATED HYPOCOTYL5 (HY5). HY5 is a positive regulator of photomorphogenesis, controlling the transcription of several light regulated genes, including many belonging to the flavonol biosynthesis pathway. These metabolites have an important role inhibiting polar auxin transport. Ultimately, HY5 functions by communicating roots of the light signal perceived by shoots. Additionally, it's known that plants cultivated in shade produce fewer lateral roots and this response is regulated by HY5, which negatively regulates two auxin transporters (PIN3 and LAX3). Hence, our goal is to evaluate whether lateral root formation depends on flavonols accumulation promoted by HY5. Our results indicate that HY5 is necessary for the correct induction of flavonols in roots and the pathway must be intact for the HY5 effect on lateral roots. Also, naringenin, or its derivatives, seem to have a role in lateral root formation, acting downstream to HY5. Besides that, the crosses with the auxin transporters mutants revealed that PIN3 and LAX3 are important for lateral root formation, probably acting downstream to flavonols. We also identified a molecular network using UHPLC-HRMS to assess the metabolic effect of naringenin. This network indicates that the complete flavonoid profile is likely important for root development, and the observed alterations, particularly in the tt6-3 mutant, may result from a metabolic diversion of the flavonoid pathway away from the flavonol subclass. Furthermore, we also evaluated the possible role of flavonols in the root meristem zoning as they might contribute to the effects of cytokinin (CK) in the transition zone of roots (zone that divides the meristematic zone from the elongation zone), which is essential for establishing an auxin minimum important for root meristem size. We observed flavonol accumulation in the root meristem of CK-treated wild type plants and the same pattern in some flavonol mutants, but without the CK treatment, suggesting an interaction of CK and flavonols. In conclusion, our results point to a cooperative role for light, auxin, CK and flavonols in the organisation and promotion of light-dependent root growth.

Key words: HY5, root development, primary root, auxin, transparent testa

Introdução

O mecanismo de percepção luminosa nas plantas e sua relação com o desenvolvimento

A luz é um sinal essencial para as plantas que afeta praticamente todo aspecto da sua fisiologia e desenvolvimento. Os organismos vegetais apresentam um sofisticado sistema para detectar e interpretar informação luminosa através da ação de múltiplos fotorreceptores, na qual podem perceber a luz em seus diversos comprimentos de onda e dessa forma se adaptarem à variações no ambiente (Leivar e Monte, 2014). Em função desse mecanismo de percepção luminosa, as plantas têm a capacidade de monitorar o ambiente para presença, intensidade, direção e época do ano, além de usar a luz como fonte de energia (De Wit, M et al., 2016), essas características conferem uma alta plasticidade que permite um ajuste fino para otimizar o desenvolvimento de acordo com a condição ambiental (Quail, P et al., 2002; Lau e Deng, 2012).

Na ausência de luz, plântulas de dicotiledôneas, como *Arabidopsis thaliana* investem no alongamento da parte aérea em detrimento do desenvolvimento dos cotilédones e da raiz, resultando em um fenótipo estiolado caracterizado por um alongamento excessivo do hipocótilo, cotilédones fechados, formação do gancho apical e uma raiz pequena (Xu, X et al., 2015). Esse processo de desenvolvimento é chamado de escotomorfogênese e depende inteiramente das reservas energéticas da semente. O alongamento exacerbado do hipocótilo é uma forma da plântula de maximizar o processo de busca por luz e a presença do gancho apical facilita a superação de obstáculos que possam estar impedindo o contato com a luz, protegendo o meristema apical caulinar. Em contrapartida, quando há luz suficiente, ocorre a ativação da fotomorfogênese que rapidamente inibe o crescimento do hipocótilo, expande os cotilédones que passam a se desenvolver, também inicia-se a produção e acúmulo de clorofila, ativando a fotossíntese. Além disso, o sistema radicular também começa a se desenvolver a fim de captar nutrientes para a plântula (Fig 1 - painel superior) (Arsovski, A et al., 2012).

A ativação da fotomorfogênese depende da atividade de diferentes conjuntos de fotorreceptores que servem como antenas nas células vegetais, absorvendo e interpretando todo o espectro luminoso. Até o momento, foram identificados cinco grupos de fotorreceptores em plantas, os fitocromos (PHYA-E em *Arabidopsis thaliana*) responsivos à luz vermelha e vermelho-extremo; os criptocromos (CRY1 e 2), fototropinas (PHOT1-2) e proteínas F-box (ZEITLUPE) para luz azul/UV-A; e UVR8 para luz UV-B (Paik, I. e Huq, E., 2019). Recentemente, evidências

sugerem a existência de um fotorreceptor para luz verde ainda não identificado (Hao, Y et al., 2023). Independente do conjunto de fotorreceptores envolvido, a luz provoca alterações conformacionais nestas proteínas que intercalam entre forma ativa e inativa (Fig 1 - painel inferior) (Möglich, A et al., 2010).

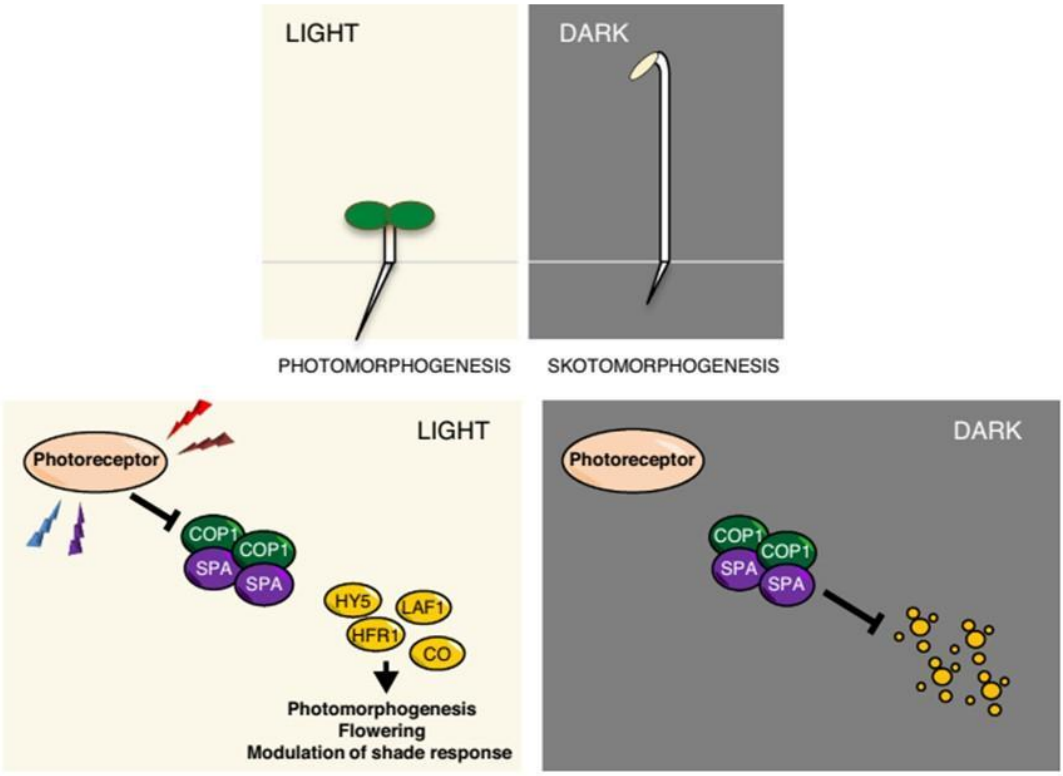

Figura 1. Regulação das respostas fotomorfogênicas é mediado pelo complexo protéico COP1-SPA. Painel superior mostra os fenótipos contrastantes de plantas cultivadas na luz contra plantas cultivadas na escuridão. Sob iluminação, plântulas apresentam raiz primária longa, cotilédones abertos e hipocótilo curto. Enquanto que na escuridão, os cotilédones permanecem fechados, a raiz não se desenvolve, o hipocótilo alonga e forma-se o gancho apical para proteger o meristema apical da parte aérea. Painel inferior demonstra o mecanismo de percepção de luz e posterior sinalização desencadeada pela luz. Fotorreceptores ativados por luz (light, à esquerda) desestabilizam o complexo COP1-SPA permitindo que proteínas indutoras da fotomorfogênese (HY5, LAF1, etc…) possam acumular e atuar no núcleo das células. (Modificado de Podolec and Ulm, 2018). Na escuridão (dark, à direita) o complexo COP1-SPA promove a degradação dos fatores fotomorfogênicos.

O papel de HY5 na regulação do desenvolvimento dependente de luz em Arabidopsis

A expressão diferencial de genes promovida pela influência da luz desencadeia respostas específicas no desenvolvimento vegetal, muitos dos quais são regulados por fatores de transcrição. Diversos fatores envolvidos na transição de desenvolvimento da escuridão para a fotomorfogênese já foram identificados em Arabidopsis (Chen e Rajewsky, 2007). Dentre eles, ELONGATED HYPOCOTYL 5 (HY5) possui um papel central na regulação do desenvolvimento dependente de luz (Oyama, T et al., 1997). HY5 é um fator de transcrição membro da família bZIP (basic leucine zipper) que foi identificado como um indutor da fotomorfogênese em função da insensibilidade à luz do mutante *hy5* (Koornneef, M et al., 1980, Ang e Deng, 1994). HY5 controla processos como proliferação celular, assimilação de nutrientes, alongamento celular e desenvolvimento do cloroplasto (Koornneef et al., 1980; Oyama, T et al., 1997; Ang, L et al., 1998; Jing, Y et al., 2013), ligando-se predominante aos promotores dos seus genes-alvo através de elementos regulatórios-cis, como G-box, motivos GAGA e GT1, resultando na ativação desses genes em resposta a luz (Tobin e Kehoe, 1994, Terzaghi e Cashmore, 1995, Millar e Kay, 1996, Chattopadhyay, S et al., 1998). Além de atuar na fotomorfogênese, HY5 ainda participa de outras rotas de sinalização relacionadas à fitormônios, defesa e biossíntese de metabólitos secundários (Gangappa e Botto, 2016). Trabalhos baseados em DAP-seq (*DNA affinity purification sequencing*) e ChIP-seq (*Chromatin immunoprecipitation sequencing*) que avaliaram potenciais sítios de ligação no genoma sugeriram que HY5 poderia influenciar cerca de até 3000 genes em Arabidopsis (Lee, J et al., 2007; Zhang, H et al., 2010; Kurihara, Y et al., 2014). Entretanto, dados recentes restringiram este número à comprovadamente somente cerca de 297 genes diretamente ativados por HY5 (Burko, Y et al., 2020). Mesmo assim, esse fator de transcrição é considerado uma proteína central na coordenação da sinalização da luz e expressão gênica, conectando diferentes processos.

A estabilidade e abundância da proteína HY5 é controlada em resposta à luz (Oyama, T et al., 1997). A percepção de luz e ativação de fotorreceptores como PHY, CRY e UVR8, induz um aumento dos níveis da proteína HY5 na célula, passando a induzir a expressão dos seus genes alvo. Um dos reguladores mais importantes que interagem com HY5 no escuro é CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), um repressor chave do desenvolvimento regulado por luz que marca HY5 para degradação proteolítica no escuro (Deng X et al, 2000).

Essa interação ocorre no núcleo celular e envolve o reconhecimento da porção N-terminal de HY5 por COP1. Na ausência de luz, COP1 se encontra no núcleo complexado com proteínas da família SUPPRESSOR OF PHYA-105 (SPAs) e HY5 é rapidamente degradado, assim como outros alvos de COP1 (Lau e Deng, 2012). Com a percepção de sinais luminosos, a atividade do complexo COP1/SPA é altamente inibida através da ação dos fotorreceptores PHYA, PHYB, CRY1, CRY2 e UVR8 que quando ativados por luz se associam com COP1-SPA competindo pelo sítio de ligação com os alvos de COP1/SPA (Ponnu, J et al., 2019; Kelvin, L et al., 2019). Esse mecanismo depende do motivo peptídico valina-prolina (VP), identificado em diversas proteínas que interagem com COP1/SPA, inclusive fotorreceptores. Portanto, a estabilidade de HY5 é regulada conforme a variação da luz, devido à relação com COP1, evidenciando um mecanismo de regulação dinâmico para modulação dos níveis do fator de transcrição dependente de luz e consequentemente um maior controle sobre alongamento da plântula (Fig 1 - painel inferior) (Gangappa e Botto, 2016).

Mutantes perda-de-função *hy5* geram fenótipos anormais em Arabidopsis, em que a planta apresenta características de estiolamento na presença de luz, como alongamento do hipocótilo, redução no acúmulo de clorofila nos cotilédones e baixo acúmulo de antocianinas (Holm, M et al., 2002). Além da parte aérea, as raízes dos mutantes também apresentam alterações morfológicas e limitações em responder a hormônios e estímulos externos (Vandenbussche et al., 2007). Uma característica fenotípica proeminente nesse mutante quando exposto à luz, é o elevado número de raízes laterais e a dinâmica de desenvolvimento destas, uma vez que apresentam taxa de crescimento acelerada e maior comprimento em comparação com o selvagem (Cluis, C et al., 2004) (Fig 2).

A proteína HY5 foi sugerida como um sinal móvel entre a parte aérea e a raiz, onde o acúmulo de HY5 na parte aérea induziria sua translocação para as raízes, onde HY5 promoveria o crescimento e a captação de nutrientes para contrabalançar a fixação de carbono fotossintético das folhas. Entretanto, trabalhos mais recentes, utilizando construções de expressão tecido-específica de uma proteína de fusão DOF-HY5 que é incapaz de ser translocada sistemicamente, mostraram que os fenótipos de crescimento do hipocótilo e da raiz primária do mutante *hy5* foram complementados por essas construções. Isso indica que HY5 age de forma indireta nesses órgãos e que a mobilidade da proteína entre a parte aérea e a raiz não é essencial para sua funcionalidade, sugerindo que um sinal à jusante, dependente de HY5, seria o

responsável pelo efeito (Burko, Y et al., 2020). O mesmo trabalho demonstrou que ao contrário do seu efeito na raiz primária, a expressão de HY5 localizada no LRP (*lateral root primordium*) é essencial para a complementação do mutante *hy5*. Isso sugere que HY5 tem um efeito direto no controle do desenvolvimento das raízes laterais enquanto seu efeito na raiz primária é indireto.

Um dos papéis regulatórios de HY5 está relacionado com a indução de genes da rota metabólica de flavonóides, que pode ocorrer de forma direta, quando ocorre ligação entre HY5 e a região promotora desses genes, ou indireta, através da associação com outros fatores de transcrição, como MYB12 (Chattopadhyay, S et al., 1998; Stracke, R et al., 2007, Dubos, C et al., 2010; Burko, Y et al., 2020), induzindo a expressão das proteínas envolvidas, sugerindo a comunicação entre fotorreceptores, HY5 e flavonóides (Wade, H et al., 2001; Dubos, C et al., 2010; Gangappa e Botto, 2016).

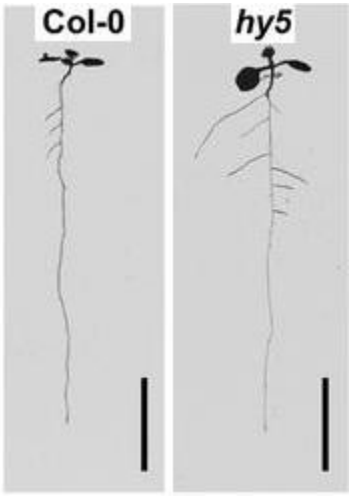

Figura 2. Fenótipo do mutante *hy5* comparado com genótipo selvagem (Col-0). Barras = 1cm (Adaptado de Van Gelderen, K et al., 2018).

O papel da luz no sistema radicular das plantas

A luz exerce um grande efeito no crescimento e desenvolvimento das raízes, induzindo o alongamento da raiz principal, formação de raízes laterais e fototropismo negativo. Apesar das raízes estarem abaixo do solo e consequentemente não receberem iluminação direta, sabe-se que a percepção da luz na parte aérea é capaz de estimular o crescimento dessas raízes, efeito esse que depende da atuação de auxina (Morelli e Ruberti, 2002) Além disso, já se observou que plantas cultivadas completamente no escuro, seja somente a parte aérea ou a plântula inteira, não

é o suficiente para induzir o desenvolvimento do sistema radicular, mesmo em quantidades ótimas de nutrientes (Miotto, Y et al., 2021). Isso sugere a existência de um sistema complexo de sinalização entre parte aérea e raízes (Stafen, C et al., 2022). Sendo assim, plantas cultivadas sob luz (fotomorfogênicas) apresentam desenvolvimento do sistema radicular, alongando sua raiz principal e aumentando o número de raízes laterais, efeitos fenotípicos que resultam da produção de auxina e açúcares na parte aérea e seu transporte para as raízes (Bhalerao, R et al., 2002; Salisbury, F et al., 2007; van Gelderen, K et al., 2017), além do movimento de outras moléculas que fazem a comunicação entre os órgãos. Algumas substâncias já foram identificadas como possíveis sinais móveis responsáveis pela sinalização sistêmica nas plantas, por exemplo sacarose, hormônios, peptídeos e fatores de transcrição (Ko e Helariutta et al., 2017).

COP1 e HY5 são componentes importantes do mecanismo de sinalização envolvido nas respostas da raiz à luz (Fig 3) (Yang e Liu, 2020). COP1, por exemplo, foi relacionado com a regulação dos transportadores de auxina PIN1 e PIN2 (PIN-FORMED) (Friml, J et al., 2003). Através da ação de COP1, foi observado que a localização celular desses transportadores na raiz é sensível à luz. Durante a exposição à luz, as proteínas PINs são encontradas na membrana plasmática das células. Por outro lado, quando há ausência de luz, elas são direcionadas para o vacúolo, onde sofrem degradação. (Laxmi, A et al., 2008). Foi proposto que COP1 atua inibindo a estabilidade de PIN1 e PIN2, observou-se que a abundância de ambas proteínas em plântulas cultivadas no escuro permanece inalterada no mutante *cop1*, indicando uma regulação de COP1 sob os PINs. Além disso, utilizando linhagens repórter GFP transformadas em *cop1*, demonstrou-se que COP1 também regula a expressão de PIN1, uma vez que na ausência de COP1, a expressão de PIN1 não sofreu nenhuma redução em plântulas crescidas na escuridão. Dessa forma, concluiu-se que COP1 regula o transporte polar de auxina inibindo a expressão de PIN1 e controlando a estabilidade de ambos, marcando o transportador para degradação (Fig 3A) (Sassi, M et al., 2012).

Quanto à HY5, demonstrou-se em *Arabidopsis* que esse fator de transcrição é translocado para as raízes, ativando sua expressão e induzindo a expressão do transportador de nitrato NRT2.1 (NITRATE TRANSPORTER 2.1), e consequentemente induzindo a absorção de nitrato do solo e regulando o crescimento da raíz (Fig 3B) (Chen, X et al., 2016). HY5 também atua suprimindo a formação de raízes laterais sob luz vermelho-extrema (FR) (van Gelderen, K et al., 2018). Observou-se que suplementando luz branca com luz FR diminui-se a densidade de raízes laterais

em plântulas selvagens de Arabidopsis, porém em plântulas que não expressam a proteína HY5 o fenótipo nessas diferentes condições de luz permanece inalterado. Além disso, sob tratamento com FR, HY5 acumula-se em primórdios de raízes laterais (estágio inicial de desenvolvimento de uma raiz lateral), assim como no córtex da raiz primária, logo acima desses primórdios e também inibe a expressão de genes envolvidos no transporte e biossíntese de auxina, como PIN3, LAX3 (LIKE AUX1 3) e ARF19 (AUXIN RESPONSE FACTOR 19) (van Gelderen, K et al., 2018).

Entender a sinalização dependente de luz em raízes em condições ambientais, na qual normalmente esse órgão está protegido pelo solo, é importante para otimizar o *design* de experimentos em laboratório, uma vez que plântulas de Arabidopsis são comumente cultivadas em placas de Petri transparentes totalmente expostas à luz, implicando em uma alteração no desenvolvimento radicular. O sistema Dark-root (D-root, Silva-Navas, J., 2015) consiste em um sistema para cultivar plântulas de Arabidopsis em que a parte aérea é iluminada, mas as raízes ficam protegidas, aproximando-se mais de uma condição ambiental. Esse sistema consiste no uso de um aparato para bloquear a exposição direta do sistema radicular à fonte de luz, uma vez que a exposição direta das raízes à iluminação altera as respostas fisiológicas da planta, distorcendo a sinalização de hormônios, acúmulo de flavonóides, balanço de espécies reativas de oxigênio entre outros (Cabrera, J et al., 2021). Embora existam outras formas descritas na literatura de como a luz poderia alcançar a raiz (Lee, H-J et al., 2016; Lee, H-J et al., 2017; Ko, D. e Helariutta, Y., 2017) maiores evidências encontram-se na teoria de uma molécula móvel responsável por carregar a informação da parte aérea para as raízes e induzir seu crescimento (Buer, C et al., 2007; Notaguchi, M et al., 2012; Matsubayashi, Y, 2014; Oh, E et al., 2018; Khondare, K et al., 2021; Stafen, C et al., 2022).

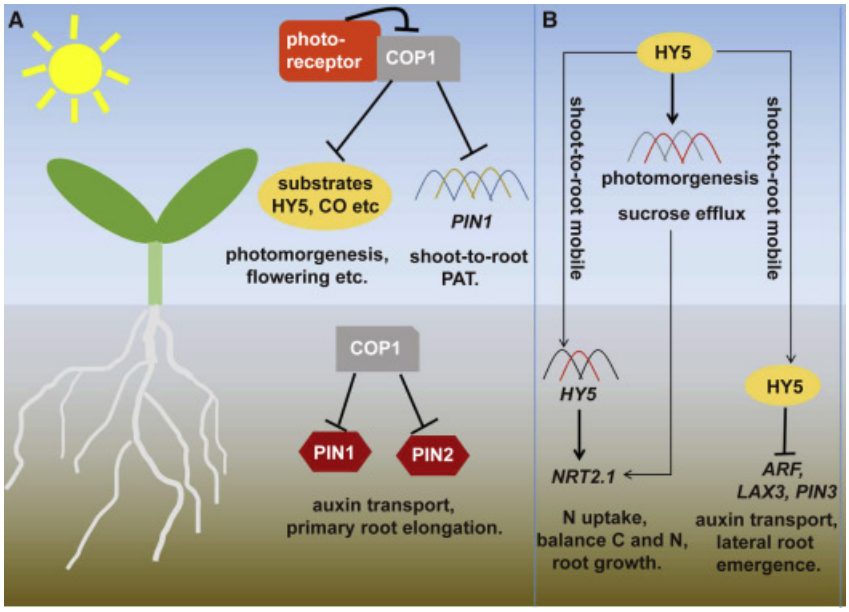

Figura 3: Respostas desencadeadas por luz e suas rotas de regulação. **(A)** Fotorreceptores regulam a fotomorfogênese inativando COP1, por sua vez, COP1 não é mais capaz de enviar seus alvos para degradação (HY5, CO, PIN1 e PIN2), além de modular abundância proteica, COP1 inibe a expressão de PIN1. **(B)** Após COP1 ser inativado, HY5 pode atuar na fotomorfogênese induzindo a expressão de NRT2.1 para captação de nitrogênio e de genes de sinalização e transporte de auxina (ARF, LAX3 e PIN3) (Obtida de Yang e Liu, 2020).

Flavonóides: uma rota de metabólica importante para a resposta às variações ambientais em plantas

Em resposta às adversidades ambientais enfrentadas pelas plantas, uma variedade de metabólitos secundários é produzida devido ao constante contato com diferentes estresses. Esses metabólitos desempenham um papel na modulação da defesa e do desenvolvimento das plantas. Um exemplo desses metabólitos são os flavonoides (Bhatia, C et al., 2018). Os flavonóides são compostos polifenólicos com papel protetivo nos tecidos vegetais contra condições ambientais adversas, incluindo alta irradiância de luz, baixas temperaturas e infecções por patógenos (Schulz, E et al., 2016). Existem evidências também sobre seu papel na defesa contra espécies reativas de oxigênio (ROS). Apesar de não haver um consenso, acredita-se que esses metabólitos atuem como uma linha de defesa adicional quando a planta passa por estresses severos (Agati, G et al., 2020). Apesar desta discrepância, sabe-se que a modulação nos níveis de ROS por flavonóides exerce efeito no desenvolvimento vegetal (Chapman e Muday, 2021). Existem diversas subclasses de flavonoides, incluindo chalconas, flavonas, isoflavonóides, flavanonas, flavonóis e

antocianinas. Em *Arabidopsis thaliana* há produção de três tipos principais de flavonóis: kaempferol, quercetina e isoramnetina (Stracke, R et al., 2010; Saito, K et al., 2013). Além do efeito protetivo, os flavonóis desempenham um papel no desenvolvimento das plantas inibindo o transporte polar de auxina (Buer, C et al., 2004; Peer, W et al., 2004; Silva-Navas, J et al 2016; Teale, W et al., 2021), especificamente o efluxo de ácido indol-3-acético (AIA) das células (Brown, D et al., 2001). A síntese de flavonóides tem início com a fenilalanina, a partir da qual a via dos flavonóis se ramifica. As enzimas envolvidas e os respectivos mutantes perda de função (tt`s) para cada são: Chalcona Sintase (CHS - tt4), a primeira enzima dedicada da rota, a Chalcone Isomerase (CHI - tt5), a Flavanona 3-Hidroxilase (F3H - tt6), a Flavonoide 3'-Hidroxilase (F3'H - *tt7*) e a flavonol sintase (FLS - *fls1*) (Kim, S et al., 2017) (Fig 4A). O acúmulo desses polifenóis ocorre em diferentes quantidades nas folhas, caule e raízes das plantas, sendo altamente dependente de luz (Ferreyra, F et al., 2012). Já foi observado que plântulas de Arabidopsis cultivadas no escuro não produzem flavonóides, diferentemente do que ocorre na presença de luz, na qual há uma grande indução na transcrição dos genes relacionados às enzimas da via de biossíntese (Buer, C et al., 2004).

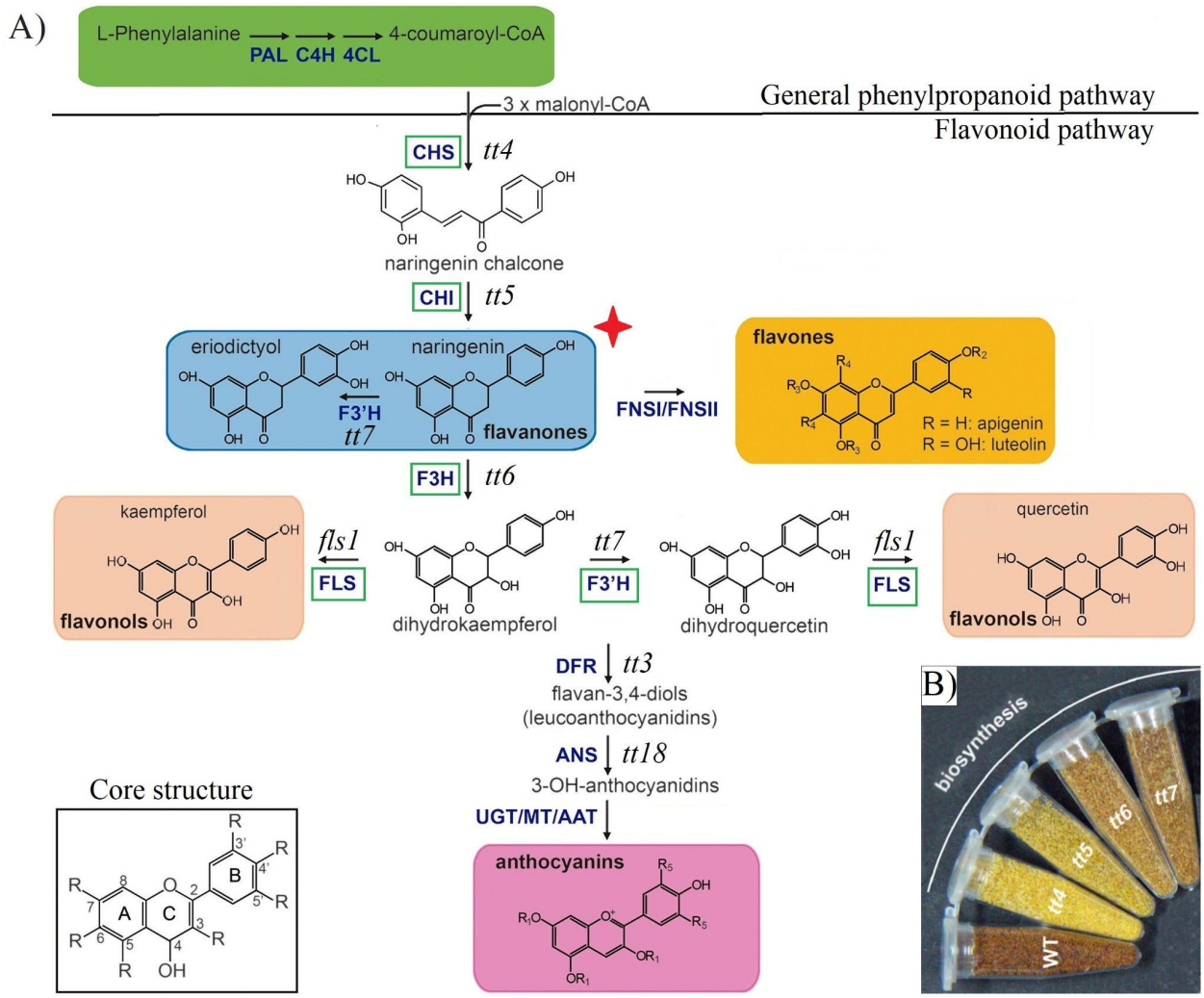

Figura 4: Rota de síntese de flavonóides em *Arabidopsis thaliana*. (A) A via começa com o metabolismo de fenilpropanóides e culmina na síntese de flavonóis. As enzimas da rota dos flavonóis estão em caixa alta e seus mutantes correspondentes em *itálico*. HY5 regula positivamente os genes (retângulos verdes) da via de biossíntese dos flavonoides. CHS – Chalcona sintase (*tt4*), CHI – Chalcona isomerase (*tt5*), F3H – Flavanona 3-hidroxilase (*tt6*), F3'H – Flavonoide 3'-Hidroxilase (*tt7*), FLS - Flavonol sintase (*fls1*). Estrela vermelha: naringenina, importante intermediário que participa de diversas outras ramificações da rota geral de flavonóides, sendo precursor da via de flavonas e isoflavonóides, além de flavonóis e antocianinas. (Adaptado de Falcone Ferreira, M et al., 2021) **(B)** Imagem representativa das sementes de cada mutante da rota de biossíntese (Adaptado de Appelhagen, I et al., 2014).

Nas raízes de Arabidopsis, foi demonstrado que esses metabólitos desempenham um papel no crescimento e na resposta fototrópica (Pollastri e Tattini, 2011). A regulação mediada por flavonóis ocorre por meio de uma redistribuição de auxina que resulta em fototropismo e um

desequilíbrio nas taxas de diferenciação e proliferação celular na zona meristemática da raiz, uma vez que afeta o transporte do hormônio nesta região e consequentemente o crescimento da raiz (Silva-Navas, J et al., 2016). A rota de biossíntese dos flavonóides é bem caracterizada em Arabidopsis devido à obtenção de mutantes perda de função para cada enzima da via (Winkel-Shirley, 2001). Esses mutantes são incapazes de acumular flavonoides, o que resulta na falta de pigmentação na camada externa das sementes, conhecida como testa, portanto foram denominados mutantes *transparent testa* (tt) (Fig 4B) (Bharti, A et al., 2003). Esses mutantes são uma ferramenta apropriada para o estudo da regulação e interação de flavonóides nas plantas, uma vez que não possuem a atividade dos genes que codificam as enzimas da via de biossíntese dos flavonóides. Dentre as mutações nas enzimas iniciais da rota de biossíntese de flavonóides, os mutantes *tt4* e *tt6* que codificam as enzimas chalcona sintase e flavanona 3-hidroxilase, respectivamente, apresentaram alterações no desenvolvimento das raízes, ambos com um aumento no número de raízes laterais quando comparado com a planta selvagem (Buer e Djordjevic, 2009; Gayomba e Muday, 2020), indicando um papel de sinalização dos flavonóides no desenvolvimento vegetal. O uso dos mutantes *transparent testa* permite estudar o papel desses metabólitos em modular rotas de sinalização que respondem a alterações no ambiente. Em função disso, dois mecanismos já foram sugeridos para tentar explicar como os flavonoides controlam o desenvolvimento da planta. Esses mecanismos estão relacionados com o transporte de auxina e o efeito antioxidante dos flavonóides (Chapman e Muday, 2020). Mutantes *tt* apresentam elevado transporte de auxina (Buer, C et al., 2013; Peer, W et al., 2004). O mecanismo proposto que relaciona flavonóides à inibição do transporte polar de auxina sugere que os flavonóides atuariam através da estabilização de dímeros das proteínas PINs, uma conformação estrutural menos ativa nas células, dessa forma, ocorre uma redução no efluxo de auxina. Acredita-se também que a interação flavonóides-PINs dependa do estado de fosforilação dos transportadores, o que modularia a capacidade de interação entre eles (Teale, W et al., 2021). Além disso, nos mutantes *tt* também se observa um aumento no nível de ROS em diferentes partes da planta, uma vez que flavonóides também atuam como moléculas antioxidantes (Gayomba e Muday, 2020; Watkins, J et al., 2014; Muhlemann, J et al., 2018). A ação dos flavonóides na modulação dos níveis de ROS regula a emergência de raízes laterais em Arabidopsis thaliana, demonstrando a variedade funcional desses metabólitos (Chapman and Muday, 2021). Levando em consideração as funções descritas dos flavonóides, a regulação de

sua síntese é controlada por fatores exógenos e endógenos através de uma rede de fatores de transcrição (Mehrtens, F et al., 2005; Wang et al., 2016). A transcrição dos genes da rota de síntese de flavonóides é altamente induzida pela luz nas raízes (Miotto, Y et al., 2020) e essa indução, tanto na parte aérea quanto nas raízes, depende de HY5, MYB11 MYB12 e MYB111 (Dubos, C et al., 2010). HY5 é um fator de transcrição chave que induz o acúmulo de flavonóides através da sua atividade promotora sob os genes da rota de biossíntese desses compostos, o que acontece majoritariamente na presença de luz (Gangappa e Botto, 2016). Já foi demonstrado que os genes codificadores das enzimas CHS (TT4), CHI (TT5), F3H (TT6) e FLS1 são induzidos por HY5 em resposta a luz (Shin, J et al., 2007) (Fig 4, retângulos verdes), sendo assim, a relação linear entre a percepção de luz, HY5 e a rota de biossíntese de flavonóides, coloca estes compostos em uma participação central das respostas fotomorfogênicas em plantas.

Anatomia e regulação hormonal no crescimento da raiz

O crescimento da raiz ocorre por meio da divisão e alongamento celular. No ápice das raízes encontra-se o meristema apical da raiz (*Root Apical Meristem* - RAM), uma região formada por um conjunto de células indiferenciadas em alta divisão celular próximas ao centro quiescente (*Quiescent centre* - QC). O QC é responsável por manter o estado indiferenciado e de alta proliferação das células circundantes, importantes para assegurar o RAM (Petricka J et al., 2012). Conforme as divisões celulares afastam as células do QC, estas passam a dar origem a diferentes tecidos e estruturas da raiz (Heidstra e Sabatini, 2014). Em Arabidopsis, a raiz consiste de quatro zonas longitudinais distintas de diferenciação e função. A zona meristemática (*Meristematic Zone* - MZ) consiste na região de divisão celular ativa; a próxima, a zona de transição (*Transition Zone* - TZ) é definida pelas células que passam de um estado de alta divisão celular para começar a alongar lentamente (Verbelen, J et al., 2006; Dello Ioio, R et al., 2007); na zona de alongamento (*Elongation Zone* - EZ), as células alongam em uma velocidade maior, esse alongamento aliado com as divisões celulares na MZ, resultam no crescimento da raiz em direção ao solo; por fim, na zona de diferenciação (*Differentiation Zone* - DZ), o alongamento cessa e surgem estruturas diferenciadas, como pelos radiculares e as estrias de Caspary (Fig 5A) (Petricka J et al., 2012). Sendo assim, podemos considerar que o eixo longitudinal da raiz contém

informação temporal, uma vez que acompanhando uma célula a partir da MZ podemos observar a passagem por todas as zonas da raiz e o desenvolvimento desta em uma estrutura especializada. Na raiz de Arabidopsis, a organização dos tecidos é formada por conjuntos de células arranjadas concentricamente originando um cilindro radialmente simétrico. A camada mais interna é composta pelo sistema vascular, responsável por conduzir nutrientes e metabólitos do solo para as folhas e vice-versa. Externo ao cilindro vascular, há o periciclo de onde se formam os primórdios de raízes laterais. Circundando o periciclo existe a endoderme, que forma uma barreira seletiva para passagem de íons devido a presença das estrias de Caspary. O próximo tecido é o córtex que fornece proteção e suporte mecânico para a raiz. Por último, a epiderme consiste da camada mais externa que envolve os outros tecidos e contém tricoblastos, posteriormente dando origem aos pelos radiculares (Fig 5A) (Petricka J et al., 2012; Dolan, L et al., 1993; Smet, S et al., 2015).

O tamanho do meristema é regulado por um equilíbrio dinâmico entre divisão celular promovida por auxina e diferenciação celular promovida por citocinina, esses dois processos devem ocorrer em taxas similares para que a raiz cresça e o tamanho do meristema não se altere no decorrer do tempo (Di Mambro. e Sabatini, 2018), portanto a ação coordenada desses fitormônios é essencial para esse controle (Verbelen, J et al., 2006). Dessa forma, a raiz é uma estrutura altamente dinâmica e os mecanismos que garantem sua atividade precisam ser regulados com precisão.

Um gene essencial na manutenção do tamanho do meristema é o gene codificador da Aux/IAA3 SHORT HYPOCOTYL2 (SHY2), que atua como repressor transcricional da sinalização de auxina (Tiwari, S et al., 2001; Taniguchi, M et al., 2007). Já se demonstrou que SHY2 atua na TZ, especificamente no tecido vascular. A indução de SHY2 ocorre através da citocinina, inibindo a expressão dos genes dos transportadores PIN e consequentemente a distribuição de auxina. Em mutantes *shy2-2* (ganho-de-função) em que a proteína SHY2 está estabilizada, a expressão das proteínas PIN está reduzida e colocaliza com o padrão de expressão SHY2 (Dello Ioio, R et al., 2008). SHY2 mantém os níveis de auxina na TZ reduzidos impedindo que a auxina sintetizada no meristema passe através da TZ, esse bloqueio favorece o acúmulo de auxina no meristema apical. Por conseguinte, no meristema, a auxina acumulada em grandes quantidades induz a ubiquitinação e posterior degradação de SHY2 através do complexo Skp1-Cul-F-box E3 ubiquitina ligase (SCF) e TRANSPORT INHIBITOR RESPONSE1 (TIR1), permitindo que as proteínas PIN exerçam sua atividade (Mockaitis e Estelle, 2008), dessa forma cria-se um looping

no fluxo aprisionando auxina na zona meristemática pela atividade de PIN2 e AUX1 (Band, L et al., 2014; Michniewicz, M et al., 2007; Blilou, I et al., 2005). Portanto, a biossíntese local de auxina no meristema, combinada com o transporte e sua repressão pela citocinina na zona de transição dão origem ao gradiente de auxina dentro da raiz (Fig 5B).

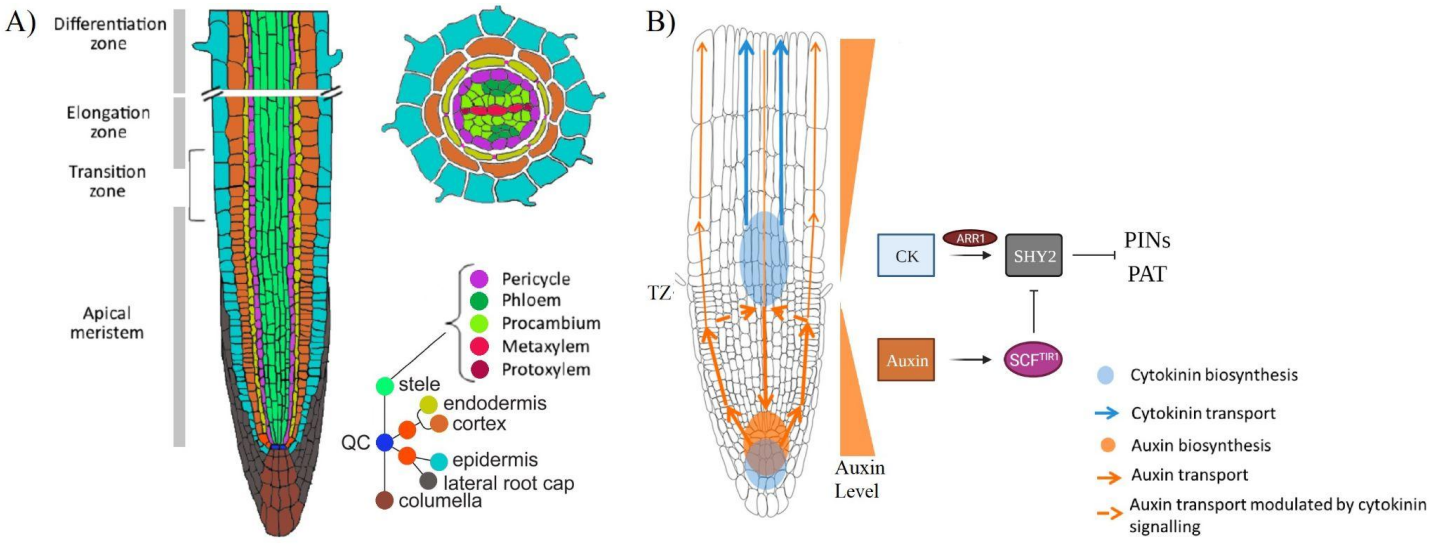

Figura 5: Anatomia e regulação hormonal de raízes. (A) Organização celular em raízes de Arabidopsis, a raiz é dividida em quatro zonas (zona de diferenciação, alongamento, transição e meristemática). A raiz é formada por camadas celulares organizadas concentricamente, a partir do centro quiescente se origina e estelo, periciclo, endoderme, córtex, epiderme, coifa e columela (Adaptado de De Smet, S et al., 2015). **(B)** Modelo esquemático mostrando o fluxo hormonal e a regulação que posiciona os gradientes de auxina e citocinina na ponta da raiz (Adaptado de Barrada, A et al., 2015).

Outra estrutura importante no sistema radicular de plantas são as raízes laterais que se originam a partir do periciclo no cilindro vascular (Fig 6A). Somente as células que foram previamente sinalizadas no meristema que formam raízes laterais, essas células são chamadas de fundadoras de raízes laterais e a sua ativação ocorre a partir de uma distância mínima da ponta da raiz (Banda, J et al., 2019). O primeiro estágio, denominado iniciação da raiz lateral, ocorre com a primeira divisão das células fundadoras na zona de diferenciação formando um primórdio de raiz lateral (Casimiro, T et al., 2003) e progride por uma série de fases que ocorrem conforme as camadas de células mais externas (endoderme, córtex e epiderme) são rompidas e atravessadas (Fig 6A) (Malamy e Benfey, 1997; Benková e Bielach, 2010). A saída da raiz lateral ocorre pela indução que auxina, produzida nas células fundadoras, promove na expressão de LAX3 e PIN3.

Esses transportadores atuam movendo a auxina do primórdio para as camadas externas de células (córtex e epiderme), o que resulta no afrouxamento da parede celular. O transporte de auxina para as próximas camadas inibe repressores da expressão de PIN3 e LAX3, o que forma um looping de feedback positivo que culmina na remodelação da parede celular liberando caminho para a emergência da raiz lateral. Dessa forma, a remodelação da parede celular juntamente com as divisões celulares no primórdio geram uma força mecânica que auxilia na saída da raiz lateral (Swarup, K et al., 2008; Péret, B et al., 2013; Vilches-Barro e Maizel, 2015). Uma vez que a raiz lateral tenha emergido, seu meristema é ativado e o crescimento subsequente resulta em uma polaridade radial e apical-basal semelhante à raiz primária (Fig 6B).

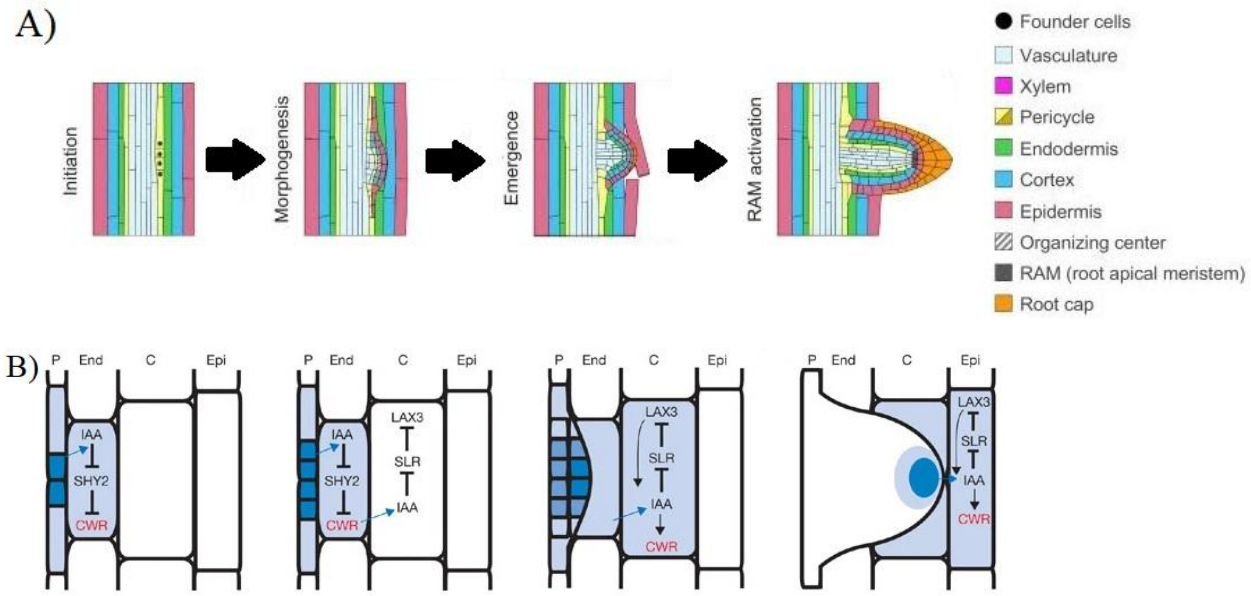

Figura 6: Desenvolvimento e emissão de uma nova raiz lateral. **(A)** Os principais estágios na formação de LR. Após formação das células fundadoras no periciclo, ocorrem as primeiras divisões anticlinais até formação do primórdio, a partir desse ponto, as divisões continuam fazendo com que o primórdio rompa as camadas celulares mais externas e emerja da raiz principal, nesse momento ocorre ativação do RAM (Obtida de Banda, J et al., 2019). **(B)** Mecanismo do mecanismo molecular que governa a emergência de raízes laterais. Auxina produzida pelas células fundadoras bloqueia a atividade de SHY2, induzindo a remodelação da parede celular. O afrouxamento da parede permite que auxina se mova para a próxima camada celular onde o mecanismo se repete. Esse movimento de auxina depende da atividade de PIN3 e LAX3, ambos têm sua expressão induzida pelo fitormônio. P: periciclo; End: endoderme; C: cortex; Epi: epiderme; IAA: auxina; SHY2 - Aux/IAA3 SHORT HYPOCOTYL2; CWR: *cell wall remodelling*; SLR: SOLITARY ROOT; LAX3: Aux1/LAX3 transportador de influxo de auxina (Obtida de Swarup, K et al., 2008).

Concluindo, o desenvolvimento das raízes segue um mecanismo regulatório preciso capaz de transformar um sinal ambiental como a luz em uma resposta fisiológica adaptativa. Esse mecanismo depende da ação inicial de fotorreceptores que percebem a qualidade e intensidade luminosa no ambiente e passam essa informação para outras proteínas, como COP1, que atua como uma trava para o crescimento das raízes na ausência de luz. Assim que COP1 é inativado pelos fotorreceptores, o principal indutor da fotomorfogênese, HY5, deixa de ser degradado e passa a se acumular nos núcleos das células, podendo ativar a transcrição de genes responsivos à luz. Entre estes, NRT2.1 que ajuda na captação de nitrogênio para auxiliar no crescimento vegetativo da planta e os genes da via de síntese de metabólitos especializados como os flavonóides que possuem diversos papéis, desde na defesa contra patógenos e estresses abióticos até no desenvolvimento por modularem o transporte de auxina. Por fim, as raízes de Arabidopsis apresentam uma estrutura celular relativamente simples em camadas de células concentricamente organizadas na qual o gradiente de auxina, juntamente com outros fitormônios como citocinina, determinam as diferentes zonas da raiz

Objetivos

1. Objetivo geral

Avaliar o papel da síntese de flavonóides no desenvolvimento fotomorfogênico de raízes em *Arabidopsis thaliana*

2. Objetivos específicos

2.1 Obtenção de combinações de mutantes em *Arabidopsis thaliana* para genes envolvidos na fotomorfogênese e síntese de flavonóides assim como linhagens de superexpressão, repórter, ou supressão para genes específicos possivelmente envolvidos nesta regulação;

2.2 Analisar as respostas à naringenina no enraizamento das combinações de mutantes gerados (*tt*'s, *hy5, pin3* e *lax3*);

2.3 Caracterizar fenotipicamente as raízes das plantas mutantes frente a diferentes condições de luz e idade

2.4 Analisar o perfil metabólico das plantas a fim de identificar os derivados de flavonóides que afetam o desenvolvimento das raízes.

Capítulo I

Avaliação do papel da síntese de flavonóides no desenvolvimento fotomorfogênico de raízes em Arabidopsis thaliana

Jonata Alex Ribeiro Christino, Yohanna Evelyn Miotto, Jürgen Klein-Vehn, Felipe de Oliveira Souza, Eduardo Jorge Pilau & Felipe dos Santos Maraschin.

Evaluation of root photomorphogenic growth dependency on light activated localized flavonoid biosynthesis

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Abstract

Plants rely on different photoreceptors to perceive light and initiate signaling pathways that result in adaptive responses, such as primary root growth and lateral root formation. While the transcription factor ELONGATED HYPOCOTYL 5 (HY5) was initially proposed as a mobile signal responsible of communicating light conditions above ground to the root and promote root development, recent studies have suggested that HY5 functions indirectly and that other HY5-dependent mobile signals responsible for the responses observed may exist. Despite that, it is known that HY5 induces the expression of enzymes from the flavonol biosynthesis pathway. Loss-of-function mutants for the key enzymatic steps of the flavonoid pathway, such as *tt5* and *tt6*, which lack chalcone isomerase and flavanone 3-hydroxylase respectively, exhibit opposite root phenotypes. This observation prompted us to hypothesise if a specific flavonoid would play a critical role in root development during photomorphogenesis. Previous works have shown that when plants are exposed to far-red light conditions, they produce fewer lateral roots. This response is also mediated by HY5, which was previously believed to repress lateral root primordia emergence by inhibiting auxin transporters LAX3 and PIN3. However, these transporters are not direct targets of HY5. The root phenotype of *tt6* is similar to that of the *hy5* mutant, with both showing an increased number of lateral roots. In contrast, *tt5* has a reduced

number of lateral roots, but this phenotype can be restored with naringenin, an intermediary in the flavonoid pathway. Based on these findings, this study proposes that light signalling controlled by HY5 in roots may involve an unknown intermediary derived from naringenin in the flavonoid pathway.

Introduction

Light perception by the shoot activates the primary root development underground, a response which alongside inhibition of hypocotyl elongation, apical hook opening, cotyledon expansion and photosynthesis activation comprises the photomorphogenic development (Gommers, C et al., 2017). For this light perception to take place, plants rely on different photoreceptors to initiate signalling pathways that ultimately result in adaptative responses. Independently of the photoreceptor acting, the molecular mechanism functions with conformational changes of these proteins when exposed to light (Möglich, A et al., 2010).

Induction of root development by shoot illumination raises the question of whether a mobile component is responsible for shoot-to-root communication. Opposing data were gathered around the transcription factor ELONGATED HYPOCOTYL 5 (HY5) acting as a mobile signal. One model suggests HY5 acts as a mobile protein that accumulates in shoots followed by its translocation and auto-activation in roots in a positive feedback loop inducing gene expression (Chen, X et al., 2016). Moreover, it was demonstrated in grafting and reporter lines experiments that scions expressing functional HY5 restore the primary root growth and nitrate uptake in *hy5* rootstocks, a result not observed with *hy5* scions, which indicates a role either for the HY5 transcript, protein or a signal dependent on HY5 to move from the shoot to the root (Chen, X et al., 2016; Lee, H et al., 2016). However, a recent study successfully unravelled more details on this matter complementing the loss-of-function *hy5* mutant with a shoot-restricted expression construct, generating a transgenic line in which the HY5 fusion protein was unable to translocate from the shoot to the root. In these plants, both hypocotyl and main root elongation phenotypes were rescued but not the lateral root phenotype (Burko, Y et al., 2020), indicating that HY5 functions indirectly and its movement through the plant is not essential for the main root growth responses, strengthening the possibility that another HY5-dependent mobile signal might be responsible for the responses observed. Furthermore, HY5 is highly expressed in lateral root primordia (Zhang, Y et al., 2019) and its local activity was shown to be crucial for the development of lateral roots and complement the *hy5* mutant (Burko, Y et al., 2020). Together, this evidence indicates that HY5 directly regulates lateral root formation, but it has an indirect effect on primary root growth.

Although initial ChIP-seq analysis suggested that HY5 would bind to thousands of genes (Lee,

J et al., 2007), a recent study reduced the list of its direct targets to a number of 297 genes in Arabidopsis (Burko, Y et al., 2020), concluding that HY5 mainly acts as a transcriptional activator, inducing those genes from the flavonol biosynthetic pathway. The regulation of the flavonol pathway genes may occur either by direct HY5 binding to their promoters or indirectly, by induction of other transcription factors, like MYB12 (Chattopadhyay, S et al., 1998; Stracke, R et al., 2007, Dubos, C et al., 2010; Burko, Y et al., 2020), which in turn, promote their transcription.

Loss-of-function mutants on genes for the flavonoid pathway display different phenotypes, these mutants are called *transparent testa* (*tt*) mutants and they are a powerful tool that helped to characterise and understand the flavonoid biosynthetic pathway in Arabidopsis (Appelhagen, I et al., 2014). The enzymatic steps of the flavonoid pathway and their corresponding mutants are displayed in Fig 1A. The mutation on *tt5-2* renders a defective chalcone isomerase (CHI) enzyme which blocks the conversion of naringenin chalcone to naringenin. Conversely, *tt6-3* carries a loss-of-function mutation in flavanone 3-hydroxylase (F3H), responsible for converting naringenin to dihydrokaempferol. These two particular mutants present contrasting root phenotypes: *tt6* (F3H) for example, shows a higher number of lateral roots in comparison to the wild-type (Buer e Djordjevic, 2009; Gayomba e Muday, 2020) whereas *tt5* (CHI) displays the opposite effect, with no formation of lateral roots suggesting a crucial role in root development. Noteworthy, the root phenotype of *tt6* shares similarity to the *hy5* mutant displaying an increased number of lateral roots.

Moreover, plants under low R/FR light ratio (simulating shade conditions) produce less lateral roots, a response also mediated by HY5 (van Gelderen, K et al., 2018). It was suggested that FR-activated phyA activates HY5 to locally repress lateral root primordia formation by repressing the expression of the auxin transporters LAX3 and PIN3. However, a curated list of direct target genes for HY5 does not include LAX3 and PIN3 (Burko, Y et al., 2020), which implies an indirect effect. As HY5 positively regulates the flavonol biosynthesis pathway, it is possible that the repressor effect on PIN3 and LAX3 occurs somehow through the induction of flavonoids.

In this study, we sought to investigate the impact of HY5-induced flavonol accumulation on primary and lateral root growth. To achieve this, we created double mutants by combining different mutations in the flavonol biosynthetic pathway with mutations in either *hy5* or the

polar auxin transporters LAX3 and PIN3. Our results indicate that HY5 effects depend on flavonol pathway genes for lateral root formation.

Materials and Methods

Plant Material and Growth conditions

Arabidopsis thaliana from the Columbia (Col-0) ecotype was used as wild-type (WT). The mutants *hy5* (SALK_056405C), *tt5-2* (GK-176H03), *tt6-3* (SALK_113904C), *pin3-3* (Friml, J et al., 2002), *lax3* (Tissier, A et al., 1999; Swarup, K et al., 2008) are in Col-0 ecotype background and were obtained from the The European Arabidopsis Stock Centre (NASC, http://arabidopsis.info/) or generously donated by the respective authors. Genotyping on T-DNA insertions was performed following the SALK (http://signal.salk.edu/index.html) instructions, and we used the one-step DNA extraction protocol to obtaining genetic material for genotyping from our crosses (Protocol Kasajima). The primers used for genotyping can be found in Supplementary Table 1. All experiments were done using homozygous lines for the T-DNA insertion.

The *35S:FLASH-HY5ox hy5* (HY5ox) and *CAB3:DOF-HY5 hy5* (CAB3:HY5) transgenic lines were kindly provided by Yogev Burko (SALK Institute, EUA). The *HY5ox* line was crossed with *tt5-2* and *tt6-3* to obtain the double homozygous *tt5HY5ox* and *tt6HY5ox* lines respectively. Moreover, double mutant lines were constructed by crossing *hy5* and the *tt* mutants with *pin3-3* and *lax3*. The reporter lines *pTT5:GUS-GFP* (TT5-GUS) and *pTT6:GUS-GFP* (TT6-GUS) were generated previously by Miotto, Y., 2019 - CAPIV.

Col-0 seeds were cultivated as described by Miotto, Y et al., 2019. Briefly, seeds were surface sterilised and cold-stratified at 4°C for two days in complete darkness to synchronise germination. Plants were grown on half strength sucrose-free Murashige and Skoog (MS) (Sigma-Aldrich, M5519) media supplemented with 1% 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 day photoperiod (16-hour light and 8-hour dark) under white light illumination (100µmol m−2 s−1)in a modified D-Root system (Silva-Navas et al., 2015). Far-red supplementation to achieve low red/far-red ratio spectrum was obtained with an additional far-red led lamp (730nm, 30W FGI Accessory Bar, Forever Green Indoors, USA) as described (van Gelderen, K et al., 2018).

Root phenotyping

Primary root growth was measured on 14 days old seedlings. Root length was measured using ImageJ (Fiji) (1.54v) setting the scale for millimeters. For lateral root density, the total number of lateral roots was counted and divided by the length of the primary root.

Naringenin treatment for lateral root quantification

For naringenin treatment, seedlings were germinated for 4 days in regular growth medium and transferred to naringenin supplemented medium to a final concentration of 50 µM. Lateral root number was quantified 3 days after treatment. MS media was prepared as described above. Naringenin (Sigma) was prepared as a 50 mM stock in EtOH. Statistical analysis of the data was completed in Prism 8 using a one-way ANOVA followed by a Tukey's multiple comparisons test or the non-parametric alternatives.

Metabolite isolation and UHPLC-HRMS analysis

Seedlings were harvested and extraction was carried out by incubation overnight in darkness at 4°C in 400µL of 80% (v/v) methanol (MeOH) and centrifuged at 16,000g for 15 min at 4°C. The supernatant was concentrated in speed-vac and resuspended in 50µL of 80% MeOH. The chromatographic chemical profile analysis of the extracts of each species was performed by UHPLC (ultra-high-performance liquid chromatography) (Shimadzu, Nexera X2) coupled to a HRMS (high resolution mass spectrometry) (Bruker, Impact II) equipped with an electrospray ionisation source, the column used in the spectrometer was an ACQUITY UPLC CSH C18 1,7µm (2,1x100mm). The mobile phase used was a solvent mixture A (water with 0.1% formic acid, v:v) and B (acetonitrile with 0.1% formic acid, v:v), both at a continuous flow rate of 0,250 mL/min according to a gradient A:B as follows: 95% A and 5% B from 0 to 6min; 50% A and 50% B from 7 to 11min; 30% A and 70% B from 12 to 17min; 5% A and 95% B from 18 to 20min; 95% A and 5% B from 21 to 25min, the last 4min step reconstitutes the column for the next analysis. The capillary voltage in the mass spectrometry was operated in positive ion mode and MS/MS spectra acquired using the scan mode for ions in the range of 50-2000m/z. Clusters from the samples were generated inserting each of the mass spectrums in the Global Natural Products Social Molecular Networking (GNPS) which creates a molecular

network grouping all compounds identified by chemical similarity. GNPS also has an inbuilt library to assign the samples to the chemicals by mass spectrum correlation. Despite the platform library, each chemical compound assigned was reviewed individually.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., CA, US). Data were tested for normal distribution by the Shapiro-Wilk test, then applied the respectively statistic test and when significant ($p \leq 0.05$) were shown in the graphs. Statistical details of each experiment (test used, replicates, sample size) can be found in the Results section and Figure-Legend sections.

Results

Flavonol biosynthesis mutant seedlings are defective on lateral root formation

In order to evaluate the effects of the flavonoid biosynthesis on the root phenotypes of photomorphogenic seedlings we first examined the primary root growth and lateral root density in loss-of-function mutants (*tt5-2* and *tt6-3)* (Fig 1B). The *tt6-3* mutants display a higher LR density than WT seedlings whereas the *tt5-2* mutant had lower density grown in regular MS. Naringenin treatment rescued the *tt5-2* mutant phenotype by increasing the number of lateral roots to *tt6-3* levels (Fig 1C). The *tt5-2* is unable to produce Naringenin whereas the *tt6-3* mutant is blocked on the conversion to downstream products of the flavonol pathway. This result suggests a role for Naringenin or a derivative flavanone compound as a promoter of lateral roots.

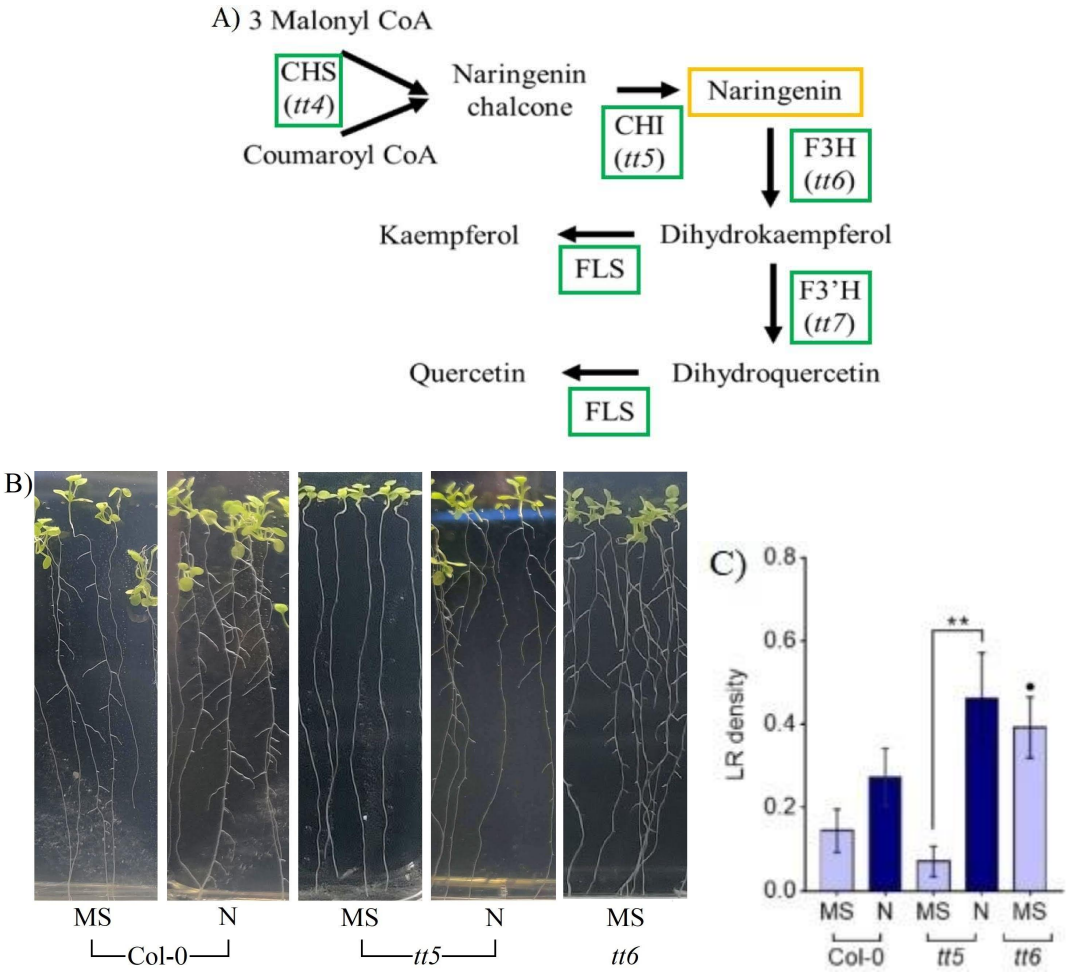

Figure 1: *tt6-3* mutant produce more LR while *tt5-2* has the opposite phenotype. **(A)** Flavonol biosynthetic pathway. Each enzyme has its respective loss-of-function mutant in (*italic*). Green boxes represent genes induced by HY5. Naringenin highlight: *tt6* is unable to carry out the conversion of naringenin to dihydrokaempferol. Representative images of each genotype highlighting their contrastant root systems **(B)**. LR density quantification on Col-0, *tt5-2* and *tt6-3* **(C)**. Naringenin (N) treatment promotes lateral root development in Col-0 and *tt5*. Naringenin is a precursor of the flavonoid biosynthesis pathway, a step in which the *tt6-3* mutant is unable to carry out due to the lack of F3H. Arabidopsis seedlings were grown using ½ MS media for 14 days or 6 days and then transferred to ½ MS media supplemented with 10uM of naringenin. The number of lateral roots was quantified 14 days after germination. • indicates statistical significance by Dunnett's test (•$p \leq 0.05$) compared to wild type (Col-0, MS) and ** indicates significance by student t test comparing control and naringenin treatment, $p \leq 0.01$.

HY5 regulates lateral root formation through interaction with flavonoids

HY5 was shown to regulate flavonol accumulation by binding directly to G-box elements in the promoter region of early genes of flavonoid biosynthetic pathway (CHS, CHI, F3H, F3'H) activating their transcription (Shin, J et al., 2007), or by inducing the expression of other transcription factors from the R2R3 MYB family, like MYB12 (Bhatia, C et al., 2021). In order to understand the relationship between the flavonoid biosynthetic pathway and the transcription factor HY5 we used transgenic lines (HY5ox *(hy5)*, CAB3:HY5 *(hy5)*), as well as crossings with *tt5* and *tt6* (*tt5 HY5ox*, *tt6 HY5ox*) to understand the genetic interaction among flavonol genes and HY5. In general, mutants showed altered root architecture in the 14 days time point observed as shown in Fig 2A. Except for *tt5-2* and HY5ox, mutants for *hy5, tt6-3, hy5 CAB3:HY5, tt5 HY5ox* and *tt6 HY5ox* displayed increased lateral root density compared to Col-0 (Fig 2B). The *tt5-2, HY5ox*, *tt5HY5ox* and *tt6HY5ox* genotypes exhibited longer roots than Col-0, while *hy5*, *tt6-3* and *hy5 CAB3:HY5* were similar to the wild-type plants (Fig 2C). Interestingly, some genotypes showed opposite development of primary root growth and lateral root formation. However, *hy5 CAB3:HY5* had no change in main root length but sustained the higher LR density of the *hy5* mutant, confirming the inability of shoot-expressed HY5 to complement the lateral root density of the *hy5* mutation as previously described (Burko, Y et al., 2020). The genotypes carrying the HY5ox construct showed longer main roots that were unaffected by the *tt6-3* mutation. Curiously, *HY5ox* increased lateral root density in the *tt5 HY5ox*, but it decreased in *tt6 HY5ox*. These results suggest that HY5ox promotes primary root growth independently of *tt5-2* and *tt6-3*, whereas its effect on lateral root formation is

dependent on the flavonoid biosynthetic pathway.

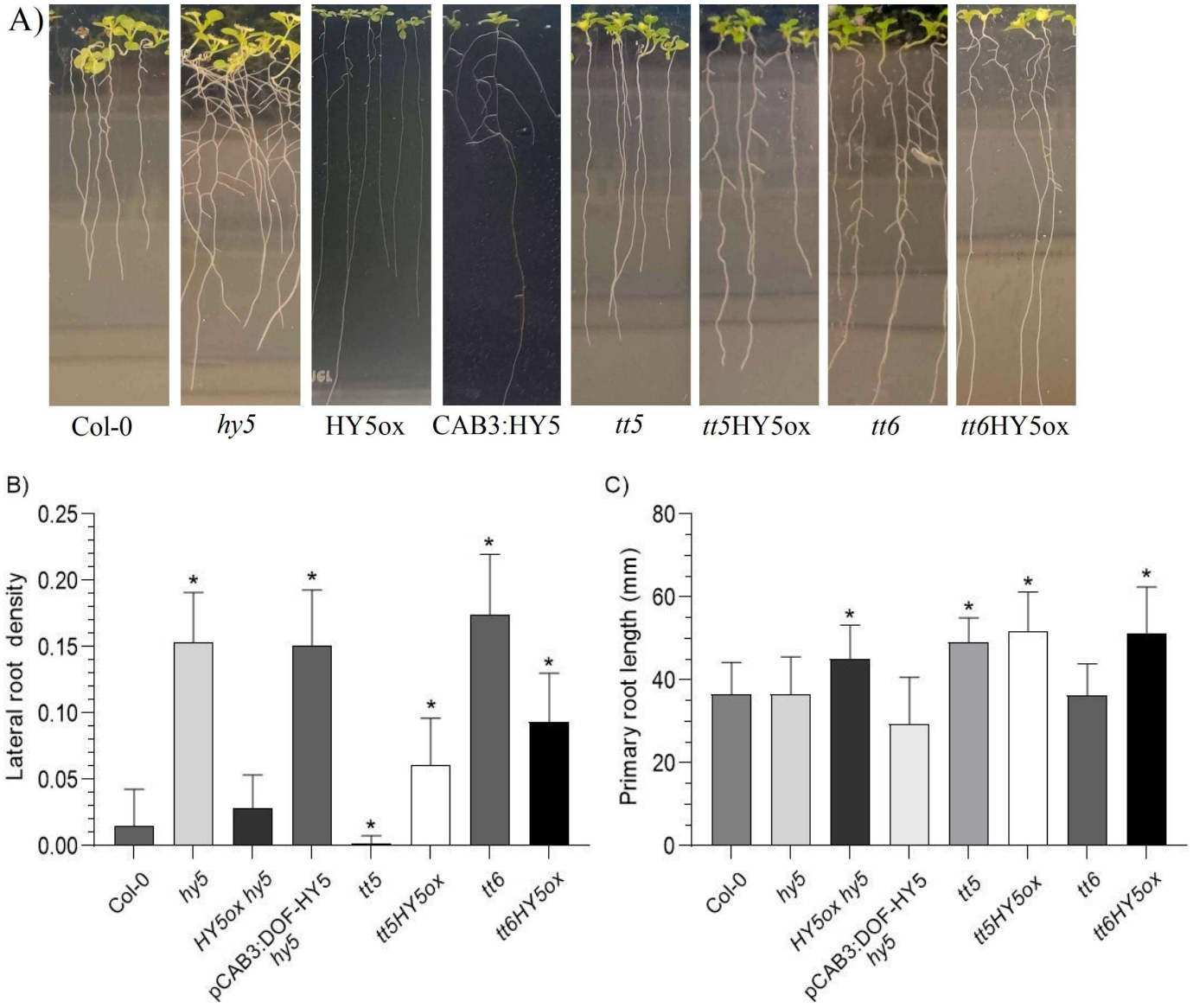

Figure 2: Flavonol and HY5 mutants have altered root phenotypes. (A) Representative pictures of root phenotype in 14-d-old seedlings (B) Primary root length (PRL) in Arabidopsis seedlings and (C) Lateral root density (LRD). Wild type (Col-0) plants and mutants (*hy5*, HY5ox, CAB3:HY5, *tt5-2,* tt6HY5ox, *tt6-3*, tt6HY5ox) were cultivated in sucrose-free 1/2 MS medium. Measurements were performed at 14 dag (days after germination). Statistical significance was determined by Kruskal–Wallis test with Dunn's post-test (*$p \leq 0.05$), the means were compared in the same light condition (100 µmol $m^{-2}s^{-1}$) against the wild-type genotype. The asterisk (*) indicates statistical significance compared to the wild type control condition (Col-0, control. Error bars show standard deviation. Primary root length and lateral root density were quantified over three replicates with $n \geq 20$.

HY5 overexpression alters naringenin effects on lateral root density

The flavonoid mutants *tt5-2* and *tt6-3* are blocked in successive catalytic steps of the flavonoid biosynthetic pathway. Therefore we tested the effect of chemical complementation with Naringenin in the lateral root density phenotype of different crossings (Fig 3). Naringenin treatment increased lateral root density in Col-0, *hy5*, *HY5ox* and *tt5-2* supporting the role of this flavonol intermediary in inducing lateral root formation. Moreover, the same pattern is observed in untreated *tt6-3* as this mutant is interrupted in the naringenin conversion step. Curiously, exogenous Naringenin causes an opposite impact on *tt6-3* when compared to the other genotypes (Fig 3), reducing lateral root formation. This observation could indicate that a fine tuning for Naringenin and its derivatives have a role regulating emergence of lateral roots. Beyond that, the response observed for *tt5HY5ox* and *tt6HY5ox* suggests that the overexpression of HY5 partially suppresses the phenotype of the flavonoid mutants to the same levels as the single mutants treated with Naringenin, indicating that overexpressed HY5 increases the metabolic flux towards the flavonoid pathway changing the sensitivity to the Naringenin treatment. The contrasting observations indicate that Naringenin or its derivative compounds would have a regulatory effect on lateral root development downstream of HY5 regulation.

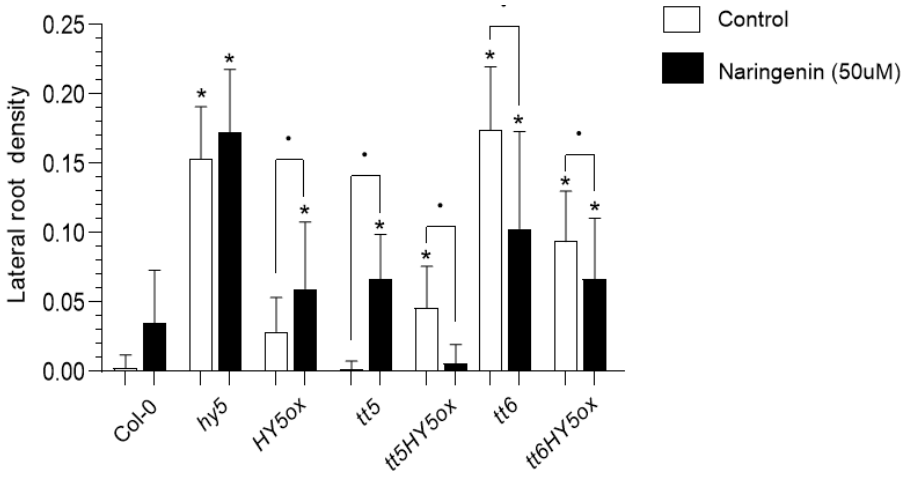

Figure 3: Effect of naringenin treatment on lateral root density. Wild type (Col-0) plants and mutants (*hy5*, *HY5ox, tt5-2, tt5HY5ox*, *tt6-3*, *tt6HY5ox*) were cultivated in sucrose-free 1/2 MS medium supplemented with 50µM of the flavonol precursor Naringenin. Measurements were performed at 14 dag (days after germination). White bars represent control condition and black bars naringenin treatment. Statistical significance was determined by Kruskal–Wallis test with Dunn's post-test (*$p \leq 0.05$), the means were compared in the same light condition (100 µmol $m^{-2}s^{-1}$) against the wild-type genotype. The asterisk (*) indicates statistical significance compared to the wild type control condition (Col-0, control) and the black dot (•) indicates statistical difference between control and

naringenin supplementation. Error bars show standard deviation. Lateral root density was quantified over three replicates with n ≥ 20.

Lateral root emergence dependency on flavonoids and auxin

Previous studies found that plants under shade conditions (Low R/FR ratio) produce less lateral roots. A mechanism was proposed where FR perceived by PhyA in the shoot activated HY5 transport to the roots where it represses lateral root emergence by repressing the auxin transporters PIN3 and LAX3 (van Gelderen, K et al., 2018). However, PIN3 and LAX3 were not found as direct targets of HY5 (Burko, Y et al., 2020). As we identified that the HY5 effect on lateral root density might be dependent on the flavonoid biosynthesis pathway we aimed to explore whether the inhibitory effect of HY5 over PIN3 and LAX3 happens through flavonoids. We generated crosses for *hy5*, *pin3-3*, *lax3*, *tt5-2* and *tt6-3* to evaluate their genetic relationship on lateral root density under white light (WL) and white light supplemented with far-red (WL+FR).

In 14-days-old seedlings, most of the mutants showed altered lateral root densities when compared to the wild type (Fig 4A). The single mutants of the flavonoid pathway presented opposite root phenotypes, *tt5-2* displays longer primary root and fewer lateral roots while *tt6-3* displays similar primary root and far more lateral root formation compared to wild type, as well as the *hy5* mutant (Fig 4A and B) (Miotto, Y., 2019 - CAPIV). Additionally, *tt6-3* was responsive to FR treatment, suggesting that flavonols might not take part in FR-dependent root regulation. Apart from that, the mutants *hy5, pin3* and *lax3* did not respond to the FR light, maintaining the lateral root density in both light treatments, which corroborates to the previous findings of FR effect being missing in *hy5, pin3-3* and *lax3-1* mutants (Fig 4B - black bars) (van Gelderen, K et al., 2018). Noteworthy to mention that the mutants *pin3-3, lax3* and the crosses *lax3tt6* and *pin3tt5* displayed reduced lateral root development, while the crosses *lax3hy5, pin3hy5, pin3tt5, pin3tt6* presented intermediary values similar to Col-0 (Fig 4B - white bars). These observations indicate that both *pin3-3* and *lax3* reduce the lateral root formation when combined with *tt6-3* suggesting that the lateral root increase observed in *tt6-3* depends on the activity of PIN3 and LAX3. Besides, the overall reduction in lateral root density observed in *pin3hy5, lax3hy5, pin3tt6* and *lax3tt6* indicates that not only the flavonol pathway is affected by the activity of the auxin transporters PIN3 and LAX3, but also the

inhibitory effect of HY5 over lateral roots depends on those transporters activity too. These results indicate that HY5, TT5 and TT6 act upstream to LAX3 and PIN3 during lateral root emergence.

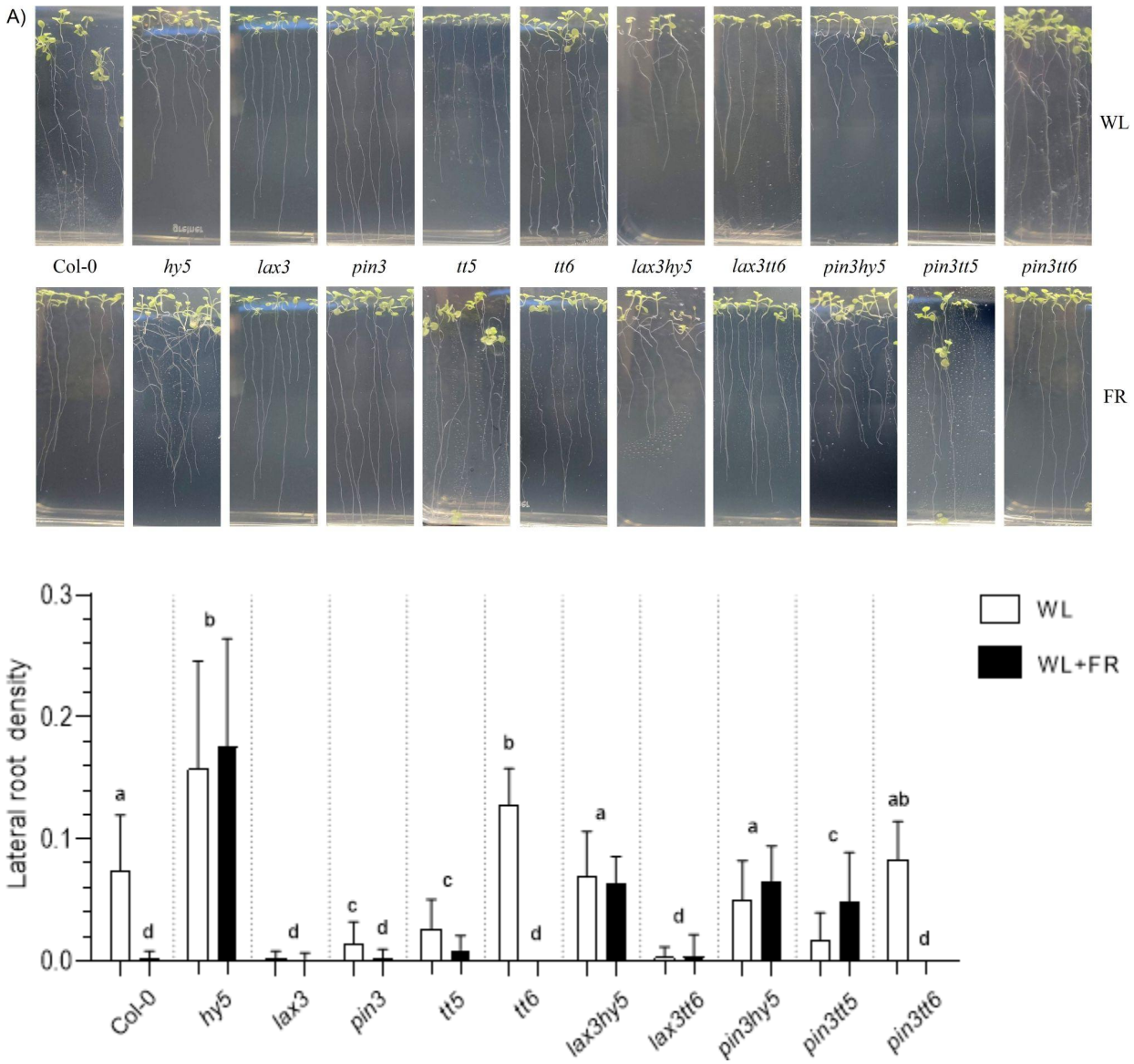

Figure 4: Auxin transporters are required for flavonoids role in lateral root formation. (A) Representative images of each mutant and crosses, upper panel seedlings cultivated on white light, lower panel white light supplemented with far-red light. (B) Lateral root density in Arabidopsis mutant seedlings. Wild type (Col-0) plants, single and double mutants were cultivated in sucrose-free 1/2 MS medium. Measurements were performed at 14 dag (days after germination). Statistical significance was determined by Kruskal–Wallis test with Dunn's post-test (*$p \leq 0.05$), the means were compared in the white light or far-red enriched light conditions (100 µmol $m^{-2}s^{-1}$) against the wild-type genotype. Bars with the same letter represent no statistical difference, while different letters indicate values that are significantly different. Error bars show standard deviation. Lateral root density was quantified over three replicates with $n \geq 20$.

HY5 regulates the expression pattern of flavonoid pathway genes

To explore the regulation of flavonol biosynthesis by HY5 we analyzed the expression pattern of CHI (TT5) and F3H (TT6) in roots by crossing the transcriptional reporter lines *pTT5:GUS-GFP* and *pTT6:GUS-GFP* with the *hy5* mutant. CHI (TT5) is broadly expressed in leaves and roots (Fig 5A) with a high GUS activity over the entire leaf tissue, following alongside the main root and lateral roots through the transition zone. No GUS signal was detected in the main root meristem of the WT. In the *hy5* background, CHI (TT5) presented a much weaker expression throughout the entire plant. In leaves, the expression was restricted to stomata whereas in roots was restricted to the main root stele and in emerged lateral roots (Fig 5A) This observation suggests that HY5 is a positive regulator of CHI (TT5) expression in leaf mesophyll and root cortical tissues whereas CHI (TT5) expression in lateral roots is not HY5-dependent. The expression pattern of *pTT6:GUS-GFP* also presented more GUS signal restricted to the leaves and root vasculature, which faded through the length of the root to the point there was no signal near the root tip (Fig 5B). Interestingly, *pTT6:GUS-GFP* expression was completely lost in the *hy5* background suggesting that HY5 is an essential positive regulator of F3H (TT6) in the whole seedling (Fig 5B). Also, during the early development of lateral roots it is possible to observe stronger GUS signal in the lateral root primordia (LRP) for both genes, supporting a role for flavonols on LR development (Fig 5A and B, iii). These results indicate that CHI (TT5) and F3H (TT6) expression is dependent on HY5, except for the stomata and lateral root expression of CHI (TT5) which are sustained in the *hy5* mutant.

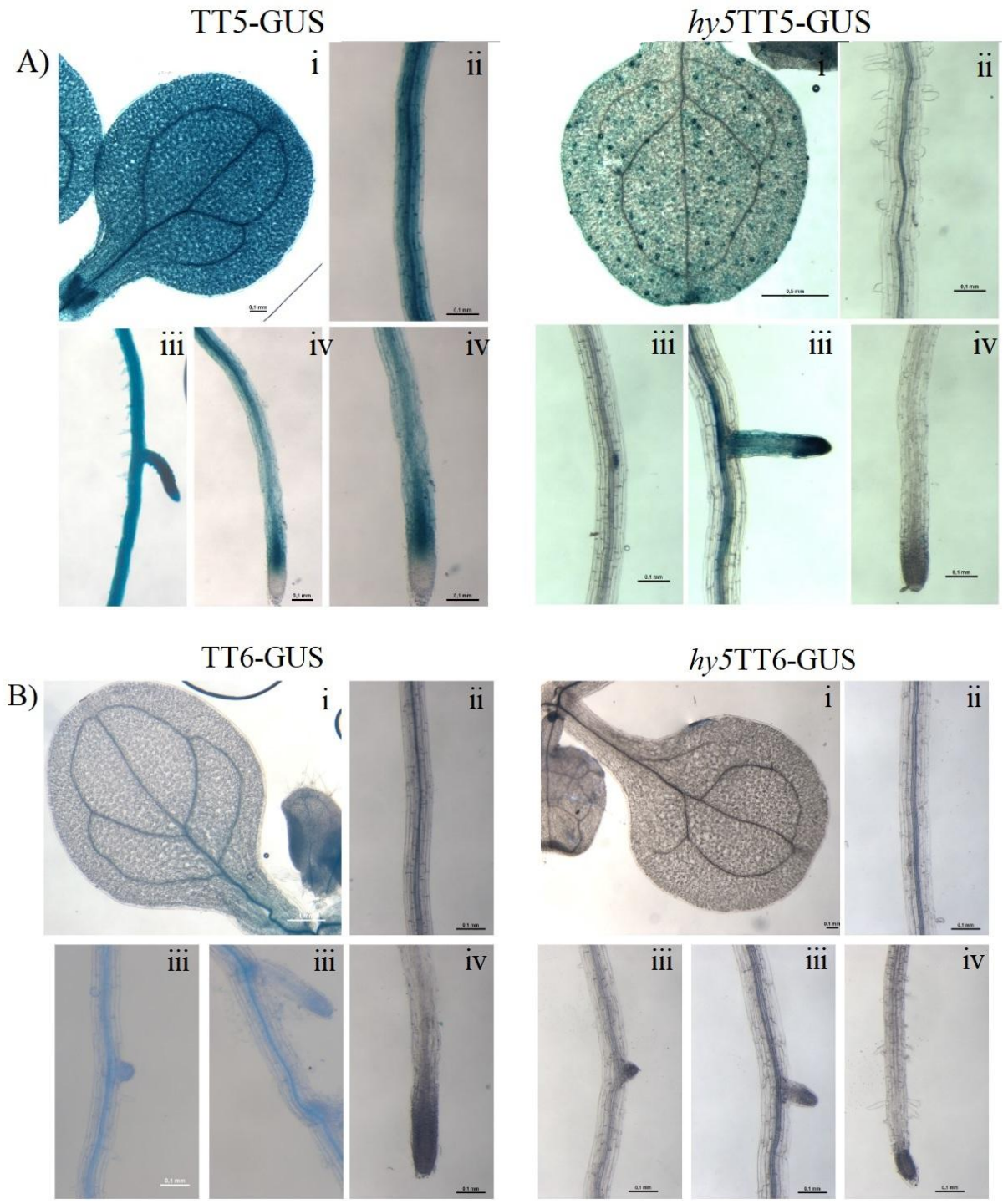

Figure 5: HY5 dependency of *pTT5-GUS-GFP* and *pTT6-GUS-GFP*. GUS staining of transcriptional reporter lines for the flavonol biosynthetic pathway genes CHI (TT5) and F3H (TT6) in the WT background (left panels) and in the *hy5* background (right panels). Plants were cultivated in sucrose-free ½ MS medium for 14 days. Expression of pTT5:GUS (A) and pTT6:GUS (B) in leaf (i), root elongation zone (ii), lateral root (iii) and root meristematic zone (iv), respectively. Scale bars = 100um.

Flavonol pathway molecular network

In order to evaluate the flavonol derivatives that might be responsible for the root phenotypes we observed, we verified the metabolite profiles in roots of the flavonol mutants mentioned above, as well as the wild type genotype (Col-0) using UHPLC/HRMS.

The molecular network comparing the genotypes and treatments is presented in Fig 6. We were able to characterise 10 different putative metabolites assigned to the flavonoid biosynthetic pathway, these metabolites were identified in two spectral families, one containing 6 nodes mainly for the initial portion of the pathway of the flavanone biosynthesis (Fig 6A), containing modified versions of naringenin, namely naringenin-7-O-glucoside (m/z 435.105) and naringenin 7-O-(6-O-malonyl-glucoside) (m/z 521.1), identified in the samples Col-0, *tt5-2* and *tt6-3* treated with exogenous Naringenin. The respective chemical structures are shown in Fig 6C. Another smaller cluster consisting of 4 nodes was identified with derivatives of compounds from the final portion of the pathway (Fig 6B), displaying kaempferol and quercetin conjugated to glycosyl groups in the Col-0 and *tt5* genotypes, curiously *tt6* samples were not present in these nodes. Kaempferol presented two modified structures, kaempferol-3-O-glucoside-7-O-rhamnoside (m/z 595.135) and kaempferol-3-O-robinoside-7-O-rhamnoside (m/z 741.183), contained in the samples Col-0 (mock and naringenin treated) and *tt5-2* treated seedlings. Besides that, another peak was observed at m/z 611.129, identified as glycosylated quercetin (quercetin 3-O-rutinoside - Rutin) and another version with a methoxy group (m/z 625.142 - quercetin 3-O-rutinoside-4-methoxy). These results indicate that the treatment with exogenous naringenin rescued the flavonol metabolic flux in *tt5-2*, as flavonols were detected. Beyond that, we couldn't quantify in *tt6-3* any form of kaempferol or quercetin, which is expected for seedlings in the mock treatment, but even in plants treated with naringenin there were no presence of those flavonols, it may be an indication that naringenin complementation does not work on this genotype, suggesting a metabolic shift in this mutant that prevented the flux towards flavonols.

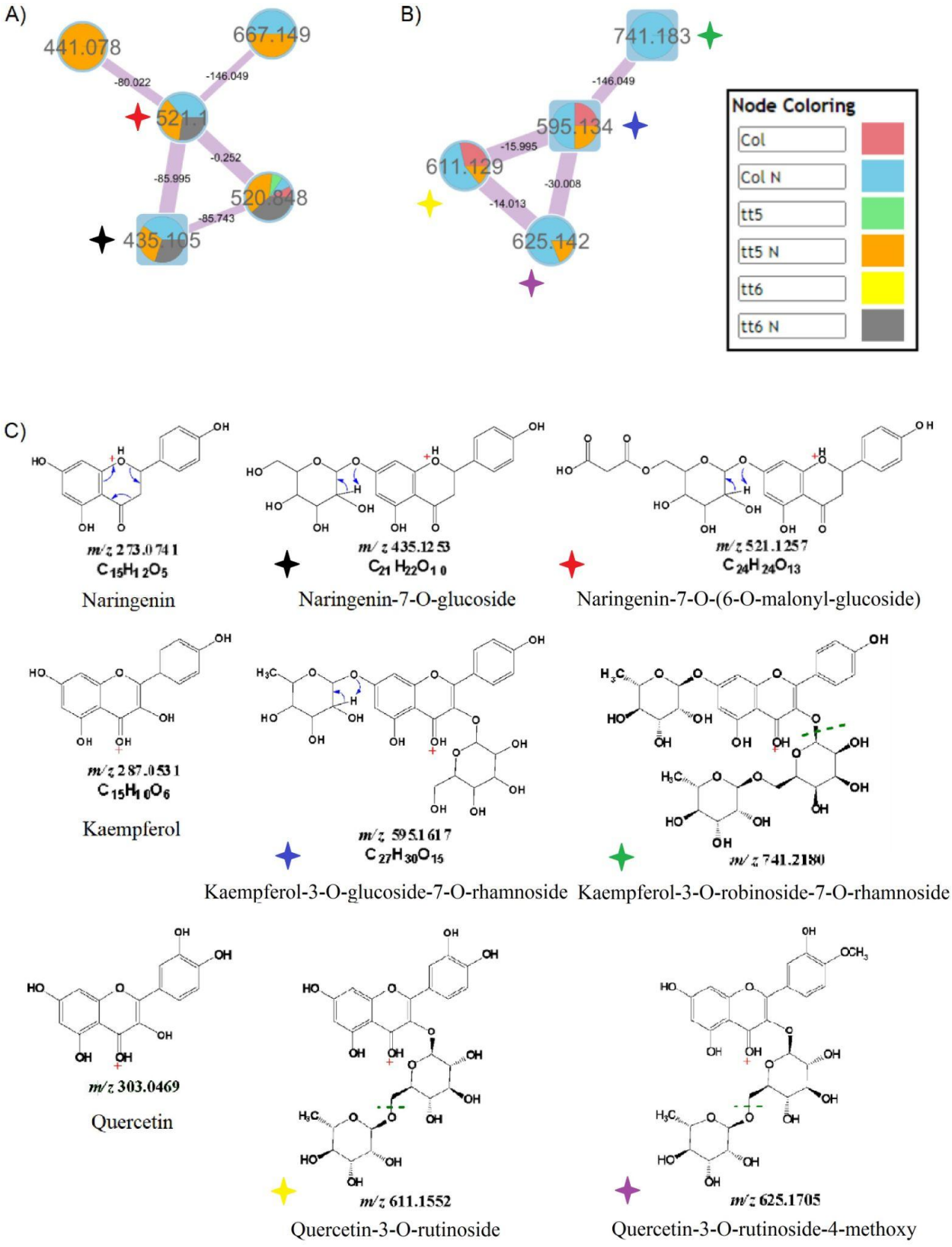

Figure 6: Molecular network of flavonoid metabolites accumulating in Col-0, *tt5* and *tt6*. Alignment and comparison of **(A)** Cluster I depicting naringenin derivatives. **(B)** Cluster II containing the glycosyl conjugated

kaempferol and quercetin. Each colour represents a genotype in control or naringenin treatment. **(C)** Chemical structures of each compound with their respective masses and names assigned. Each node has different colours according to each genotype in both treatments, triplicate files were used for the analysis, the nodes were labelled with the respective group colours and compound masses, the lines contain the mass differences between the connected nodes. Coloured four point stars were added for reader's ease, linking each structure to its respective node.

Discussion

Plants adjust their root architecture to maximise nutrient uptake, influencing directly on the fitness of plants (Muller, B et al., 2019; Santos Teixeira and Tusscher, 2019). Light signals coordinate the shoot demand for water and nutrients to counterbalance the photosynthetic output (Li, H et al., 2021). The light-activated transcription factor HY5 is one of these signals controlling the biosynthesis of flavonols linking shoot-to-root signalling through the modulation of polar auxin transport (Sibout, R et al., 2006; Silva-Navas, J et al., 2016; Chapman and Muday, 2021). Both *hy5* and *tt6-3* mutants present altered root phenotype with increased lateral root formation (Oyama, T et al., 1997; Buer, C et al., 2009). This observation prompted us to elucidate this mechanism through a genetic and chemical approach to determine whether the regulation on lateral roots by HY5 and flavonols act in the same pathway. Moreover, we investigated the flavonoid profile in *tt* mutants in order to evaluate the metabolite responsible for the observed root phenotypes. The experiments presented here suggest that Naringenin (a flavanone), or a downstream flavanone byproduct, positively regulates lateral root formation. Interestingly, a previous work has found that kaempferol acts as a repressor of lateral roots (Chapman and Muday, 2021), evidencing the functional diversity of these metabolites. Here, we evaluated the root phenotype of different mutants, in the *tt5-2* mutant there was a reduction in lateral roots, which was chemically rescued by Naringenin (Fig 3) and genetically by crossing with *HY5ox* (Fig 2 and 3). On the other hand, *tt6-3* originally produced more lateral roots, which was reduced when chemically treated with Naringenin or crossed with overexpressing HY5 transgenic line. This result could indicate that the *tt6* mutation leds to a shift towards another branch of the flavonoid pathway, since Naringenin is a molecular hub serving as a common substrate for a wide range of enzymes, thus, depending on the conditions, different flavanone derivatives can be synthesised (Liu, W et al., 2021; Wang, L et al., 2022). By crossing *tt* mutants with HY5ox in order to increase the metabolic flux, we wanted to evaluate the genetic link between these genes. Our results indicated that overexpressing HY5 had the same effect as Naringenin treatment and the inhibitory effect of HY5 on lateral roots (observed in the HY5ox transgenic line) depends on an intact flavonol biosynthesis pathway (Fig 2). Then, using reporter lines to visualise the expression pattern of flavonols genes also revealed a dependency from flavonols on HY5 activity, as expected of a

transcription factor known to induce the expression of flavonol genes (Bathia, C et al., 2021). CHI (TT5) and F3H (TT6) expression reduced drastically (Fig 5) in the *hy5* mutant, although the expression domains remained unaltered, suggesting that HY5 regulation is limited to modulate expression levels. Despite the tight HY5 regulation over flavonoid gene expression, it is important to highlight that CHI (TT5) expression seems to be more specific to lateral root sites while F3H (TT6) is restricted to the vasculature. It did not escape our attention that naringenin is mobile in plants (Buer, C et al., 2007; Buer, C et al., 2008), which aligns with our observations on TT6-GUS (Fig 5). Moreover, the expression of TT5 on early stages of LR formation is in agreement with previous studies that revealed the localization of some flavonoid genes and showed a kaempferol increase in LRPs to scavenge reactive oxygen species (ROS) and consequently promoting lateral root emergence (Chapman and Muday, 2021). Taken together, these results converge to a mechanism in which flavonols act downstream of HY5 repressing lateral root growth via inhibition of auxin transport (Fig 7).

Lateral roots were suggested to be suppressed by HY5 under FR light, a mechanism which the transcription factor would downregulate the expression of the auxin transporters PIN3 and LAX3 (van Gelderen, K et al., 2018), albeit these transporters are not recognized as HY5 direct target genes. Flavonoids are potent endogenous inhibitors of auxin transport (Brown, D et al., 2001; Teale and Palme, 2018; Teale, W et al., 2021). Therefore, as we found a relationship between HY5 and flavonols, we checked the hypothesis that flavonols would also participate in FR responses. Our findings confirm the previous findings showing that *hy5, pin3* and *lax3* are all insensitive to the low R/FR effect on lateral roots. On the other hand, the *tt* mutants responded to the low R/FR treatment, reducing their lateral root number, implying that flavonoids are not involved in this response in this pathway (Fig 4). Nevertheless, our data support a link between HY5 and auxin transport. Strikingly, we showed that the higher LR density of *hy5* and *tt6-3* was suppressed by the *pin3* and *lax3* mutations, revealing that PIN3 and LAX3 act downstream to HY5 and flavonols in lateral root emergence.

The wide range of flavonoid functions is mainly due to changes made to its core structure by various enzymes, resulting in hydroxylation, methylation, glycosylation, or acylation. These modifications are important for moving the molecules through the plant and storing it in cells, since it was observed that flavonoids are stored as glycosides in the vacuoles (Sugiyama and

Yazaki, 2014; Kuhn, B et al., 2016; Alseekh, S et al., 2020), based on that, increasing evidence has been discovered about the enzymes responsible for carrying out the modifications on flavonoids and the glycosides derivatives as well, gaining insights into the developmental roles they are involved (Jones, P et al., 2003; Böttcher, C et al., 2008; Yonekura-Sakakibara, K et al., 2012; Yin, R et al., 2013; Kuhn, B et al., 2016). However, the exact flavonoid derivative responsible for physiological responses still a puzzle. Our findings in the molecular network (Fig 6) detected mainly naringenin, kaempferol and quercetin alongside their respective glycosylated forms. These modifications were predominantly 7-O-glycosides additions which is in agreement with the most frequent modifications found in Arabidopsis (Graham, T, 1998). We identified two forms of naringenin conjugated to glucose residues (naringenin-7-O-glucoside and naringenin-7-O-glucoside(6-O-malonyl-glucoside)), present in all three treated genotypes. Intringuily, however, is the absence of the *tt6-3* mutant from mock treatment in the network, since the pathway is interrupted on this conversion step and it should accumulate naringenin. Previous studies have quantified flavonols on *tt* mutants, it is interesting that only the intermediary naringenin was not detected either in wild type or *tt6* mutant (Peer, A et al., 2001). This lack of naringenin was attributed to a leaky phenotype on the mutant allele used in this previous work, however the allele we used in this article (SALK_113904C) is not leaky and even so we also could not detect the metabolite on *tt6*. A possible explanation is a partial complementation by another enzyme, like FLAVONOL SYNTHASE (FLS1, functions at later stages), which is a member of the same family, 2-oxoglutarate-dependent dioxygenases. Although inefficiently it was found *in vitro* that these enzymes were able to carry out the F3H conversion (Owens, D et al., 2008). Another explanation, more importantly, would be a metabolic shift towards Eriodictyol production carried out by F3'H (TT7) (Pucker, B et al., 2020). This alteration can lead to a different flavonoid profile. A third reason that could explain this is that *tt6-3* actually has a different flavonol glycosylation profile that may account for the observed changes. It is still unclear if the modifications that flavonols receive have distinct functions, although there is some evidence in the literature. For example, the *rol1-2* mutant displays a modified flavonol glycosylation profile, which causes alterations in auxin transport and results in growth defects in its shoot tissues (Kuhn, B et al., 2016). Apart from that, the root phenotype of *tt5-2* was chemically rescued by the treatment with naringenin (Fig 3), bringing more insights into

flavonoids as developmental modulators.

Nevertheless, the genetic and chemical identification of Naringenin or its derivatives as an endogenous promoter of LR is in agreement with previous studies (Buer and Djordjevic, 2009). In fact, kaempferol-3-o-7-o-rhamnoside and *tt7* (which accumulates high levels of kaempferol) was implicated as an PAT and lateral root inhibitor (Yin, R et al., 2013; Chapman and Muday, 2021) and the mutant *tt6-3* assessed in our article does not produce this derivative, presenting more lateral roots. Regardless of this evidence, the question that remains is whether the *tt6-3* phenotype is a consequence of a kaempferol deficiency or naringenin activity.

Therefore, even though our results from the molecular network do not indicate the existence of a specific intermediary responsible for the root phenotypes in the *tt* mutants, we raised two hypothesis that the whole flavonol profile may be necessary for the correct root system development, as flavonoids are a large family of compounds with various subclasses each possessing influence on different parts of the plants and developmental stages (1), or it is a role from a specific flavonoid, namely naringenin or kaempferol and their derivatives (2). However, because *tt6-3* from neither treatments (mock or naringenin) presented any final flavonol (kaempferol and quercetin) detected, we lean towards the hypothesis of naringenin or a flavanone derivative acting as a lateral root inducer.

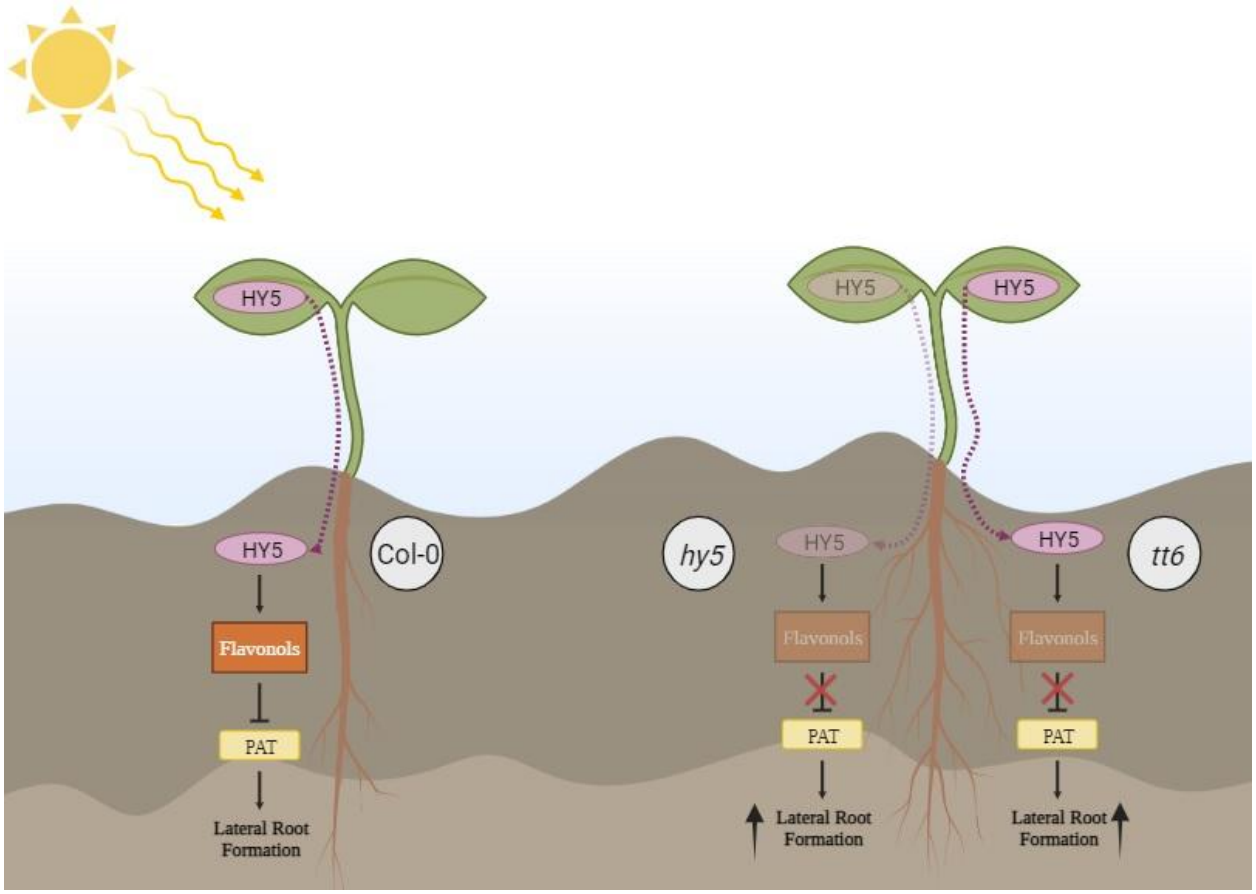

Figure 7: Flavonoids effect on the lateral root regulatory circuit in response to light. Light-dependent stabilisation of HY5 induces flavonols accumulation, which in turn modulates the polar auxin transport in Arabidopsis roots, ultimately, this mechanism inhibits lateral root production. On the other hand, in the absence of HY5 or F3H (TT6), the blockage on PAT is missing either for a lack of gene expression induction promoted by HY5 or for the absence of the repressors flavonols, consequently PAT increases and lateral roots develop and emerge in high numbers. Faded boxes mean absence of protein or metabolites, dashed arrow represents HY5 movement from shoot-to-root. Created in **BioRender.com**

References

Appelhagen, I., Thiedig, K., Nordholt, N. et al. Update on transparent testa mutants from Arabidopsis thaliana: characterisation of new alleles from an isogenic collection. Planta 240, 955–970 (2014). https://doi.org/10.1007/s00425-014-2088-0

Bhatia, C., Gaddam, S. R., Pandey, A., & Trivedi, P. K. (2021). COP1 mediates light-dependent regulation of flavonol biosynthesis through HY5 in Arabidopsis. *Plant Science*, *303*, 110760. https://doi.org/10.1016/j.plantsci.2020.110760

Buer, C. S., Muday, G. K., & Djordjevic, M. A. (2007). Flavonoids Are Differentially Taken Up and Transported Long Distances in Arabidopsis. Plant Physiology, 145(2), 478–490. https://doi.org/10.1104/pp.107.101824

Buer, C. S., Muday, G. K., & Djordjevic, M. A. (2008). Implications of long-distance flavonoid movement inArabidopsis thaliana. Plant Signaling & Behavior, 3(6), 415–417. https://doi.org/10.4161/psb.3.6.5440

Buer, C. S., & Djordjevic, M. A. (2009). Architectural phenotypes in the transparent testa mutants of Arabidopsis thaliana. *Journal of Experimental Botany*, *60*(3), 751–763. https://doi.org/10.1093/jxb/ern323

Burko, Y., Gaillochet, C., Seluzicki, A., Chory, J., & Busch, W. (2020). Local HY5 Activity Mediates Hypocotyl Growth and Shoot-to-Root Communication. *Plant Communications*, *1*(5), 100078. https://doi.org/10.1016/j.xplc.2020.100078

Burko, Y., Seluzicki, A., Zander, M., Pedmale, U. V., Ecker, J. R., & Chory, J. (2020). Chimeric Activators and Repressors Define HY5 Activity and Reveal a Light-Regulated Feedback Mechanism. *The Plant Cell*, *32*(4), 967–983. https://doi.org/10.1105/tpc.19.00772

Chattopadhyay, S., Ang, L., Puente, P., Deng, X., & Wei, N. (1998). Arabidopsis bZIP Protein HY5 Directly Interacts with Light-Responsive Promoters in Mediating Light Control of Gene Expression. *The Plant Cell*, *10*(5), 673–683. https://doi.org/10.1105/tpc.10.5.673

Chen, X., Yao, Q., Gao, X., Jiang, C., Harberd, N. P., & Li, W. (2016). Shoot-to-Root Mobile Transcription Factor HY5 Coordinates Plant Carbon and Nitrogen Acquisition. *Current Biology*, *26*(5), 640–646. https://doi.org/10.1016/j.cub.2015.12.066

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., & Lepiniec, L. (2002). MYB transcription factors in Arabidopsis. *Trends in Plant Science*, *15*(10), 573–581. https://doi.org/10.1016/j.tplants.2010.06.005

Gayomba, S. R., & Muday, G. K. (2020). Flavonols regulate root hair development by modulating accumulation of reactive oxygen species in the root epidermis. *Development*. https://doi.org/10.1242/dev.185819

Gommers, C. M. M., & Monte, E. (2017). Seedling Establishment: A Dimmer Switch-Regulated Process between Dark and Light Signaling. *Plant Physiology*, *176*(2), 1061–1074. https://doi.org/10.1104/pp.17.01460

Holm, M., Ma, L., Qu, L., & Deng, X. W. (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. *Genes & Development*, *16*(10), 1247–1259. https://doi.org/10.1101/gad.969702

Lee, J. E., He, K., Stolc, V., Lee, H., Figueroa, P., Gao, Y., Tongprasit, W., Zhao, H., Lee, I., & Deng, X. W. (2007). Analysis of Transcription Factor HY5 Genomic Binding Sites Revealed Its Hierarchical Role in Light Regulation of Development. *The Plant Cell*, *19*(3), 731–749. https://doi.org/10.1105/tpc.106.047688

Maraschin, F. D. S. (2020). *Efeitos da sinalização dependente de luz no desenvolvimento de raízes em Arabidopsis thaliana*. https://lume.ufrgs.br/handle/10183/212108

Miotto, Y. E., Da Costa, C. T., De Oliveira, B. H. N., Guzman, F., Margis, R., De Almeida, R. M., Offringa, R., & Maraschin, F. D. S. (2019). Identification of root transcriptional responses to shoot illumination in Arabidopsis thaliana. *Plant Molecular Biology*, *101*(4–5), 487–498. https://doi.org/10.1007/s11103-019-00918-7

Möglich, A., Yang, X., Ayers, R. A., & Moffat, K. (2010). Structure and Function of Plant Photoreceptors. *Annual Review of Plant Biology*, *61*(1), 21–47. https://doi.org/10.1146/annurev-arplant-042809-112259

Pucker, B., Reiher, F., & Schilbert, H. M. (2020). Automatic Identification of Players in the Flavonoid Biosynthesis with Application on the Biomedicinal Plant Croton tiglium. Plants, 9(9), 1103. https://doi.org/10.3390/plants9091103

Shin, J., Park, E., & Choi, G. (2007). PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene

promoters in Arabidopsis. *Plant Journal*, *49*(6), 981–994. https://doi.org/10.1111/j.1365-313x.2006.03021.x

Silva-Navas, J., Moreno-Risueno, M. A., Manzano, C., Pallero-Baena, M., Navarro-Neila, S., Téllez-Robledo, B., García-Mina, J. M., Baigorri, R., Gallego, F., & Del Pozo, J. C. (2015). D-Root: a system for cultivating plants with the roots in darkness or under different light conditions. *Plant Journal*, *84*(1), 244–255. https://doi.org/10.1111/tpj.12998

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., & Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. *Plant Journal*, *50*(4), 660–677. https://doi.org/10.1111/j.1365-313x.2007.03078.x

Teale, W. H., & Palme, K. (2018). Naphthylphthalamic acid and the mechanism of polar auxin transport. Journal of Experimental Botany, 69(2), 303–312. https://doi.org/10.1093/jxb/erx323

Teale, W. H., Pasternak, T., Bosco, C. D., Dovzhenko, A., Kratzat, K., Bildl, W., Schwörer, M., Falk, T., Ruperti, B., Schaefer, J. V., Shahriari, M., Pilgermayer, L., Li, X., Lübben, F., Plückthun, A., Schulte, U., & Palme, K. (2021). Flavonol‐mediated stabilization of PIN efflux complexes regulates polar auxin transport. The EMBO Journal, 40(1). https://doi.org/10.15252/embj.2020104416

Van Gelderen, K., Kang, C., Paalman, R., Keuskamp, D. H. G., Hayes, S. M., & Pierik, R. (2018). Far-Red Light Detection in the Shoot Regulates Lateral Root Development through the HY5 Transcription Factor. *The Plant Cell*, *30*(1), 101–116. https://doi.org/10.1105/tpc.17.00771

Zhang, Y., Wang, C., Xu, H., Shi, X., Zhen, W., Hu, Z., Huang, J., Zheng, Y., Huang, P., Zhang, K., Xiao, X., Hao, X., Wang, X., Zhou, C., Wang, G., Li, C., & Zheng, L. (2019). HY5 Contributes to Light-Regulated Root System Architecture Under a Root-Covered Culture System. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.01490

Zhi, C., Testerink, C., & Zhang, Y. (2021). How roots and shoots communicate through stressful times. Trends in Plant Science, 26(9), 940–952. https://doi.org/10.1016/j.tplants.2021.03.005

Supplementary Information

Supplementary table is available at:

Supplementary Table 1

Capítulo II

- Flavonols role on the root apical meristem zoning of *Arabidopsis thaliana* -

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Flavonols role on the root apical meristem zoning of Arabidopsis thaliana

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Abstract

The architecture of the root system is capable of adapting to its environment by modifying its development. The early photomorphogenic development of the root is dependent on light signals activated in the shoot. The root apical meristem (RAM) is the site where cell proliferation occurs, from this site forward cells elongate and differentiate, moving away from the quiescent centre (QC). Genes from the flavonol biosynthesis pathway are induced by light in roots and the expression domain of these genes is established early during root development, displaying a clear boundary in the transition zone (TZ) towards the elongation zone. The TZ separates the RAM from the elongation zone and its positioning is defined by an auxin minimum achieved through CK signalling. We aimed to evaluate whether the spatial distribution of the expression of the flavonoid pathway genes is an important event for the establishment of root photomorphogenic growth. By treating different tt mutants with CK in order to evaluate root and RAM length we checked the role of flavonols on the RAM formation. Our findings suggest that the effect of CK is dependent on an intact flavonoid pathway, and CK affects the distribution of root flavonoids, which in turn, controls the correct balance of the auxin/cytokinin responses in the root meristem.

Introduction

The root system architecture is capable of adjusting to the environment, modifying primary root growth. The balance between cell proliferation and elongation determines the growth of the root, with the root apical meristem (RAM) being the location where cell proliferation occurs (Giehl, R et al., 2014). As cells leave the meristematic zone by proliferation, moving away from the quiescent centre (QC), eventually they begin to differentiate and elongate (Perilli, S et al., 2012; Scheres, B et al., 2002; Bennett and Scheres, 2010). Root development depends on the interaction between auxin and cytokinin (CK) phytohormones. These phytohormones interact to coordinate root growth, one of the main roles of cytokinin is to inhibit cell proliferation while auxin induces divisions in the RAM. Mutations in genes related to auxin production, transport, and signalling affect cytokinin responses, which supports a model where the two hormones interact to control root growth (De Rybel, B et al., 2014; Dello Ioio, R et al., 2008; El-Showk, S et al., 2013; Schaller, G et al., 2015).

The cytokinin-responsive transcription factor ARR1 (*ARABIDOPSIS RESPONSE REGULATOR 1*) triggers the expression of *Aux/IAA3* (SHY2, SHORT HYPOCOTYL 2) that suppresses auxin transport in the root meristem. This suppression negatively regulates both transcriptionally and post-transcriptionally, genes of the PIN-FORMED (PIN) auxin transporters, causing cytokinin to stimulate cell differentiation by altering auxin distribution (Marhavý, P et al., 2011; Ruzicka, K et al., 2009; Zhang, W et al., 2011). Conversely, auxin promotes SHY2 protein degradation, which maintains auxin transporter activity and meristem cell divisions and induces the expression of GH3.17 to regulate auxin catabolism in the external root tissues.

Mobile signalling molecules are essential for defining cell identity zones in different organs. Auxin serves as one of these molecules, interacting with cytokinin signalling in Arabidopsis roots to position the transition zone (TZ), a region above the meristematic zone responsible for separating high proliferative cells from maturing cells in the elongation zone (Dello Ioio, R et al., 2008). This boundary is formed by an auxin minimum that depends on cytokinin signalling (Di Mambro, R et al., 2017; Salvi, E et al., 2020). Moreover, SHY2 expression was found to be highly induced by light (Miotto, Y et al., 2019). The *shy2-2* mutant, which expresses a degradation-resistant version of SHY2 protein (Colón-Carmona, A et al., 2000), is considered a

gain-of-function mutant and results in shorter roots (Li, T et al., 2020).

The genes from enzymes of the flavonol biosynthesis pathway are highly light-induced in roots (Miotto, Y et al., 2021) and the expression domain of these genes is established early during root development and display a clear boundary in the TZ towards the elongation zone (Lewis, D et al., 2011; Peer, W et al., 2001; Kuhn, B.M et al., 2011). The highly specific light inducibility of the flavonoid pathway genes in the roots and the striking overlap of their expression domains with the transition zone in the root meristem indicate a possible link between flavonols and cytokining-dependent root zoning (Silva-Navas, J et al., 2016).

In this report, we aimed to investigate if the light-mediated activation of root growth is dependent on flavonoids for the correct positioning of the TZ in Arabidopsis. Our findings suggest that cytokinin effect is dependent on an intact flavonoid pathway and this phytohormone affects the distribution of root flavonoids which, in turn, controls the correct balance of the auxin/cytokinin responses in the root meristem.

Materials and Methods

Plant material and growth conditions

Arabidopsis Columbia (Col-0) was used as wild-type (WT), and the mutants *tt4-15* (*GK-545D04*), *tt5-2* (GK-176H03), *tt6-3* (SALK_113904C), *fls1* (*SALK_009992*), *shy2-2* (Tian Q. et al., 2002) are in Col-0 ecotype background and were obtained from the The European Arabidopsis Stock Centre (NASC, http://arabidopsis.info/).

The reporter lines *pTT5:GUS-GFP* (TT5-GUS), *pTT6:GUS-GFP* (TT6-GUS) and *pTT7:GUS-GFP* (TT7-GUS) were generated previously by Miotto, Y., 2019 - CAPIV and the translation reporter line pFLS1:FLS1-GFP (FLS1-GFP) was courteously shared by (Kuhn, B et al., 2011). Petri dishes were kept vertically and grown under long days photoperiod (21 °C ± 3 °C, 16 hour light and 8-hour dark).

Root phenotyping

For measuring primary root elongation, plants were cultivated vertically in square petri dishes in LD light conditions (only shoots illuminated, D-Root system) and pictures were taken 14 days after germination. Root length and root apical meristem were measured using ImageJ (Fiji) (1.54v) setting the scale for millimetres or micrometres respectively, root meristem length was measured as the distance between the quiescent centre and the first elongating cortical cells.

Cytokinin treatment for root meristem measurement

For cytokinin treatment, a stock solution of 10 mM 6-benzylaminopurine (BAP, Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and it was diluted in the medium for a final concentration of 0.1 µM. Plants were first cultivated in sucrose-free ½ MS for 4 days, afterwards seedlings were transferred either to ½ MS supplemented with BAP or just to a new ½ MS plate (mock) and growth was resumed for the indicated time.

Flavonol staining and imaging

Flavonol accumulation was measured by staining with diphenylboric acid 2-aminoethyl ester (DPBA). A staining solution with DPBA, 0.25 % (w/v), 20 % (v/v) ethanol, 0.01 % (v/v) Triton X-100) was used for staining. DPBA staining was performed as described previously (Johnatan Vilasboa, 2022). Seven-days-old seedlings were imaged with a Leica DMR HC microscope with a Zeiss Axiocam HRc digital camera equipped with epifluorescence (excitation filter 450–490 nm) using the image capture program Axiovision SE 64 v. 4.9.1.

GUS Staining and imaging 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 K3Fe(CN)6 , 1 mM K4Fe(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 95% (v/v) ethanol at room temperature before imaging with a Leica M165 FC stereomicroscope with a Leica DFC 500 digital camera using the program LAS version 4.1. For cropping and organising images, Microsoft PowerPoint and CorelDRAW Graphics Suite 2020 softwares were used.

Quantification of auxin and its derivatives

Results

Cytokinin signalling depends on a functional flavonol biosynthesis pathway

The expression of flavonol biosynthesis genes, like Chalcone Synthase (CHS, TT4) and Flavonol Synthase1 (FLS1), is induced by light in Arabidopsis roots. This process is dependent on cytokinin which promotes flavonol accumulation in the transition zone (Silva-Navas, J et al., 2016). The flavonol accumulation in this region coincides with the border dividing the meristematic zone from the elongation zone. The separation between root zones and maintenance of the transition zone depends on an equilibrium formed by an auxin minimum and cytokinin signalling (Di Mambro, R et al., 2017; Salvi, E et al., 2020). Therefore, we aimed to evaluate the correlation of flavonol accumulation and transition zone establishment. We measured root length and root apical meristem length in response to exogenous cytokinin 6-benzylaminopurine (BAP) treatment in seedlings mutant for the flavonol biosynthetic pathway (*tt4-11, tt5-2, tt6-3* and *fls1)*, a gain-of-function mutant (*shy2-2*) presenting a constitutive response to cytokinin and compared them to wild type (Col-0). The wild type and *fls1* plants displayed a reduction in their root length in response to BAP while *tt4-11, tt5-2 and tt6-3* mutants were partially insensitive to BAP treatment (Fig 1A), whereas the *shy2-2* mutant showed the strongest root length inhibition in BAP (Fig 1A). All the mutant meristems were smaller in size than Col-0 even in the control condition. BAP treatment led to a reduction in meristem size in Col-0 whereas the flavonoid mutants were insensitive to cytokinin (Fig 1B). Comparing the relative cytokinin responses within each genotype we observed that *tt4-11* displayed the weakest response, reducing the root length in BAP treatment by -11.9%, whilst *shy2-2* displayed the greatest reduction of -38%. As for *tt5-2, tt6-3* and *fls1*, displayed similar results to Col-0 with reductions of -24% *tt5-2* and *tt6-3*, -29.73% for *fls1* and -26% for Col-0 (Fig 1C). Concerning the relative response in the meristem size, *tt5-2* and *tt6-3* had a smaller reduction in presence of cytokinin, while the other genotypes presented a reduction similar to Col-0 (Fig 1D). These results suggest that cytokinin sensitivity of the primary roots depends on an intact flavonoid pathway leading to a reduction of meristem size.

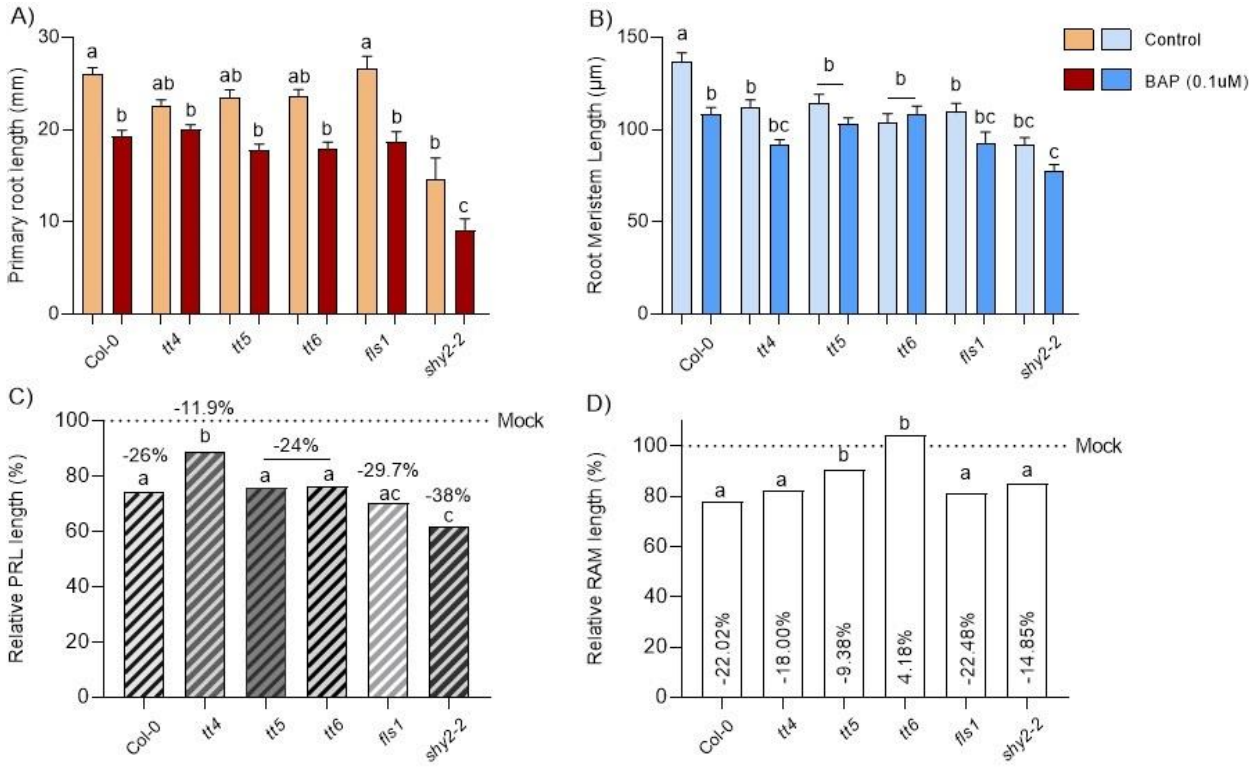

Figure 1: Root elongation and meristem size maintenance by cytokinin seem to need a functional flavonol biosynthesis pathway. Measurements on primary root length (PRL) **(A)**, root meristem (RAM) length (B), relative reduction of PRL **(C)** and meristem size **(D)** relative to control condition (Mock). Wild type (Col-0) plants and mutants (*tt4, tt5-2, tt6-3, fls1, shy2-2*) were cultivated in ½ MS medium supplemented with 0.1µM BAP. Plants were grown in ½ MS for 4 days, then transferred to a medium containing the hormone, measurements were taken on the 7th day. Statistical significance was determined by Kruskal–Wallis test with Dunn's post-test (*$p \leq 0.05$), the means were compared in the same light condition (100 µmol $m^{-2}s^{-1}$) against the wild-type. * indicates statistical significance compared to the wild type control condition (Col-0, MS) and • indicates statistical difference between control and BAP supplementation. Error bars show standard deviation.

Cytokinin influences flavonol accumulation in Arabidopsis roots meristematic zone

In order to evaluate the tissue localization of flavonols in the seedlings roots loss of function mutants from the flavonol biosynthetic pathway were treated with BAP and the flavonoid localization was evaluated in the seedlings by DPBA staining. BAP-treated Col-0 seedlings presented a stronger DPBA fluorescence in the root meristem, while *tt6-3* and *fls1* did not seem to respond to the CK treatment (Fig 2A and B). In agreement with its role as an activator of cytokinin response (Tian, Q et al., 2002), the *shy2-2* gain-of-function mutant shows constitutive higher accumulation of flavonoids in the root. Surprisingly, besides increasing the total DPBA fluorescence signal, the cytokinin treatment altered the accumulation localization of the flavonoids in the root. Normally, the DPBA signal is lower in the central meristematic zone (Fig

2A, Col-0), but in the BAP treatment we visualised an increased signal invading the central root meristem (Fig 2B). Interestingly, *tt6* and *fls1* showed an evenly distributed signal also in the control condition, suggesting a possible regulation of flavonol transport in roots by cytokinin. These observations support that cytokinins promote flavonol accumulation in roots and suggest that they increase in flavonol transport and distribution.

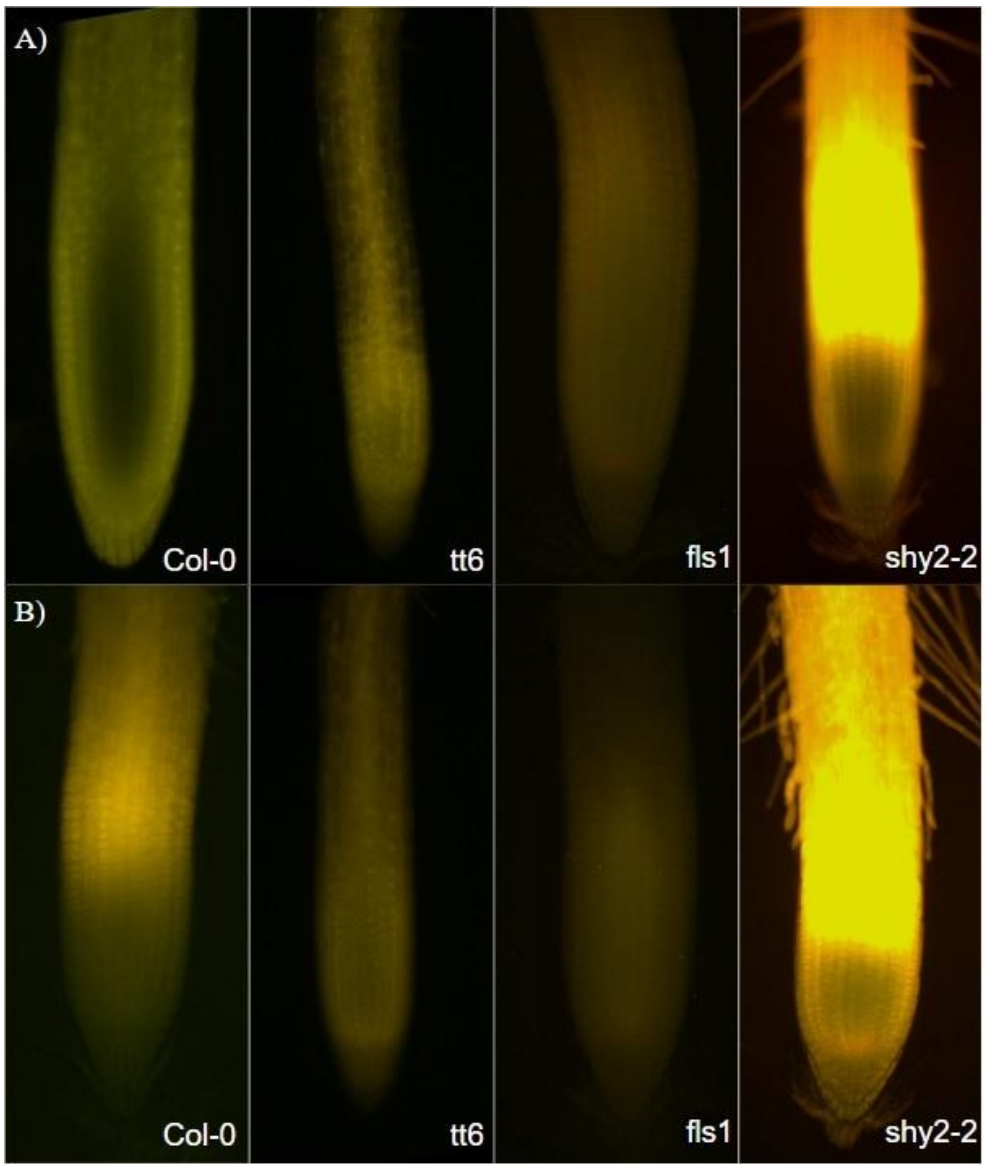

Figure 2: Cytokinin promotes flavonol accumulation in the root meristem. DPBA staining of 7 days-old grown roots from seedlings *Col-0, tt6, fls1 and shy2-2* grown in control condition (½ MS) **(A)** or supplemented with 0.1uM BAP **(B)**. Seedlings were transferred to BAP containing medium on the 4 DAG. Statistics were calculated using one-way ANOVA with Tukey's multiple comparisons test (p-value <0.0001, n=15). Different letters represent statistical significance between groups.

Expression of flavonol pathway genes is induced by cytokinin.

The increased flavonol accumulation induced by CKs in roots led us to evaluate the effect of BAP treatment on the transcription of the genes of the flavonol biosynthesis pathway. The transcriptional reporter lines *pTT5:GUS-GFP, pTT6:-GUS-GFP* and *pTT7:GUS-GFP*, as well as the translational reporter line *pFLS:FLS1-GFP* were exposed to exogenous CK treatment. Fig 3A shows representative images of *pTT5:GUS-GFP* and *pTT6:-GUS-GFP*. The *pTT5:GUS-GFP* expression was broadly spread through the whole seedling (Fig 3A, i) in the control condition, showing a slightly stronger GUS signal in the shoots' vasculature while in roots the signal faded away along the elongation zone and intensified again in the transition zone (Fig 3A, ii and iii). BAP treatment led to a quantitative increase of *pTT5:GUS-GFP* expression in the whole seedling and we did notice a spatial alteration in TT5 expression, as it shifted into the meristem (Fig 3B, i-iii). As for *pTT6:-GUS-GFP*, the signal was restricted to the leaf vasculature and root stele (Fig 3C, i-iii) displaying a weaker overall expression which was insensitive to BAP treatment (Fig 3D, i-iii). Curiously, *pTT6:-GUS-GFP* expression was reduced towards the root, the signal weakened in the elongation zone and completely disappeared near the root apical meristem (Fig 3C and D, iii).

In order to have a more detailed visualisation of the reporter lines expression under cytokinin effect, we evaluated GFP fluorescence via confocal microscopy. Fig 3E shows a cytokinin-dependent increase in *pTT5:GUS-GFP* and *pTT7:GUS-GFP* expression in the transition zone, while *pFLS:FLS1-GFP* seedlings did not respond to the treatment. Ultimately, these results indicate that cytokinin levels might be important for the controlled expression and localization of flavonol accumulation in Arabidopsis roots for maintenance of meristem and transition zones.

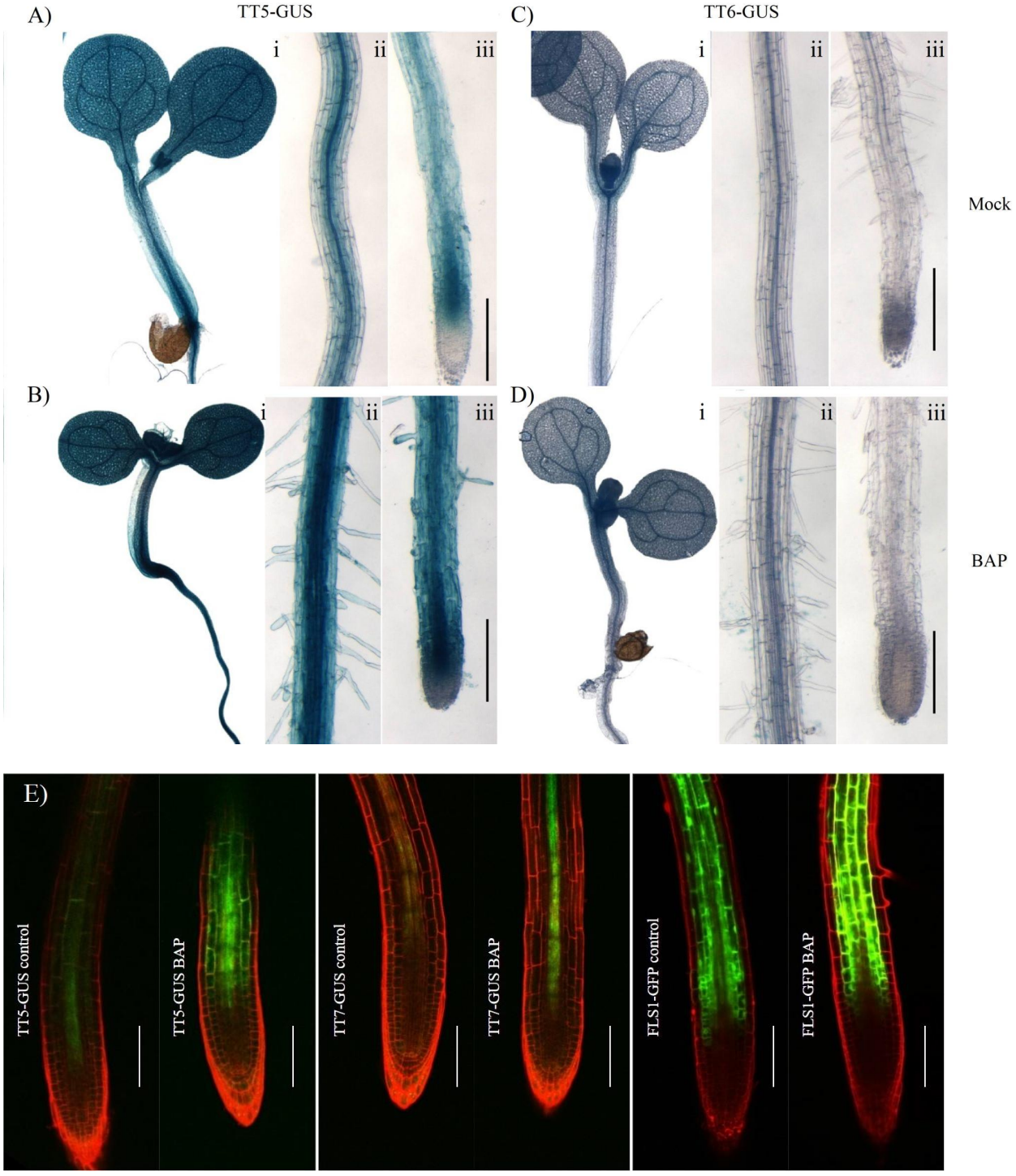

Figure 3: Expression of flavonol genes is stimulated by BAP treatment. Expression pattern of *pTT5:GUS-GFP* in control condition **(A)** and cytokinin treatment **(B)**. *pTT6:GUS-GFP* in control **(C)** and cytokinin treatment **(D)**. **(i)** whole imaging on shoot and initial portion of root, **(ii)** elongation zone, **(iii)** root tip. 7 days-old grown seedlings cultivated in ½ MS (control) and ½ MS containing 0.1µM BAP. Scale bar represents 200um. **(E)** Confocal microscopy images of 6 days-old roots carrying the *pTT5:GUS-GFP, pTT7:GUS-GFP* and *pFLS1:FLS1-GFP* constructs. Green and red fluorescence represent GFP expression and propidium iodide staining respectively. Scale bars = 100um.

Absence of Flavonoids Enhances Auxin Response in Root Development

Flavonols are known to act as inhibitors of the polar auxin transport, regulating root development (Brown, D et al., 2001; Silva-Navas, J et al., 2016; Teale and Palme, 2017; Teale, W et al., 2021). The interplay between auxin and cytokinin-signalling in the root meristem zonation led us to quantify the levels of auxin and its precursors and conjugates in Col-0, *tt5-2* and *tt6-3*. All three genotypes presented similar concentrations for the majority of the compounds analyzed, including IAA (Fig4A and Supplementary Fig. 1). The *tt6-3* mutant did not show any statistical difference of the metabolites quantified (Fig 4A-C). Interestingly, the *tt5-2* mutant presented a higher accumulation of the IAA conjugated forms IAA-glutamate (IAA-Glu) and IAA-glucose (IAA-Glc) (Fig4B and C). Coincidentally, IAA-Glu is the product of IAA inactivation by GH3.17 which is a main suppressor of auxin levels in the RAM (Di Mambro, R et al., 2017) directly activated by CK signals. This result implies that CHI (TT5) might participate in the spatial distribution of IAA inactivation in the RAM mediated by CK.

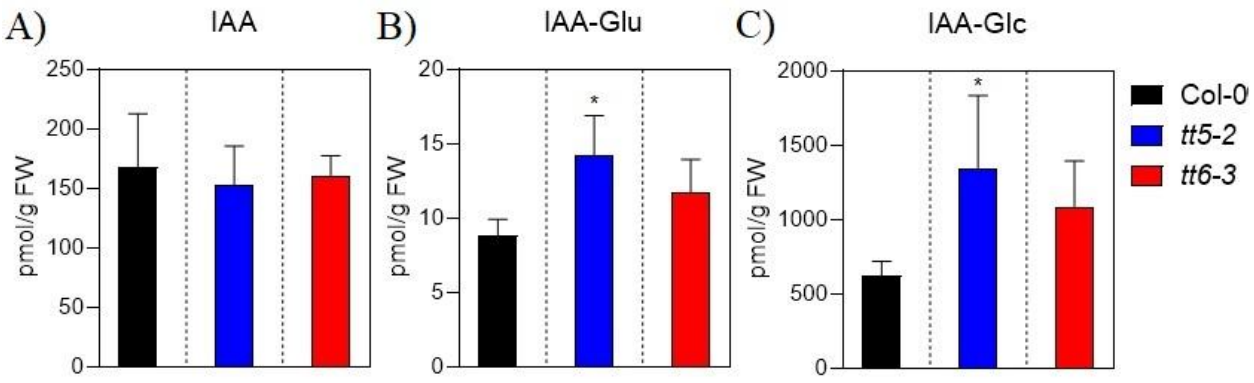

Figure 4: *tt5* affects levels of auxin conjugates. Quantification of IAA, IAA-Glu and IAA-Glc is shown for WT (Col-0, black column), *tt5-2* (blue column), and *tt6-3* (red column), grown in dark root conditions (LD) for 7 days, FW, fresh weight. Statistically significant difference (one-way ANOVA) with $p<0,05$. * indicates statistical significance groups between genotypes.

Discussion

In this work, we searched for a link between flavonol-mediated responses in the root zonation and its correlation to CK signals. By screening the root phenotypes of mutants of the flavonoid pathway we uncovered a plausible link between the flavonoids and the establishment of the RAM. The data presented here shows that flavonol-deficient mutants' roots were resistant to CK treatment, indicating the requirement of an intact pathway for a correct CK response. Flavonol accumulation in roots is induced in response to CK (Silva-Navas, J et al., 2016), since mutants defective in CK receptors do not present any flavonol accumulation in the RAM. Furthermore, it is known that treatment with exogenous auxin promotes flavonol accumulation in roots, mainly by the activity of the transcription factor WRKY23. In other words, if the root meristem depends on an auxin/CK equilibrium, flavonols are controlled by both phytohormones (Grunewald, W et al., 2012). Therefore, we analysed the flavonol accumulation on *tt* mutants treated with CK (Fig 2), our findings show no difference on accumulation intensity for *tt6-3* and *fls1* mutants, but surprisingly, we observed an altered distribution of these compounds even in the control condition, penetrating the root meristem which normally lacks flavonols. It is important to note that *fls1* has a mutation on the flavonol synthase (FLS) enzyme of the pathway, and consequently, it does not produce any flavonol, the pathway is blocked on the dihydroflavonol conversion, as for *tt6-3*, it was expected low fluorescence signal from naringenin (Buer and Djordjevic, 2009). Additionally, *shy2-2* presented the strongest responses in our analysis, supporting the hypothesis that flavonols take part in the CK-signalling, these observations are in agreement with previous works as *shy2-2* has the cytokinin signalling constitutively induced (Silva-Navas, J et al., 2016).

These results give insights into a spatial regulation of CK over flavonols. Next, we showed the gene expression spatial localization using GUS reporter lines. Under CK application, TT5-GUS had a stronger expression throughout the whole plant and it seems to penetrate to some extent the root meristem, while TT6-GUS did not respond to the application (Fig 3), which is in agreement with our previous results (Fig 2). An interesting detail is the fading signal along the root length suggesting that F3H might be absent and the biosynthetic pathway incomplete near the root tip (Fig 3C and D, iii). Prior studies showed that other flavonol enzymes are not expressed in all root cells, for example CHS expression was limited to lateral

root primordia, this indicates that precursors have to move along the plant (Chapman and Muday, 2021). Consistent with this idea, there is evidence demonstrating that naringenin is one of these mobile precursors (Buer, C et al., 2007; Buer, C et al., 2008). Therefore, despite the expression of flavonol enzymes not covering the entire root tissues, the precursors compensate by moving to the sites where they can be further converted. Thus, the TT6-GUS expression pattern is in agreement with these findings.

One key aspect of auxin's activity is the establishment and maintenance of auxin gradients, which are necessary for proper plant growth and development. Auxin gradients refer to the variation of auxin concentration within different regions of the plant, which create distinct zones of high and low auxin concentration, known as auxin maxima and minima, respectively (Pěnčík, A et al., 2013). In plants, only a small fraction of the synthesised auxin pool remains in its free form (Ludwig-Müller, J et al., 2011). Recently, IAA-Asp and IAA-Glu were suggested to act as storage conjugates and have different roles in auxin homeostasis (Hayashi, K et al., 2021). Beyond that, flavonols are important inhibitors of polar auxin transport, recent evidence has shown that flavonols stabilise PIN complexes by interacting with PIN1 and reducing their capacity to mediate cellular auxin efflux (Teale, W et al., 2021). Here, we have shown that the lack of flavonols in *tt5-2* leads to the increase in the conjugates IAA-Glu and IAA-Glc. IAA-Glc presented the highest concentration among all auxin forms (Fig 5), which is in agreement with this conjugate storage role (Dzievit, K et al., 2021; Hayashi, K et al., 2021). Furthermore, in *tt4* mutants it was also observed an increase in polar auxin transport (Peer, W et al., 2004). We conclude that in the absence of flavonols the polar auxin transport is maximised and consequently more auxin is converted to its storage forms.

In summary, this study has brought insights into the regulatory network involving CK, auxin and flavonols. Based on that, we propose a model for this mechanism (Fig 5). Our data points to a scenario where flavonols are induced by CK through SHY2 and/or another mechanism, which can enhance PINs inhibition by SHY2 modulating auxin transport in the meristem. Furthermore, flavonols can also target auxin to conjugation inactivating the phytohormone even more. Ultimately, both CK and flavonols gradients in the root are essential for establishing the auxin minimum in the correct position.

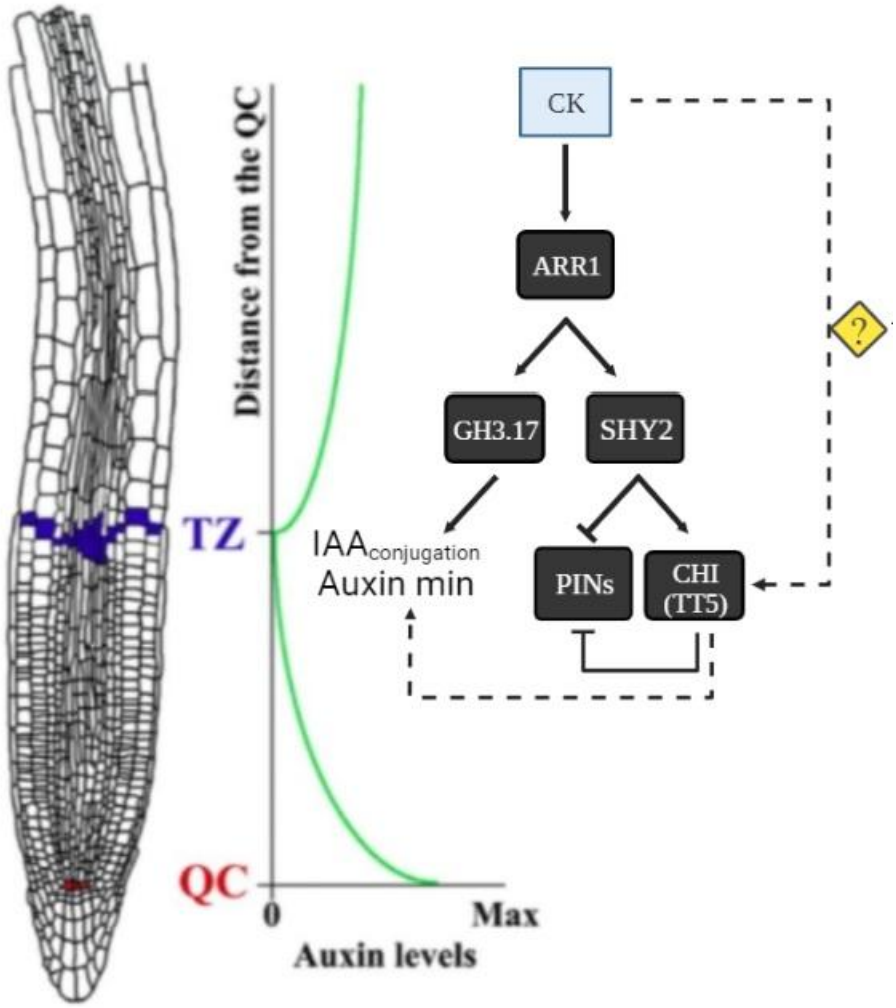

Figure 5: CHI (TT5) role on auxin-cytokinin interplay in root zoning. SHY2 is induced by cytokinin through the activity of ARR1. PIN efflux carriers are inhibited by SHY2 regulating negatively the polar auxin transport in the transition zone, which ensures a maximum auxin level in the root meristem. CHI seems to participate in this regulatory system, CK induces CHI expression through an unknown mechanism. SHY2 also was found to regulate CHI positively. In this model PINs would be further inhibited through SHY2/CK-induced CHI. The expression domain of CHI supports this hypothesis. Also, CHI seems to participate in auxin homeostasis, targeting the hormone to conjugation, whether it is stored or degraded remains unknown.

References

Bennett, T., & Scheres, B. (2010). Root development—two meristems for the price of one? Current Topics in Developmental Biology, 91, 67-102. https://doi.org/10.1016/S0070-2153(10)91003-X

Bizet, F., Hummel, I., & Bogeat-Triboulot, M. (2015). Length and activity of the root apical meristem revealed in vivo by infrared imaging. Journal of Experimental Botany, 66(5), 1387–1395. https://doi.org/10.1093/jxb/eru488

Colón-Carmona, A., Chen, D. L., Yeh, K., & Abel, S. (2000). Aux/IAA Proteins Are Phosphorylated by Phytochrome in Vitro. Plant Physiology, 124(4), 1728–1738. https://doi.org/10.1104/pp.124.4.1728

De Rybel, B., Adibi, M., Breda, A. S., Wendrich, J. R., Smit, M. E., Novák, O., Yamaguchi, N., Yoshida, S., Van Isterdael, G., ..., & Weijers, D. (2014). Integration of growth and patterning during vascular tissue formation in Arabidopsis. Science, 345(6197), 1255215. https://doi.org/10.1126/science.1255215

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M. T., Aoyama, T., Costantino, P., & Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science, 322(5906), 1380-1384. https://doi.org/10.1126/science.1164147

Di Mambro, R., De Ruvo, M., Pacifici, E., Salvi, E., Li, M., Benfey, P. N., Busch, W., Novák, O., Ljung, K., Di Paola, L., Marée, A. F. M., Costantino, P., Grieneisen, V. A., & Sabatini, S. (2017). Auxin minimum triggers the developmental switch from cell division to cell differentiation in the Arabidopsis root. Proceedings of the National Academy of Sciences of the United States of America, 114(36). https://doi.org/10.1073/pnas.1705833114

El-Showk, S., Ruonala, R., & Helariutta, Y. (2013). Crossing paths: cytokinin signalling and crosstalk. Development, 140(7), 1373-1383. https://doi.org/10.1242/dev.086371

Kuhn, B., Geisler, M., Bigler, L., & Ringli, C. (2011). Flavonols Accumulate Asymmetrically and Affect Auxin Transport in Arabidopsis. Plant Physiology, 156(2), 585–595. https://doi.org/10.1104/pp.111.175976

Lewis, D., Ramirez, M. V., Miller, N. H., Vallabhaneni, P., Ray, W. K., Helm, R. F., Winkel, B. S., & Muday, G. K. (2011). Auxin and Ethylene Induce Flavonol Accumulation through Distinct

Transcriptional Networks. Plant Physiology, 156(1), 144–164. https://doi.org/10.1104/pp.111.172502

Li, T., Kang, X., Lei, W., Yao, X., Zou, L., Zhang, D., & Lin, H. (2020). SHY2 as a node in the regulation of root meristem development by auxin, brassinosteroids, and cytokinin. Journal of Integrative Plant Biology, 62(10), 1500–1517. https://doi.org/10.1111/jipb.12931

Marhavý, P., Bielach, A., Abas, L., Abuzeineh, A., Duclercq, J., Tanaka, H., Pařezová, M., Petrášek, J., Friml, J., Kleine-Vehn, J., & Benková, E. (2011). Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis. Developmental Cell, 21(4), 796–804. https://doi.org/10.1016/j.devcel.2011.08.014

Miotto, Y. E., Da Costa, C. T., De Oliveira, B. H. N., Guzman, F., Margis, R., De Almeida, R. M., Offringa, R., & Maraschin, F. D. S. (2019). Identification of root transcriptional responses to shoot illumination in Arabidopsis thaliana. Plant Molecular Biology, 101(4–5), 487–498. https://doi.org/10.1007/s11103-019-00918-7

Miotto, Y. E., Da Costa, C. T., Offringa, R., Kleine-Vehn, J., & Maraschin, F. D. S. (2021). Effects of Light Intensity on Root Development in a D-Root Growth System. Frontiers in Plant Science, 12. https://doi.org/10.3389/fpls.2021.778382

Peer, W. A., Brown, D. R. N., Tague, B. W., Muday, G. K., Taiz, L., & Murphy, A. S. (2001). Flavonoid Accumulation Patterns of Transparent Testa Mutants of Arabidopsis. Plant Physiology, 126(2), 536–548. https://doi.org/10.1104/pp.126.2.536

Perilli, S., Di Mambro, R., & Sabatini, S. (2012). Growth and development of the root apical meristem. Current Opinion in Plant Biology, 15(1), 17-23. https://doi.org/10.1016/j.pbi.2011.10.006

Ricardo F. H. Giehl, Benjamin D. Gruber, Nicolaus von Wirén. (2014). It's time to make changes: Modulation of root system architecture by nutrient signals. Journal of Experimental Botany, 65(3), 769–778. https://doi.org/10.1093/jxb/ert421

Rost, T. L., & Baum, S. F. (1988). On the Correlation of Primary Root Length, Meristem Size and Protoxylem Tracheary Element Position in Pea Seedlings. American Journal of Botany, 75(3), 414. https://doi.org/10.2307/2443988

Růžička, K., Simaskova, M., Duclercq, J., Petrášek, J., Zažímalová, E., Simon, S., Friml, J., Van Montagu, M., & Benková, E. (2009). Cytokinin regulates root meristem activity via modulation

of the polar auxin transport. Proceedings of the National Academy of Sciences of the United States of America, 106(11), 4284–4289. https://doi.org/10.1073/pnas.0900060106

Salvi, E., Di Mambro, R., & Sabatini, S. (2020). Dissecting mechanisms in root growth from the transition zone perspective. Journal of Experimental Botany, 71(8), 2390–2396. https://doi.org/10.1093/jxb/eraa079

Scheres, B., Benfey, P., & Dolan, L. (2002). Root development. The Arabidopsis Book, 1, e0077. https://doi.org/10.1199/tab.0101

Schaller, G. E., Bishopp, A., & Kieber, J. J. (2015). The Yin-Yang of Hormones: Cytokinin and Auxin Interactions in Plant Development. The Plant Cell, 27(1), 44-63. https://doi.org/10.1105/tpc.114.133595

Silva-Navas, J., Moreno-Risueno, M. A., Manzano, C., Téllez-Robledo, B., Navarro-Neila, S., Carrasco, V. M. S., Pollmann, S., Gallego, F. O., & Del Pozo, J. C. (2016). Flavonols Mediate Root Phototropism and Growth through Regulation of Proliferation-to-Differentiation Transition. The Plant Cell, 28(6), 1372–1387. https://doi.org/10.1105/tpc.15.00857

Tian, Q., Uhlir, N. J., & Reed, J. S. (2002). Arabidopsis SHY2/IAA3 Inhibits Auxin-Regulated Gene Expression. The Plant Cell, 14(2), 301–319. https://doi.org/10.1105/tpc.010283

Vilasboa, J., Da Costa, C. T., De Araujo Mariath, J. E., & Fett-Neto, A. G. (2022). Histochemical Localization of Phenolic Compounds and Reactive Oxygen Species in Eucalypt Microcuttings. Methods in Molecular Biology, 29–42. https://doi.org/10.1007/978-1-0716-2185-1_3

Zhang, W., To, J. P., Cheng, C. H., Schaller, G. E., & Kieber, J. J. (2011). Type-A response regulators are required for proper root apical meristem function through post-transcriptional regulation of PIN auxin efflux carriers. Plant Journal, 68(1), 1–10. https://doi.org/10.1111/j.1365-313x.2011.04668.x

Considerações finais

Os dados apresentados nessa dissertação avaliam a participação de flavonóides no mecanismo de regulação do desenvolvimento radicular em *Arabidopsis thaliana*. Nossos resultados trazem evidências para melhorar o entendimento do efeito que os flavonóides exercem nas raízes durante a fotomorfogênese. O uso dos mutantes *tt* como ferramenta genética para caracterizar funcionalmente esses compostos é de grande valia, uma vez que podemos avaliar diferentes etapas da rota e estabelecer hierarquia entre interações gênicas. Através dos cruzamentos gerados neste trabalho verificamos que HY5 de fato é importante para modular os níveis de expressão dos genes da rota dos flavonóides, ambos sendo necessários para a correta formação de raízes laterais. Além disso, verificamos a partir da combinação com mutantes perda-de-função para transportadores de auxina que estas proteínas são essenciais para a expressão do fenótipo que observamos *hy5* e *tt6-3*. Dessa forma concluímos que a regulação promovida por HY5 conta com a atividade dos flavonóides, que por sua vez, inibem a ação de PIN3 e LAX3, resultando na redução do transporte polar de auxina.

Os fenótipos contrastantes nos mutantes *tt* nos levou a investigar o perfil de flavonóides nessas plantas, uma vez que esses metabólitos existem em formas modificadas em Arabidopsis, principalmente como glicosídeos. Questionamos se o perfil de flavonóides poderia nos indicar um metabólito específico que participasse da rota regulatória que faz o ajuste fino o crescimento da raiz em resposta a estímulos ambientais. A utilização de UHPLC-HRMS é uma ferramenta poderosa que nos permitiu identificar um grupo de flavonóides e suas respectivas modificações. Apesar de ser uma ferramenta poderosa, nossa análise apresentou um caráter semi-quantitativo devido às condições específicas das nossas amostras, entretanto, ainda assim foi possível observar um perfil diferencial entre os genótipos. Futuramente, um aumento na coleta de biomassa das raízes e aprimoramento na extração dos metabólitos pode refinar ainda mais esse conjunto de dados, possibilitando um detalhamento maior na análise.

No capítulo II, observamos que (1) os flavonóis são essenciais para a resposta da citocinina durante o crescimento das raízes; (2) a citocinina não regula os níveis de expressão de todos os genes dos flavonóis, mas altera o padrão de acúmulo desses metabólitos no meristema radicular; e (3) um intermediário específico pode ser responsável pela regulação do eixo

flavonol-fitormônio, já que o mutante *tt5-2* apresentou níveis mais elevados de IAA conjugada a resíduos de glicose, enquanto que *tt6-3* apresentou quantidades similares a Col-0. Concluindo, esses resultados sugerem que os flavonóis participam de um circuito intricado de regulação auxina-citocinina na morfogênese radicular de Arabidopsis.

Referências

Agati, G., Brunetti, C., Fini, A., Gori, A., Guidi, L., Landi, M., Sebastiani, F., & Tattini, M. (2020). Are Flavonoids Effective Antioxidants in Plants? Twenty Years of Our Investigation. Antioxidants, 9(11), 1098. https://doi.org/10.3390/antiox9111098

Ang, L., Chattopadhyay, S., Zhao, J., Oyama, T., Okada, K., Batschauer, A., & Deng, X. W. (1998). Molecular Interaction between COP1 and HY5 Defines a Regulatory Switch for Light Control of Arabidopsis Development. *Molecular Cell*, *1*(2), 213–222. https://doi.org/10.1016/s1097-2765(00)80022-2

Ang, L., & Deng, X. W. (1994). Regulatory hierarchy of photomorphogenic loci: allele-specific and light-dependent interaction between the HY5 and COP1 loci. *The Plant Cell*, *6*(5), 613–628. https://doi.org/10.1105/tpc.6.5.613

Arsovski, A. A., Galstyan, A., Guseman, J. M., & Nemhauser, J. L. (2012). Photomorphogenesis. *The Arabidopsis Book*, *10*, e0147. https://doi.org/10.1199/tab.0147

Band, L. R., Wells, D. M., Fozard, J. R., Ghetiu, T., French, A. S., Pound, M. P., Wilson, M. T., Yu, L., Li, W., Hijazi, H., Oh, J., Pearce, S. H. S., Perez-Amador, M. A., Yun, J., Kramer, E. M., Alonso, J. M., Godin, C., Vernoux, T., Hodgman, C., . . . Bennett, M. J. (2014). Systems Analysis of Auxin Transport in the *Arabidopsis* Root Apex. *The Plant Cell*, *26*(3), 862–875. https://doi.org/10.1105/tpc.113.119495

Barrada, A., Montané, M., Robaglia, C., & Menand, B. (2015). Spatial Regulation of Root Growth: Placing the Plant TOR Pathway in a Developmental Perspective. International Journal of Molecular Sciences, 16(8), 19671–19697. https://doi.org/10.3390/ijms160819671

Benková, E., & Bielach, A. (2010). Lateral root organogenesis — from cell to organ. *Current Opinion in Plant Biology*, *13*(6), 677–683. https://doi.org/10.1016/j.pbi.2010.09.006

Bhalerao, R. P., Eklöf, J., Ljung, K., Marchant, A., Bennett, M. J., & Sandberg, G. (2002). Shoot-derived auxin is essential for early lateral root emergence in*Arabidopsis*seedlings. *Plant Journal*, *29*(3), 325–332. https://doi.org/10.1046/j.0960-7412.2001.01217.x

Bharti, A. K., & Khurana, J. P. (2003). Molecular characterization of transparent testa (tt) mutants of Arabidopsis thaliana (ecotype Estland) impaired in flavonoid biosynthetic pathway. *Plant Science*, *165*(6), 1321–1332. https://doi.org/10.1016/s0168-9452(03)00344-3

Bhatia, C., Pandey, A., Gaddam, S. R., Hoecker, U., & Trivedi, P. K. (2018). Low Temperature-Enhanced Flavonol Synthesis Requires Light-Associated Regulatory Components in Arabidopsis thaliana. *Plant and Cell Physiology*, *59*(10), 2099–2112. https://doi.org/10.1093/pcp/pcy132

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., & Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in Arabidopsis roots. *Nature*, *433*(7021), 39–44. https://doi.org/10.1038/nature03184

Brown, D. R. N., Rashotte, A. M., Murphy, A. S., Normanly, J., Tague, B. W., Peer, W. A., Taiz, L., & Muday, G. K. (2001). Flavonoids Act as Negative Regulators of Auxin Transport in Vivo in Arabidopsis. *Plant Physiology*, *126*(2), 524–535. https://doi.org/10.1104/pp.126.2.524

Buer, C. S., & Djordjevic, M. A. (2009). Architectural phenotypes in the transparent testa mutants of Arabidopsis thaliana. *Journal of Experimental Botany*, *60*(3), 751–763. https://doi.org/10.1093/jxb/ern323

Buer, C. S., Kordbacheh, F., Truong, T. T., Hocart, C. H., & Djordjevic, M. A. (2013). Alteration of flavonoid accumulation patterns in transparent testa mutants disturbs auxin transport, gravity responses, and imparts long-term effects on root and shoot architecture. *Planta*, *238*(1), 171–189. https://doi.org/10.1007/s00425-013-1883-3

Buer, C. S., & Muday, G. K. (2004). The *transparent testa4* Mutation Prevents Flavonoid Synthesis and Alters Auxin Transport and the Response of Arabidopsis Roots to Gravity and Light[W]. *The Plant Cell*, *16*(5), 1191–1205. https://doi.org/10.1105/tpc.020313

Buer, C. S., Muday, G. K., & Djordjevic, M. A. (2007). Flavonoids Are Differentially Taken Up and Transported Long Distances in Arabidopsis. *Plant Physiology*, *145*(2), 478–490. https://doi.org/10.1104/pp.107.101824

Burko, Y., Seluzicki, A., Zander, M., Pedmale, U. V., Ecker, J. R., & Chory, J. (2020). Chimeric Activators and Repressors Define HY5 Activity and Reveal a Light-Regulated Feedback Mechanism. *The Plant Cell*, *32*(4), 967–983. https://doi.org/10.1105/tpc.19.00772

Casimiro, I., Beeckman, T., Graham, N. M., Bhalerao, R. P., Zhang, H., Casero, P. J., Sandberg, G., & Bennett, M. J. (2003). Dissecting Arabidopsis lateral root development. *Trends in Plant Science*, *8*(4), 165–171. https://doi.org/10.1016/s1360-1385(03)00051-7

Chapman, J. M., & Muday, G. K. (2021). Flavonols modulate lateral root emergence by scavenging reactive oxygen species in Arabidopsis thaliana. *Journal of Biological Chemistry*, *296*, 100222. https://doi.org/10.1074/jbc.ra120.014543

Chattopadhyay, S., Ang, L., Puente, P., Deng, X., & Wei, N. (1998). Arabidopsis bZIP Protein HY5 Directly Interacts with Light-Responsive Promoters in Mediating Light Control of Gene Expression. *The Plant Cell*, *10*(5), 673–683. https://doi.org/10.1105/tpc.10.5.673

Chen, K. J., & Rajewsky, N. (2007). The evolution of gene regulation by transcription factors and microRNAs. *Nature Reviews Genetics*, *8*(2), 93–103. https://doi.org/10.1038/nrg1990

Cluis, C. P., Mouchel, C., & Hardtke, C. S. (2004). The*Arabidopsis*transcription factor HY5 integrates light and hormone signaling pathways. *Plant Journal*, *38*(2), 332–347. https://doi.org/10.1111/j.1365-313x.2004.02052.x

De Smet, S., Cuypers, A., Vangronsveld, J., & Remans, T. (2015). Gene Networks Involved in Hormonal Control of Root Development in Arabidopsis thaliana: A Framework for Studying Its Disturbance by Metal Stress. *International Journal of Molecular Sciences*, *16*(8), 19195–19224. https://doi.org/10.3390/ijms160819195

De Wit, M., Galvão, V. C., & Fankhauser, C. (2016). Light-Mediated Hormonal Regulation of Plant Growth and Development. *Annual Review of Plant Biology*, *67*(1), 513–537. https://doi.org/10.1146/annurev-arplant-043015-112252

Di Mambro, R., & Sabatini, S. (2018). Developmental Analysis of Arabidopsis Root Meristem. *Springer EBooks*, 33–45. https://doi.org/10.1007/978-1-4939-7747-5_3

Dolan, L., Janmaat, K., Willemsen, V., Linstead, P., Poethig, S., Roberts, K., & Scheres, B. (1993). Cellular organisation of the Arabidopsis thaliana root. *Development*, *119*(1), 71–84. https://doi.org/10.1242/dev.119.1.71

Dubos, C., Stracke, R., Grotewold, E., Weisshaar, B., Martin, C., & Lepiniec, L. (2002). MYB transcription factors in Arabidopsis. *Trends in Plant Science*, *15*(10), 573–581. https://doi.org/10.1016/j.tplants.2010.06.005

Ferreyra, M. L. F., Rius, S. P., & Fernie, A. R. (2012). Flavonoids: biosynthesis, biological functions, and biotechnological applications. Frontiers in Plant Science, 3. https://doi.org/10.3389/fpls.2012.00222

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., & Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical–basal axis of Arabidopsis. *Nature*, *426*(6963), 147–153. https://doi.org/10.1038/nature02085

Gangappa, S. N., & Botto, J. F. (2016). The Multifaceted Roles of HY5 in Plant Growth and Development. *Molecular Plant*, *9*(10), 1353–1365. https://doi.org/10.1016/j.molp.2016.07.002

Gayomba, S. R., & Muday, G. K. (2020). Flavonols regulate root hair development by modulating accumulation of reactive oxygen species in the root epidermis. *Development*. https://doi.org/10.1242/dev.185819

Han, X., Huang, X., & Deng, X. W. (2020). The Photomorphogenic Central Repressor COP1: Conservation and Functional Diversification during Evolution. *Plant Communications*, *1*(3), 100044. https://doi.org/10.1016/j.xplc.2020.100044

Hao, Y., Zeng, Z., Zhang, X., Xie, D., Li, X., Ma, L., Liu, M., & Liu, H. (2023). Green means go: Green light promotes hypocotyl elongation via brassinosteroid signaling. *The Plant Cell*. https://doi.org/10.1093/plcell/koad022

Heidstra, R., & Sabatini, S. (2014). Plant and animal stem cells: similar yet different. *Nature Reviews Molecular Cell Biology*, *15*(5), 301–312. https://doi.org/10.1038/nrm3790

Holm, M., Ma, L., Qu, L., & Deng, X. W. (2002). Two interacting bZIP proteins are direct targets of COP1-mediated control of light-dependent gene expression in *Arabidopsis*. *Genes & Development*, *16*(10), 1247–1259. https://doi.org/10.1101/gad.969702

Ioio, R. D., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., & Sabatini, S. (2007a). Cytokinins Determine Arabidopsis Root-Meristem Size by Controlling Cell Differentiation. *Current Biology*, *17*(8), 678–682. https://doi.org/10.1016/j.cub.2007.02.047

Ioio, R. D., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., & Sabatini, S. (2007b). Cytokinins Determine Arabidopsis Root-Meristem Size by Controlling Cell Differentiation. *Current Biology*, *17*(8), 678–682. https://doi.org/10.1016/j.cub.2007.02.047

Jing, Y., Zhang, D. H., Wang, X., Tang, W., Wanqing, W., Huai, J., Xu, G., Chen, D., Li, Y., & Lin, R. (2013). *Arabidopsis*Chromatin Remodeling Factor PICKLE Interacts with Transcription Factor HY5 to Regulate Hypocotyl Cell Elongation. *The Plant Cell*, *25*(1), 242–256. https://doi.org/10.1105/tpc.112.105742

Kim, S., Hwang, G., Lee, S., Zhu, J. Y., Paik, I., Nguyen, T. T., Kim, J., & Oh, E. (2017). High Ambient Temperature Represses Anthocyanin Biosynthesis through Degradation of HY5. *Frontiers in Plant Science*, *8*. https://doi.org/10.3389/fpls.2017.01787

Ko, D., & Helariutta, Y. (2017). Shoot–Root Communication in Flowering Plants. *Current Biology*, *27*(17), R973–R978. https://doi.org/10.1016/j.cub.2017.06.054

Kondhare, K. R., Patil, N., & Banerjee, A. (2021). A historical overview of long-distance signalling in plants. *Journal of Experimental Botany*, *72*(12), 4218–4236. https://doi.org/10.1093/jxb/erab048

Koornneef, M., & Van Der Veen, J. (1980). Induction and analysis of gibberellin sensitive mutants in Arabidopsis thaliana (L.) heynh. *Theoretical and Applied Genetics*, *58*(6), 257–263. https://doi.org/10.1007/bf00265176

Lau, O. S., & Deng, X. W. (2012a). The photomorphogenic repressors COP1 and DET1: 20 years later. *Trends in Plant Science*, *17*(10), 584–593. https://doi.org/10.1016/j.tplants.2012.05.004

Lau, O. S., & Deng, X. W. (2012b). The photomorphogenic repressors COP1 and DET1: 20 years later. *Trends in Plant Science*, *17*(10), 584–593. https://doi.org/10.1016/j.tplants.2012.05.004

Laxmi, A., Pan, J., Morsy, M., & Chen, R. (2008). Light Plays an Essential Role in Intracellular Distribution of Auxin Efflux Carrier PIN2 in Arabidopsis thaliana. PLOS ONE, 3(1), e1510. https://doi.org/10.1371/journal.pone.0001510

Lee, H. S., Ha, J., Kim, S. H., Choi, H. Y., Kim, Z. H., Han, Y., Kim, J. H., Oh, Y., Fragoso, V., Shin, K., Hyeon, T., Choi, H. D., Oh, K. T., Baldwin, I. T., & Park, C. G. (2016). Stem-piped light activates phytochrome B to trigger light responses in *Arabidopsis thaliana* roots. *Science Signaling*, *9*(452). https://doi.org/10.1126/scisignal.aaf6530

Leivar, P., & Monte, E. (2014). PIFs: Systems Integrators in Plant Development. *The Plant Cell*, *26*(1), 56–78. https://doi.org/10.1105/tpc.113.120857

Li, C., Qi, L., Zhang, S., Dong, X., Jing, Y., Cheng, J., Feng, Z., Peng, J., Li, H., Zhou, Y., Wang, X., Han, R. P., Duan, J., Terzaghi, W., Lin, R., & Li, J. (2021). Mutual upregulation of HY5 and TZP in mediating phytochrome A signaling. *The Plant Cell*, *34*(1), 633–654. https://doi.org/10.1093/plcell/koab254

Malamy, J. E., & Benfey, P. N. (1997). Organization and cell differentiation in lateral roots of Arabidopsis thaliana. *Development*, *124*(1), 33–44. https://doi.org/10.1242/dev.124.1.33

Maraschin, F. D. S. (2020). *Efeitos da sinalização dependente de luz no desenvolvimento de raízes em Arabidopsis thaliana*. https://lume.ufrgs.br/handle/10183/212108

Matsubayashi, Y. (2014). Posttranslationally Modified Small-Peptide Signals in Plants. *Annual Review of Plant Biology*, *65*(1), 385–413. https://doi.org/10.1146/annurev-arplant-050312-120122

Mehrtens, F., Kranz, H., Bednarek, P., & Weisshaar, B. (2005). The Arabidopsis Transcription Factor MYB12 Is a Flavonol-Specific Regulator of Phenylpropanoid Biosynthesis. Plant Physiology, 138(2), 1083–1096. https://doi.org/10.1104/pp.104.058032

Michniewicz, M., Brewer, P. B., & Friml, J. (2007). Polar Auxin Transport and Asymmetric Auxin Distribution. *The Arabidopsis Book*. https://doi.org/10.1199/tab.0108

Millar, A. J., & Kay, S. A. (1996). Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(26), 15491–15496. https://doi.org/10.1073/pnas.93.26.15491

Miotto, Y. E., Da Costa, C. T., Offringa, R., Kleine-Vehn, J., & Maraschin, F. D. S. (2021). Effects of Light Intensity on Root Development in a D-Root Growth System. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.778382

Mockaitis, K., & Estelle, M. (2008). Auxin Receptors and Plant Development: A New Signaling Paradigm. *Annual Review of Cell and Developmental Biology*, *24*(1), 55–80. https://doi.org/10.1146/annurev.cellbio.23.090506.123214

Möglich, A., Yang, X., Ayers, R. A., & Moffat, K. (2010). Structure and Function of Plant Photoreceptors. *Annual Review of Plant Biology*, *61*(1), 21–47. https://doi.org/10.1146/annurev-arplant-042809-112259

Morelli, G., & Ruberti, I. (2002). Light and shade in the photocontrol of Arabidopsis growth. *Trends in Plant Science*, *7*(9), 399–404. https://doi.org/10.1016/s1360-1385(02)02314-2

Muhlemann, J. K., Younts, T. L. B., & Muday, G. K. (2018). Flavonols control pollen tube growth and integrity by regulating ROS homeostasis during high-temperature stress. *Proceedings*

of the National Academy of Sciences of the United States of America, *115*(47). https://doi.org/10.1073/pnas.1811492115

Notaguchi, M., Wolf, S., & Lucas, W. J. (2012). Phloem-Mobile*Aux*/*IAA*Transcripts Target to the Root Tip and Modify Root Architecture[F]. *Journal of Integrative Plant Biology*, *54*(10), 760–772. https://doi.org/10.1111/j.1744-7909.2012.01155.x

Oh, E., Seo, P. J., & Kim, J. (2018). Signaling Peptides and Receptors Coordinating Plant Root Development. *Trends in Plant Science*, *23*(4), 337–351. https://doi.org/10.1016/j.tplants.2017.12.007

Osterlund, M. T., Hardtke, C. S., Zhao, J., & Deng, X. W. (2000). Targeted destabilization of HY5 during light-regulated development of Arabidopsis. *Nature*, *405*(6785), 462–466. https://doi.org/10.1038/35013076

Oyama, T., Shimura, Y., & Okada, K. (1997). The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes & Development*, *11*(22), 2983–2995. https://doi.org/10.1101/gad.11.22.2983

Paik, I., & Huq, E. (2019). Plant photoreceptors: Multi-functional sensory proteins and their signaling networks. *Seminars in Cell & Developmental Biology*, *92*, 114–121. https://doi.org/10.1016/j.semcdb.2019.03.007

Peer, W. A., Bandyopadhyay, A., Blakeslee, J. J., Makam, S. N., Chen, R., Masson, P., & Murphy, A. S. (2004). Variation in Expression and Protein Localization of the PIN Family of Auxin Efflux Facilitator Proteins in Flavonoid Mutants with Altered Auxin Transport in *Arabidopsis thaliana* [W]. *The Plant Cell*, *16*(7), 1898–1911. https://doi.org/10.1105/tpc.021501

Péret, B., Middleton, A. M., French, A. S., Larrieu, A., Bishopp, A., Njo, M. F., Wells, D. M., Porco, S., Mellor, N., Band, L. R., Casimiro, I., Kleine-Vehn, J., Vanneste, S., Sairanen, I., Leontie, L., Sandberg, G., Ljung, K., Beeckman, T., Benková, E., . . . Bennett, M. J. (2013). Sequential induction of auxin efflux and influx carriers regulates lateral root emergence. Molecular Systems Biology, 9(1), 699. https://doi.org/10.1038/msb.2013.43

Petricka, J. J., Winter, C. M., & Benfey, P. N. (2012). Control of *Arabidopsis* Root Development. *Annual Review of Plant Biology*, *63*(1), 563–590. https://doi.org/10.1146/annurev-arplant-042811-105501

Podolec, R., & Ulm, R. (2018). Photoreceptor-mediated regulation of the COP1/SPA E3 ubiquitin ligase. *Current Opinion in Plant Biology*, *45*, 18–25. https://doi.org/10.1016/j.pbi.2018.04.018

Pollastri, S., & Tattini, M. (2011). Flavonols: old compounds for old roles. *Annals of Botany*, *108*(7), 1225–1233. https://doi.org/10.1093/aob/mcr234

Ponnu, J., & Hoecker, U. (2021). Illuminating the COP1/SPA Ubiquitin Ligase: Fresh Insights Into Its Structure and Functions During Plant Photomorphogenesis. *Frontiers in Plant Science*, *12*. https://doi.org/10.3389/fpls.2021.662793

Quail, P. H. (2002a). Phytochrome photosensory signalling networks. *Nature Reviews Molecular Cell Biology*, *3*(2), 85–93. https://doi.org/10.1038/nrm728

Quail, P. H. (2002b). Phytochrome photosensory signalling networks. *Nature Reviews Molecular Cell Biology*, *3*(2), 85–93. https://doi.org/10.1038/nrm728

Saito, K., Yonekura-Sakakibara, K., Nakabayashi, R., Higashi, Y., Yamazaki, M., Tohge, T., & Fernie, A. R. (2013). The flavonoid biosynthetic pathway in Arabidopsis: Structural and genetic diversity. Plant Physiology and Biochemistry, 72, 21–34. https://doi.org/10.1016/j.plaphy.2013.02.001

Salisbury, F. J., Hall, A., Grierson, C. S., & Halliday, K. J. (2007). Phytochrome coordinates Arabidopsis shoot and root development. *Plant Journal*, *50*(3), 429–438. https://doi.org/10.1111/j.1365-313x.2007.03059.x

Sassi, M., Lu, Y., Zhang, Y., Wang, J., Dhonukshe, P., Blilou, I., Dai, M., Li, J., Gong, X., Jaillais, Y., Yu, X., Traas, J., Ruberti, I., Wang, H., Scheres, B., Vernoux, T., & Xu, J. (2012). COP1 mediates the coordination of root and shoot growth by light through modulation of PIN1- and PIN2-dependent auxin transport in *Arabidopsis*. *Development*, *139*(18), 3402–3412. https://doi.org/10.1242/dev.078212

Schulz, E., Tohge, T., Zuther, E., Fernie, A. R., & Hincha, D. K. (2016). Flavonoids are determinants of freezing tolerance and cold acclimation in Arabidopsis thaliana. *Scientific Reports*, *6*(1). https://doi.org/10.1038/srep34027

Shin, J., Park, E., & Choi, G. (2007). PIF3 regulates anthocyanin biosynthesis in an HY5-dependent manner with both factors directly binding anthocyanin biosynthetic gene promoters in Arabidopsis. *Plant Journal*, *49*(6), 981–994. https://doi.org/10.1111/j.1365-313x.2006.03021.x

Silva-Navas, J., Moreno-Risueno, M. A., Manzano, C., Pallero-Baena, M., Navarro-Neila, S., Téllez-Robledo, B., García-Mina, J. M., Baigorri, R., Gallego, F., & Del Pozo, J. C. (2015). D-Root: a system for cultivating plants with the roots in darkness or under different light conditions. *Plant Journal*, *84*(1), 244–255. https://doi.org/10.1111/tpj.12998

Silva-Navas, J., Moreno-Risueno, M. A., Manzano, C., Téllez-Robledo, B., Navarro-Neila, S., Carrasco, V. M. S., Pollmann, S., Gallego, F. O., & Del Pozo, J. C. (2016). Flavonols Mediate Root Phototropism and Growth through Regulation of Proliferation-to-Differentiation Transition. *The Plant Cell*, *28*(6), 1372–1387. https://doi.org/10.1105/tpc.15.00857

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., & Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. *Plant Journal*, *50*(4), 660–677. https://doi.org/10.1111/j.1365-313x.2007.03078.x

Stracke, R., Jahns, O., Keck, M., Tohge, T., Niehaus, K., Fernie, A. R., & Weisshaar, B. (2010). Analysis of PRODUCTION OF FLAVONOL GLYCOSIDES‐dependent flavonol glycoside accumulation inArabidopsis thaliana plants reveals MYB11‐, MYB12‐ and MYB111‐independent flavonol glycoside accumulation. New Phytologist, 188(4), 985–1000. https://doi.org/10.1111/j.1469-8137.2010.03421.x

Sullivan, J., & Deng, X. W. (2003). From seed to seed: the role of photoreceptors in Arabidopsis development. *Developmental Biology*, *260*(2), 289–297. https://doi.org/10.1016/s0012-1606(03)00212-4

Taniguchi, M., Sasaki, N., Tsuge, T., Aoyama, T., & Oka, A. (2007). ARR1 Directly Activates Cytokinin Response Genes that Encode Proteins with Diverse Regulatory Functions. *Plant and Cell Physiology*, *48*(2), 263–277. https://doi.org/10.1093/pcp/pcl063

Teale, W. H., Pasternak, T., Bosco, C. D., Dovzhenko, A., Kratzat, K., Bildl, W., Schwörer, M., Falk, T., Ruperti, B., Schaefer, J. V., Shahriari, M., Pilgermayer, L., Li, X., Lübben, F., Plückthun, A., Schulte, U., & Palme, K. (2021). Flavonol‐mediated stabilization of PIN efflux complexes regulates polar auxin transport. *The EMBO Journal*, *40*(1). https://doi.org/10.15252/embj.2020104416

Terzaghi, W., & Cashmore, A. R. (1995). Light-Regulated Transcription. *Annual Review of Plant Physiology and Plant Molecular Biology*, *46*(1), 445–474. https://doi.org/10.1146/annurev.pp.46.060195.002305

Tiwari, S. B., Wang, X., Hagen, G., & Guilfoyle, T. J. (2001). AUX/IAA Proteins Are Active Repressors, and Their Stability and Activity Are Modulated by Auxin. *The Plant Cell*, *13*(12), 2809–2822. https://doi.org/10.1105/tpc.010289

Tobin, E. M., & Kehoe, D. M. (1994). Phytochrome regulated gene expression. *Seminars in Cell Biology*, *5*(5), 335–346. https://doi.org/10.1006/scel.1994.1040

Van Gelderen, K., Kang, C., Paalman, R., Keuskamp, D. H. G., Hayes, S. M., & Pierik, R. (2018). Far-Red Light Detection in the Shoot Regulates Lateral Root Development through the HY5 Transcription Factor. *The Plant Cell*, *30*(1), 101–116. https://doi.org/10.1105/tpc.17.00771

Van Gelderen, K., Kang, C., & Pierik, R. (2017). Light Signaling, Root Development, and Plasticity. *Plant Physiology*, *176*(2), 1049–1060. https://doi.org/10.1104/pp.17.01079

Vandenbussche, F., Habricot, Y., Condiff, A. S., Maldiney, R., Van Der Straeten, D., & Ahmad, M. (2007). HY5 is a point of convergence between cryptochrome and cytokinin signalling pathways in Arabidopsis thaliana. *Plant Journal*, *49*(3), 428–441. https://doi.org/10.1111/j.1365-313x.2006.02973.x

Verbelen, J., De Cnodder, T., Le, J., Vissenberg, K., & Baluška, F. (2006a). The Root Apex of*Arabidopsis thaliana*Consists of Four Distinct Zones of Growth Activities. *Plant Signaling & Behavior*, *1*(6), 296–304. https://doi.org/10.4161/psb.1.6.3511

Verbelen, J., De Cnodder, T., Le, J., Vissenberg, K., & Baluška, F. (2006b). The Root Apex of*Arabidopsis thaliana*Consists of Four Distinct Zones of Growth Activities. *Plant Signaling & Behavior*, *1*(6), 296–304. https://doi.org/10.4161/psb.1.6.3511

Vilches-Barro, A., & Maizel, A. (2015). Talking through walls: mechanisms of lateral root emergence in Arabidopsis thaliana. *Current Opinion in Plant Biology*, *23*, 31–38. https://doi.org/10.1016/j.pbi.2014.10.005

Wade, H. K., Bibikova, T. N., Valentine, W. J., & Jenkins, G. J. (2001). Interactions within a network of phytochrome, cryptochrome and UV-B phototransduction pathways regulate chalcone synthase gene expression in *Arabidopsis* leaf tissue. *Plant Journal*, *25*(6), 675–685. https://doi.org/10.1046/j.1365-313x.2001.01001.x

Wang, Y., Wang, Y., Song, Z., & Zhang, H. (2016). Repression of MYBL2 by Both microRNA858a and HY5 Leads to the Activation of Anthocyanin Biosynthetic Pathway in Arabidopsis. *Molecular Plant*, *9*(10), 1395–1405. https://doi.org/10.1016/j.molp.2016.07.003

Watkins, J. M., Hechler, P. J., & Muday, G. K. (2014). Ethylene-Induced Flavonol Accumulation in Guard Cells Suppresses Reactive Oxygen Species and Moderates Stomatal Aperture. *Plant Physiology*, *164*(4), 1707–1717. https://doi.org/10.1104/pp.113.233528

Winkel-Shirley, B. (2001). Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. *Plant Physiology*, *126*(2), 485–493. https://doi.org/10.1104/pp.126.2.485

Xu, X., Paik, I., Zhu, L., & Huq, E. (2015). Illuminating Progress in Phytochrome-Mediated Light Signaling Pathways. *Trends in Plant Science*, *20*(10), 641–650. https://doi.org/10.1016/j.tplants.2015.06.010

Yang, Y., & Liu, H. (2020). Coordinated Shoot and Root Responses to Light Signaling in Arabidopsis. *Plant Communications*, *1*(2), 100026. https://doi.org/10.1016/j.xplc.2020.100026