UNIVERSIDAD MICHOACANA DE SAN NICOLÁS DE HIDALGO

INSTITUTO DE INVESTIGACIONES QUÍMICO–BIOLÓGICAS

MAESTRÍA EN CIENCIAS EN BIOLOGÍA EXPERIMENTAL

"Participación del péptido CLE14 y el receptor PEPR2 sobre procesos de viabilidad y regeneración celular en la raíz de *Arabidopsis thaliana*"

Tesis que presenta:

L.BT. Adrián Ávalos Rangel

Para obtener el grado de:

Maestro en Ciencias en Biología Experimental

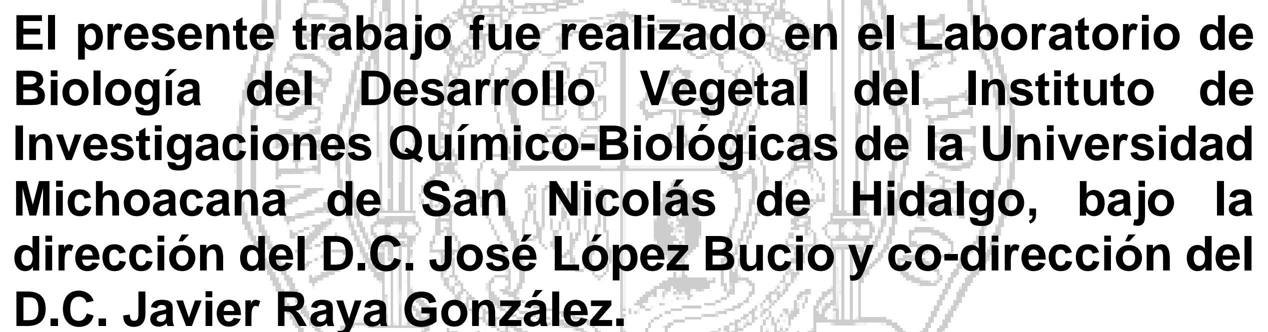
Asesor de tesis:

D.C. José López Bucio

Co-asesor de tesis:

D.C. Javier Raya González

Morelia, Michoacán, Marzo 2023



El presente trabajo fue realizado en el Laboratorio de Biología del Desarrollo Vegetal del Instituto de Investigaciones Químico-Biológicas de la Universidad Michoacana de San Nicolás de Hidalgo, bajo la dirección del D.C. José López Bucio y co-dirección del D.C. Javier Raya González.

DEDICATORIA

A mis Padres y abuelos

Agradecimientos

A mis padres, Arturo Ávalos y María del Rocío Rangel, por su apoyo incondicional, consejos y enseñanzas que me han ayudado a ser la persona que soy hoy en día.

A mi familia, a mis abuelos Arturo, José Salud y Josefina, mis tíos Ricardo, Gaude, Leticia, José, Jerónimo, Sofía y Yolanda, mis primos Alejandro, María y Alberto, por su invaluable apoyo y consejos, siendo un pilar muy importante en mi vida.

Al D.C. José López Bucio, por abrirme la oportunidad de ser parte de su excelente equipo de investigación, apoyándome en continuar con mi formación académica, por compartir sus conocimientos y consejos, al igual que agradezco su vital apoyo para desarrollar mi trabajo de tesis además del apoyo y facilidades otorgadas.

Al D.C. Javier Raya González, por apoyarme en todo momento durante toda mi estancia en el laboratorio, así como agradezco sus consejos y conocimientos compartidos, así como el tiempo dedicado para el desarrollo del presente trabajo.

Al D.C. Homero Reyes de la Cruz, D.C. Jesús Salvador López Bucio y el D.C. Ernesto García Pineda, por acceder a ser parte de mi comité sinodal y por sus atenciones.

Al D.C. Luis Herrera Estrella y a la D.C. Dolores Gutiérrez Alanís por brindarnos el apoyo y proporcionarnos el material biológico para el desarrollo de este proyecto.

Al D.C. Ramón Pelagio Flores y al D.C. León Francisco Ruíz Herrera por su apoyo en el laboratorio para realizar diferentes experimentos, así como en otras actividades que me han brindado su gran ayuda.

Al M.C. Luis María Suarez Rodríguez por sus valiosos consejos y apoyo en diferentes momentos en mi estadía en el instituto.

A mis compañeros y amigos de laboratorio: Fernanda, Yeimi, Pedro, Kiran, Liz, Elizabet, Ivan, Joseline, Roberto, Diego y Pepe por brindarme su apoyo y amistad incondicional, haciendo mi estancia en el laboratorio más agradable.

A mis amigos, Andrea**,** Ximena, Monserrat, Ambar, Cristian, Alfonso, Roberto Guerra, Rosa Elena, Carla, Marcos, Adrián, Emily, Claudia, Esther, Aixa e Itzel por su invaluable amistad durante estos años y siempre estar para mí en todo momento.

Al Arq. Leonel Muñoz Bolaños, por su gran apoyo y valiosos consejos que me ha brindado durante todo este tiempo que me han ayudado en gran medida.

Al Lic. Jorge Villalpando Fierro por sus atenciones y apoyo que me ha brindado.

Al Consejo Nacional de Ciencia y Tecnología (CONACyT), por la beca otorgada que permitió mi manutención durante la realización de este trabajo.

ÍNDICE

RESUMEN 1
ABSTRACT 3
1. INTRODUCCIÓN 5
2. ANTECEDENTES 6
2.1. Regulación hormonal del desarrollo de las plantas 6
2.2. La raíz: estructura y funciones 7
2.3. El meristemo apical de la raíz 8
2.4. Respuesta de la raíz ante el agobio ambiental 9
2.5. Respuestas fisiológicas y moleculares ante un daño al ADN 11
2.6. Regeneración celular 11
2.7. Callogénesis 13
2.8. Comunicación celular mediada por péptidos y receptores con actividad de cinasa 14
2.9. Clasificación de los receptores tipo cinasa 16
2.10. Funciones de los receptores tipo cinasa 17
3. JUSTIFICACIÓN 19
4. HIPÓTESIS 20
5. OBJETIVOS 20
5.1. Objetivo general 20
5.2. Objetivos específicos 20
6. MATERIALES Y MÉTODOS 20
6.1. Material biológico y reactivos 20
6.2. Preparación de semilla y condiciones de crecimiento 21
6.4. Ensayos de corte de raíz y regeneración 22
6.5. Tinción de yoduro de propidio (IP) y detección de la proteína verde fluorescente 22
6.6. Análisis estadístico 22
7. RESULTADOS 23
7.1. La aplicación de CLE14 o su sobre-expresión inhiben el crecimiento de la raíz y promueven la diferenciación celular en *Arabidopsis* 23

7.2. CLE14 se expresa durante la regeneración de la punta de la raíz de *Arabidopsis* ………………………………………………………………………………………… **25**

7.3. El tratamiento con CLE14 disminuye la expresión del factor de transcripción ERF115 durante la regeneración de la raíz **.. 27**

7.5. Los explantes de brotes *35S:CLE14* manifiestan una disminución en la formación de callos **.. 30**

7.6. El corte de la raíz induce la expresión del receptor con actividad de cinasa PEPR2**... 31**

7.7. El corte de la raíz induce la expresión de PEPR2 en el follaje **....................... 33**

7.8. La expresión de PEPR2 se induce por herida en los cotiledones**.................... 34**

7.9. El daño celular por agentes genotóxicos induce la expresión de PEPR2 en la provasculatura de la raíz**. 35**

7.10. El promotor de PEPR2 se induce de manera constitutiva por ácido jasmónico**…… 36**

8. DISCUSIÓN Y CONCLUSIONES.. 38

9. LITERATURA CITADA 42

10. ADDENDUM …………………..……………………………………………………… 53

ÍNDICE DE FIGURAS

Figura 1. Los péptidos de la familia CLE y su papel en el desarrollo vegetal y la adaptación al estrés**.** **15**

Figura 2. Estructura general de los receptores con actividad de cinasa de *Arabidopsis thaliana*. **17**

Figura 3. La aplicación de CLE 14 detiene el crecimiento de la raíz de *Arabidopsis* y promueve la ramificación de la raíz y la formación de pelos radiculares. **24**

Figura 4. La sobre-expresión de CLE14 inhibe el crecimiento radicular en *Arabidopsis*.......... **25**

Figura 5. CLE14 se expresa en células del borde después de la escisión de la punta de la raíz**.** **26**

Figura 6. Efecto de CLE14 sobre la expresión de *pERF115:GUS-GFP* durante la regeneración de la raíz**.** **28**

Figura 7. Regeneración de la punta de la raíz primaria de *Arabidopsis* después del corte con un bisturí**.** **29**

Figura 8. La sobreexpresión de CLE14 interfiere con la formación de callos**.** **31**

Figura 9. Inducción de la expresión del receptor PEPR2 durante el corte y regeneración de la raíz. **32**

Figura 10. Expresión sistémica de PEPR2 en el follaje en respuesta al corte de la raíz.......... **33**

Figura 11. Inducción de la expresión de PEPR2 en respuesta a herida en los cotiledones.......... **34**

Figura 12. Efecto de la zeocina sobre la expresión de PEPR2 en el meristemo de la raíz.......... **36**

Figura 13. Expresión de PEPR2 en plantas de *Arabidopsis* tratadas con ácido jasmónico.......... **37**

RESUMEN

CLE14 pertenece a una familia de péptidos que son secretados por las plantas y que interactúan con receptores con actividad de cinasa y repetidos ricos en leucina (LRR-RLK) en su estructura, importantes para la comunicación celular. En este trabajo, a través de enfoques farmacológicos, genéticos y de biología celular, se investigó la función de CLE14 para determinar el equilibrio entre la división celular y la diferenciación, así como su impacto en la callogénesis y la regeneración de la punta de la raíz.

Las concentraciones nanomolares de CLE14 o su sobreexpresión en *Arabidopsis* reprimen el crecimiento de la raíz primaria y desencadenan la ramificación de la raíz y la formación de pelos radiculares. La aplicación farmacológica del péptido en el medio de crecimiento disminuye la expresión del factor de transcripción de respuesta a etileno *ETILENE RESPONSE FACTOR 115* (ERF115) durante el proceso de regeneración. Después de la escisión de la punta de la raíz primaria, la expresión de *pCLE14:GUS-GFP* se localizó específicamente en la capa celular adyacente al corte y en la capa celular más externa de la cofia. Las mutantes *cle14* tuvieron una regeneración de la punta de la raíz comparable con las plantas normales, mientras que las plántulas *35S:CLE14* no logran regenerar la punta de la raíz ante el corte con un bisturí. La desdiferenciación celular se analizó en explantes de tallo de plantas normales, *cle14* y *35S:CLE14* cultivados en medios inductores de callos. Los resultados mostraron una producción de biomasa celular comparable para plantas normales y *cle14*, pero una callogénesis drásticamente reducida para los explantes *35S:CLE14*.

CLE14 interactúa con el receptor PEPR2 en plantas expuestas a estrés por deficiencia de fosfato y esto induce la pérdida del meristemo. Para conocer la relación entre PEPR2 con los procesos de regeneración, se analizaron plantas transgénicas que expresan la construcción *pPEPR2:GUS* en respuesta al corte de la raíz. En las raíces de las plantas que no sufrieron daño, el receptor no se expresa en el meristemo o en la zona de diferenciación, en cambio, una vez realizado el corte de la punta de la raíz, se manifestó una fuerte expresión, evidenciada por la coloración azul producto de la actividad de la

beta glucuronidasa en los tejidos adyacentes al corte y también en regiones distales al daño, incluyendo el meristemo apical del follaje.

La expresión de PEPR2 se indujo por herida en los cotiledones y también en la raíz en respuesta al tratamiento con zeocina, un antibiótico que induce la muerte celular en células de la pro-vasculatura. Finalmente, la aplicación de ácido jasmónico indujo la expresión de PEPR2 de manera constitutiva, indicando un papel importante de esta fitohormona en los procesos de adaptación al daño celular. En conjunto, nuestros resultados proporcionaron información valiosa sobre el vínculo entre el péptido CLE14 y el receptor PEPR2 en los procesos de organogenesis en plantas, así como en la respuesta a herida o daño celular y en la señalización del ácido jasmónico.
Palabras clave: Regeneración celular, material biologíco, raíz, semillas.

ABSTRACT

CLE14 belongs into a family of plant-secreted peptides that interact with leucine-rich repeat receptor-like receptor kinases (LRR-RLK) important for cell communication in plants. Here, through pharmacological, genetic and cell biology approaches, the function of CLE14 was investigated to determine the balance between cell division and differentiation, as well as its impact on callogenesis and root tip regeneration. Nanomolar concentrations of CLE14 or its overexpression in *Arabidopsis* repress taproot growth and trigger root branching and root hair formation. The pharmacological application of the peptide in the growth medium decreases the expression of the ethylene response transcription factor *ETHYLENE RESPONSE FACTOR 115* (ERF115) during the regeneration process. After resection of the primary root tip, *pCLE14:GUS-GFP* expression localized specifically to the cell layer adjacent to the cut and to the outermost cell layer of the root cap and *cle14* mutants had root tip regeneration comparable to normal plants, whereas *35S:CLE14* seedlings fail to regenerate the missing root tip after excision. Dedifferentiation of proliferative tissue was analyzed in WT, *cle14* mutants, and *35S:CLE14* stem explants grown in callus-inducing media. The results showed comparable biomass production for normal and *cle14* plants, but drastically reduced callus production for *35S:CLE14* explants.

CLE14 interacts with the PEPR2 receptor in plants exposed to phosphate deficiency stress and this induces meristem loss. To know the relationship between PEPR2 in regeneration processes, transgenic plants expressing the *pPEPR2:GUS* construct in response to root cutting were analyzed. In the roots of plants that did not suffer damage, the receptor is not expressed in the meristem or in the differentiation zone, however, once the cut of the root tip was made, a strong expression was manifested, evidenced by the blue coloration product of the activity of beta glucuronidase in the tissues adjacent to the cut and also in regions distal to the damage, including the shoot apical meristem.

PEPR2 expression was induced by wounding in the cotyledons and also in the root in response to treatment with zeocin, an antibiotic that induces cell death in highly proliferative cells of the pro-vasculature. Finally, the application of jasmonic acid induced the expression of PEPR2 constitutively, indicating an important role of this phytohormone

in the adaptation processes to cell damage. Together, our results provided valuable insights into the link between the CLE14 peptide and the PEPR2 receptor in plant-mediated organogenesis, wounding response and jasmonic acid signaling.

1. INTRODUCCIÓN

Las plantas manifiestan una gran capacidad para adaptarse a los cambios en las condiciones ambientales a través de la regeneración tisular y la organogénesis *de novo*. El daño físico de las hojas, tallos y raíces puede ocurrir por el ataque de herbívoros y patógenos, así como durante la exploración del suelo o la exposición a contaminantes, para lo cual la cicatrización de heridas o el reemplazo de tejidos dañados orquestan la respuesta adaptativa (Ikeuchi *et al*. 2016; Mathew y Prasad 2021).

Los péptidos de la familia CLAVATA3/EMBRYO SURROUNDING (CLE) interaccionan con receptores membranales con actividad de cinasa activando rutas de transducción de señales mediante eventos de fosforilación (Cock y McCormick 2001; Sharma *et al*. 2003; Fletcher 2020; Willoughby y Nimchuk 2021). Dichas interacciones modulan procesos críticos durante el desarrollo de la planta, incluida la actividad meristemática (Hirakawa *et al.* 2021), las respuestas nutricionales de las raíces (Gutiérrez-Alanís *et al*. 2017) y la senescencia (Zhang *et al*. 2022). En particular, el péptido CLE14 actúa como un represor del crecimiento de la raíz y como inductor de la formación de pelos radiculares en *Arabidopsis*, arroz y tomate (Hayashi *et al*. 2018; 2019), así como en la represión de la actividad del meristemo radicular en condiciones de limitación de fosfato, actuando a través de los receptores CLAVATA2/PEPR2 (Gutiérrez-Alanís *et al*. 2017).

La regeneración completa de plantas a partir de esquejes es una herramienta importante para la multiplicación de material vegetal, teniendo un impacto positivo en la horticultura y la floricultura (Ikeuchi *et al.* 2016). Este proceso implica la formación de células pluripotentes derivadas de diferentes partes, a partir de las cuales es factible producir tallos o raíces a través del manejo del equilibrio entre las auxinas y las citocininas (Ikeuchi *et al*. 2016; Mathew y Prasad 2021). La regeneración de brotes a partir del tejido madre implica la desdiferenciación de las células y la entrada en mitosis, aspecto que comúnmente conduce a la formación de callos (Ikeuchi *et al*. 2016; Mathew y Prasad 2021).

Un avance importante hacia la comprensión de la regeneración de la raíz fue el hallazgo de que después del corte de la punta de la raíz en *Arabidopsis* que elimina la parte más distal, incluida la cofia y las células madre del meristemo, las células dentro del tejido

sobreviviente se transdiferencian para desarrollar un nuevo centro quiescente necesario para reconstruir los tejidos faltantes (Heyman *et al*. 2013; 2016; Ruiz-Aguilar *et al*. 2020). Este proceso implica cambios en los gradientes de auxinas, así como la señalización de ácido jasmónico y brasinoesteroides (Zhang *et al*. 2019; Zhou *et al*. 2019; Canher *et al*. 2020; Takahashi y Umeda 2022). Actualmente, se desconocen las funciones de los péptidos CLE en procesos de regeneración o callogénesis.

2. ANTECEDENTES

2.1. Regulación hormonal del desarrollo de las plantas

Las plantas son organismos extraordinariamente sensibles y adaptables, que a diferencia de los animales pueden cambiar su forma en respuesta a los estímulos del ambiente, mediante cambios discretos en los niveles de fitohormonas, compuestos de naturaleza química que influyen sobre el crecimiento y desarrollo. El concepto de "fitohormona" hace referencia a un compuesto bioactivo que proviene y/o actúa en un vegetal modificando procesos celulares en concentraciones pequeñas, usualmente nano o micromolares. Dichas substancias pueden actuar directamente sobre las células que las sintetizan y pueden translocarse a otros tejidos a través de proteínas transportadoras presentes en las membranas. Las principales hormonas descritas son las auxinas, las citocininas, el etileno, las giberelinas, el ácido abscísico, el ácido jasmónico, los brasinosteroides, así como péptidos pequeños que actúan como ligandos para la activación de respuestas del crecimiento, desarrollo e inmunidad (Blázquez *et al*. 2020).

La especie vegetal empleada tradicionalmente para el análisis genético de los procesos del desarrollo es *Arabidopsis thaliana* (*Arabidopsis*), una planta dicotiledónea perteneciente a la familia *Brassicaceae* (Meinke *et al*. 1998). El estadio vegetativo de *Arabidopsis* consiste en una roseta de alrededor de 8 cm de diámetro, de donde surgen posteriormente tallos florales que alcanzan una altura de entre 30 a 40 cm, dependiendo de la nutrición y otros factores del crecimiento. La planta adulta produce flores con 4 sépalos que se alternan con 4 pétalos, y en el centro se localiza el ovario, rodeado por los estambres productores de polen. Al ser hermafrodita, las flores de *Arabidopsis* se

auto-fecundan, dando lugar a la formación de un fruto denominado silicua, el cual puede contener de 30 a 60 semillas (Meinke *et al.* 1998).

Los estudios con *Arabidopsis,* extensivos durante la segunda mitad del siglo XX, posicionaron a esta planta como modelo central en investigación científica debido a que reúne diversas características, incluyendo su crecimiento rápido, la posibilidad de crecerla *in vitro* utilizando cajas de Petri, la producción de una alta cantidad de semillas en condiciones de laboratorio, se cuenta con la secuencia completa de su genoma, y hay disponibles líneas transgénicas reporteras en muchos de sus genes y mutantes en prácticamente cada uno de sus genes. Por lo tanto, la convierten en un excelente modelo para el estudio de la regulación hormonal del desarrollo, y las rutas de transducción de señales implicadas en la percepción de estímulos ambientales (Koorneef y Meinke 2010).

Gracias al estudio de *Arabidopsis* se han descubierto los principales sistemas de señalización hormonal, al respecto nos referimos a las vías de las auxinas y del ácido jasmónico, que son de relevancia para este trabajo.

2.2. La raíz: estructura y funciones

En las plantas, la organogénesis ocurre primordialmente después de la germinación con la formación del sistema aéreo conformado por tallos, ramas y hojas, así como del sistema radical. Las raíces generalmente se encuentran debajo de la superficie del suelo, donde crecen y responden a una amplia variedad de estímulos ambientales, brindan soporte estructural al follaje y son esenciales para la adquisición de agua y nutrientes. La supervivencia de la planta depende de un correcto desarrollo y crecimiento de la raíz. Sin embargo, el estudio detallado de los procesos asociados a su morfogénesis representa un desafío, debido a que estas son poco accesibles. La mayor parte de las especies vegetales presentan sistemas radiculares complejos y su observación a menudo requiere de medidas invasivas o destructivas (Petricka *et al.* 2012).

El sistema radicular de *Arabidopsis* está conformado por la raíz primaria que es el eje principal de crecimiento del cual surgen raíces laterales a partir de las células del periciclo. Tanto de la raíz primaria como de las raíces laterales se forman prolongaciones de las células epidérmicas conocidas como pelos radiculares, las cuáles extienden la

superficie de absorción (Motte *et al*. 2019; Maqbool *et al*. 2022).
En los ápices de crecimiento de la raíz primaria, así como de las raíces laterales se definen tres regiones subsecuentes, la zona meristemática, la zona de elongación y la zona de diferenciación. Las células presentes en el meristemo mantienen una capacidad de proliferación celular alta y un crecimiento lento, aspectos coordinados por el centro quiescente, formado por 4 células y cuya posición y mediante eventos de señalización, mantiene el estado indiferenciado de las células madre adyacentes. El meristemo se caracteriza por la presencia de células mitóticamente activas, donde se encuentra el centro quiescente (CQ), y las células madre que dan origen a los tejidos, en la zona de elongación las células provenientes del meristemo disminuyen su actividad proliferativa para comenzar a crecer y posteriormente alcanzan una morfología y función determinadas en la zona de diferenciación (Perilli *et al*. 2012). En la zona de elongación, las células aumentan hasta 20 veces su tamaño y se forma una gran vacuola central que ayuda a mantener el equilibrio osmótico. Cuando la punta de la raíz se encuentra frente a una barrera mecánica, las raíces giran y pueden desviar su crecimiento a través del crecimiento diferencial de sus células. En la zona de diferenciación, las células adquieren su función final y esta región se identifica fácilmente por la presencia de pelos radiculares (Ivanov y Dubrosvsky 2013).

2.3. El meristemo apical de la raíz

El meristemo apical de la raíz se forma durante la embriogénesis, y su principal función es proporcionar células para el crecimiento indeterminado. Está formado por el centro quiescente rodeado por un nicho de células madre, que producen todos y cada uno de los tejidos incluyendo el xilema, el floema, el periciclo, la endodermis, la corteza y la epidermis, las cuales se extienden longitudinalmente en torno al eje de crecimiento (Di Mambro y Sabatini 2018).

El centro quiescente es esencial para el mantenimiento del meristemo y su identidad puede ser controlada a través de dos vías de señalización paralelas: (i) la ruta PLETHORA (PLT) y (ii) la ruta SHORT-ROOT (SHR) / SCARECROW (SCR). Mutaciones en estos genes provocan la pérdida del centro quiescente y el arresto prematuro del crecimiento de la raíz. Los genes *PLT* codifican para factores de transcripción del tipo

AP2, mientras que *SHR* y *SCR* codifican miembros de la familia de factores de transcripción tipo GRAS. Los niveles más altos de proteínas PLT se encuentran en la región del nicho de células madre, donde promueven la división celular, mientras que la proteína SHR se mueve desde la endodermis hacia las capas celulares adyacentes para activar la transcripción de *SCR* que da como resultado la división asimétrica y la mitosis (Aida *et al*. 2004; Shaar-Moshe y Brady 2022).

La alta tasa mitótica del meristemo implica que el ciclo celular debe contar con todos los nutrientes necesarios para que ocurra la duplicación del ADN durante la fase S, aspecto tremendamente delicado, ya que antibióticos producidos por microorganismos o agentes tóxicos como salinidad o metales interfieren con la replicación causando la muerte celular, y este daño, es suficiente para inducir la diferenciación del meristemo y comprometer el crecimiento de la raíz (Raya-González *et al*. 2018). Se han identificado mutaciones que comprometen la viabilidad de las células meristemáticas, por ejemplo, las causadas en los genes que codifican para la subunidad 18 del complejo MEDIADOR (MED), o en la subunidad mayor de la RNA polimerasa II, las cuáles desarrollan una raíz de menor tamaño que las plantas normales (Raya-González *et al*. 2018; 2022). Los reportes mencionados proporcionaron las primeras evidencias de la importancia de la transcripción para la viabilidad celular y el crecimiento de la raíz.

2.4. Respuesta de la raíz ante el agobio ambiental

Por su característica sésil, las plantas responden y se adaptan a una amplia gama de factores ambientales a través de procesos bioquímicos, fisiológicos y moleculares muy dinámicos, proceso conocido como plasticidad vegetal (De Jong *et al*. 2020). El estrés abiótico reduce el crecimiento y el rendimiento por debajo de los niveles óptimos y puede incluso comprometer la reproducción y sobrevivencia de las plantas, aquí se incluyen el estrés por temperatura (frío o calor), la sequía, la salinidad, la escasez de nutrientes o la presencia de metales o agentes tóxicos (Zhang *et al*. 2022). Por otra parte, el daño por herida es uno de los principales tipos de estrés, provocado tanto por factores ambientales como el viento, la lluvia, el granizo o biológicos, como el ataque por herbívoros o insectos. Las heridas representan una amenaza constante para la supervivencia de las plantas

debido a que no solo se destruyen físicamente los tejidos, sino que además propician una vía de entrada para la invasión de patógenos (Hilleary y Gilroy 2018).

Las respuestas moleculares al estrés implican interacciones con múltiples vías de señalización. Cuando las plantas perciben daño en alguno de sus órganos, se activan respuestas de defensa que incluyen la producción de especies de oxígeno reactivas (ROS, por sus siglas en inglés) y las especies de nitrógeno reactivas (RNS, por sus siglas en inglés), lo que modula la actividad enzimática y la expresión génica (Suzuki y Mittler 2012). Sin embargo, una consecuencia secundaria de la producción de ROS, es su daño a macromoléculas como el ADN y proteínas, así, para contrarrestar el estrés oxidativo, se producen compuestos antioxidantes como los carotenoides, xantofilas, glutatión, tocoferol y ascorbato, y ocurre la activación de enzimas como superóxido dismutasa, catalasas y peroxidasas que detoxifican las ROS producidas (Pitzschke *et al*. 2006).

Las hormonas son reguladores importantes de las respuestas al estrés abiótico. El ácido abscísico (ABA), el etileno (Et) y el ácido jasmónico (AJ) se consideran mensajeros celulares importantes ante una señal de estrés. Bajo condiciones de sequía, se evita la pérdida de agua inhibiendo la transpiración mediante el cierre de estomas, una respuesta fisiológica dependiente de ABA (Chen *et al*. 2020). De igual manera, el etileno y el AJ están involucrados en un gran número de respuestas asociadas al estrés abiótico, incluyendo sequía, ozono, hipoxia y anoxia, calor, frío, heridas y luz U.V. (Waadt *et al*. 2022). Algunas moléculas como el óxido nítrico (NO, por sus siglas en inglés) y los gradientes de Ca^{2+} intracelulares están implicados en múltiples respuestas de defensa de tipo biótico y abiótico. De hecho, las células guardianas se abren o cierran bajo el control del ABA en respuesta a estrés por sequía mediante cambios en los niveles de NO y Ca^{2+} y una activación de la señalización dependiente de proteínas cinasas activadas por mitógeno (MAPK), enzimas involucradas en múltiples procesos de desarrollo y crecimiento vegetal, así como en respuesta a estrés biótico y abiótico (Neill *et al*. 2008). Por lo tanto, la capacidad de las plantas de reconocer y responder a las diversas señales ambientales a través de la modulación de los niveles espacio-temporal de las fitohormonas y moléculas señalizadoras determina su adaptación

2.5. Respuestas fisiológicas y moleculares ante un daño al ADN

La capacidad de un organismo para reparar el daño del ADN de manera oportuna es esencial para la integridad y el mantenimiento de su genoma. Diferentes tipos de estrés, incluyendo la exposición a distintos mutágenos ambientales como la radiación ultra-violeta, metales pesados, y antibióticos secretados por microorganismos de la rizósfera causan estrés genotóxico y comprometen la integridad del genoma. Cuando se detecta daño, el ciclo celular se detiene y los mecanismos de reparación entran en acción (Balestrazzi *et al.* 2011).

El ADN puede sufrir ruptura sencilla o doble de su cadena, lo cual puede conducir a la activación de procesos de muerte celular programada y diferenciación. Dependiendo del daño se activan respuestas moleculares específicas a través de elementos que actúan como sensores, mediadores y efectores (Gentric *et al*. 2021). La cinasa de detección de daños en el ADN *ATAXIA-TELANGIECTASIA MUTATED* (*ATM)* se activa mediante rupturas de ADN de doble cadena, actuando principalmente en los puntos de control G1/S y G2/M del ciclo celular. ATM al igual que ATR (RAD3-RELATED) son blancos de *SUPPRESSOR OF GAMMA RESPONSE 1* (*SOG1*), los cuales en conjunto controlan los genes relacionados con la respuesta de daño y genes implicados en los puntos de control del ciclo celular (Gentric *et al*. 2021). Estos son algunos de los mecanismos por los cuales las plantas logran adaptarse y sobrevivir a las diferentes condiciones de crecimiento.

2.6. Regeneración celular

La célula es la unidad básica para la regeneración de tejidos, fenómeno que puede ocurrir en múltiples niveles de organización biológica. La lesión más pequeña requiere de la reparación de una sola célula, mientras que un trauma severo podría requerir el reemplazo de un órgano completo a través de la morfogénesis coordinada mediante señalización entre distintos tipos de tejido, lo que se conoce como transdifferenciación (Thowfeequ *et al.* 2007).

La regeneración ocurre ampliamente en el reino animal, aunque la restauración tisular varía considerablemente. En los invertebrados, se pueden regenerar órganos completos,

por ejemplo, en las planarias del género *Turbellaria,* el organismo logra sobrevivir a traumatismos severos y compensan la pérdida de células a través de la re-especificación celular y entrada a mitosis (Reddien 2018). Dependiendo del grado de daño, el proceso regenerativo implica varios pasos, entre ellos puede ocurrir la cicatrización de la herida, el cambio de identidad celular (i.e. formación de blastema) y la diferenciación. Mediante el uso de distintos organismos modelo, incluyendo a *Drosophila*, *Hydra*, *Xenopus*, y ratón, se ha revelado que la apoptosis puede ser una fuerza impulsora de la proliferación celular que conduce a la formación de blastema y la regeneración de tejidos, un fenómeno que se conoce como "proliferación compensadora inducida por apoptosis" (Fan y Bergmann 2008; Krasovec *et al.* 2022).

Las plantas, además de poseer la capacidad de reemplazar los órganos perdidos, muestran una sorprendente habilidad para regenerar partes dañadas, siendo ésta una de las características más importantes para su supervivencia. Las especies arbóreas pueden regenerar nueva corteza y vasculatura después de una herida (Xu 2018). Esto ocurre por la activación de genes específicos del floema, como *ALTERED PHLOEM DEVELOPMENT* (*APL*), *KANADI* (*KAN*) y *DNA binding with one finger* (*DOF*) y del cambium como *CLAVATA1* (*CLV1*), *CLV2*, *AINTEGUMENTA1* (*ANT1*), *SHR* y *SCR* que contribuyen en el establecimiento de la identidad de los tejidos regenerados (Pulianmackal *et al.* 2014).

El proceso de regeneración de raíces a partir de explantes de tallos o ramas ha sido de utilidad en tecnologías agrícolas, como los esquejes para la propagación vegetativa. Aquí ocurren tres fases sucesivas: una señalización temprana al momento de la herida, acumulación localizada de auxinas en el sitio de daño y transición del destino celular que involucra dos tipos de células con diferentes funciones, la célula convertidora que percibe las señales tempranas para influir sobre las células vecinas y la célula competente para la regeneración que experimenta la transición del destino guiada por auxinas (Xu 2018).

El potencial regenerativo es variable dependiendo de los tejidos, por ejemplo, la eliminación de la punta de la raíz regenera un meristemo completo si el corte se realiza a la altura del centro quiescente, pero si el corte se realiza en la zona de formación de pelos radiculares, la herida cicatriza y el crecimiento termina (Efroni *et al.* 2016). A

diferencia de la regeneración de un centro quiescente que ocurre cuando el meristemo sufre un daño por tratamiento con láser, la reprogramación de la identidad celular perdida en el meristemo de la raíz después de su eliminación con corte por navaja, no requiere de células madre pre-existentes, más bien sucede por un cambio de identidad de las células adyacentes que adquieren totipotencia (Efroni *et al.* 2016).

Mediante análisis finos en respuesta a la pérdida del meristemo en puntas de raíces de *Arabidopsis thaliana* se han identificado diferentes elementos genéticos implicados en estos procesos regenerativos, incluyendo el complejo heterotrimérico conformado por los factores de transcripción ETHYLENE RESPONSE FACTOR 115 (ERF115) y PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1) (Heyman *et al.* 2016). El complejo ERF115-PAT1 dirige la regeneración de las plantas para reemplazar de manera eficiente las células dañadas por otras nuevas (Heyman *et al.* 2016). Un fenómeno similar se observó en plántulas de *Arabidopsis* expuestas al compuesto genotóxico bleomicina, cuya capacidad de unirse al DNA y promover su ruptura, desencadena la muerte de las células en rápida división, en especial las de la pro-vasculatura. Se observó que plantas tratadas con bleomicina sobre-expresan los genes ERF115 y PAT1, principalmente en las células vecinas al daño, un patrón de expresión similar al que sucede durante el programa regenerativo de la punta de la raíz. Estos resultados muestran la relevancia del complejo ERF115-PAT1 sobre el potencial regenerativo ante un daño en tejidos u órganos (Heyman *et al.* 2016).

2.7. Callogénesis

La aplicación de diferentes reguladores del crecimiento en medios de cultivo se ha utilizado durante mucho tiempo para propagar plantas *in vitro* y es la base del cultivo de tejidos. Con las ventajas de la totipotencia de las células vegetales, es factible obtener segmentos de tallos, hojas o raíces e inducir la organogénesis para producir plantas completas, en este sistema, la aplicación de auxinas conduce a la formación de raíces en tanto que las citocininas producen hojas (Yan *et al.* 2020; Zhai y Xu 2021; Temmerman *et al.* 2022). La combinación de altas concentraciones de auxinas y citocininas induce la producción de callos in vitro, que aparece inicialmente a partir de tejido diferenciado y

adquiere las características de un tipo de neoplasia o tumor, que es generalmente indiferenciado.

En los callos derivados del tejido de *Arabidopsis*, las células se asemejan al meristemo de las raíces y tiene el potencial de producir brotes y raíces (Rosspopoff *et al*. 2017; Hanano *et al*. 2020). Se ha documentado que los tratamientos con citocininas cambian la identidad de los primordios de las raíces laterales para formar meristemos de follaje (Rosspopoff *et al*. 2017). Aunque la inducción de callos se ha utilizado como una estrategia para la multiplicación celular y propagación de materiales vegetales, se conoce muy poco de los mecanismos moleculares que conducen a las transiciones en los estados diferenciado-indiferenciado, así como a la adquisición de nuevas características.

2.8. Comunicación celular mediada por péptidos y receptores con actividad de cinasa

La comunicación celular en las plantas implica el reconocimiento de una amplia gama de estímulos bióticos y abióticos que afectan el crecimiento y desarrollo, así como la respuesta inmune. En las últimas dos décadas se han logrado avances muy importantes en el entendimiento de los mecanismos de percepción de señales entre las que participan hormonas como las auxinas, citocininas, etileno, brasinoesteroides y péptidos. (Murphy *et al*. 2012; Richardson y Torii 2013; Shinohara y Matsubayashi 2015).

En las plantas, diversos péptidos de entre 12 a 14 aminoácidos de la familia CLAVATA3/EMBRYO SURROUNDING (CLE) actúan a través de su unión a proteínas de la membrana plasmática que son estructural y funcionalmente similares a receptores con actividad de cinasa codificados en los genomas de los animales, y que desempeñan funciones muy importantes en el desarrollo y en la adaptación ante el agobio ambiental Por esta razón se les ha denominado receptores similares a proteínas cinasas o *Receptor-Like Kinases* (RLK), por sus siglas en inglés (**Fig. 1**).

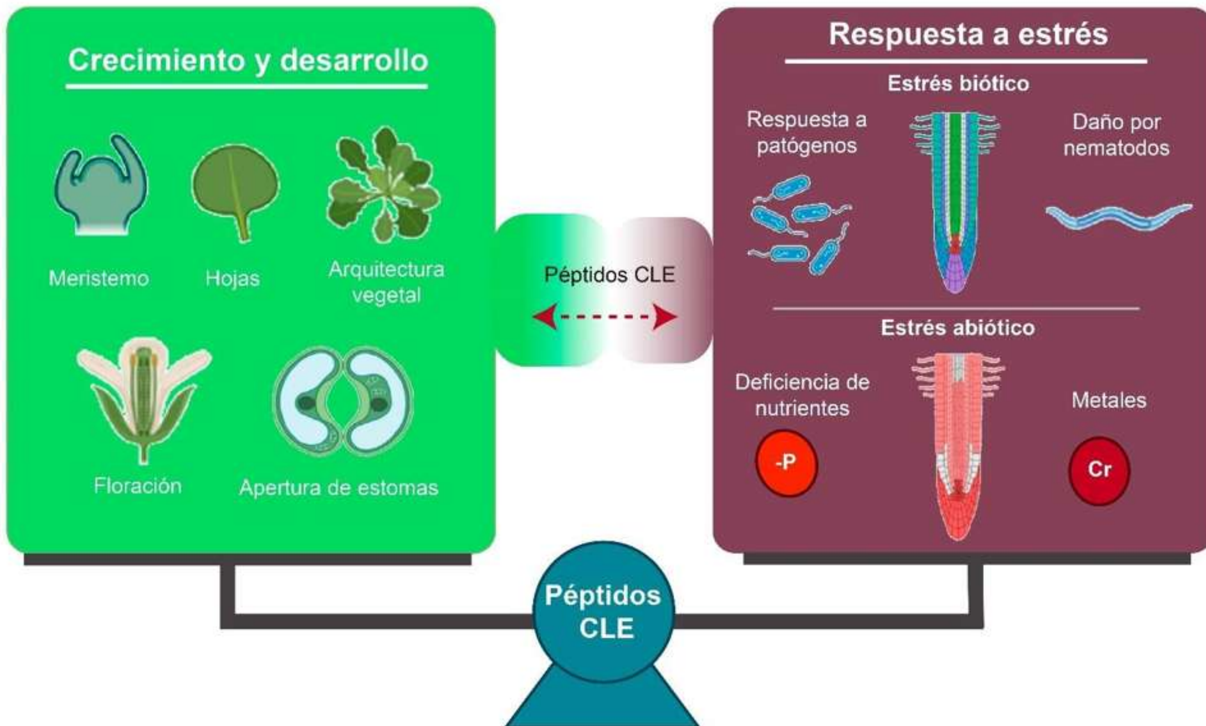


Figura 1. Los péptidos de la familia CLE y su papel en el desarrollo vegetal y la adaptación al estrés. El balance entre crecimiento y respuesta al estrés depende de una percepción correcta de señales en el ambiente. Los péptidos pequeños de la familia CLE actúan como ligandos celulares y afectan el funcionamiento de meristemos, el desarrollo foliar, la floración y apertura de estomas, o la respuesta a patógenos, deficiencia de nutrientes y toxicidad por metales.

La primera RLK se encontró en el maíz (Walker y Zhang 1990), y hasta la fecha se han descrito en un gran número de especies vegetales (Zhu *et al*. 2023) con al menos 610 miembros en *Arabidopsis* y 1131 en arroz (Shiu *et al*. 2004). Un RLK típico consiste de un solo dominio transmembranal, un dominio cinasa intracelular y una región extracelular de composición variable, a través de la cual se detectan las señales externas como fitohormonas y péptidos pequeños. Los receptores RLK facilitan la comunicación intercelular durante crecimiento de las plantas, desarrollo y respuesta al estrés. Por ejemplo, un péptido pequeño bien estudiado es CLAVATA1 (CLV1), que se asocia con el receptor CLAVATA3 y regula la homeostasis del meristemo apical del follaje. La señalización mediada por RLK promueve la activación transcripcional de genes implicados en la defensa contra patógenos, como en el caso del reconocimiento de las bacterias, en que un epítope de un componente estructural del flagelo (flagelina22;

FLG22) se une al receptor Flagelin Sensible 2 (FLS2) para iniciar el reclutamiento de los correceptores BAK1 para formar heterodímeros y la posterior transfosforilación de su dominio citoplásmico.

2.9. Clasificación de los receptores tipo cinasa

La mayoría de los RLK vegetales tienen tres dominios proteicos importantes: el dominio extracelular; el dominio transmembranal; y el dominio citoplasmático con actividad de cinasa. El dominio extracelular se encuentra en el extremo amino terminal y está conectado con el péptido señal, puede detectar un estímulo externo, polimeriza con otros receptores homólogos o heterólogos, y luego Inicia el proceso de transmisión de la señal. El dominio transmembranal atraviesa la membrana citoplasmática y contiene entre 22-28 aminoácidos y es responsable de fijar las proteínas a la membrana. El dominio cinasa intracelular está ubicado en el extremo carboxilo terminal y está altamente conservado, con sitios de fosforilación de serina/treonina, y transmite la transmisión de la señal a otras proteínas cascada abajo a través de eventos de fosforilación. Hay muchos tipos de dominios extracelulares y según sus características, los RLK se pueden dividir en al menos once subfamilias, de las cuáles las proteínas con dominios extracelulares con repetidos ricos en leucina (LRR-RLKs) son los más abundantes con al menos 223 miembros (Ou *et al.* 2021) (**Fig. 2**). Además de los RLK típicos, hay un gran número de RLK atípicos denominados cinasas citoplasmáticas similares a receptores que se fijan en la membrana plasmática, pero carecen de dominios de unión extracelular.

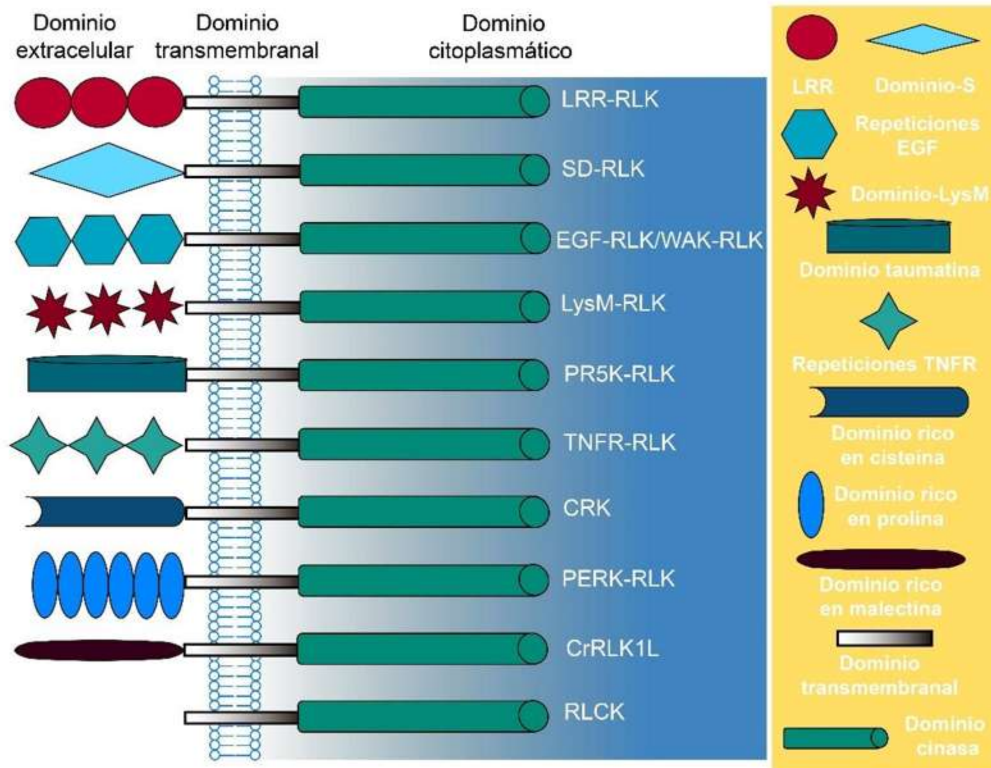


Figura 2. Estructura general de los receptores con actividad de cinasa de *Arabidopsis thaliana*. El dominio citoplásmico es la región más conservada de las proteínas y posee actividad de cinasa. El dominio extracelular es variable y une a diferentes ligandos, entre ellos péptidos pequeños de la familia CLE.

2.10. Funciones de los receptores tipo cinasa

El crecimiento y desarrollo de las plantas ocurre gracias a la actividad mitótica que se realiza en los meristemos de la raíz y del follaje. En estos sitios, el ciclo celular se mantiene constante, lo que permite la división celular, por lo tanto, la producción de biomasa durante todo el ciclo de vida del organismo. En *Arabidopsis*, la actividad mitótica del meristemo del follaje está controlada por el receptor CLAVATA 1 (CLV1), un miembro de la subfamilia LRR-RLK mediante el reconocimiento del dodecapéptido CLV3 y cuya interacción afecta la expresión del factor de transcripción WUSCHEL (WUS) que se expresa en la región central del meristemo y promueve a su vez la expresión de CLV3 (Clark *et al*. 1993; 1997). CLV1 recluta a otros receptores tipo cinasa para formar un complejo receptor/co-receptor, y posteriormente media las señales de retroalimentación

CLV3-WUS, modulando el tamaño del meristemo y el número de órganos florales que se producen (Suzaki *et al*. 2004).

En el meristemo de la raíz se han descrito varios tipos de receptores qué a través la interacción con diferentes ligandos, promueven o afectan la integridad tisular. Los péptidos de la familia CLV3/EMBRYO SURROUNDING REGION-related (CLE) interaccionan con diferentes RLKs con efectos distintivos sobre los meristemos y el crecimiento de la raíz. Por ejemplo, el péptido CLE40 interacciona con la cinasa ACR4 y restringe la expresión del factor de transcripción WOX5 en el centro quiescente, afectando el nicho de células iniciales (Stahl *et al.* 2009). El péptido ROOT GROWTH FACTOR (RGF1), interactúa con una familia de 5 receptores, denominados RGF1 INSENSITIVE (RGI) con dominios extracelulares ricos en leucina. El fenotipo de una mutante quíntuple *rgi1/2/3/4/5*, es similar al de las mutantes con pérdida de función en las proteínas cinasas activadas por mitógenos MPK3 y MPK6, MKK4 y MKK5, o YDA, mostrando un fenotipo de raíz corta, que se asocia con una actividad mitótica reducida y una menor expresión de los factores de transcripción PLETHORA 1 (PLT1) / PLT2 en el meristemo (Lu *et al*. 2020; Shao *et al*. 2021). En un trabajo reciente, Pelagio-Flores y col. (2020) mostraron que la mutación y la sobreexpresión del receptor CRK28, respectivamente, afecta de manera contrastante el crecimiento y desarrollo de *Arabidopsis*. Las mutantes *crk21-1* produjeron raíces de mayor longitud y pelos radiculares cortos, en tanto que las sobreexpresoras 35S:CRK28 presentaron raíces cortas con pelos radiculares muy largos, fenotipo similar al observado con la aplicación de diferentes dodecapeptidos.

La familia RLK contiene varias proteínas que participan en el desarrollo de las hojas. El receptor ERECTA (ER) pertenece a la subfamilia LRR-RLK, incluyendo también a ERECTA-LIKE1 (ERL1) y ERL2. Desempeñan un papel importante en la regulación de la morfología foliar, desarrollo de los estomas y respuesta al estrés biótico y abiótico. Los péptidos EPIDERMAL PATTERNING FACTOR-LIKE2 (EPFL2) y EPFL9 ricos en cisteína, interactúan con ERECTA, así promueven el crecimiento de los cotiledones, la producción de óvulos en etapa reproductiva, la formación de las semillas y el crecimiento

de los frutos (Kawamoto *et al*. 2020; Fujihara *et al*. 2021). En el arroz y jitomate la expresión de un homólogo de ERECTA confirió tolerancia al calor (Shen et al. 2015).

El receptor tipo cinasa mejor conocido y ampliamente estudiado, denominado BRASSINOSTEROID RESPONSE 1 (BRI 1), se identificó en la búsqueda de genes que median la respuesta de *Arabidopsis* a los brasinosteroides. BRI1 es un LRR-RLK típico que se dimeriza con sus co-receptores SOMATIC EMBRYOGENESIS RECEPTOR KINASE 3 (SERK3) y BAK1 (Clouse *et al*. 1996, Zheng *et al*. 2019; Hohman *et al*. 2020). BAK1 y SERK3 actúan como correceptores de varios receptores RLK y actúan en diversos procesos de crecimiento y desarrollo de las plantas activando al factor transcripcional BRI1-EMS-SUPPRESSOR1 (BES1). SERK2 es un componente de la señalización por brasinosteroides en arroz, ya que su sobreexpresión aumenta el tamaño del grano y confiere resistencia al estrés salino (Dong *et al*. 2020). El crecimiento retardado de la raíz en las líneas de *Arabidopsis* que sobreexpresan la construcción *35S:CRK28* correlacionó con el aumento en la expresión de genes inducibles por ácido abscísico y de los factores de transcripción ABI4 y ABI5 (Pelagio-Flores *et al*. 2020). Hasta la fecha, no existe información detallada sobre la participación de los receptores con actividad de cinasa y los péptidos que estos reconocen sobre procesos de viabilidad celular, regeneración y callogénesis, por lo que lograr algunos avances en este campo sería de gran relevancia para entender sus funciones en las plantas.

3. JUSTIFICACIÓN

Con base en los antecedentes, este trabajo parte de la premisa de que existe una relación funcional entre la regulación de la señalización por CLE14 y procesos de estrés abiótico en el desarrollo de la raíz de *Arabidopsis*. Queda por determinarse hasta qué punto existe una correspondencia funcional en relación a procesos de daño, regeneración celular y/ formación de callos. Por lo anterior, este trabajo se plantea para esclarecer si la señalización dependiente de CLE14 está implicada en procesos de la viabilidad y regeneración celular en *Arabidopsis thaliana*.

4. HIPÓTESIS

La señalización dependiente de CLE14 participa en procesos de viabilidad y regeneración celular en *Arabidopsis thaliana*.

5. OBJETIVOS

5.1. Objetivo general

Determinar la función del péptido CLE14 y el receptor PEPR2 en procesos de viabilidad y regeneración celular en *Arabidopsis thaliana*

5.2. Objetivos específicos

I. Determinar el efecto del péptido CLE14 y su sobreexpresión en el desarrollo de la raíz de *A. thaliana*.

II. Analizar el papel de *CLE14* en la regeneración de la punta de la raíz y la formación de callos en explantes de tallo de *A. thaliana*.

III. Estudiar la regulación de la expresión de *PEPR2* en respuesta a diferentes factores inductores de estrés.

6. MATERIALES Y MÉTODOS

6.1. Material biológico y reactivos

Se utilizaron semillas de *Arabidopsis thaliana* del ecotipo silvestre Columbia 0 (Col-0), así como de las líneas transgénicas *pERF115:GUS-GFP* (Heyman *et al*. 2016), *CLE14:GUS-GFP* (Gutiérrez-Alanís *et al.* 2018) *PEPR2:GUS* (Wu *et al*. 2016), para determinar la función del péptido CLE14, sobre el procesos de regeneración celular, los Dres. Alanís y Herrera nos compartieron semillas de plantas que sobreexpresan el marco de lectura abierto de CLE14 bajo el control del promotor fuerte y constitutivo del virus del

mosaico de la coliflor 35S (*35S:CLE14*). Para el medio suplementado con el péptido CLE14 se solicitó una muestra del péptido sintético de 12 amino ácidos a los Dres. Dolores Alanís y Luis Herrera Estrella, adscritos al Laboratorio Nacional de Genómica para la Biodiversidad del CINVESTAV, campus Irapuato, quienes nos proporcionaron amablemente una muestra (Genscript) que tiene una pureza del 98%, las concentraciones evaluadas del péptido se realizaron en un rango de 0,001 a 1 µM. El yoduro de propidio (IP) y el 5-bromo-4-cloro-3-indolil-β-D-glucurónido (X-Gluc) se adquirieron en la casa comercial Sigma-Aldrich.

6.2. Preparación de semilla y condiciones de crecimiento

Las semillas fueron sembradas y crecidas en medio de cultivo para tejido vegetal bajo condiciones axénicas. Para esto, se utilizaron sales comerciales Murashige y Skoog (MS) 0.2x base del medio de cultivo, adicionado con sacarosa como fuente energética y agar como gelificante. El pH del medio de cultivo se ajustó a pH 7.0 para posteriormente ser sometido a un proceso de esterilización en una autoclave bajo una presión de 15 lbs por 20 minutos. El medio de cultivo, se vertió en cajas Petri estériles, y una vez gelificado, las semillas de *Arabidopsis* previamente desinfectadas con etanol y cloro fueron sembradas y germinadas sobre el medio de cultivo, todo esto en una campana de flujo laminar. Las placas Petri con las semillas de *Arabidopsis* se colocan en una cámara de crecimiento vegetal en posición vertical bajo condiciones óptimas de crecimiento con un fotoperiodo de 16 horas de luz y 8 horas de oscuridad, a una intensidad de luz de 100 µmol m^2s^{-1} a 22°C de temperatura.

6.3. Análisis histoquímico de la actividad de la β-glucuronidasa

Las líneas *pERF115:GUS-GFP*, *PEPR2:GUS* y *CLE14:GUS-GFP* se transfirieron a cajas de microtítulo con X-Gluc al 0.1% (5bromo-4-cloro-3-indolil-ß-D-glucoronido) en amortiguador de fosfatos (NaH_2PO_4 Na_2HPO_4, 0.1 M, pH 7) y se incubaron por 6 horas a 37 °C. Posteriormente, se retira la solución de X-Gluc y las plantas se clarifican con una solución de HCl 0.24 N en metanol al 20% (v/v) durante 60 minutos a 62 °C, después la solución se removió y se agregó NaOH al 7% (v/v) en metanol al 60% (v/v) por 25 minutos a temperatura ambiente. La solución fue retirada y el tejido se deshidrató con

tratamientos de etanol al 40, 20 y 10% (v/v) por 20 minutos cada uno. Una vez retirado el etanol al 10%, se adicionó glicerol al 50% (v/v) y se colocaron en portaobjetos para su análisis mediante microscopía de contraste de interferencia diferencial (DIC; por sus siglas en inglés) en un microscopio Leica DM 5500 B.

6.4. Ensayos de corte de raíz y regeneración

Se utilizaron plantas de *Arabidopsis* de cuatro días de edad y las puntas de las raíces se cortaron con un bisturí estéril con ayuda de un microscopio estereoscópico (Leica MZ6) en condiciones axénicas. Los cortes se realizaron en la punta de la raíz por encima de las células del QC. El análisis de la estructura del meristema apical de la raíz (RAM), la viabilidad y la regeneración celular se realizó cada 24 h durante 3 días después del corte. Para esto, las raíces de las plantas se tiñeron con yoduro de propidio (YP) y se colocaron en portaobjetos para ser observadas con un microscopio confocal (Olympus FV1200; Olympus Corp., Tokio, Japón).

6.5. Tinción con yoduro de propidio (IP) y detección de la proteína verde fluorescente

Para el análisis de las líneas transgénicas *CLE14:GUS-GFP* y *pERF115:GUS-GFP* se tomaron las plantas del medio y se incluyeron en una solución de yoduro de propidio al 0.1% (v/v) sobre un portaobjetos y finalmente se analizaron en un microscopio confocal Olympus Fluo-View FV1000-PME. Para la detección de la fluorescencia de GFP se utiliza una longitud de onda de excitación de 488 nm y el registro de un haz de 509 nm, para el YP se utiliza la longitud de onda de 568 nm para la excitación y una ventana de emisión de 585-610 nm.

6.6. Análisis estadístico

Los datos obtenidos de los experimentos se analizaron estadísticamente con el software STATISTICA 10 (Statsoft, 2010), aplicándoles análisis de varianza, ANOVA de una vía y pruebas de significancia de Tukey con una $P<0.05$.

7. RESULTADOS

7.1. La aplicación de CLE14 o su sobre-expresión inhiben el crecimiento de la raíz y promueven la diferenciación celular en *Arabidopsis*

En estudios previos, se analizó farmacológicamente la bioactividad de CLE14 en el crecimiento de la raíz y la formación de pelos radiculares, lo que indicó efectos contrastantes del péptido en estas características (Hayashi *et al*. 2018; 2019). Con el objetivo de conocer más a detalle las propiedades de regulación de la arquitectura de la raíz por CLE14, en una primera serie de ensayos, se crecieron plantas de *Arabidopsis* del ecotipo Col-0 en cajas de Petri que contenían medio nutritivo de Murashige y Skoog (MS) al 0.2% sin o con un suministro de CLE14 en concentración final de 1 μM. La aplicación del péptido causó la represión del crecimiento de la raíz primaria y la formación de raíces laterales (**Fig. 3a, b**). Un análisis detallado de la región meristemática por microscopía confocal evidenció el adelgazamiento de la raíz y la formación de pelos radiculares en la punta (**Fig. 3c**).

Una estrategia para conocer la función de genes específicos en procesos del desarrollo y la morfogénesis es la sobreexpresión en plantas transgénicas. Los Dres. Alanís y Herrera nos compartieron semillas de plantas que sobreexpresan el marco de lectura abierto de CLE14 bajo el control del promotor fuerte y constitutivo del virus del mosaico de la coliflor 35S (*35S:CLE14*). La comparación del crecimiento de las plantas normales (Col-0) con las transgénicas, crecidas lado a lado en la misma caja de Petri conteniendo medio nutritivo de Murashige y Skoog (MS) al 0.2x, mostró el fenotipo de raíz corta en la línea transgénica (**Fig. 4a, b**). Estos resultados indican que CLE14 ejerce efectos antagónicos inhibiendo el crecimiento y promoviendo la diferenciación celular epidérmica en la raíz de *Arabidopsis*.

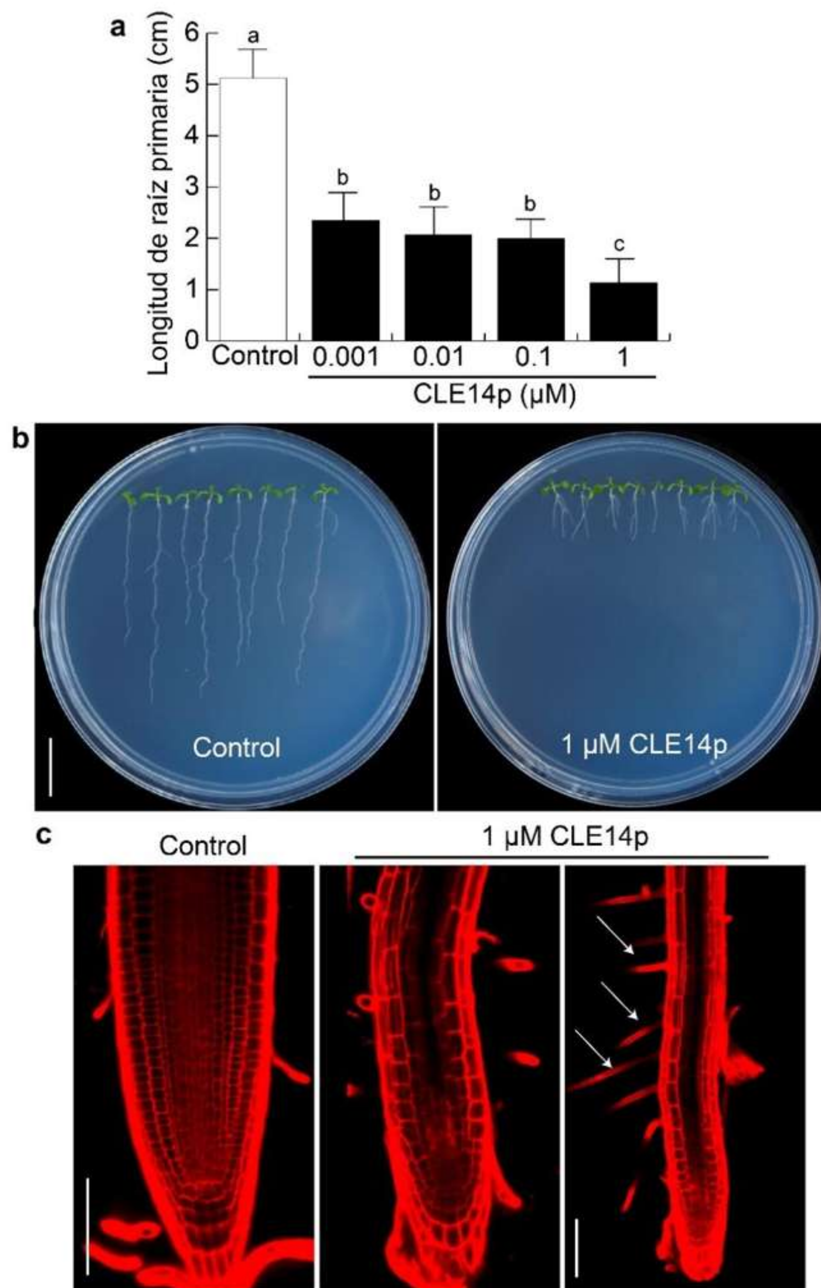


Figura 3. La aplicación de CLE 14 detiene el crecimiento de la raíz de *Arabidopsis* y promueve la ramificación de la raíz y la formación de pelos radiculares. a-b) Efecto de la aplicación de CLE14 a los medios de crecimiento sobre el fenotipo de las plántulas de *Arabidopsis* de 10 días después de la germinación. c) Estructura del meristemo de la raíz de plántulas cultivadas en medio carente de CLE14 (izquierda) o suplementadas con 1 μM de CLE14 (imágenes media y derecha). Se muestran imágenes representativas de plantas incubadas con yoduro de propidio para denotar en color rojo la estructura tisular (n=12). Las flechas blancas indican los pelos radiculares. Las letras en (a) indican medias con diferencia significativa (P<0.05). El experimento se realizó al menos tres veces con resultados similares. Barra de escala en (b)= 1 cm y en (c)= 50 μm.

.

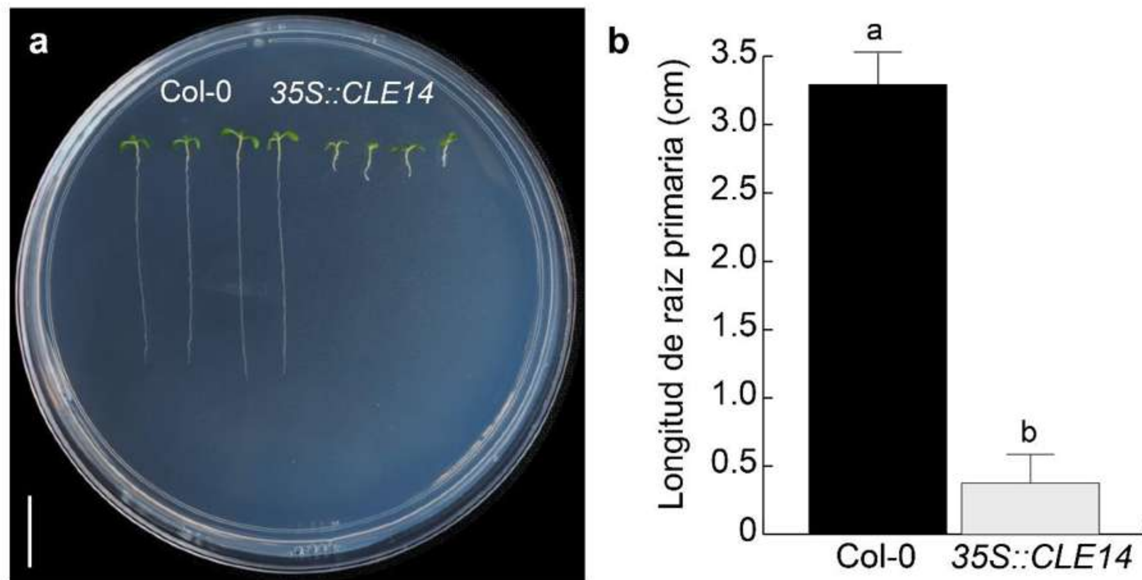


Figura 4. La sobre-expresión de CLE14 inhibe el crecimiento radicular en *Arabidopsis*. Se muestran plantas de 8 días del ecotipo Col-0 y transgénicas *35S:CLE14* germinadas y crecidas en la misma placa. La gráfica muestra la media y la desviación estándar de 12 plántulas analizadas. El experimento se repitió tres veces. Las imágenes de las placas son representativas de 4 cajas independientes. Diferentes letras en (b) indican medias con diferencia significativa ($P<0.05$). Barra de escala= 1 cm.

7.2. CLE14 se expresa durante la regeneración de la punta de la raíz de *Arabidopsis*

Las plantas manifiestan una fuerte capacidad para recuperar los tejidos faltantes después del daño, a través de la reversión del estado diferenciado y la adquisición de identidad celular pluripotente (Heyman *et al*. 2013; 2016; Ruiz-Aguilar *et al*. 2021; Takahashi *et al*. 2022). La eliminación de la punta de la raíz de *Arabidopsis* con un bisturí se ha utilizado para caracterizar el potencial de regeneración, mediante dicha estrategia ha sido posible observar la reconstrucción de los tejidos faltantes a través de la activación de la división celular (Ruiz-Aguilar *et al*. 2021). En plantas intactas, el análisis de la expresión de *pCLE14:GUS-GFP* mediante tinción con yoduro de propidio y detección de fluorescencia de la proteína verde fluorescente (GFP) por microscopía confocal, mostró la señal verde en la capa más externa de la columela, que permaneció desde los 4 hasta los 7 días posteriores a la germinación, tiempo

en que se realizó el estudio (dag, **Fig. 5a-d**). La eliminación de la punta de la raíz permitió detectar la proteína verde fluorescente en la capa celular adyacente al corte (**Fig. 5e, f**), y a medida que el proceso de regeneración avanza, a partir de 2-3 días después del corte, se formó una nueva punta de raíz, y la expresión se restable, restringiéndose a la capa celular externa de la cofia (**Fig. 5g-h**). Estos resultados muestran la inducción de la expresión de CLE14 en respuesta a un daño celular y durante el proceso regenerativo.

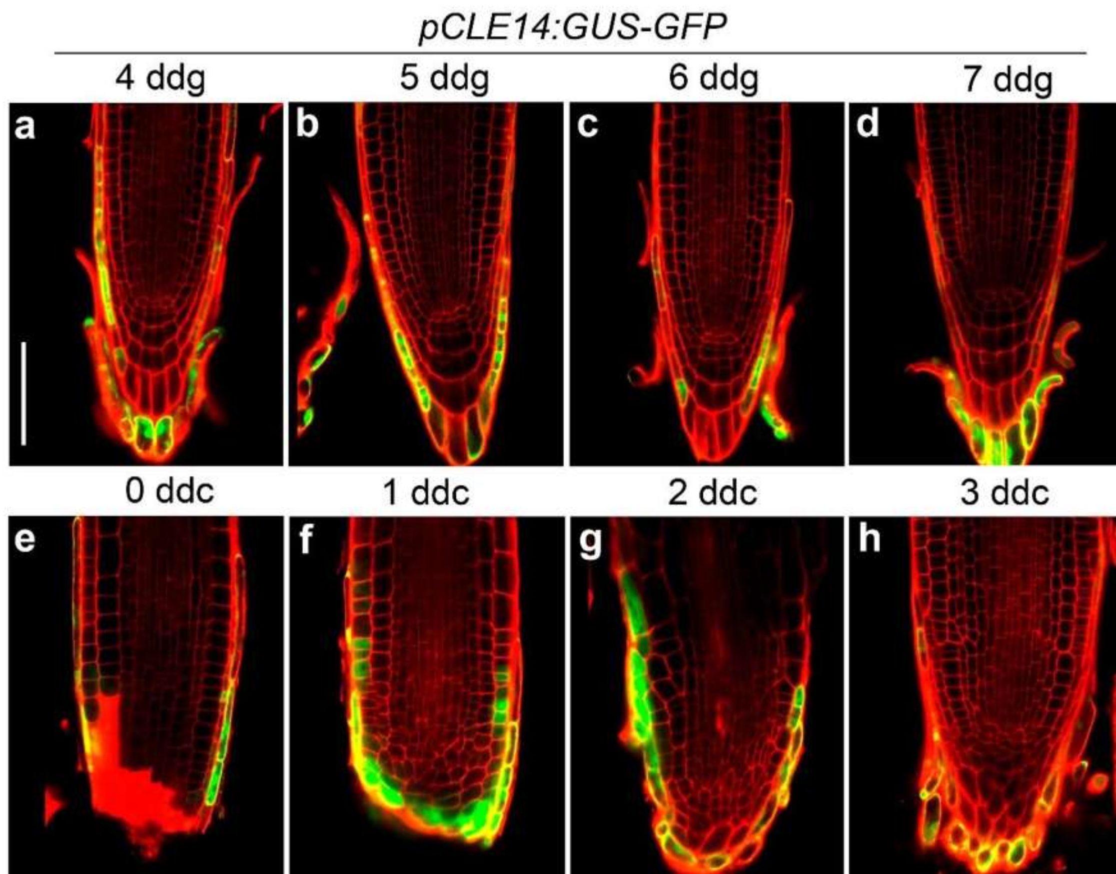


Figura 5. CLE14 se expresa en células del borde después de la escisión de la punta de la raíz. Imágenes por microscopia confocal del ápice de la raíz primaria de *Arabidopsis* que expresan *pCLE14:GUS-GFP* teñidas con yoduro de propidio 4-7 días después de la germinación (a-d), y los cambios en la fluorescencia de GFP durante la regeneración de la punta de la raíz (e-h). Las imágenes son representativas de seis individuos independientes. El experimento se repitió tres veces con resultados comparables. Barra de escala= 100 µm.

7.3. El tratamiento con CLE14 disminuye la expresión del factor de transcripción ERF115 durante la regeneración de la raíz

El factor de transcripción de respuesta a etileno ETILENE RESPONSE FACTOR 115 (ERF115) mantiene la función del meristemo al promover la renovación celular después de la pérdida de células madre (Heyman *et al*. 2016). La muerte celular en el meristemo de la raíz causada por heridas, antibióticos o estrés ambiental promueve la actividad de ERF115 en células que están en contacto directo con las células dañadas, promoviendo divisiones que reponen las células madre colapsadas. La participación de ERF115 en la recuperación completa del nicho de células madre se ha documentado tras la escisión de la punta de la raíz, en mutantes con muerte celular espontánea en el meristemo y en raíces expuestas a metales como cromato (Heyman *et al*. 2016; Raya-González *et al*. 2018; Ruiz-Aguilar *et al*. 2020). Con el objetivo de dilucidar una posible influencia de CLE14 sobre la expresión de ERF115 durante el proceso de regeneración, se comparó la formación de la punta de la raíz ante el corte de la misma con un bisturí desde las 24 h después del corte hasta los 4 días, tiempo que permitió la observación de una nueva cofia. En los diferentes tiempos analizados, en plantas transgénicas que expresan *pERF115:GUS-GFP* teñidas con yoduro de propidio, se logró detectar la fluorescencia del gen reportero por microscopía confocal, mostrando ésta la mayor expresión en los tejidos en proximidad con el sitio de corte, los cuáles disminuyeron a medida que la regeneración avanza (**Fig. 6a-e**). El tratamiento con CLE14 en concentración de 1 µM disminuyó la señal verde en las diferentes etapas (**Fig. 6f-j**), lo que sugiere que CLE14 actúa como un regulador negativo sobre la expresión de ERF115 durante la regeneración celular. Se puede observar, además, que esa disminución en la expresión de ERF115 inducida por esa concentración del péptido CLE14, no impidió que se completara el proceso de regeneración.

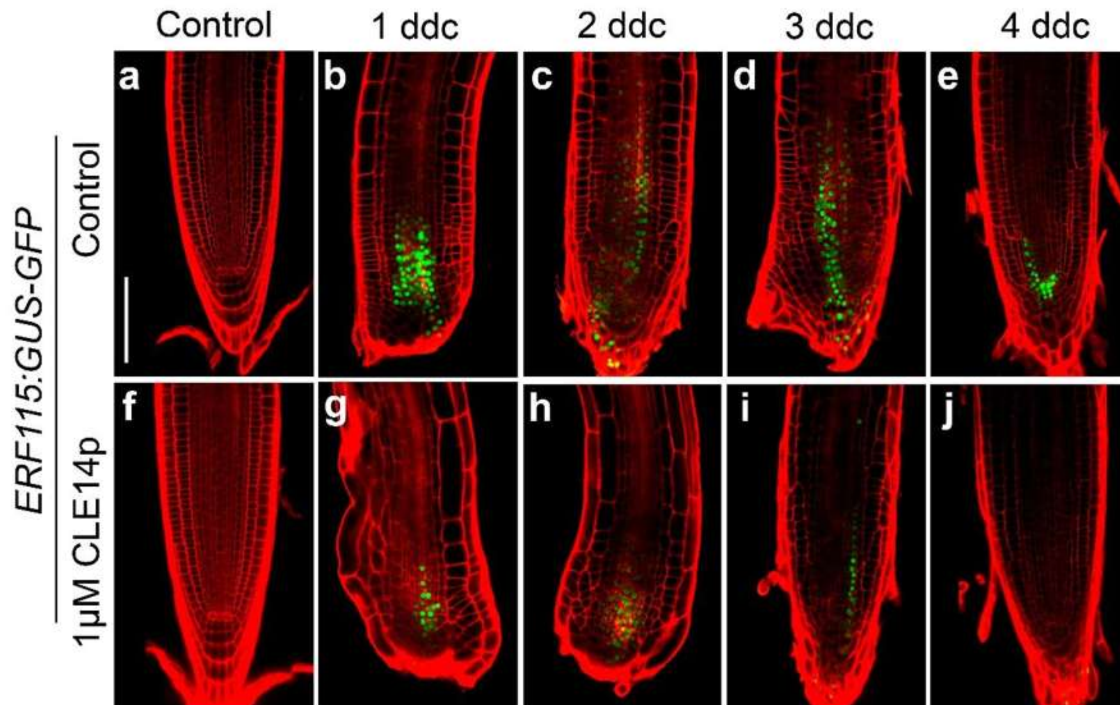


Figura 6. Efecto de CLE14 sobre la expresión de *pERF115:GUS-GFP* durante la regeneración de la raíz. Imágenes por microscopia confocal de la regeneración del ápice de la raíz primaria de plantas de *Arabidopsis* que expresan p*ERF115:GUS-GFP* teñidas con yoduro de propidio en los días 1-4 después del corte con un bisturí (a-e). (f-j) Cambios en la fluorescencia de GFP en respuesta al tratamiento con una concentración de CLE14 de 1 µM. Las imágenes son representativas de seis individuos independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 100 µm.

7.4. La sobreexpresión de CLE14 impide la regeneración de la punta de la raíz después del corte

La herida causada por el corte con el bisturí promueve una fuerte reacción defensiva necesaria para mantener fuera microbios potencialmente peligrosos a través del sellado de la herida o, alternativamente, para impulsar la regeneración del tejido faltante y reiniciar el crecimiento (Zhang *et al.* 2019; Zhou *et al.* 2019). A continuación, comparamos el proceso de regeneración de la punta de la raíz después de cortar en plantas silvestres (Col-0), mutantes *cle14* y sobre-expresoras *35S:CLE14*. Las imágenes representativas a los 1, 3 y 5 días después del corte (ddc) mostraron que las mutantes *cle14* tienen un buen potencial de regeneración, comparable en tiempo y características del tejido regenerado con el que se observa en plantas normales Col-0, mientras que las plántulas *35S:CLE14* no logran

regenerar la punta de la raíz faltante (**Fig. 7a-l**). Estos resultados indican que la sobreexpresión de CLE14 interfiere con el proceso regenerativo.

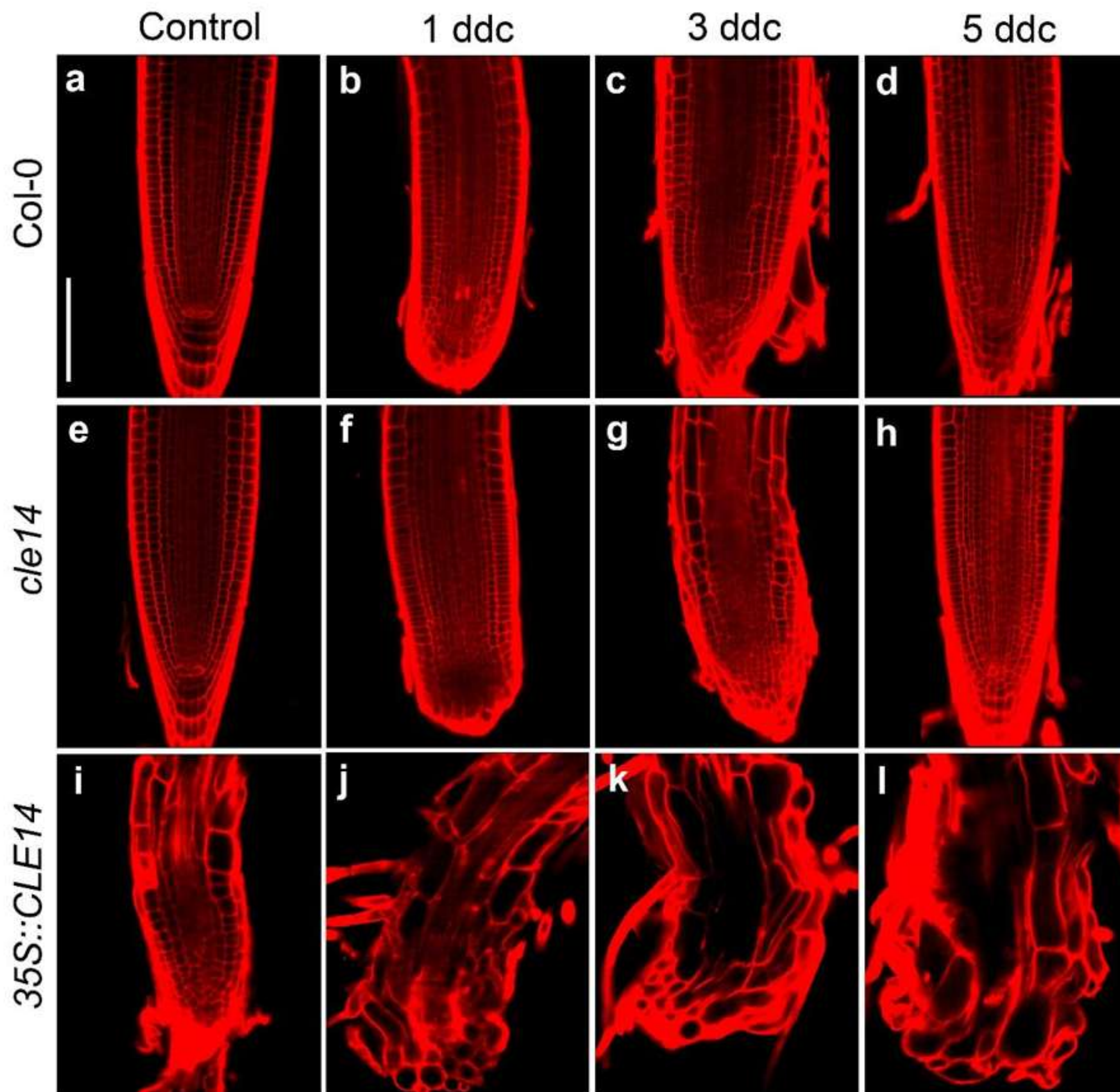


Figura 7. Regeneración de la punta de la raíz primaria de *Arabidopsis* después del corte con un bisturí. Imágenes por microscopia confocal de raíces de plantas normales (Col-0), la mutante *cle14* y la línea sobre-expresora *35S::CLE14* teñidas con yoduro de propidio a los 1, 3 y 5 días después del corte. Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 100 µm.

7.5. Los explantes de brotes *35S:CLE14* manifiestan una disminución en la formación de callos

La regeneración de brotes a partir de tejido diferenciado, ya sea de la raíz o del follaje, implica la desdiferenciación de las células y la entrada en mitosis, un proceso promovido por las auxinas y las citocininas (Ikeuchi *et al*. 2013). La aplicación de dichas hormonas al medio de cultivo, permite la obtención de masas celulares indiferenciadas a partir de explantes de brotes de *Arabidopsis* nos permitió comparar el proceso de desdiferenciación y proliferación subyacente a la formación de callos. La biomasa del callo se cuantificó a los 25 días después de la transferencia de los explantes provenientes de plantas normales (Col-0), *cle14* y *35S:CLE14* en medio inductor de callos. Los datos muestran una biomasa equiparable para plantas normales y *cle14*, pero una reducción drástica en la línea *35S:CLE14* (**Fig. 8a**). Las imágenes tomadas directamente de los cultivos mostraron claramente la respuesta reducida de formación de callos en los causada por la sobreexpresión de CLE14 (**Fig. 8b**). Los resultados anteriores indican que CLE14 interfiere con el proceso de división celular que posibilita la callogenesis.

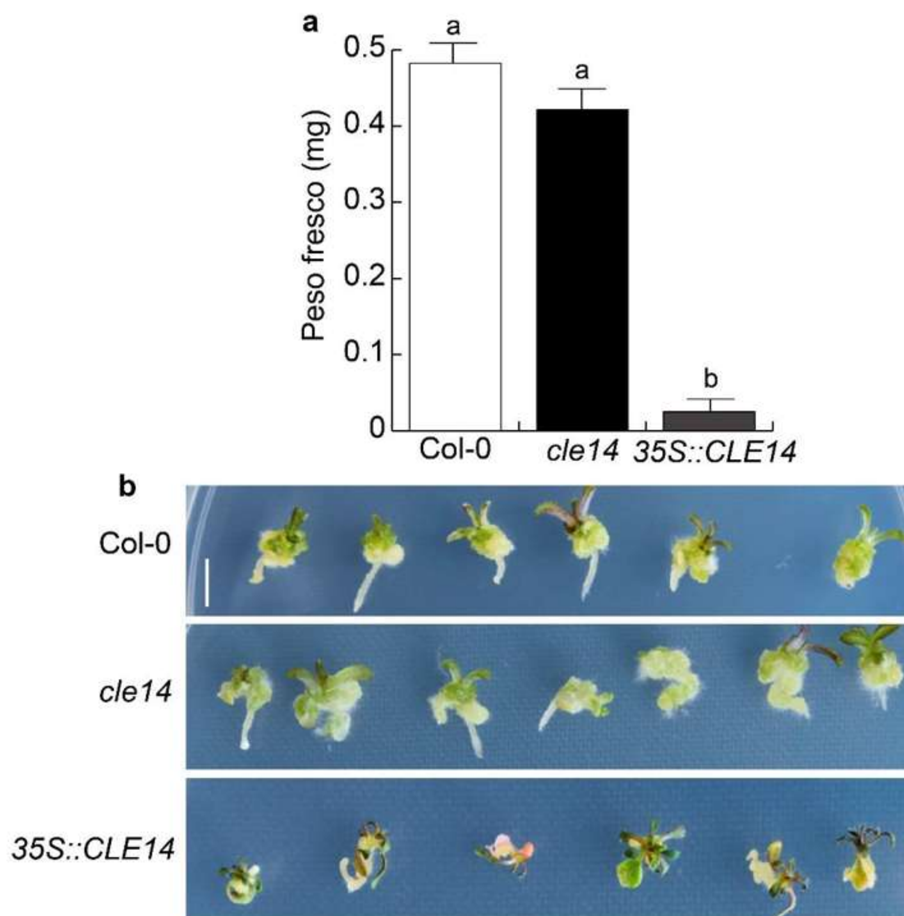


Figura 8. La sobreexpresión de CLE14 interfiere con la formación de callos. (a) Mediciones de peso fresco de biomasa de callos producidos en explantes Col-0, *cle14* y *35S::CLE14* cultivados en medio inductor de callos. (b) Imágenes de explantes productores de callo, provenientes de plantas normales (Col-0), mutantes *cle14* y sobre-expresoras *35S::CLE14*. Diferentes letras en (a) indican medias con diferencia significativa ($P<0.05$). El experimento se repitió tres veces con resultados similares. Barra de escala= 2 mm.

7.6. El corte de la raíz induce la expresión del receptor con actividad de cinasa PEPR2

El gen PEPR2 codifica para un receptor con actividad de cinasa que contribuye a las respuestas de defensa en *Arabidopsis* (Yamaguchi *et al*. 2010) y su expresión aumenta en respuesta a deficiencia de fosfato (Gutiérrez-Alanis *et al*. 2017,; Wu et *al.* 2016). En las plantas bajo estrés nutricional el módulo CLE14/PEPR2 conduce al acortamiento de la raíz y a la diferenciación del meristemo (Gutiérrez-Alanis, *et al*. 2017). Lo anterior nos motivó a caracterizar la regulación de la expresión de PEPR2 en plantas transgénicas que expresan la construcción *pPEPR2:GUS* en

respuesta al corte de la raíz y durante el proceso de regeneración. En las raíces de las plantas que no sufrieron daño, el receptor no se expresa en el meristemo o en la zona de diferenciación, en cambio, una vez realizado el corte de la punta de la raíz, se manifestó una fuerte expresión, evidenciada por la coloración azul producto de la actividad de la beta glucoronidasa en los tejidos adyacente al corte, que perdura hasta el momento de la regeneración completa de la raíz (**Fig. 9a-f**). Un análisis más detallado de la zona de diferenciación de la raíz, denotó la expresión en la zona cubierta por pelos radiculares y sitios de formación de raíces laterales (**Fig. 9g-l**). Estos resultados indican que la expresión de PEPR2 es inducible por heridas y puede ocurrir en lugares de la planta distantes al sitio donde ocurrió el daño.

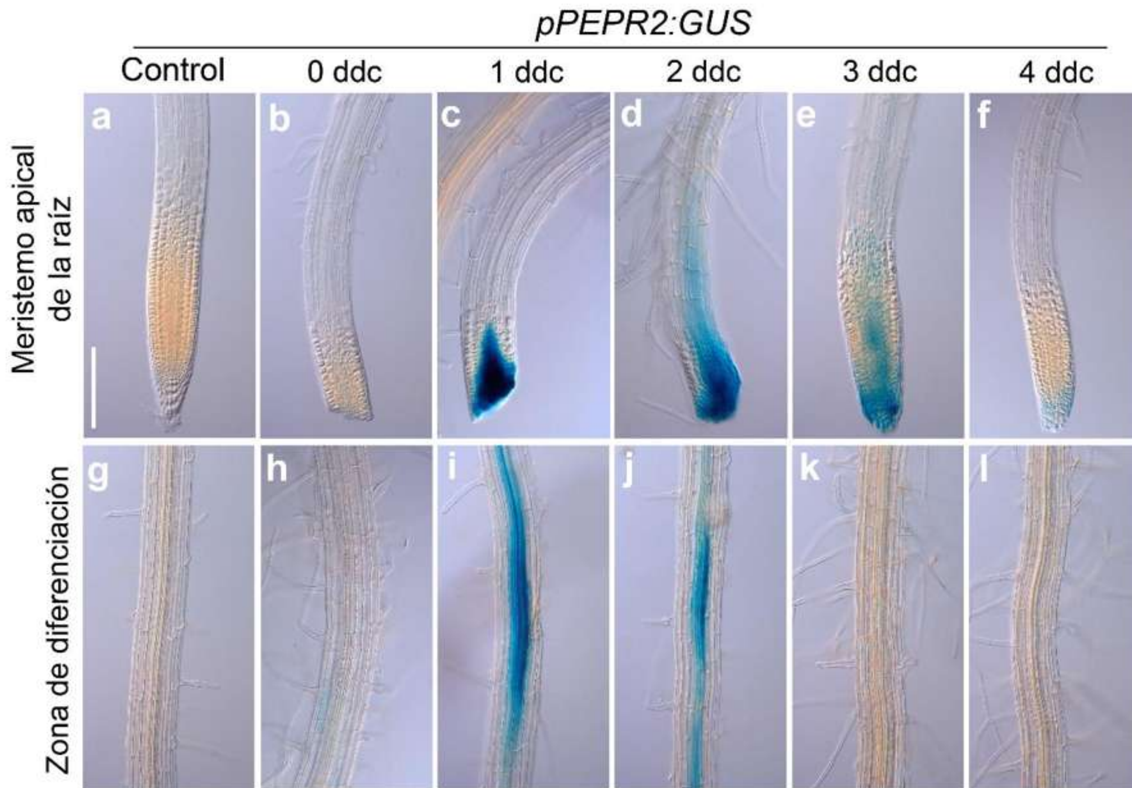


Figura 9. Inducción de la expresión del receptor PEPR2 durante el corte y regeneración de la raíz. Imágenes tomadas mediante microscopía de campo claro antes y después del corte de la punta de la raíz, mostrando la expresión de *pPEPR2:GUS* durante la regeneración (a-f) y en la zona de diferenciación (g-l). Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 100 µm.

7.7. El corte de la raíz induce la expresión de PEPR2 en el follaje

El receptor con actividad de cinasa atPEPR1 participa en la detección de la sistemina y del péptido PEP1, los cuales son péptidos pequeños que se producen en las hojas dañadas por herbivoría, lo que induce la expresión de inhibidores de proteasas a través de la acumulación de ácido jasmónico (Huffaker *et al*. 2006; Yamaguchi *et al*. 2006; Holmes *et al*. 2018). A continuación examinamos la expresión de *pPEPR2:GUS* en respuesta al corte de la raíz en los tres días subsecuentes, en los tejidos aledaños al meristemo del follaje y en los cotiledones. En las plantas que no sufrieron daño, o al momento del corte, el receptor no se expresa en el meristemo del follaje ni en los cotiledones, pero a las 24 y 48h, se incrementó notablemente la expresión tanto en el meristemo (**Fig. 10a-e**) como en los cotiledones (**Fig. 10f-j**) y dicha expresión correlaciona y termina con la regeneración de la raíz (**Fig. 10a-j**). Con base en lo anterior, podemos concluir que la expresión de *PEPR2* en el follaje ocurre de manera sistémica ante el corte de la raíz y se mantiene durante el proceso de regeneración.

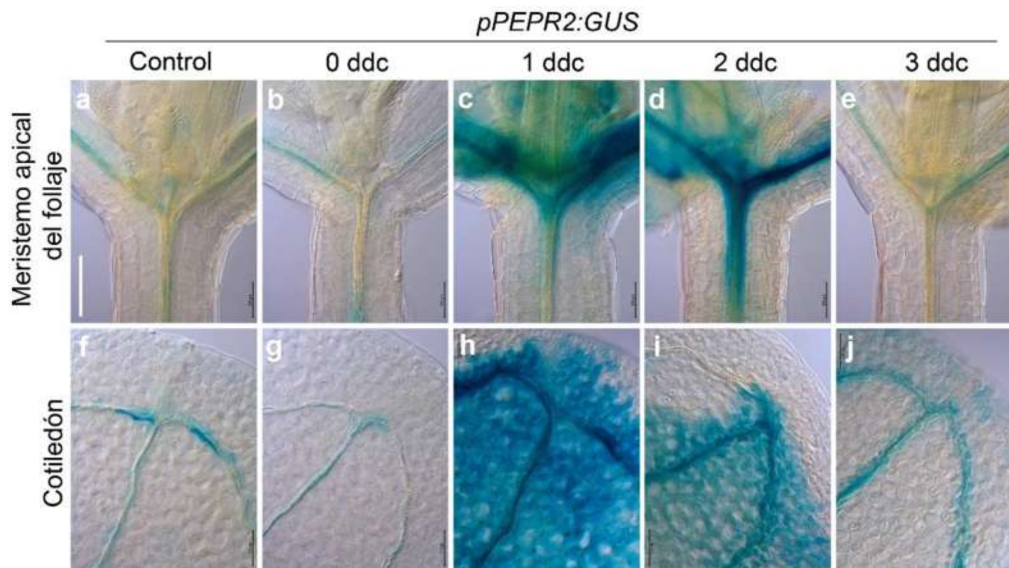


Figura 10. Expresión sistémica de PEPR2 en el follaje en respuesta al corte de la raíz. Imágenes tomadas mediante microscopía de campo claro antes y después del corte de la punta de la raíz, mostrando la expresión de *pPEPR2:GUS* en el meristemo del follaje (a-e) y en los cotiledones (f-j). Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 100 µm.

7.8. La expresión de PEPR2 se induce por herida en los cotiledones

Las heridas provocadas en las hojas causan la acumulación localizada de ácido jasmónico y en esta reacción defensiva participan péptidos que contribuyen en la inmunidad (Yamaguchi *et al.* 2006; Holmes *et al*. 2018). Para investigar la posible participación de PEPR2 en la respuesta a herida en el follaje, se realizaron heridas con una aguja esterilizada en uno de los dos cotiledones de plantas transgénicas de *Arabidopsis* que expresan *pPEPR2:GUS* y se comparó la actividad del gen reportero mediante una detección histoquímica a las 3, 6 y 12 h después de la herida con las plantas que no fueron dañadas. Observamos qué desde las tres horas de haber pinchado uno de los cotiledones, se induce fuertemente la expresión de PEPR2 en el cotiledón dañado. Interesantemente, la expresión de PEPR2 se extiende hacia los peciolos e inclusive hasta el segundo cotiledón y perdura hasta las 24 h (**Fig. 11a-h**). Estos resultados resaltan el patrón de expresión local y sistémica de PEPR2 inducible por herida, lo cual podría contribuir con el sellado de la herida, con la regeneración del tejido dañado o con procesos de comunicación a larga distancia.

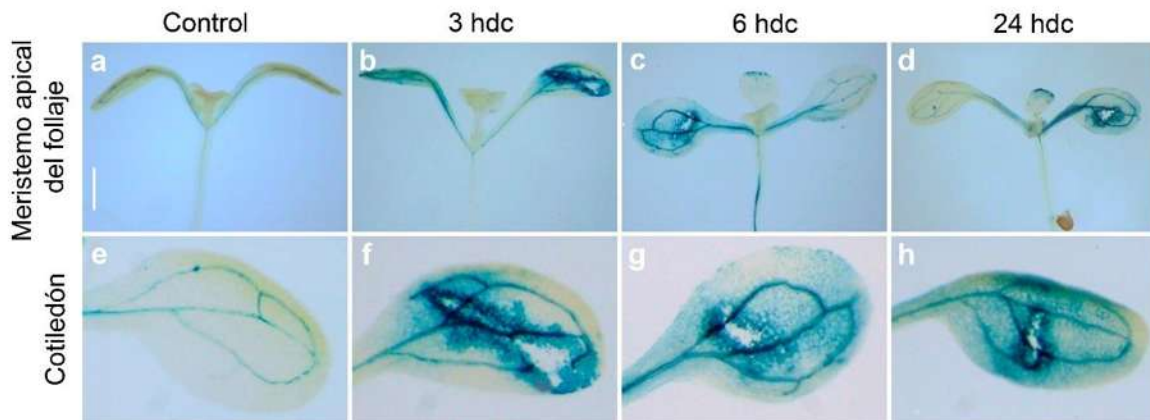


Figura 11. Inducción de la expresión de PEPR2 en respuesta a herida en los cotiledones. Imágenes tomadas mediante microscopía de campo claro en cotiledones intactos (a, e) y heridos (b-d; f-h), mostrando la expresión de *pPEPR2:GUS*. Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 500 µm.

7.9. El daño celular por agentes genotóxicos induce la expresión de *PEPR2* en la provasculatura de la raíz.

La zeocina es un antibiótico glicopeptídico de *Streptomyces verticillus* de amplio espectro, efectivo frente a la mayoría de bacterias, hongos filamentosos, levaduras y células vegetales y animales, que provoca la muerte celular intercalándose en la cadena de ADN e induciendo en ella roturas de doble cadena (Chankova *et al*. 2007). La aplicación de zeocina y otros antibióticos causa la muerte de células altamente proliferativas en el meristemo de la raíz de *Arabidopsis,* utilizándose como herramientas farmacológicas para estudiar procesos de viabilidad celular en respuesta al daño del ADN (Fulcher y Sablowski, 2009). Para conocer si el daño en la raíz reportado ante el tratamiento con zeocina podría inducir la expresión de PEPR2, se realizaron análisis comparando la estructura de los meristemos de plantas control y transferidas a un medio con 10 µM de zeocina a las 24 y 72h. Los resultados indican que la exposición de la raíz a la zeocina causa la inducción de la diferenciación, evidenciada por la formación de pelos radiculares que no se observa en las plantas control. Este cambio en la estructura del meristemo coincide con la expresión clara y específica de *pPEPR2:GUS* en la región de la pro-vasculatura, en donde ocurre daño celular, y que se magnifica mayormente a las 72h (**Fig. 12a-d**). Estos resultados sugieren una posible participación de PEPR2 en la adaptación al daño celular y al estrés genotóxico causado por la zeocina.

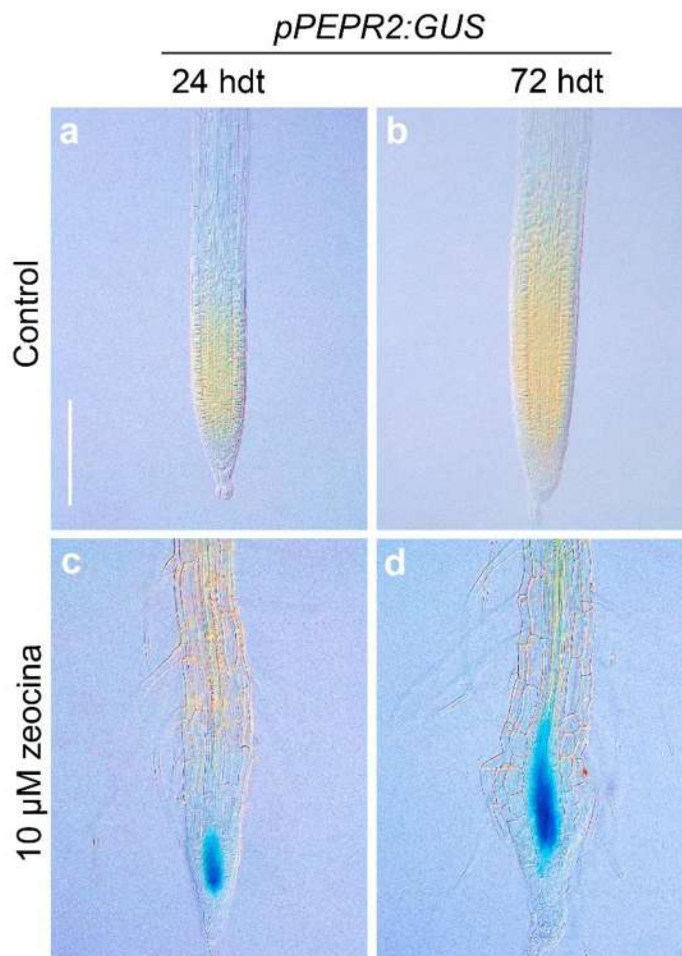


Figura 12. Efecto de la zeocina sobre la expresión de PEPR2 en el meristemo de la raíz. Imágenes tomadas mediante microscopía de campo claro en meristemos de la raíz primaria de plantas control (a, b) y transferidas a un medio con 10 µM de zeocina (c, d), a las 24 (a, c) y 72 h (b,d), respectivamente, mostrando la expresión de *pPEPR2:GUS*. Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 200 µm.

7.10. El promotor de PEPR2 se induce de manera constitutiva por ácido jasmónico

La función principal del ácido jasmónico es regular las respuestas de las plantas ante el estrés abiótico y biótico, así como el crecimiento y desarrollo (Ghorbel *et al.* 2021). Como ya hemos visto, las heridas en la raíz y las hojas inducen fuertemente la expresión de PEPR2, por lo que cabe la posibilidad de que dicha inducción ocurre por incrementos en los niveles de ácido jasmónico. Para evaluar lo anterior, se comparó la expresión de *pPEPR2:GUS* en plantas intactas de *Arabidopsis* crecidas

en un medio control o suplementado con una concentración de ácido jasmónico de 4 μM. El registró de la expresión del gen reportero en el follaje, el tallo y la raíz mostró la inducción de la expresión de PEPR2 en todos los tejidos (**Fig. 13a-j**), indicando un patrón de inducción constitutiva por el ácido jasmónico.

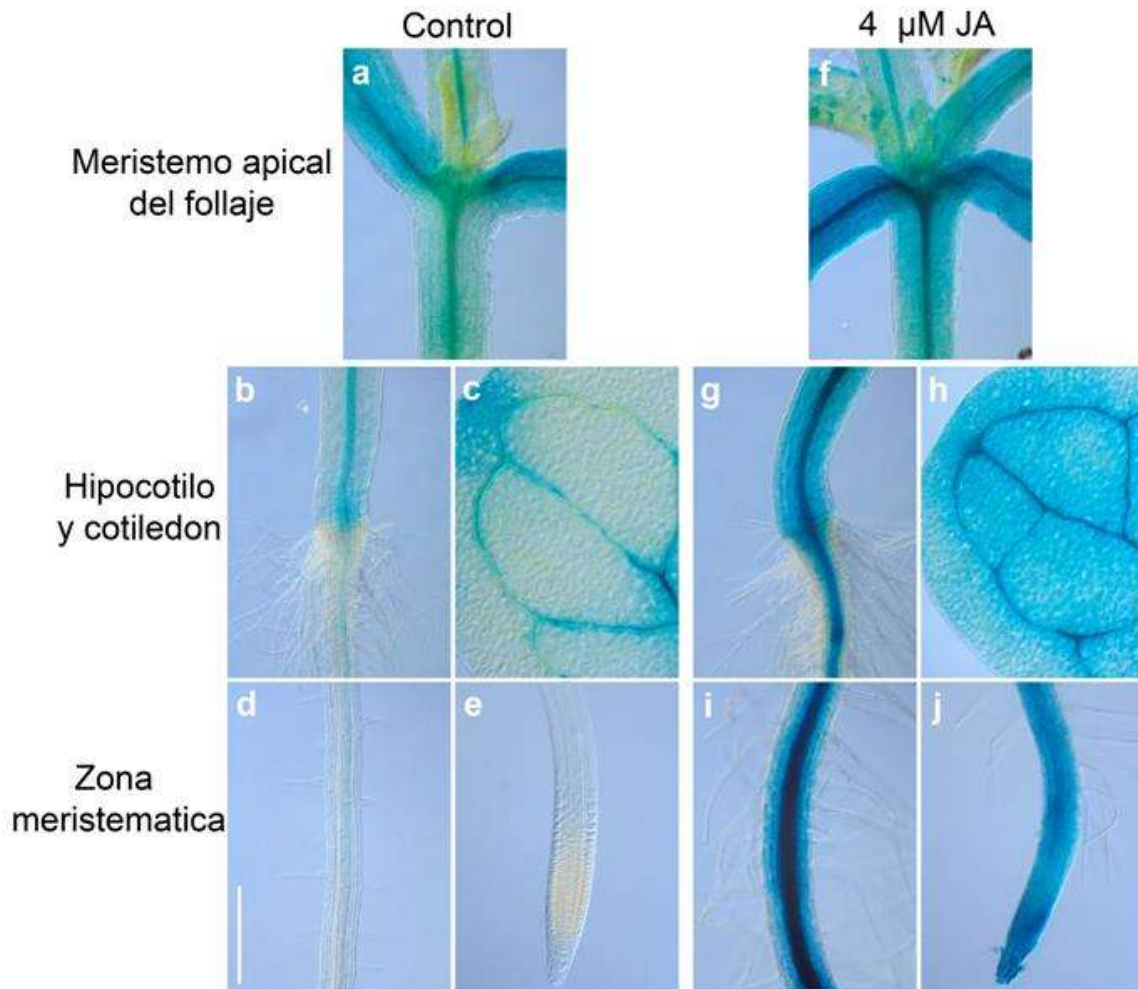


Figura 13. Expresión de PEPR2 en plantas de *Arabidopsis* tratadas con ácido jasmónico. Imágenes tomadas mediante microscopía de campo claro en meristemos del follaje, segmentos de tallo, cotiledones y raíz primaria de plantas control (a-e) y suplementadas con 4 μM de ácido jasmónico (f-j), mostrando la expresión de *pPEPR2:GUS*. Las imágenes son representativas de seis registros independientes. El experimento se repitió tres veces con resultados similares. Barra de escala= 200 μm.

8. DISCUSIÓN Y CONCLUSIONES

La integración del desarrollo, la defensa y la adaptación de las plantas ante los cambios continuos en el ambiente implica la coordinación de una sofisticada red de respuestas que ocurren de manera local y sistémica. En la raíz, ocurre la detección de los niveles de agua y nutrientes, pH, presencia de metales, moléculas de microorganismos a través de receptores y mecanismos específicos de reconocimiento (Ravelo *et al*. 2023). Uno de los grupos clave en la recepción de estímulos son las proteínas receptoras con actividad de cinasa, elementos ubicuos tanto en plantas como animales, de los cuáles en *Arabidopsis* se han identificado más de 610 miembros y 1131 en arroz (Shiu *et al*. 2004). Esta enorme abundancia, así como su distribución tanto en monocotiledóneas como dicotiledóneas sugiere la existencia de un gran número de ligandos, entre los cuales destacan pequeños péptidos de la familia CLE.

Estudios previos ya habían sugerido la importancia de la interacción de péptidos CLE con algunos receptores con actividad de cinasa, destacando la relación de CLE14, un péptido conformado por 12 amino ácidos, con los receptores PEPR1 y CLAVATA, cuya interacción en condiciones de limitación de fosfato, causa la terminación del ciclo celular en el meristemo y su posterior diferenciación (Gutiérrez-Alanis *et al*. 2017). Estos autores, nos proporcionaron amablemente materiales valiosos para realizar la presente investigación, particularmente con la donación de una cantidad significativa del péptido sintético, así como semillas homocigas de plantas mutantes y transgénicas que sobre-expresan el péptido CLE14.

En un primer análisis, la incorporación del péptido al medio de crecimiento de plantas de *Arabidopsis* cambió drásticamente la arquitectura de la raíz. Nuestros resultados son consistentes con lo reportado por Meng y Feldman (2010), quienes mostraron que CLE14 detiene irreversiblemente el crecimiento de la raíz primaria a través de la reducción de la división celular y la expresión de *Ciclina B1* en el meristemo, y con los de Hayashi *et al*. (2018) donde CLE14 deterioró el crecimiento de la raíz y promovió la formación de pelos radiculares. Estos datos confirman el importante papel de CLE14 como inhibidor de la proliferación celular y como

inductor de la diferenciación, que podría ser compartido por otros miembros de la familia CLE, ya que se ha reportado que la sobre-expresión *CLE8, CLE12, CLE19 y CLE22* conduce a la diferenciación del meristemo radicular (Fiers *et al*. 2005; Ito *et al*. 2006; Strabala *et al*. 2006; Jun *et al*. 2010). El patrón de expresión de CLE14 en cofia de la raíz se reportó por primera vez por Meng y Feldman (2011). Es importante mencionar que, bajo condiciones óptimas de crecimiento, CLE14 se expresa específicamente en la última capa celular de la cofia, la cual se sabe que entra a procesos de muerte celular programada como un proceso de recambio celular, sugiriendo que CLE14 podría estar implicado en estos procesos. Aquí observamos su inducción específica en la capa celular inmediata adyacente al sitio de corte durante la regeneración, donde el yoduro de propidio marca un parche rojo en las células dañadas. Por lo tanto, es posible que CLE14 pueda estar involucrado en el proceso de reconstrucción y adquisición de destinos celulares a medida que avanza la construcción de la punta de la raíz o en la cicatrización de heridas después del daño.

Zhang *et al*. (2019) mostraron la inducción del factor de transcripción de respuesta a etileno *ETILENE RESPONSE FACTOR 115* (ERF115) por el ácido jasmónico, una hormona que actúa como centinela tanto para la defensa de las plantas. La herida provoca la biosíntesis rápida y señalización dependiente del ácido jasmónico que conduce al reforzamiento de la pared celular, la producción de fitoalexinas e inhibidores de proteasas en tejido foliar (Glauser *et al.* 2008; Koo *et al*. 2009). A diferencia de lo descrito sobre las respuestas de defensa en hojas, poco se sabe del mecanismo inductor en la raíz. Un aspecto parece ser común, esto es, la herida de la raíz con navaja al momento del corte, potencia la expresión de *pCLE14:GUS-GFP* en células adyacentes al corte, lo que podría conducir a la cicatrización de la herida, ya que como pudimos observar en los estudios de regeneración, la sobreexpresión de CLE14 interfiere con el proceso de reconstitución de la punta de la raíz conduciendo al sellado de la herida.

A pesar de su reconocida utilidad en la investigación y sus aplicaciones para la propagación asexual de plantas, existe muy poca información sobre las bases

moleculares de la formación de callos. La correlación encontrada en nuestro trabajo de que el péptido CLE14 compromete el funcionamiento del meristemo, inhibe la regeneración, así como la formación de callos en explantes de hipocotilo revela los puntos en común en estos procesos. El equilibrio entre la división celular y la diferenciación es crítico para la adaptación al estrés abiótico, en plantas bajo escasez de fosfato, el meristemo de la raíz se agota y sus células se diferencian produciendo pelos radiculares a través de la acción de CLE14 y sus receptores CLV2 y PEPR2 (Gutiérrez-Alanis *et al.* 2017). Además de PEPR2, el genoma de *Arabidopsis* codifica también para PEPR1, y ambos están implicados en respuestas de daño celular inducida por organismos patógenos (Ortiz-Morea *et al.* 2016). Nuestros resultados apoyan la hipótesis de que el destino celular y la organogénesis dependen de las interacciones de varios péptidos que actúan como ligandos cuyos blancos moleculares quedan por esclarecerse.

Las células vegetales detectan la presencia de microorganismos potencialmente patógenos en el apoplasto a través de receptores localizados en la membrana plasmática. Los receptores activados desencadenan cascadas de señalización mediadas por fosforilación que protegen a la célula de la infección. Se cree que la señalización activada por señales exógenas, como la flagelina bacteriana, puede ser amplificada por señales endógenas, como hormonas o segundos mensajeros que amplifican la señal. Por ejemplo, la percepción de flagelina y otras moléculas microbianas da como resultado una mayor expresión de péptidos de la familia CLE que potencian la respuesta inmune. Las fitohormonas como el metil-jasmonato también inducen la expresión de varios péptidos CLE, lo que sugiere relaciones de retroalimentación locales y sistémicas.

La evaluación de la respuesta inmune por el receptor AtPEP1 en mutantes afectadas en el receptor de jasmonato CORONATINE-INSENSITIVE 1 (COI1) se compromete la resistencia a patógenos (Holmes *et al.* 2018). Nuestros resultados también demostraron que la expresión de PEPR2 se induce por herida, estrés genotóxico, y tratamientos con ácido jasmónico, sugiriendo que la percepción de esta fitohormona juega un papel importante en las respuestas inmunes moduladas

por AtPEP1 y AtPEP2. Aún queda por determinarse si las mutantes en el gen AtPEP2 o su sobreexpresión en plantas transgénicas comprometen o maximizan las respuestas de crecimiento e inmunidad inducidas por factores ambientales y hormonas como el ácido jasmónico.

Interesantemente, el corte en la punta de la raíz principal, así como la herida en uno de los cotiledones, activó la expresión sistémica del receptor PEPR2 en tejidos ú órganos distantes. Se ha descrito que el péptido CLE25 actúa en la transmisión de señales de deficiencia de agua de la raíz hacia el follaje, lo que conduce en la acumulación de la hormona ABA en las hojas y por lo tanto el control de la apertura y cierre de estomas (Takahashi *et al*. 2018). CLE25 producido en la raíz es movilizado hacia las hojas y en asociación con el receptor BARELY ANY MERISTEM (BAM) activan respuestas de estrés a la deshidratación (Takahashi *et al*. 2018). Poniendo en contexto nuestros resultados con lo descrito anteriormente, sugiere fuertemente que existe una señal a larga distancia, posiblemente algún(os) péptido(s) CLE, que viaja de la zona del estímulo hacia regiones distantes, para la activación de la biosíntesis y/o señalización de hormonas, como el AJ, posiblemente como una respuesta de alerta de la planta en tejidos no dañados.

En conclusión, se demostró que el péptido CLE14, se encuentra implicado en múltiples procesos fisiológicos, celulares y moleculares de las plantas, regulando procesos de viabilidad celular, callogenesis y regeneración *de novo* de la raíz de *Arabidopsis.* Las perspectivas de esta investigación si la sobreexpresión del receptor de CLE14, PEPR2, comprometen o maximizan las respuestas de crecimiento e inmunidad inducidas por factores ambientales y hormonas como el ácido jasmónico.

9. LITERATURA CITADA

Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Nussaume L, Noh YS, Amasino R, Scheres B (2004). The PLETHORA genes mediate patterning of the Arabidopsis root stem cell niche. *Cell* 119(1):109-20. doi: 10.1016/j.cell.2004.09.018.

Balestrazzi A, Confalonieri M, Macovei A, Donà M, Carbonera D (2011). Genotoxic stress and DNA repair in plants: emerging functions and tools for improving crop productivity. *Plant Cell Rep* 30(3):287-95. doi: 10.1007/s00299-010-0975-9.

Blázquez MA, Nelson DC, Weijers D (2020). Evolution of plant hormone response pathways. *Annu Rev Plant Biol* 71:327-353. doi: 10.1146/annurev-arplant-050718-100309.

Canher B, Heyman J, Savina M, Devendran A, Eekhout T, Vercauteren I, Prinsen E, Matosevich R, Xu J, Mironova V, De Veylder L (2020). Rocks in the auxin stream: Wound-induced auxin accumulation and *ERF115* expression synergistically drive stem cell regeneration. *Proc Natl Acad Sci U S A* 117(28):16667-16677. doi: 10.1073/pnas.2006620117.

Chankova SG, Dimova E, Dimitrova M, Bryant PE (2007). Induction of DNA double-strand breaks by zeocin in *Chlamydomonas reinhardtii* and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response. *Radiation and Environmental Biophysics* **46** (4): 409-16.

Chen K, Li GJ, Bressan RA, Song CP, Zhu JK, Zhao Y (2020). Abscisic acid dynamics, signaling, and functions in plants. *J Integr Plant Biol* 62(1):25-54. doi: 10.1111/jipb.12899.

Clark SE, Running MP, Meyerowitz EM (1993). CLAVATA1, a regulator of meristem and flower development in Arabidopsis. *Development* 119(2):397-418. doi: 10.1242/dev.119.2.397.

Clark SE, Williams RW, Meyerowitz EM (1997). The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* 89(4):575-85. doi: 10.1016/s0092-8674(00)80239-1.

Clouse SD, Langford M, McMorris TC (1996). A brassinosteroid-insensitive mutant in *Arabidopsis thaliana* exhibits multiple defects in growth and development. *Plant Physiol* 111(3):671-8. doi: 10.1104/pp.111.3.671.

Cock JM, McCormick S (2001). A large family of genes that share homology with CLAVATA3. *Plant Physiol* 126(3):939-942. doi: 10.1104/pp.126.3.939.

Cock JM, Vanoosthuyse V, Gaude T (2002). Receptor kinase signalling in plants and animals: distinct molecular systems with mechanistic similarities. *Curr Opin Cell Biol* 14(2):230-236. doi: 10.1016/s0955-0674(02)00305-8.

Di Mambro R, Sabatini S (2018). Developmental analysis of Arabidopsis root meristem. *Methods Mol Biol* 1761:33-45. doi: 10.1007/978-1-4939-7747-5_3.

De Jong M, Leyser O (2012). Developmental plasticity in plants. Cold Spring Harb *Symp Quant Biol* 77:63-73. doi: 10.1101/sqb.2012.77.014720.

Dong N, Yin W, Liu D, Zhang X, Yu Z, Huang W, Liu J, Yang Y, Meng W, Niu M, Tong H (2020). Regulation of brassinosteroid signaling and salt resistance by SERK2 and potential utilization for crop improvement in rice. *Front Plant Sci* 11:621859. doi: 10.3389/fpls.2020.621859.

Efroni I, Mello A, Nawy T, Ip PL, Rahni R, DelRose N, Powers A, Satija R, Birnbaum KD (2016). Root regeneration triggers an embryo-like sequence guided by hormonal interactions. *Cell* 165(7):1721-1733. doi: 10.1016/j.cell.2016.04.046.

Fan Y, Bergmann A (2008). Apoptosis-induced compensatory proliferation. The Cell is dead. Long live the Cell! *Trends Cell Biol* 18(10):467-73. doi: 10.1016/j.tcb.2008.08.001.

Fiers M, Golemiec E, Xu J, van der Geest L, Heidstra R, Stiekema W, Liu CM (2005). The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the

root meristem in Arabidopsis through a CLAVATA2- dependent pathway. *Plant Cell* 17:2542–2553. doi: 10.1105/tpc.105.034009.

Fletcher JC (2020). Recent advances in Arabidopsis CLE peptide signaling. *Trends Plant Sci* 25:1005-1016. doi: 10.1016/j.tplants.2020.04.014.

Fujihara R, Uchida N, Tameshige T, Kawamoto N, Hotokezaka Y, Higaki T, Simon R, Torii KU, Tasaka M, Aida M (2021). The boundary-expressed *EPIDERMAL PATTERNING FACTOR-LIKE2* gene encoding a signaling peptide promotes cotyledon growth during *Arabidopsis thaliana* embryogenesis. *Plant Biotechnol* (Tokyo) 38(3):317-322. doi: 10.5511/plantbiotechnology.21.0508a.

Fulcher N, Sablowski R (2009). Hypersensitivity to DNA damage in plant stem cell niches. *Proc Natl Acad Sci U S A* 106(49):20984-8. doi: 10.1073/pnas.0909218106.

Glauser G, Grata E, Dubugnon L, Rudaz S, Farmer EE, Wolfender JL (2008). Spatial and temporal dynamics of jasmonate synthesis and accumulation in Arabidopsis in response to wounding. *J Biol Chem* 283(24):16400-7. doi: 10.1074/jbc.M801760200.

Gentric N, Genschik P, Noir S (2021). Connections between the cell cycle and the DNA damage response in plants. *Int J Mol Sci* 22(17):9558. doi: 10.3390/ijms22179558.

Ghorbel M, Brini F, Sharma A, Landi M (2021). Role of jasmonic acid in plants: the molecular point of view. *Plant Cell Rep* 40(8):1471-1494. doi: 10.1007/s00299-021-02687-4.

Gutiérrez-Alanís D, Yong-Villalobos L, Jiménez-Sandoval P, Alatorre-Cobos F, Oropeza-Aburto A, Mora-Macías J, Sánchez-Rodríguez F, Cruz-Ramírez A, Herrera-Estrella L (2017). Phosphate starvation-dependent iron mobilization induces CLE14 expression to trigger root meristem differentiation through CLV2/PEPR2 signaling. *Dev Cell* 41:555-570.e3. doi: 10.1016/j.devcel.2017.05.009.

Hanano S, Tomatsu H, Ohnishi A, Kobayashi K, Kondo Y, Betsuyaku S, Takita E, Ogata Y, Ozawa K, Suda K, Hosouchi T, Nagase T, Suzuki H, Sakurai N, Masumoto

H, Fukuda H, Shibata D (2020). An artificial conversion of roots into organs with shoot stem characteristics by inducing two transcription factors. *iScience* 24;23(7):101332. doi: 10.1016/j.isci.2020.101332.

Hayashi N, Tetsumura T, Sawa S, Wada T, Tominaga-Wada R (2018). CLE14 peptide signaling in Arabidopsis root hair cell fate determination. *Plant Biotechnol* (Tokyo) 35(1):17-22. doi: 10.5511/plantbiotechnology.18.0122a.

Hayashi N, Rongkavilit N, Tetsumura T, Sawa S, Wada T, Tominaga-Wada R (2019). Effect of the CLE14 polypeptide on *GLABRA2* homolog gene expression in rice and tomato roots. *Plant Biotechnol* (Tokyo) 36(3):205-208. doi: 10.5511/plantbiotechnology.19.0725a.

Heyman J, Cools T, Vandenbussche F, Heyndrickx KS, Van Leene J, Vercauteren I, Vanderauwera S, Vandepoele K, De Jaeger G, Van Der Straeten D, De Veylder L (2013). ERF115 controls root quiescent center cell division and stem cell replenishment. *Science* 342(6160):860-3. doi: 10.1126/science.1240667.

Heyman J, Cools T, Canher B, Shavialenka S, Traas J, Vercauteren I, Van den Daele H, Persiau G, De Jaeger G, Sugimoto K, De Veylder L (2016). The heterodimeric transcription factor complex ERF115-PAT1 grants regeneration competence. *Nat Plants* 2(11):16165. doi: 10.1038/nplants.2016.165.

Hilleary R, Gilroy S (2018). Systemic signaling in response to wounding and pathogens. *Curr Opin Plant Biol* 43:57-62. doi: 10.1016/j.pbi.2017.12.009.

Hirakawa Y (2021). CLAVATA3, a plant peptide controlling stem cell fate in the meristem. *Peptides*142:170579. doi: 10.1016/j.peptides.2021.170579.

Holmes DR, Grubb LE, Monaghan J (2018). The jasmonate receptor COI1 is required for AtPep1-induced immune responses in *Arabidopsis thaliana*. *BMC Res Notes* 11(1):555. doi: 10.1186/s13104-018-3628-7.

Hohmann U, Ramakrishna P, Wang K, Lorenzo-Orts L, Nicolet J, Henschen A, Barberon M, Bayer M, Hothorn M (2020). Constitutive activation of leucine-rich repeat receptor kinase signaling pathways by BAK1-INTERACTING RECEPTOR-LIKE KINASE3 chimera. *Plant Cell* 32(10):3311-3323. doi: 10.1105/tpc.20.00138.

Huffaker A, Pearce G, Ryan CA (2006). An endogenous peptide signal in Arabidopsis activates components of the innate immune response. *Proc Natl Acad Sci U S A* 103(26):10098-103. doi: 10.1073/pnas.0603727103.

Ikeuchi M, Sugimoto K, Iwase A (2013). Plant callus: mechanisms of induction and repression. *Plant Cell* 25(9):3159-3173. doi: 10.1105/tpc.113.116053.

Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016). Plant regeneration: cellular origins and molecular mechanisms. *Development* 143:1442-1451. doi: 10.1242/dev.134668.

Ito Y, Nakanomyo I, Motose H, Iwamoto K, Sawa S, Dohmae N, Fukuda H (2006). Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 313(5788):842-5. doi: 10.1126/science.1128436.

Ivanov VB, Dubrovsky JG (2013). Longitudinal zonation pattern in plant roots: conflicts and solutions. *Trends Plant Sci* 18(5):237-43. doi: 10.1016/j.tplants.2012.10.002.

Jun J, Fiume E, Roeder AH, Meng L, Sharma VK, Osmont KS, Baker C, Ha CM, Meyerowitz EM, Feldman LJ, Fletcher JC (2010). Comprehensive analysis of CLE polypeptide signaling gene expression and overexpression activity in Arabidopsis. *Plant Physiol* 154(4):1721-36. doi: 10.1104/pp.110.163683.

Kawamoto N, Del Carpio DP, Hofmann A, Mizuta Y, Kurihara D, Higashiyama T, Uchida N, Torii KU, Colombo L, Groth G, Simon R (2020). A peptide pair coordinates regular ovule initiation patterns with seed number and fruit size. *Curr Biol* 30(22):4352-4361.e4. doi: 10.1016/j.cub.2020.08.050

Koo AJ, Gao X, Jones AD, Howe GA (2009). A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. *Plant J* 59(6):974-986. doi: 10.1111/j.1365-313X.2009.03924.x.

Koornneef M, Meinke D (2010). The development of Arabidopsis as a model plant. *Plant J* 61(6):909-21. doi: 10.1111/j.1365-313X.2009.04086.x.

Krasovec G, Horkan HR, Quéinnec É, Chambon JP (2022). The constructive function of apoptosis: More than a dead-end job. *Front Cell Dev Biol* 10:1033645. doi: 10.3389/fcell.2022.1033645.

Lu X, Shi H, Ou Y, Cui Y, Chang J, Peng L, Gou X, He K, Li J (2020). RGF1-RGI1, a peptide-receptor complex, regulates Arabidopsis root meristem development via a MAPK signaling cascade. *Mol Plant* 13(11):1594-1607. doi: 10.1016/j.molp.2020.09.005.

Mathew MM, Prasad K (2021). Model systems for regeneration: *Arabidopsis*. *Development* 148(6): dev195347. doi: 10.1242/dev.195347.

Maqbool S, Hassan MA, Xia X, York LM, Rasheed A, He Z (2022). Root system architecture in cereals: progress, challenges and perspective. *Plant J* 110(1):23-42. doi: 10.1111/tpj.15669.

Meinke DW, Cherry JM, Dean C, Rounsley SD, Koornneef M (1998). *Arabidopsis thaliana*: a model plant for genome analysis. Science 282(5389):662, 679-82. doi: 10.1126/science.282.5389.662.

Meng L, Feldman LJ (2010). CLE14/CLE20 peptides may interact with CLAVATA2/CORYNE receptor-like kinases to irreversibly inhibit cell division in the root meristem of Arabidopsis. *Planta* 232(5):1061-1074. doi: 10.1007/s00425-010-1236-4.

Meng L, Feldman LJ (2011). CLE genes may act in a variety of tissues/cells and involve other signaling cascades in addition to CLV3-WUS-like pathways. *Plant Signal Behav* 6(1):105-8. doi: 10.4161/psb.6.1.14186.

Motte H, Vanneste S, Beeckman T (2019). Molecular and environmental regulation of root development. *Annu Rev Plant Biol* 70:465-488. doi: 10.1146/annurev-arplant-050718-100423.

Murphy E, Smith S, De Smet I (2012). Small signaling peptides in Arabidopsis development: how cells communicate over a short distance. *Plant Cell* 24(8):3198-217. doi: 10.1105/tpc.112.099010.

Neill S, Barros R, Bright J, Desikan R, Hancock J, Harrison J, Morris P, Ribeiro D, Wilson I (2008). Nitric oxide, stomatal closure, and abiotic stress. *J Exp Bot* 59(2):165-76. doi: 10.1093/jxb/erm293.

Ou Y, Kui H, Li J (2021). Receptor-like kinases in root development: Current progress and future directions. *Mol Plant* 14(1):166-185. doi: 10.1016/j.molp.2020.12.004.

Pitzschke A, Forzani C, Hirt H. Reactive oxygen species signaling in plants. *Antioxid Redox Signal* 8(9-10):1757-64. doi: 10.1089/ars.2006.8.1757.

Pelagio-Flores R, Muñoz-Parra E, Barrera-Ortiz S, Ortiz-Castro R, Saenz-Mata J, Ortega-Amaro MA, Jiménez-Bremont JF, López-Bucio J (2019). The cysteine-rich receptor-like protein kinase CRK28 modulates Arabidopsis growth and development and influences abscisic acid responses. *Planta* 27;251(1):2. doi: 10.1007/s00425-019-03296-y.

Perilli S, Di Mambro R, Sabatini S (2012). Growth and development of the root apical meristem. *Curr Opin Plant Biol* 15(1):17-23. doi: 10.1016/j.pbi.2011.10.006.

Petricka JJ, Winter CM, Benfey PN (2012). Control of Arabidopsis root development. *Annu Rev Plant Biol* 63:563-90. doi: 10.1146/annurev-arplant-042811-105501.

Pulianmackal, A. J., Kareem, A. V., Durgaprasad, K., Trivedi, Z. B., & Prasad, K. (2014). Competence and regulatory interactions during regeneration in plants. *Frontiers in plant science*, 5, 142. doi: 10.3389/fpls.2014.00142.

Ravelo-Ortega, Raya-González J, López-Bucio J (2023). Compounds from rhizosphere microbes that promote plant growth. *Curr Opin Plant Biol* 73:102336.

Raya-González J, Oropeza-Aburto A, López-Bucio JS, Guevara-García ÁA, de Veylder L, López-Bucio J, Herrera-Estrella L (2018). MEDIATOR18 influences Arabidopsis root architecture, represses auxin signaling and is a critical factor for cell viability in root meristems. *Plant J* 96(5):895-909. doi: 10.1111/tpj.14114.

Raya-González J, Ávalos-Rangel A, Ruiz-Herrera LF, Valdez-Alarcón JJ, López-Bucio J (2022). The RNA polymerase II subunit NRPB2 is required for indeterminate

root development, cell viability, stem cell niche maintenance, and de novo root tip regeneration in Arabidopsis. *Protoplasma* 259(5):1175-1188. doi: 10.1007/s00709-021-01732-z.

Reddien PW (2018). The cellular and molecular basis for planarian regeneration. *Cell* 175(2):327-345. doi: 10.1016/j.cell.2018.09.021.

Richardson LG, Torii KU (2013). Take a deep breath: peptide signalling in stomatal patterning and differentiation. *J Exp Bot* 64(17):5243-51. doi: 10.1093/jxb/ert246.

Rosspopoff O, Chelysheva L, Saffar J, Lecorgne L, Gey D, Caillieux E, Colot V, Roudier F, Hilson P, Berthomé R, Da Costa M, Rech P (2017). Direct conversion of root primordium into shoot meristem relies on timing of stem cell niche development. *Development* 144(7):1187-1200. doi: 10.1242/dev.142570.

Ruiz-Aguilar B, Raya-González J, López-Bucio JS, Reyes de la Cruz H, Herrera-Estrella L, Ruiz-Herrera LF, Martínez-Trujillo M, López-Bucio J (2020). Mutation of *MEDIATOR 18* and chromate trigger twinning of the primary root meristem in Arabidopsis. *Plant Cell Environ* 43(8):1989-1999. doi: 10.1111/pce.13786.

Shaar-Moshe L, Brady SM (2022). SHORT-ROOT and SCARECROW homologs regulate patterning of diverse cell types within and between species. *New Phytol* 2022 Dec 1. doi: 10.1111/nph.18654.

Sharma VK, Ramirez J, Fletcher JC (2003). The Arabidopsis CLV3-like (CLE) genes are expressed in diverse tissues and encode secreted proteins. *Plant Mol Biol* 51(3):415-25. doi: 10.1023/a:1022038932376.

Shao Y, Yu X, Xu X, Li Y, Yuan W, Xu Y, Mao C, Zhang S, Xu J (2020). The YDA-MKK4/MKK5-MPK3/MPK6 cascade functions downstream of the RGF1-RGI ligand-receptor pair in regulating mitotic activity in root apical meristem. *Mol Plant* 13(11):1608-1623. doi: 10.1016/j.molp.2020.09.004.

Shinohara H, Matsubayashi Y (2015). Reevaluation of the CLV3-receptor interaction in the shoot apical meristem: dissection of the CLV3 signaling pathway from a direct ligand-binding point of view. *Plant J* 82(2):328-36. doi: 10.1111/tpj.12817.

Shiu SH, Bleecker AB (2001). Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc Natl Acad Sci U S A* 98(19):10763-8. doi: 10.1073/pnas.181141598.

Shiu SH, Karlowski WM, Pan R, Tzeng YH, Mayer KF, Li WH (2004). Comparative analysis of the receptor-like kinase family in Arabidopsis and rice. *Plant Cell* 16(5):1220-34. doi: 10.1105/tpc.020834.

Shen H, Zhong X, Zhao F, Wang Y, Yan B, Li Q, Chen G, Mao B, Wang J, Li Y, Xiao G, He Y, Xiao H, Li J, He Z (2015). Overexpression of receptor-like kinase ERECTA improves thermotolerance in rice and tomato. *Nat Biotechnol* 33(9):996-1003. doi: 10.1038/nbt.3321.

Stahl Y, Wink RH, Ingram GC, Simon R (2009). A signaling module controlling the stem cell niche in Arabidopsis root meristems. *Curr Biol* 19(11):909-14. doi: 10.1016/j.cub.2009.03.060.

Strabala TJ, O'donnell PJ, Smit AM, Ampomah-Dwamena C, Martin EJ, Netzler N, Nieuwenhuizen NJ, Quinn BD, Foote HC, Hudson KR (2006). Gain-of-function phenotypes of many CLAVATA3/ESR genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. *Plant Physiol* 140(4):1331-44. doi: 10.1104/pp.105.075515.

Suzaki T, Sato M, Ashikari M, Miyoshi M, Nagato Y, Hirano H (2004). The gene *FLORAL ORGAN NUMBER1* regulates floral meristem size in rice and encodes a leucine-rich repeat receptor kinase orthologous to Arabidopsis *CLAVATA1*. *Development* 131(22):5649-5657. doi: 10.1242/dev.01441.

Suzuki N, Mittler R (2012). Reactive oxygen species-dependent wound responses in animals and plants. *Free Radic Biol Med* 53(12):2269-76. doi: 10.1016/j.freeradbiomed.2012.10.538.

Takahashi N, Umeda M (2022). Brassinosteroids are required for efficient root tip regeneration in *Arabidopsis*. *Plant Biotechnol* (Tokyo) 39(1):73-78. doi: 10.5511/plantbiotechnology.21.1103a.

Temmerman A, Marquez-Garcia B, Depuydt S, Bruznican S, De Cuyper C, De Keyser A, Boyer FD, Vereecke D, Struk S, Goormachtig S (2022). MAX2-dependent competence for callus formation and shoot regeneration from Arabidopsis thaliana root explants. *J Exp Bot* 73(18):6272-6291. doi: 10.1093/jxb/erac281.

Thowfeequ S, Myatt EJ, Tosh D (2007). Transdifferentiation in developmental biology, disease, and in therapy. *Dev Dyn* 236(12):3208-17. doi: 10.1002/dvdy.21336.

Waadt R, Seller CA, Hsu PK, Takahashi Y, Munemasa S, Schroeder JI (2022). Plant hormone regulation of abiotic stress responses. *Nat Rev Mol Cell Biol* 23(10):680-694. doi: 10.1038/s41580-022-00479-6.

Walker JC, Zhang R (1990). Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. *Nature* 345(6277):743-746. doi: 10.1038/345743a0.

Willoughby AC, Nimchuk ZL (2021). WOX going on: CLE peptides in plant development. *Curr Opin Plant Biol* 63:102056. doi: 10.1016/j.pbi.2021.102056.

Wu Y, Xun Q, Guo Y, Zhang J, Cheng K, Shi T, He K, Hou S, Gou X, Li J (2016). Genome-wide expression pattern analyses of the Arabidopsis leucine-rich repeat receptor-like kinases. *Mol Plant* 9(2):289-300. doi: 10.1016/j.molp.2015.12.011.

Yan A, Borg M, Berger F, Chen Z (2020). The atypical histone variant H3.15 promotes callus formation in *Arabidopsis thaliana*. *Development* 147(11):dev184895. doi: 10.1242/dev.184895.

Xu L (2018). De novo root regeneration from leaf explants: wounding, auxin, and cell fate transition. *Curr Opin Plant Biol* 41:39-45. doi: 10.1016/j.pbi.2017.08.004.

Yamaguchi Y, Pearce G, Ryan CA (2006). The cell surface leucine-rich repeat receptor for AtPep1, an endogenous peptide elicitor in Arabidopsis, is functional in transgenic tobacco cells. *Proc Natl Acad Sci U S A* 103(26):10104-9. doi: 10.1073/pnas.0603729103.

Yamaguchi Y, Huffaker A, Bryan AC, Tax FE, Ryan CA (2010). PEPR2 is a second receptor for the Pep1 and Pep2 peptides and contributes to defense responses in Arabidopsis. *Plant Cell* 22(2):508-22. doi: 10.1105/tpc.109.068874.

Zhai N, Xu L (2021). Pluripotency acquisition in the middle cell layer of callus is required for organ regeneration. *Nat Plants* 7(11):1453-1460. doi: 10.1038/s41477-021-01015-8.

Zhang H, Zhu J, Gong Z, Zhu JK (2022). Abiotic stress responses in plants. *Nat Rev Genet* 23(2):104-119. doi: 10.1038/s41576-021-00413-0.

Zhang G, Zhao F, Chen L, Pan Y, Sun L, Bao N, Zhang T, Cui CX, Qiu Z, Zhang Y, Yang L, Xu L (2019). Jasmonate-mediated wound signalling promotes plant regeneration. *Nat Plants* 5(5):491-497. doi: 10.1038/s41477-019-0408-x.

Zhang Z, Liu C, Li K, Li X, Xu M, Guo Y (2022). CLE14 functions as a "brake signal" to suppress age-dependent and stress-induced leaf senescence by promoting JUB1-mediated ROS scavenging in Arabidopsis. *Mol Plant* 15(1):179-188. doi: 10.1016/j.molp.2021.09.006.

Zheng B, Bai Q, Wu L, Liu H, Liu Y, Xu W, Li G, Ren H, She X, Wu G (2019). EMS1 and BRI1 control separate biological processes via extracellular domain diversity and intracellular domain conservation. *Nat Commun* 10(1):4165. doi: 10.1038/s41467-019-12112-w.

Zhou W, Lozano-Torres JL, Blilou I, Zhang X, Zhai Q, Smant G, Li C, Scheres BA (2019). Jasmonate signaling network activates root stem cells and promotes regeneration. *Cell* 177(4):942-956.e14. doi: 10.1016/j.cell.2019.03.006.

Zhu Q, Feng Y, Xue J, Chen P, Zhang A, Yu Y (2023). Advances in receptor-like protein kinases in balancing plant growth and stress responses. *Plants* 12, 427. https://doi.org/10.3390/ plants12030427.

10. ADDENDUM

Publicaciones relacionadas con este trabajo:

Raya-González J, **Ávalos-Rangel A**, Ruiz-Herrera LF, Valdez-Alarcón JJ, López-Bucio J (2022). The RNA polymerase II subunit NRPB2 is required for indeterminate root development, cell viability, stem cell niche maintenance, and de novo root tip regeneration in Arabidopsis. *Protoplasma* 259(5):1175-1188. doi: 10.1007/s00709-021-01732-z.

Esparza-Reynoso, S., **Ávalos-Rangel, A.**, Pelagio-Flores, R., & López-Bucio, J. (2023). Reactive oxygen species and NADPH oxidase-encoding genes underly the plant growth and developmental responses to Trichoderma. *Protoplasma*, 1-13. doi: 10.1007/s00709-023-01847-5.

Ávalos-Rangel, A., Ruiz-Herrera LF., Gutiérrez-Alanis, D., Herrera-Estrella L., Raya-González, J., López-Bucio J. "CLE14 peptide impairs root tip regeneration and callogenesis in Arabidopsis". En proceso de publicación.

Protoplasma (2022) 259:1175–1188
https://doi.org/10.1007/s00709-021-01732-z

ORIGINAL ARTICLE

The RNA polymerase II subunit NRPB2 is required for indeterminate root development, cell viability, stem cell niche maintenance, and *de novo* root tip regeneration in Arabidopsis

Javier Raya-González[1] · Adrián Ávalos-Rangel[2] · León Francisco Ruiz-Herrera[2] · Juan José Valdez-Alarcón[3] · José López-Bucio[2]

Received: 18 August 2021 / Accepted: 19 December 2021 / Published online: 3 January 2022
© The Author(s), under exclusive licence to Springer-Verlag GmbH Austria, part of Springer Nature 2022

Abstract

The RNA polymerase II drives the biogenesis of coding and non-coding RNAs for gene expression. Here, we describe new roles for its second-largest subunit, NRPB2, on root organogenesis and regeneration. Down-regulation of *NRPB2* activates a determinate developmental program, which correlated with a reduction in mitotic activity, cell elongation, and size of the root apical meristem. Noteworthy, *nrpb2-3* mutants manifest cell death in pro-vascular cells within primary root tips of plants grown in darkness or exposed to light, which triggers the expression of the regeneration gene marker ERF115 in neighbor cells close to damage. Auxin and stem cell niche (SCN) gene expression as well as structural analysis revealed that NRPB2 maintains SCN activity through distribution of PIN transporters in root tissues. Wild-type seedlings regenerated the root tip after excision of the QC and SCN, but *nrpb2-3* mutants did not rebuild the missing tissues, and this process could be genotypified using *pERF115:GFP*, *DR5:GFP,* and *pWOX5:GFP* reporter constructs. The levels of reactive oxygen species increased in the mutants four days after germination and strongly decreased at later times, whereas nitric oxide accumulated as the root tip differentiates. These results show the importance of the transcriptional machinery for root organogenesis, cell viability, and regenerative capacity for reconstruction of tissues and organs upon injury.

Keywords *Arabidopsis thaliana* · RNA Pol II · root system configuration · stem cell niche maintenance · root tip regeneration

Introduction

The phenotype of living beings is tightly controlled at the transcriptional level since the amount of RNA being produced in time and space enables further stages of regulation of gene expression. Lesions in RNA polymerase II, the protein complex that recognizes gene promoters and drives RNA biosynthesis, may have a profound effect on growth, development, and adaptation, and given their pleiotropic consequences on the phenotype, very little is known about the specific functions of the polypeptides that comprise the whole holoenzyme on the biology of plants (Kaufmann et al. 2010).

Signaling between shoot and root is critical for plant adaptation to the ever-changing environment. Reconfiguration of root architecture controls the plant's ability to obtain water and nutritional resources, compete with neighbors and respond to biotic and abiotic stimuli. Integration of environmental signals into cellular programs required for adaptation

Handling Editor: Peter Nick

✉ Javier Raya-González
javier.raya@umich.mx

✉ José López-Bucio
jbucio@umich.mx

1 Facultad de Químico Farmacobiología, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Tzintzuntzan 173, Col. Matamoros, 58240 Morelia, Michoacán, México

2 Instituto de Investigaciones Químico Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B3, Ciudad Universitaria, 58030 Morelia, Michoacán, México

3 Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México

occurs through multiple signal transduction pathways that influence root organogenesis (Sánchez-Calderón et al. 2005; Durgaprasad et al. 2019; Raya-González et al. 2019). Within the root apical meristem (RAM), the stem cell niche (SCN) modulates the indeterminate developmental program that strongly depends on continuous cell replenishment and may persist during the entire plant life cycle. The SCN consists of the quiescent center (QC), a four-cell cluster with low mitotic activity that instructs the neighboring initial cells to divide, giving rise to all cell types that comprise the root body. SCN identity, maintenance, and cell positioning occur through the activity of several transcription factors, including WUSCHEL-RELATED HOMEOWOX 5 (WOX5), SCARECROW (SCR), SHORT ROOT (SHR), and the AP2-family transcription factors PLETHORA1-4 (Aida et al. 2004; Sánchez-Calderón et al. 2005; Galinha et al. 2007).

During plant phase transitions, distinct internal and/or external inputs may cause meristem exhaustion and root tip differentiation as the conforming cells are highly sensitive to agents that damage DNA, but also to physical stress caused by nematodes, wounding, and/or mechanical damage. However, during this process cell regeneration might be activated for cell replenishment in order to replace the missing tissues and support root growth (Sánchez-Calderón et al. 2005; Fulcher and Sablowski 2009; Hashimura and Ueguchi 2011; Raya-González et al. 2018; Marhava et al. 2019; Zhou et al. 2019).

Cell regeneration is essential for survival owing to the sessile lifestyle of plants. Ablation of initial cells and the excision of the SCN in the Arabidopsis primary root tip have been used to study regeneration and to unravel the genetic and hormonal components involved in this process (Sena et al. 2009; Heyman et al. 2013, 2016; Zhang et al. 2018). After root tip excision, auxins are re-mobilized and accumulate in wound-adjacent, neighbor cells via PIN-FORMED (PIN) efflux transporters to fine tune restorative cell division and cell re-specification (Hashimura and Ueguchi 2011; Hoermayer et al. 2020). Besides auxin, the transcription factor ERF115, a member of ETHYLENE RESPONSE FACTOR (ERF) family, has arisen as a key player in cell division and regeneration in response to multiple inputs, including genotoxic stress, cell ablation, root tip excision, healing, mechanical damage, and cell death triggered by specific gene mutations (Heyman et al. 2013, 2016; Raya-González et al. 2018, 2019; Zhang et al. 2018; Canher et al. 2020; Hoermayer et al. 2020; Matosevich et al. 2020).

Recently, mutation of the *HALTED PRIMARY ROOT* (*HPR1*) locus in Arabidopsis unveiled an interesting mechanism by which death of pro-vasculature cells within root meristems was sufficient to change primary root growth from indeterminate to determinate. Although the molecular identity of HPR1 remains unknown, the study of the root phenotype of the mutant helped to clarify the relationship among root cell viability and the auxin response (Raya-González et al. 2019). Moreover, mutations of the gene encoding Mediator18 (MED18) sub-unit of the MEDIATOR transcriptional complex also lead to spontaneous programed cell death (PCD) in the meristem pro-vasculature cells that exacerbates with age, light exposure or treatment with genotoxics, which correlated with expression of *ERF115* and *CYCLIN D6* (*CYCD6*) (Heyman et al. 2016; Raya-González et al. 2018).

MEDIATOR is a multi-subunit protein complex that acts as a bridge between transcription factors and RNA polymerase II (Pol II) (Kidd et al. 2009). In Arabidopsis, Pol II is integrated by 12 subunits, being NRPB1, NRPB2, and NRPB3 the three major polypeptides of the catalytic site (Larkin et al. 2009). Arabidopsis plants with alterations on C-terminal domain (CTD) of NRPB1 manifest cell cycle disruption and decreased size of root and shoot meristems (Zhang et al. 2018), whereas NRPB3 has been involved with the pattering of stomata and differentiation through physic interaction with FAMA and INDUCER OF CBF EXPRESSION (ICE1) transcription factors (Chen et al. 2016). Interruption of *NRPB2* in three different alleles, namely *nrpb2-1*, *nrpb2-2*, and *nrpb2-3,* affects embryo, leaf, and floral organ development (Onodera et al. 2008; Zheng et al. 2009). Lack of maternal transmission in *nrpb2-1* and *nrpb2-2* generates small unfertilized ovules, which leads to lethality (Onodera et al. 2008; Zheng et al. 2009). However, *nrpb2-3* having a G-to-A mutation in the coding region is a weak allele because the NRPB2 protein still accumulates, but at a reduced level when compared to the WT, which enables its study (Kim et al. 2011). MED18 and the RNA Pol II, through their second-largest subunit, NRPB2, functionally interact to control non-coding RNA biogenesis in Arabidopsis (Kim et al. 2011). However, nothing is known about the roles of NRPB2 during root development. Regarding its critical function as part of the transcriptional machinery, it is possible that NRPB2 could act as a key regulator of root system processes such as cell viability and/or regeneration.

Here, we provide evidence for a critical role of NRPB2 in root development, cell viability, and root tip regeneration. Arabidopsis seedlings with diminished *NRPB2* expression show several defects on root architecture, including short primary roots, full differentiation of the root meristem, and higher root hair formation, which correlates with an altered local auxin distribution, low mitotic activity and reduction in cell size. Cellular damage within the root meristem of *nrpb2* mutants was accompanied by an induced expression of *ERF115* and changes in detection of oxidative and nitrosative molecular species. Cell tissue regeneration assays further showed that NRPB2 is required for *de novo* root tip meristem reconstruction after excision. These data demonstrate the critical function of RNA Pol II components in root organogenesis and regeneration.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana WT (Col-0), the transgenic *Arabidopsis* lines *CycB1:uidA* (Colón-Carmona et al. 1999), *EXP7:uidA* (Cho and Cosgrove 2002), *DR5:GFP* (Ottenschlager et al. 2003); *pERF115:GFP* (Heyman et al. 2016), *pPIN1::PIN1-GFP* (Benková et al. 2003), *pPIN2::PIN2-GFP* (Blilou et al. 2005), *pPIN3::PIN3-GFP* (Blilou et al. 2005), *pWOX5:GFP* (Ding and Friml 2010), *pSHR::SHR-GFP* (Cruz-Ramírez et al. 2012), *pSCR::SCR-YFP* (Cruz-Ramírez et al. 2012) and the mutant line *nrpb2-3* (Zheng et al. 2009), were used in this work. Seeds were disinfected by using 95% (v/v) ethanol and 20% (v/v) commercial bleach. After five washes with distilled water, seeds were plated on Murashige and Skoog (MS) medium 0.2X, with 0.6% sucrose and 1% agar. Plates were placed in a plant growth chamber at 21 °C in a 16 h light/8 h darkness cycle and light intensity of 200 μmol m^{-2} s^{-1}. Primary root length was scored using a ruler, and root meristem length, root width, and distance from QC to first root hair were scored from microscope images of Arabidopsis root tips.

Root tip excision and regeneration

Root tips were excised by hand using a sterile scalpel and a stereoscope microscope in a laminar flow cabinet. The excisions were performed at the QC, and the seedlings were transferred to fresh medium. Expression analysis of genetic markers was performed for the WT and *nrpb2-3* seedlings every 24 h for 3 d after cutting. The seedlings were stained with propidium iodide and placed on microscope slides to be visualized by confocal microscopy. Root tip regeneration was measured via counting the number of seedlings that regenerated a functional SCN, whereas a negative score was used when roots collapsed and failed to reconstitute the root meristem. The experiment included at least 40 seedlings for each treatment/line and was replicated at least three times

Propidium iodide staining and GFP detection

RAM structure, cell viability, and cell regeneration were assessed in roots of seedlings submerged into a propidium iodide (PI) solution (10 mg/ml) during 1 min. After two washes with distilled water, seedlings were placed on microscope slides and visualized with a confocal microscope (Olympus FV1200, Tokyo, Japan). Wavelengths specific for IP (568 nm excitation; 585-610 nm emission) and GFP (500-523 nm emission; 488 nm excitation) were used and recorded separately when necessary. For GFP/PI pictures, the two images were merged to generate the final image. Fluorescence quantification was scored as relative fluorescence.

Detection of reactive oxygen species and nitric oxide

Reactive oxygen species (ROS) and nitric oxide (NO) levels were detected and visualized by using the specific probes 2',7'-dichlorofluorescein diacetate (H_2DCF-DA) and 4,5-diaminofluorescein diacetate (DAF-2DA), respectively. WT and *nrpb2-3* Arabidopsis seedlings were incubated in 10 μM dilutions of H_2DCF-DA or DAF-2DA during 30 min in darkness conditions. Seedlings were washed three times and placed on microscopy slides, and fluorescence signals were observed and detected by a confocal microscope.

Histochemical analysis

β-glucuronidase (GUS) histochemical detection was performed incubating overnight Arabidopsis seedlings at 37 °C in phosphate buffer pH 7.4 supplied with the enzymatic substrate (0.5 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucoronide dissolved in 0.24 N sodium phosphate, pH 7.0). Seedlings with GUS activity were cleared and fixed by using the protocol described by Malamy and Benfey (1997), in which seedlings were incubated by 60 min in a solution 0.24N HCl/20% methanol at 63 °C. Then, the seedlings were transferred to 7% NaOH/60% ethanol solution by 30 min at room temperature, and finally, a dehydration process was performed by using ethanol treatments at 40, 20, and 10% (v/v) for 20 min period each at room temperature. Seedlings were stored and mounted with glycerol 50% on microscope slides and visualized by differential interference contrast microscope (DIC), using a LEICA DM500B microscope. For each transgenic line, the root tips of at least 20 stained seedlings were recorded and analyzed.

Starch staining

Arabidopsis WT and *nrpb2-3* seedlings were germinated and grown by 2, 4, and 8 d in MS 0.2X medium, cleared and fixed by using the protocol described by Malamy and Benfey (1997), and incubated with lugol solution 10 s. Then, seedlings were mounted in 50% glycerol solution and visualized by differential interference contrast microscope (DIC), using a LEICA DM500B microscope. For each genotype, at least fifteen root tips from WT and *nrpb2-3* were analyzed and recorded.

Results

NRPB2 controls Arabidopsis primary root growth

Two homozygous mutants with NRPB2 loss-of-function have been isolated, *nrpb2-1* and *nrpb2-2* (Onodera et al. 2008). However, both are embryo-lethal, indicating the essential role of this RNA Pol II subunit for plant survival. In this work, we used a self-fertile, weak allele previously reported, *nrpb2-3*, in which NRPB2 transcript is still detected, but at a reduced level (Zheng et al. 2009). Arabidopsis wild-type (WT; Columbia-0 [Col-0]) and *nrpb2-3* seedlings were germinated and grown on MS agar-solidified media for 10 days, and primary root elongation was recorded every 24 h after germination (Fig. 1a–b). Sustained root growth was observed in WT seedlings during the analysis, consistent with their indeterminate developmental program, whereas *nrpb2-3* mutants not only showed a slower root growth rate, but also primary root elongation stopped 4 d after germination, accompanied by an induction of root hair development and more lateral roots emerging close to the root tip (Fig. 1a–c, f). These defects were also manifested when root elongation

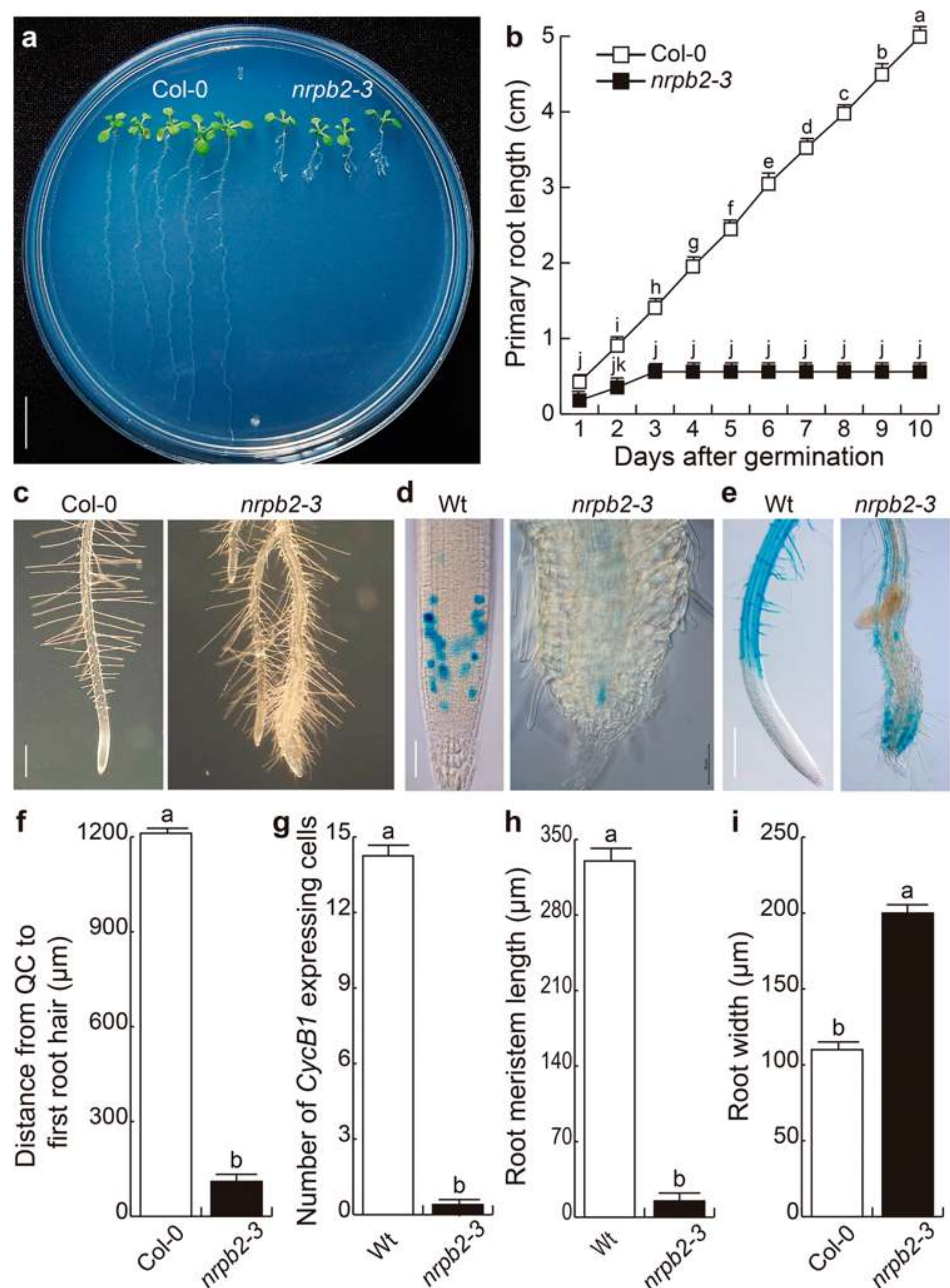


Fig. 1 NRPB2 supports indeterminate root development in *Arabidopsis thaliana.* Wild-type (Wt; Col-0) and *nrpb2-3* mutant seedlings were germinated and grown for 10 d on 0.2x Murashige and Skoog (MS) medium. (a) Photograph of representative Wt and *nrpb2-3* seedlings grown side by side. (b) Primary root growth of Wt and *nrpb2-3* plants recorded every 24 h for 10 d. (c) Photographs of representative Wt and *nrpb2-3* root tips. (d, e) Wt and *nrpb2-3* roots expressing *CycB1:uidA* (d) and *EXP7:uidA* (e) gene constructs. (f) Meristem and elongation zones measured from QC to first root hair. (g) Number of *CycB1* expressing cells recorded as blue spots. (h) Root meristem length. (i) Root width. Bars represent means ± SE from 30 seedlings analyzed. Different letters indicate means that differ statistically ($P<0.05$). The experiments were repeated three times with similar results. Scale bar = 1 cm (a); 200 µm (c, e); 50 µm (d).

occurred under darkness where *nrpb2-3* seedlings had reduced growth rates and altered root meristem structure (Fig. S1).

Root growth is modulated by cell division, elongation, and differentiation. To determine whether these processes are altered in *nrpb2-3* mutants, the expression of *CycB1:uidA* and *EXP7:uidA* gene markers were analyzed in 10-d WT and *nrpb2-3* seedlings via out-crossing mutant seedlings with pollen from transgenic Arabidopsis plants expressing the markers. CycB1 monitors cell cycle activity on root meristems (Colón-Carmona et al. 1999), whereas *EXP7:uidA* reveals the differentiated status of root epidermal cells (trichoblasts) that give rise to root hairs (Cho and Cosgrove 2002). Reduction in the primary root meristems in *nrpb2-3* correlated with a weak expression of *CycB1:uidA* and advancement of *EXP7:uidA* expression toward the root tip (Fig. 1d, e). The number of cells expressing *CycB1:uidA* and the root meristem length inversely correlated with root tip width in the mutants (Fig. 1g–i). This suggests that *NRPB2* plays a key role in root morphogenesis by positive modulation of cell proliferation processes in the meristem.

NRPB2 supports cell integrity in root meristems

The alterations in *nrpb2-3* mutants on root development prompted us to analyze root cell organization, morphology, and integrity in root tips. With this aim, WT and *nrpb2-3* seedlings were stained with propidium iodide (PI) and root tips were visualized by confocal microscopy. In living cells, PI stains the cell walls, which allows visualization and analysis of each cell tissue, whereas in dead cells PI freely penetrate, what is observed by red patchy patterns (Truernit and Haseloff 2008). The RAM from Arabidopsis WT and *nrpb2-3* seedlings was analyzed every 48 h during the first 10 days after germination by confocal microscopy. WT roots showed comparable root tip structure, without

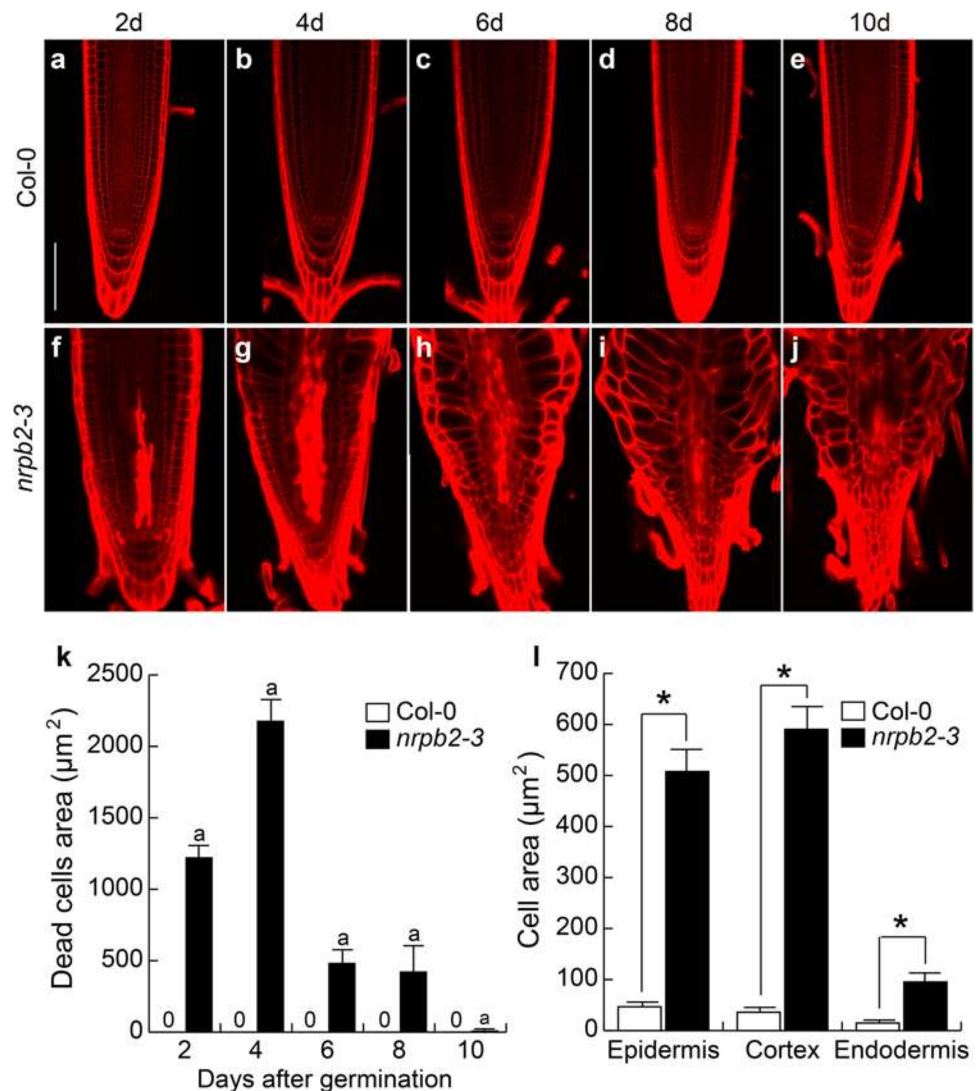


Fig. 2 NRPB2 is required for cell viability in root meristems. Wt (Col-0) and *nrpb2-3* seedlings were grown on 0.2x MS medium, and cell integrity was analyzed 2 d (a, f), 4 d (b, g), 6 d (c, h), 8 d (d, i), and 10 d (e, j) after germination. (k) Kinetics of dead cell area. (l) Area of epidermis, cortex, and endodermis cell layers. Representative photographs are shown from at least 10 seedlings of each condition/line, stained with IP, and visualized by confocal microscopy. Bars represent means ± SE from 10 seedlings analyzed. Different letters indicate means that differ statistically ($P<0.05$). The experiments were repeated three times with similar results. Scale bar = 100 µm.

any apparent phenotypical change during the experiment (Fig. 2a–e). Interestingly, *nrpb2-3* primary root tips, lateral root tips, and adventitious root tips showed extensive patches of cell death 2 days after germination, indicating that reduction in *NRPB2* expression compromises viability of vascular precursor cells within root meristems (Figs. 2f–j and S2). Kinetic analysis of cell death and cell size indicated that the meristem consumption and root tip differentiation advances with time (Fig. 2k, l). Previously, we reported that light is a conditional factor for cell damage in root meristem, which impedes primary root elongation in *med18* mutants (Raya-González et al. 2018). In *nrpb2-3* seedlings, even growth under darkness caused cell damage symptoms and a reduction in root growth (Fig. S1). These results suggest that NRPB2 is required for cell viability in Arabidopsis root meristems.

NRPB2 influences auxin signaling and controls stem cell niche maintenance in Arabidopsis

Auxin is a major plant growth regulator that orchestrates root morphogenesis. To determine whether auxin signaling is involved in *nrpb2-3* root phenotype, the auxin-inducible gene marker *DR5:GFP* was introduced into *nrpb2-3* background by out-crossing and homozygous mutant seedlings harboring the marker were analyzed by confocal microscopy. From here, 4-d old seedlings were used for further experiments, since *nrpb2-3* line showed halted root growth that coincides with depletion of the root meristem (Fig. 1a– e). In WT seedlings, *DR5:GFP* expression was visualized in QC, columella, and pro-vasculature cell tissues, as well as in the lateral root formation zone and emerging lateral roots (Figs. 3a and S3). For *nrpb2-3* mutants, *DR5:GFP* expression in primary roots was extended toward the epidermis, lateral root cap, and along the vascular tissues and lateral root primordia, suggesting its influence on root system development through auxin signaling (Figs. 3e, and S3). *In vivo* detection of auxin efflux transporters via confocal microscopy in *pPIN1::PIN1-GFP*, *pPIN2::PIN2-GFP*, and *pPIN3::PIN3-GFP* in the WT and *nrpb2-3* seedlings, indicates that NRPB2 influences both the level and positioning of the auxin transporters within root tissues, since GFP detection driven by the reporter fusions strongly decreased in the mutants (Fig. S4).

Local auxin levels maintain distal stem cells in root tips involving WOX5, SHR, and SCR transcription factors (Sabatini et al. 2003; Tian et al. 2014). As the auxin signaling appears to be modified in *nrpb2-3* root tips, we now examined the expression of the QC and SCN markers. For QC activity, we analyzed the expression and quantified the number of *pWOX5:GFP*-expressing cells from root tips of 4 d-old seedlings. In WT plants around four GFP-expressing cells were found, whereas in *nrpb2-3* around twenty cells showing *WOX5* expression at the proximal meristem (PM) above the QC could be quantified (Fig. 3b, f, j), suggesting that NRPB2 restricts WOX5 expression to the QC. Because WOX5 moves from QC to columella stem cells toward maintaining their un-differentiated status (Pi et al. 2015), it could explain the alteration of columella cell morphology and structure in *nrpb2-3* seedlings, which marks the transit from cell division to differentiation, as supported by starch staining with lugol (Fig. S5). We next examined the expression of *pSHR::SHR-GFP* and *pSCR::SCR-YFP* in WT and *nrpb2-3* seedlings. SHR and SCR are expressed in stele and endodermis, respectively (Fig. 3c–d) (Galinha et al. 2007). *SHR* and *SCR* expression was drastically reduced in *nrpb2-3* mutants (Fig. 3g–h, k–l), being more pronounced for *SCR-YFP* (Fig. 3h), which was consistent with fluorescence quantification compared with WT roots (Fig. 3l). Together, these results indicate that NRPB2 is a key regulator of root meristem activity and maintenance trough modulation of WOX5, SHR, and SCR stem cell niche genes in *Arabidopsis thaliana*.

NRPB2 influences expression of ERF115 in Arabidopsis root tips

In root tips, cell damage promotes regeneration through ERF115 activity (Sena et al. 2009; Heyman et al. 2016; Raya-González et al. 2018). To assess whether cell damage in *nrpb2-3* activates a cell regeneration program, we analyzed the expression of ERF115 by transferring *pERF115:GFP* gene construct into *nrpb2-3* background and comparing the expression with the WT where *ERF115* is not expressed in standard growth conditions (Fig. 4a–c). A strong and specific *ERF115* expression could be found on adjacent cells of injured tissues in *nrpb2-3* mutants (Fig. 4d–g).

Arabidopsis root tip resection has been used to study cell regeneration in plants (Sena et al. 2009; Heyman et al. 2013). We employed this strategy to investigate the role of NRPB2 on root tip recovery by evaluating the capacity of WT and *nrpb2-3* seedlings to regenerate a functional root meristem. Compared with un-excised SCN of WT plants (Fig. 5a–e), a functional SCN was regenerated from 3 days after root tip excision and accompanied with transcriptional induction of ERF115 adjacent to the excision site that was later reduced as the new SCN becomes functional (Fig. 5k–o). In un-excised SCN in *nrpb2-3* mutant seedlings, ERF115 expression was induced in primary root meristems as mentioned above (Fig. 5f–j). However, although a strong ERF115 expression in adjacent cells correlates with the cell damage after root excision in *nrpb2-3* seedlings, it failed to re-establish a functional SCN as only ~3% of *nrpb2-3* roots were able to regenerate a novel root tip compared with 100%

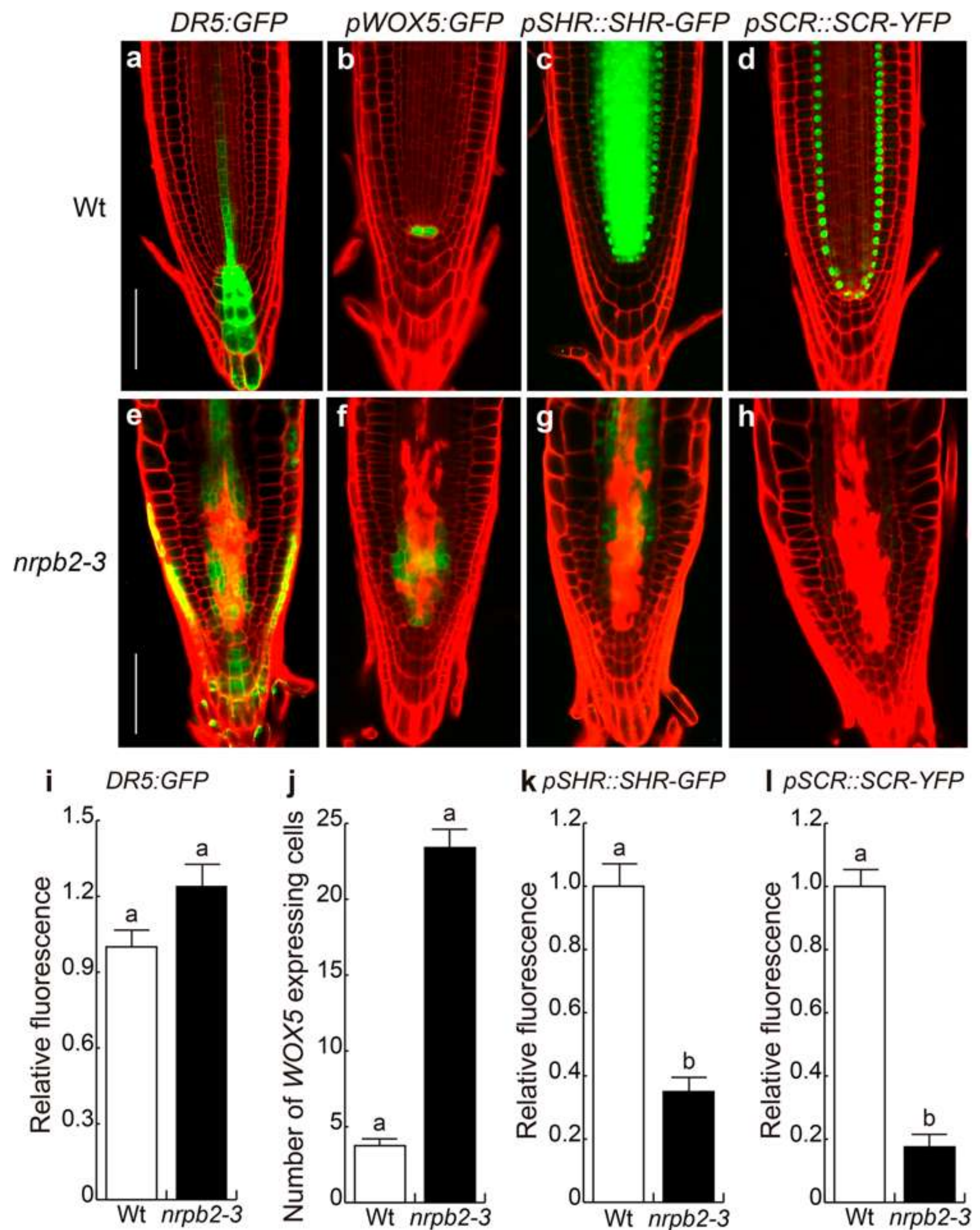


Fig. 3 NRPB2 determines auxin signaling and SCN gene expression in Arabidopsis root tips. Wt and *nrpb2-3* seedlings expressing *DR5:GFP* (a, e), *pWOX5:GFP* (b, f), *pSHR::SHR-GFP* (c, g), and *pSCR::SCR-YFP* (d, h) gene constructs were germinated and grown for 4 d in 0.2x MS medium, stained with propidium iodide (PI) , and visualized by confocal microscopy. Graphs (i, k, l) illustrate expression measured as relative fluorescence for *DR5:GFP* (i), *pSHR::SHR-GFP* (k), and (l) *pSCR::SCR-YFP*. (j) Number of *WOX5* expressing cells in root meristems of Wt and *nrpb2-3*. Bars represent means ± SE from 10 seedlings analyzed. Different letters indicate means that differ statistically ($P<0.05$). The experiments were repeated three times with similar results. Scale bar = 50 µm.

of WT roots (Figs. 5p–t, and Fig. S6), indicating that *de novo* root tip formation critically depends of NRPB2.

Changes in the auxin response during regeneration of the Arabidopsis root tip

Local auxin biosynthesis and accumulation via PIN-mediated transport are necessary for root tip regeneration after wounding (Matosevich et al. 2020). To assess whether auxin response is deficient in *nrpb2-3* root tips after root excision, we monitored *DR5:GFP* expression in WT and *nrpb2-3* mutants every 24 h during 3 d in 4 d-old WT and *nrpb2-3* seedlings and after excision of the root tip at the QC. In un-excised SCN WT plants, *DR5:GFP* expression was comparable at all indicated points (Fig. 6a–e), whereas in *nrpb2-3*, a stronger GFP expression was observed, which disappeared at the end of the experiment, which correlated with root tip differentiation and the lack of regeneration (Fig. 6f–j). The fact that root tip excision induced *DR5:GFP* expression on the adjacent cells of damaged areas, 24 and 48 h after excision (Fig. 6k–o), indicated that the loss of SCN by wounding activated the formation of a novel auxin pattern that triggered SCN re-specification and further regeneration. Interestingly, when auxin response was analyzed in *nrpb2-3* root tips after excision, *DR5:GFP* expression was increased, but it was confined to vascular tissues (Fig. 6p–t), indicating that *nrpb2-3* was unable to

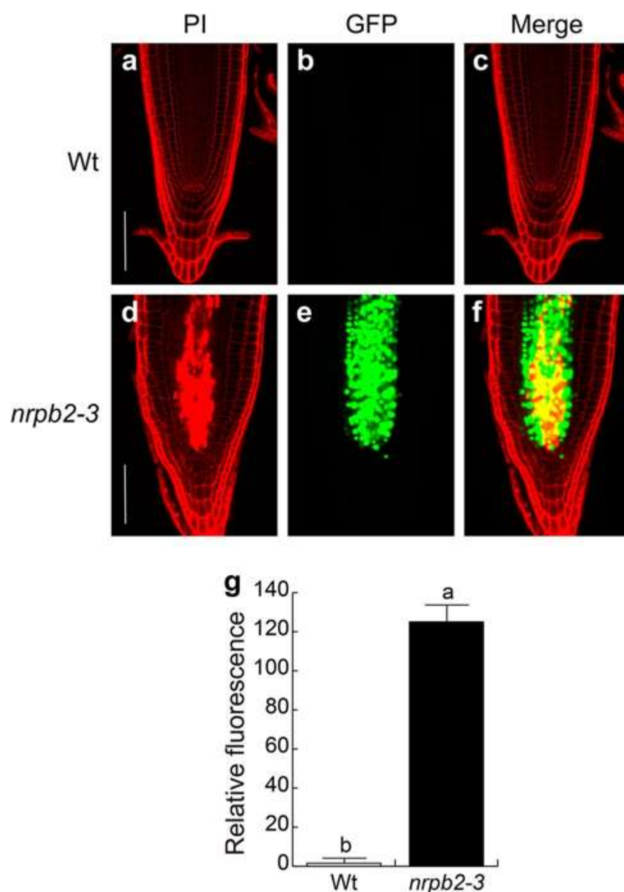


Fig. 4 Cell damage in *nrpb2-3* mutant triggers *ERF115* expression. Wt (a-c) and *nrpb2-3* (d-f) seedlings harboring *pERF115:GFP* gene construct were germinated and grown for 4 d in MS 0.2X and stained with IP. Then, ERF115 expression was visualized by confocal microscopy. Graph in (g) represents the expression of *ERF115* measured as relative fluorescence. Different letters indicate that differ statistically ($P<0.05$). The experiment was repeated three times with similar results. Scale bar = 50 µm.

re-create auxin patterning after excision, and that it most likely induces a cell differentiation program.

Expression of the quiescent center marker, *pWOX5:GFP* during root tip regeneration

To understand how NRPB2 mutation affects the spatial dynamics of cell identity within the stem cell niche during root regeneration, we analyzed the *pWOX5:GFP* reporter, which marks cells with QC identity (Fig. 7a–e). Starting at 1 dpe (days post excision), WOX5 was co-expressed within the zone proximal to cell damage. Between 2 dpe and 3 dpe, it marked the new domain corresponding to QC tissue (Fig. 7k–o). Next, we analyzed *WOX5* expression in *nrpb2-3* root tips. In excised seedlings, *WOX5* was co-expressed in QC and neighboring cells, but disappeared in an age-dependent manner (Fig. 7f–j). Similarly, the *WOX5* expression was observed in *nrpb2-3* seedlings after excision, disappearing after 3d, suggesting that *nrpb2-3* is affected in re-establishment of a functional SCN (Fig. 7p–t).

Mutation of NRPB2 changes the levels of reactive oxygen species and nitric oxide in root tips

Accumulation of reactive oxygen and nitrogen species (ROS; RNS) is a hallmark of aerobic metabolism and might be related to cell death (Zhao 2007). By using fluorescence probes and confocal microscopy, we analyzed ROS and nitric oxide (NO) levels in root tips at four and eight-days after germination in WT and *nrpb2-3* Arabidopsis seedlings. ROS signal could be detected in several cell tissues, including epidermis, cortex, stem cell niche, and root cap of the WT (Fig. 8a). Interestingly, four-day-old *nrpb2-3* seedlings showed higher ROS levels than WT seedlings, which decreased at a time of full meristem consumption (Fig. 8a, c). NO signal could be detected in epidermal and cortex layers in WT roots at the two times assayed, whereas in *nrpb2-3* mutants, the fluorescence was increased in the epidermal layer and a new signal pattern was found in a central region, which correlates with the cell death patches (Fig. 8b, d). Thus, ROS and NO levels change in *nrpb2-3* mutants during cell differentiation programs ending with meristem exhaustion caused by NRPB2 dysfunction.

Discussion

The RNA Polymerase II (Pol II) is a macromolecular complex, which interacts with gene promoters and biosynthesizes the encoded RNA messengers of the open reading frames. In this report, we employed a range of strategies to unravel the role of the second largest subunit of Pol II, NRPB2, on root system architecture, cell viability, and cell regeneration processes in *Arabidopsis thaliana*. To the best of our knowledge, no previous studies on this matter have been done with this subunit, since its complete loss-of-function led to lethality at the embryo stage.

An advantage of having at hand a non-lethal allele, such as *nrpb2-3*, whose corresponding mutant seedlings are able to grow and self-fertilize producing homozygous progeny is the possibility to perform detailed phenotypical and molecular comparisons with the WT. Reduction on NRPB2 protein levels resulted in repression of primary root elongation, death of pro-vasculature cells, and impeded the regeneration of the root tip after excision, suggesting that NRPB2 is necessary for the expression of major genes for cell division/regeneration.

The main observable effect of knocking down *NRPB2* in Arabidopsis seedlings is a determinate primary root growth, which is tightly related to cell death in pro-vasculature cells

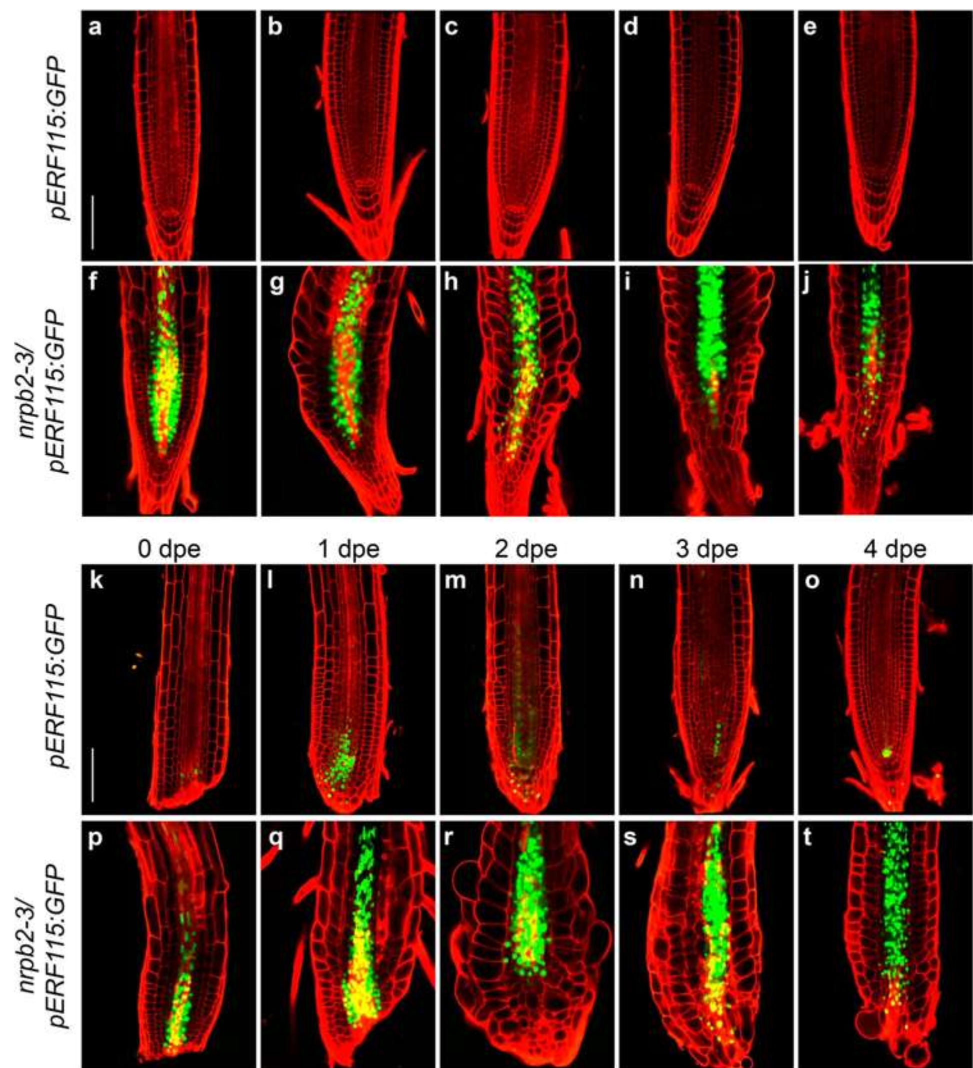


Fig. 5 *ERF115* expression during root tip regeneration in Wt and *nrpb2-3* seedlings. *ERF115* expression in 4d-old Wt (a-e, k-o) and *nrpb2-3* (f-j, p-t) seedlings after root tip excision for the indicated time points. (a-e) Un-excision of Wt seedlings and after excision of the root tip (k-o). (f-j) Un-excision of *nrpb2-3* seedlings and after excision of the root tip (p-t). Representative photographs from at least 10 seedlings of each condition, stained with PI, and visualized by confocal microscopy. The experiment was repeated two times with similar results. dpe= days post excision. Scale bar = 100 µm.

and alteration of auxin signaling, a phenomenon previously reported for *nrpb2* mutants at several embryo developmental stages (Xiong et al. 2019). Our data are also consistent with the phenotype of an Arabidopsis mutant termed *constitutive auxin response with DR5:GFP* (*CARD1*), which encodes for NRPB1, the RNA Pol II's largest subunit (Zhang et al. 2018). Interestingly, *nrpb2-3* and *card1* both show higher auxin responses at the meristem and share several root phenotypes, including reduction in root growth and patterning of stem cells through regulation of transcription factors involved in morphogenesis (Zhang et al. 2018). CARD1 and NRPB2 form the catalytic site of RNA Pol II. The fact that the corresponding mutants produce short roots whose meristems disappear a few days after germination indicates their critical role for proper root development.

The magnified *DR5:GFP* expression in *nrpb2-3* seedlings in neighbor tissues to cell death areas is coincident with recent reports that vasculature stem cell damage by laser or genotoxic stress triggers auxin biosynthesis in the cells immediately adjacent to the wound, or induces its accumulation in the endodermis, which in turn confers a capacity for regeneration of vasculature stem cells. *DR5:GFP* patterns inversely correlated with *in vivo* detection of PIN1, 2 and 3. The weaker *PIN1*, *PIN2,* and *PIN3* expression in *nrpb2-3* may be simply explained if the corresponding genes are directly regulated by NRPB2 or alternatively from the structural damage imposed to meristems.

Upon root exposure to biotic and abiotic stress that trigger cell damage, ERF115 expression is induced and this appears to depend on auxin signaling, which is required for further tissue recovery (Canher et al. 2020; Hoermayer et al. 2020). Here, we found an over induction of ERF115 in *nrpb2-3* root meristems, which suggests a possible mechanism to replenish the missing tissues. However, *nrpb2-3* seedlings fail to rebuild the root tip after excision of the SCN, thus the seedlings require functional transcriptional machinery for tissue regeneration.

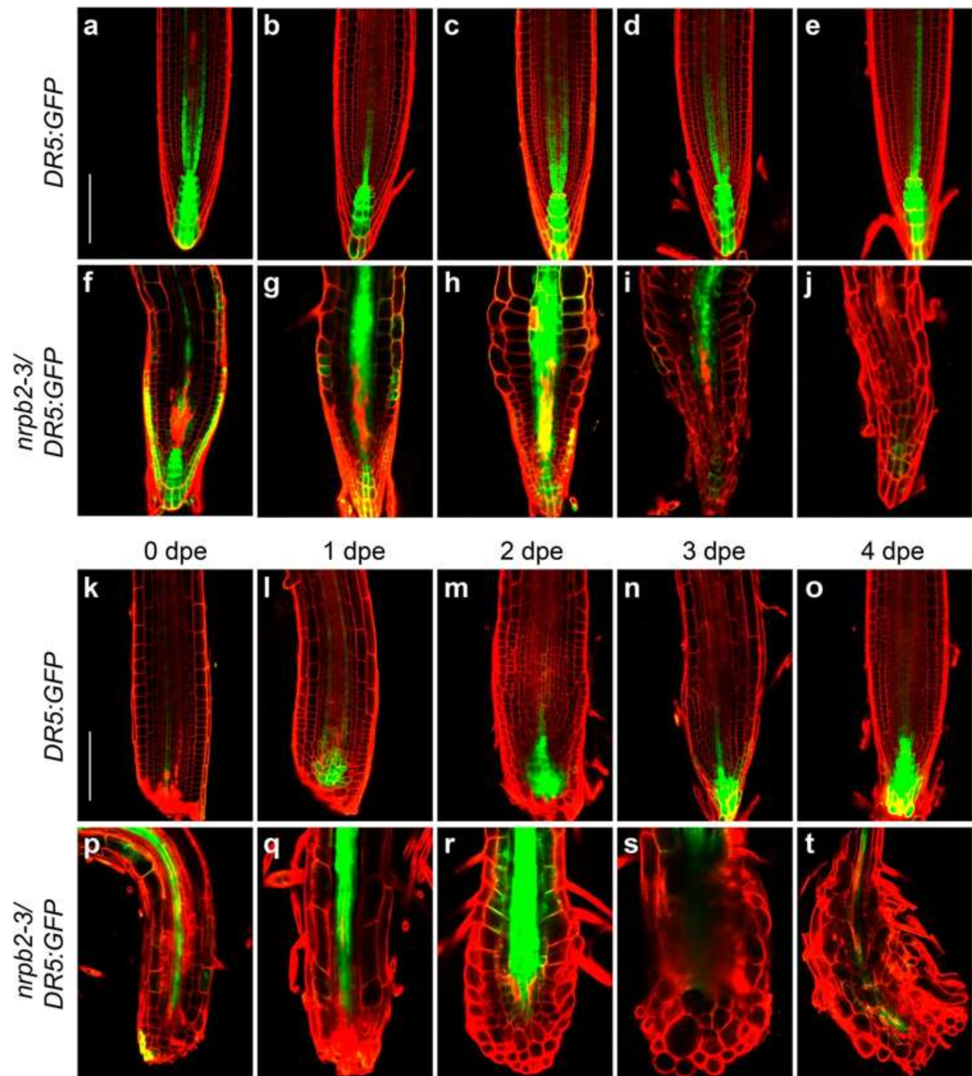


Fig. 6 Auxin signaling and *de novo* root tip regeneration in Wt and *nrpb2-3* mutants. *DR5:GFP* expression during root regeneration in 4d-old Wt (a-e, k-o) and *nrpb2-3* (f-j, p-t) seedlings after root tip excision for the indicated time points. (a-e) Un-excision of Wt seedlings and root tips after excision (k-o). (f-j) Un-excision of *nrpb2-3* seedlings and root tips after excision (p-t). Representative photographs of at least 10 seedlings of each condition/line, stained with IP, and visualized by confocal microscopy. The experiment was repeated three times with similar results. dpe= days post excision. Scale bar = 100 µm.

It has been shown that production and accumulation of ROS and nitric oxide regulate root system configuration by modulating the polar transport and redistribution of auxin (Méndez-Bravo et al. 2010; Su et al. 2016). ROS determines the boundaries between the meristem and cell elongation zone of the primary root and act in concert with NO for growth (Tsukagoshi et al. 2010; Fernández-Marcos et al. 2011). In consonance with NO detection and the phenotype of *nrpb2-3* seedlings, application of NO donors to Arabidopsis seedlings induced root meristematic defects and growth inhibition affecting the PIN-FORMED 1 (PIN1)-auxin translocation (Fernández-Marcos et al. 2011), which is also consistent with the reduction in PIN1 observed in the *nrpb2-3* meristems. Therefore, NRPB2 appears to be an important factor in transducing the NO signaling either as a driver of NO production or as a consequence of the differentiation of the root tip that correlates with more NO in root tip cells and reduction in PIN transporters.

Previously, we showed that the loss-of-function of MED18 triggers cell death in root meristems and affects auxin signaling (Raya-González et al. 2018), two alterations also manifested in *nrpb2-3* mutants, suggesting that commonalities exist between the transcriptional machinery and the Mediator complex in gene regulation. On the other side, Kim et al. (2011) showed that *med20*a and *nrpb2-3* mutants exhibit comparable morphological phenotypes and by microarray-based transcript profiling, they found that both mutants share several sets of genes regulated differentially, including miRNAs, suggesting a degree of overlap between Mediator and RNA Pol II in gene expression. Recently, PLT2 arose as a key player in root tip regeneration specified by its expression gradient (Kareem et al. 2015; Durgaprasad et al. 2019). PLT2 expression is triggered early, 8h after root tip excision, around cell damage areas, and down-regulation of PLT2 in distinct sets of *plt2* mutants failed to reconstruct the root tip after resection, which leads to cell differentiation (Durgaprasad et al. 2019). It is tempting to speculate that PLT2

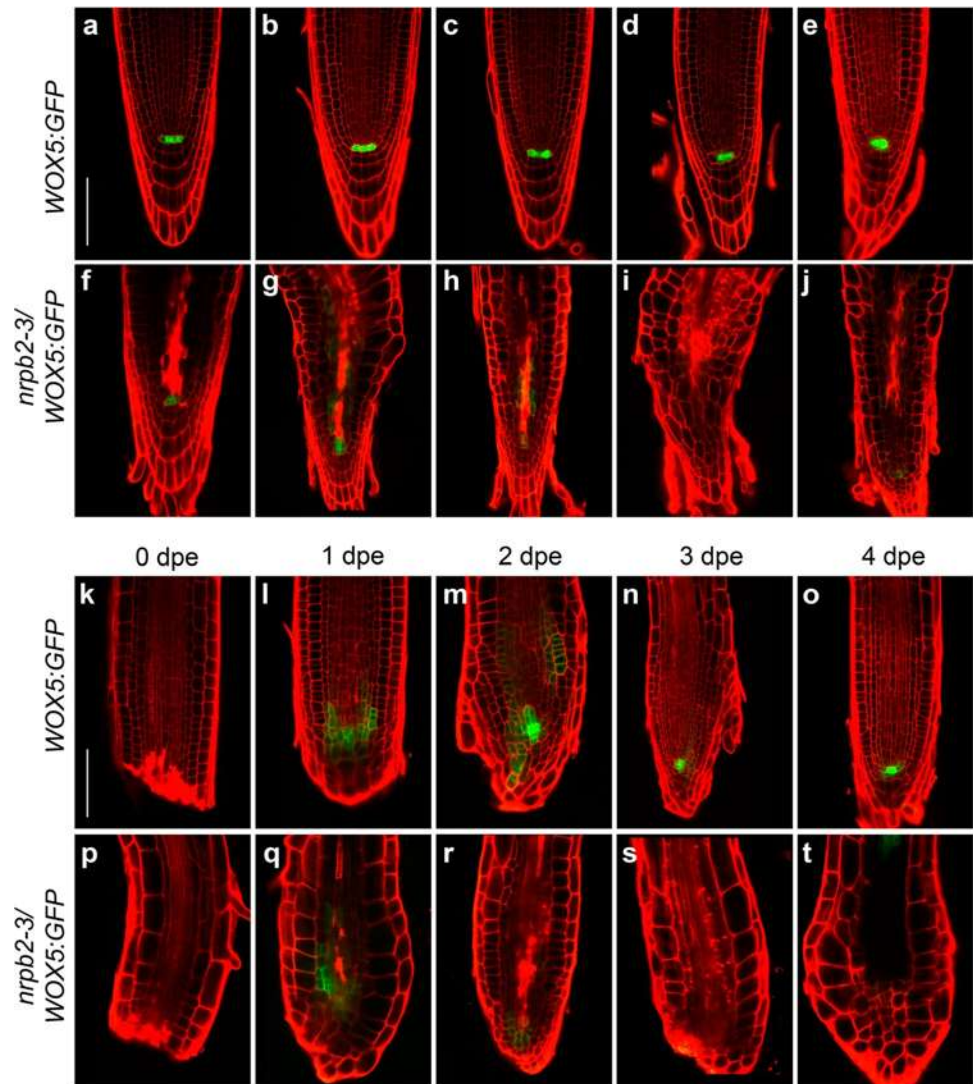


Fig. 7 Quiescent center re-specification during root tip regeneration in Wt and *nrpb2-3* seedlings. *WOX5* expression in 4d-old Wt (a-e, k-o) and *nrpb2-3* (f-j, p-t) seedlings after root tip excision for the indicated time points. (a-e) Un-excision of Wt seedlings and after excision of the root tip (k-o). (f-j) Un-excision of *nrpb2-3* seedlings and after excision of the root tip (p-t). Representative photographs from at least 10 seedlings of each condition, stained with PI, and visualized by confocal microscopy. The experiment was repeated three times with similar results. dpe= days post excision. Scale bar = 100 µm.

acts or is influenced by NRPB2 during root tip regeneration, but this remains to be investigated.

Although several questions remain to be answered about the molecular mechanisms by which NRPB2 controls root system configuration and regeneration, our work supports that: (i) NRPB2 activity is essential for cell division, cell size, and cell differentiation, three key processes involved in root patterning; (ii) NRPB2 regulates auxin signaling as well as oxidative and nitrosative signaling pathways that control PIN transporters in root tips; (iii) NRPB2 protects root meristems against damage to maintain an SCN functional and an active RAM, and (iv) NRPB2 function is required for auxin redistribution and cell re-specification to grant root tip reconstruction after excision. A model is provided that shows the role of NRPB2 on root morphogenesis (Fig. S7), in which NRPB2 acts as a positive regulator to control stem cell niche maintenance, cell division, and cell viability modulating the auxin response pathway and the activity of WOX5, SHR, and SCR transcription factors. As previously proposed (Heyman et al. 2016; Raya-González et al. 2018; Durgaprasad et al. 2019), and on the basis of results provide here, NRPB2 acting through the auxin pathway, and ERF115-PLT2 signaling, for root tip rebuild after tissue cell damage and/or loss, which grants a correct root growth and development as well as the possibility to adapt to damage caused by biotic or abiotic factors.

To the best of our knowledge, NRPB2 is one of a few master genes already identified, which orchestrates the balance between death and regeneration in highly proliferative, root meristem cells, which may be of tremendous adaptive importance as the root grows and explores the soil in search of nutrients and water resources and to drive discrete movements as it encounters obstacles or pollutants or as a response to the rhizosphere microbiota.

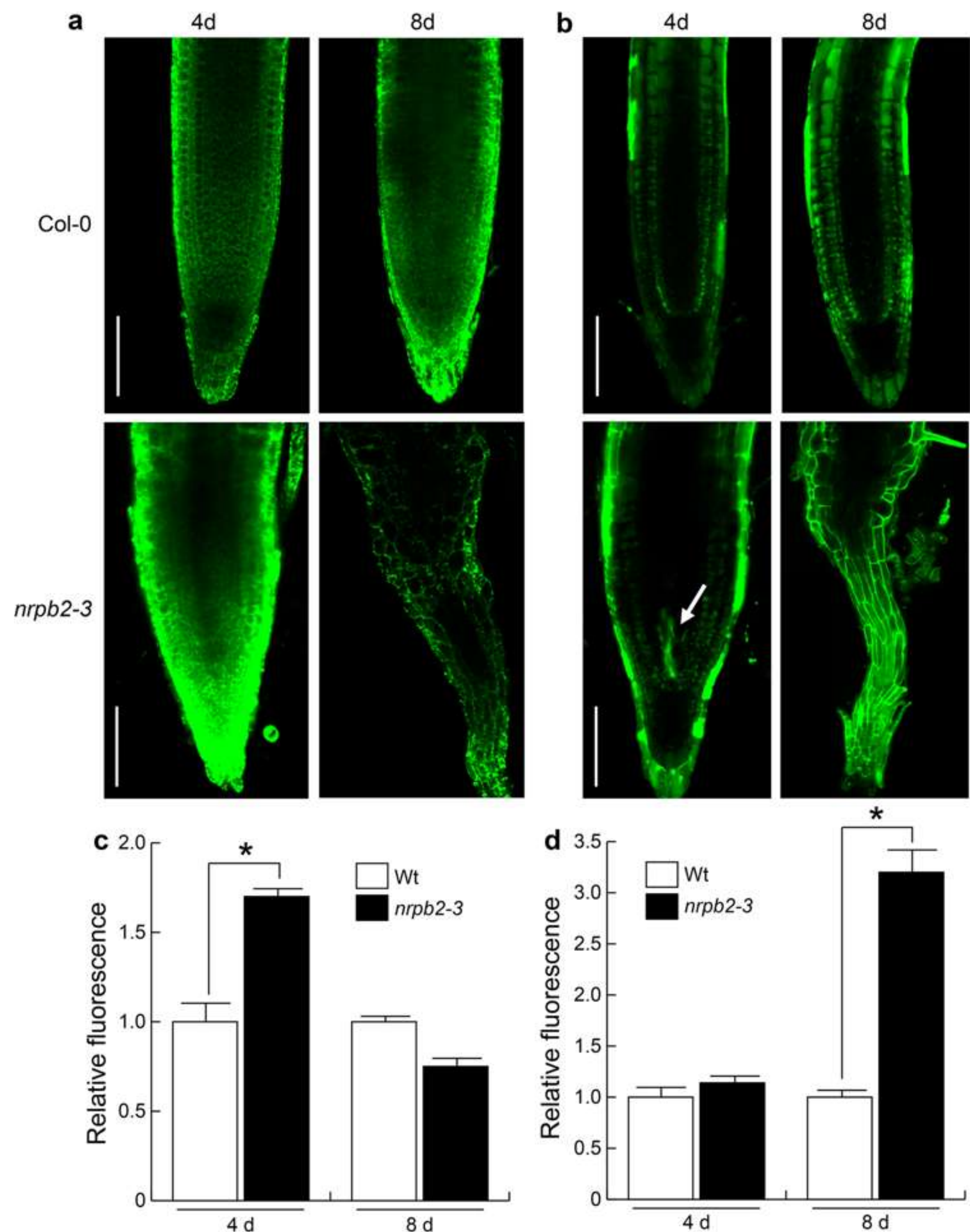


Fig. 8 Detection of reactive oxygen species (ROS) and nitric oxide (NO) in root tips of Wt and *nrpb2-3* seedlings. (a) Detection of ROS using the specific probe H2DCF-DA by confocal microscopy in four and eight-day-old Wt (Col-0) and *nrpb2-3* seedlings. (b) Detection of NO via the specific probe, DAF-2DA. Seedlings were germinated and grown on Petri plates containing agar-solidified 0.2x MS medium and processed at the indicated times. Graphs in (c, d) represent the ROS (c) and NO (d) levels measured as relative fluorescence. Asterisks indicate that differ statistically ($P<0.05$). Photographs were chosen from 5 seedlings of each genetic background. Note that cell damage zone showed by *nrpb2-3* mutant in the root tip correlates with NO accumulation (white arrow). The experiment was repeated two times with similar results. Scale bar = 100 µm.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00709-021-01732-z.

Acknowledgements The authors thank to Prof. Xuemei Chen for sharing the *nrpb2-3* mutant, Prof. Lieven de Veylder for providing *pERF115:GFP* line and Profs. Ben Scheres and Alfredo Cruz Ramirez for donation of Arabidopsis transgenic lines. We thank Dr. Jesús Salvador López Bucio for critical reading and correction of this manuscript. This work was funded by grants from SEP-CONACYT A1-S-34768, and FORDECYT-PRONACES/376120/2020).

Author contributions J.R.G., A.A.R., J.V.A., and L.F.R.H. performed the experiments. J.R.G., A.A.R., and J.L.B. analyzed the data. J.R.G. and J.L.B. designed the experiments and wrote the manuscript. All authors approved the manuscript.

Declarations

Competing interest statement The authors declare no competing financial interest.

References

Aida M, Beis D, Heidstra R, Willemsen V, Blilou I, Galinha C, Scheres B (2004) The *PLETHORA* genes mediate patterning of the Arabidopsis root stem cell niche. Cell 119:109–120

Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertova D, Jurgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell 115:591–602

Blilou I, Xu J, Wildwater M, Willemsen V, Papanov 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 433:39–44

Canher B, Heyman J, Savina M, Devendran A, Eekhout T, Vercauteren I, Prinsen E, Matosevich R, Xu J, Mironova M, De Veylder L (2020) Rocks in the auxin stream: wound-induced auxin accumulation and *ERF115* expression synergistically drive stem cell regeneration. Proc Natl Acad Sci 117:16667–16677

Chen L, Guan L, Qian P, Xu F, Wu Z, Wu Y, He K, Gou X, Li J, Hou S (2016) NRPB3, the third largest subunit of RNA polymerase II, is essential for stomata patterning and differentiation in Arabidopsis. Development 143:1600–1611

Cho H-T, Cosgrove DJ (2002) Regulation of root hair initiation and expansin gene expression in Arabidopsis. Plant Cell 14:3237–3253

Colón-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin–GUS fusion protein. Plant J 20:503–508

Cruz-Ramírez A, Díaz-Triviño S, Blilou I et al (2012) A bistable circuit involving SCARECROW-RETINOBLASTOMA integrates cues to inform asymmetric stem cell divisions. Cell 150:1002–1015

Ding Z, Friml J (2010) Auxin regulates distal stem cell differentiation in Arabidopsis roots. Proc Natl Acad Sci U S A 107:12046–12051

Durgaprasad K, Roy MV, Venugopal A, Kareem A, Raj K, Willemsen V, Mähönen AP, Scheres B, Prasad K (2019) Gradient expression of transcription factor imposes a boundary on organ regeneration potential in plants. Cell Rep 29:453–463

Fernández-Marcos M, Sanz L, Lewis DR, Muday GK, Lorenzo O (2011) Nitric oxide causes root apical meristem defects and growth inhibition while reducing PIN-FORMED 1 (PIN1)-dependent acropetal auxin transport. Proc Natl Acad Sci U S A 108:18506–18511

Fulcher N, Sablowski R (2009) Hypersensitivity to DNA damage in plant stem cell niches. Proc Natl Acad Sci 106:20984–20988

Galinha C, Hofhuis H, Luijten M, Willemsen V, Blilou I, Heidstra R, Scheres B (2007) PLETHORA proteins as dose-dependent master regulators of Arabidopsis root development. Nature 449:1053–1057

Hashimura Y, Ueguchi C (2011) The Arabidopsis *MERISTEM DISORGANIZATION 1* gene is required for the maintenance of stem cells through the reduction of DNA damage. Plant J 68:657–669

Heyman J, Cools T, Vandenbussche F et al (2013) *ERF115* controls root quiescent center cell division and stem cell replenishment. Science 342:860–863

Heyman J, Cools T, Canher B et al (2016) The heterodimeric transcription factor complex ERF115–PAT1 grants regeneration competence. Nat Plants 2:1–7

Hoermayer L, Montesinos JC, Marhava P, Benková E, Yoshida S, Friml J (2020) Wounding-induced changes in cellular pressure and localized auxin signalling spatially coordinate restorative divisions in roots. Proc Natl Acad Sci 117:15322–15331

Kareem A, Durgaprasad K, Sugimoto K et al (2015) *PLETHORA* genes control regeneration by a two-step mechanism. Curr Biol 25:1017–1030

Kaufmann K, Pajoro A, Angenent G (2010) Regulation of transcription in plants: mechanisms controlling developmental switches. Nat Rev Genet 11:830–842

Kidd BN, Edgar CI, Kumar KK, Aitken EA, Schenk PM, Manners JM, Kazan K (2009) The mediator complex subunit PFT1 is a key regulator of jasmonate-dependent defense in Arabidopsis. Plant Cell 21:2237–2252

Kim YJ, Zheng B, Yu Y, Won SY, Mo B, Chen X (2011) The role of Mediator in long noncoding RNA production in *Arabidopsis thaliana*. EMBO J 30:814–822

Larkin RM, Hagen G, Guilfoyle TJ (2009) *Arabidopsis thaliana* RNA polimerase II subunits related to yeast and human RPB5. Gene 231:41–47

Malamy JE, Benfey PN (1997) Organization and cell differentiation in lateral roots of *Arabidopsis thaliana*. Development 124:33–44

Marhava P, Hoermayer L, Yoshida S, Marhavý P, Benková E, Friml J (2019) Re-activation of stem cell pathways for pattern restoration in plant wound healing. Cell 177:957–969

Matosevich R, Cohen I, Gil-Yarom N, Modrego A, Friedlander-Shani L, Verna C, Scarpella E, Efroni I (2020) Local auxin biosynthesis is required for root regeneration after wounding. Nat Plants 6:1020–1030

Méndez-Bravo A, Raya-González J, Herera-Estrella L, López-Bucio J (2010) Nitric oxide is involved in alkamide-induced lateral root development in Arabidopsis. P Cell Physiol 51:1612–1626

Onodera Y, Nakagawa K, Haag JR, Pikaard D, Mikami T, Ream T, Ito Y, Pikaard CS (2008) Sex-biased lethality or transmission of defective transcription machinery in Arabidopsis. Genetics 180:207–218

Ottenschlager I, Wolff P, Wolverton C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells. Proc Natl Acad Sci 100:2987–2991

Pi L, Aichinger E, van der Graaff E, Llavata-Peris CI, Weijers D, Hennig L, Groot E, Laux T (2015) Organizer-derived WOX5 signal maintains root columella stem cells through chromatin-mediated repression of *CDF4* expression. Dev Cell 33:576–588

Raya-González J, Oropeza-Aburto A, López-Bucio JS, Guevara-García ÁA, De Veylder L, López-Bucio J, Herrera-Estrella L (2018) MEDIATOR18 influences Arabidopsis root architecture, represses auxin signaling and is a critical factor for cell viability in root meristems. Plant J 96:895–909

Raya-González J, Ortiz-Castro R, López-Bucio J (2019) Determinate root development in the *halted primary root1* mutant of Arabidopsis correlates with death of root initial cells and an enhanced auxin response. Protoplasma 256:1657–1666

Sabatini S, Heidstra R, Wildwater M, Scheres B (2003) SCARECROW is involved in positioning the stem cell niche in the *Arabidopsis* root meristem. Genes Dev 17:354–358

Sánchez-Calderón L, López-Bucio J, Chacón-López A, Cruz-Ramírez A, Nieto-Jacobo F, Dubrovsky JG, Herrera-Estrella L (2005) Phosphate starvation induces a determinate developmental program in the roots of *Arabidopsis thaliana*. Plant Cell Physiol 46:174–184

Sena G, Wang X, Liu HY, Hofhuis H, Birnbaum KD (2009) Organ regeneration does not require a functional stem cell niche in plants. Nature 457:1150–1153

Su C, Liu L, Liu H et al (2016) H_2O_2 regulates root system architecture by modulating the polar transport and redistribution of auxin. J Plant Biol 59:260–270

Tian H, Wabnik K, Niu T et al (2014) WOX5-IAA17 feedback circuit-mediated cellular auxin response is crucial for the patterning of root stem cell niches in Arabidopsis. Mol Plant 7:277–289

Truernit E, Haseloff J (2008) A simple way to identify non-viable cells within living plant tissue using confocal microscopy. Plant Methods 4:15

Tsukagoshi H, Busch W, Benfey PN (2010) Transcriptional regulation of ROS controls transition from proliferation to differentiation in the root. Cell 143:606–616

Xiong F, Liu HH, Duan CY, Zhang BK, Wei G, Zhang Y, Li S (2019) Arabidopsis JANUS regulates embryonic pattern formation through Pol II-mediated transcription of *WOX2* and *PIN7*. Iscience 19:1179–1188

Zhang QQ, Li Y, Fu ZY et al (2018) Intact Arabidopsis RPB1 functions in stem cell niches maintenance and cell cycling control. Plant J 95:150–167

Zhao J (2007) Interplay among Nitric oxide and reactive oxygen species. Plant Signal Behav 2:544–547

Zheng B, Wang Z, Li S, Yu B, Liu JY, Chen X (2009) Intergenic transcription by RNA Polymerase II coordinates Pol IV and Pol V in siRNA-directed transcriptional gene silencing in Arabidopsis. Genes Dev 23:2850–2860

Zhou W, Lozano-Torres JL, Blilou I, Zhang X, Zhai Q, Smant G, Li C, Scheres B (2019) A jasmonate signaling network activates root stem cells and promotes regeneration. Cell 177:942–956

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Protoplasma
https://doi.org/10.1007/s00709-023-01847-5

ORIGINAL ARTICLE

Reactive oxygen species and NADPH oxidase-encoding genes underly the plant growth and developmental responses to *Trichoderma*

Saraí Esparza-Reynoso[1] · Adrián Ávalos-Rangel[1] · Ramón Pelagio-Flores[2] · José López-Bucio[1]

Received: 15 September 2022 / Accepted: 21 February 2023
© The Author(s), under exclusive licence to Springer-Verlag GmbH Austria, part of Springer Nature 2023

Abstract
The modulation of plant growth and development through reactive oxygen species (ROS) is a hallmark during the interactions with microorganisms, but how fungi and their molecules influence endogenous ROS production in the root remains unknown. In this report, we correlated the biostimulant effect of *Trichoderma atroviride* with *Arabidopsis* root development via ROS signaling. *T. atroviride* enhanced ROS accumulation in primary root tips, lateral root primordia, and emerged lateral roots as revealed by total ROS imaging through the fluorescent probe H2DCF-DA and NBT detection. Acidification of the substrate and emission of the volatile organic compound 6-pentyl-2H-pyran-2-one appear to be major factors by which the fungus triggers ROS accumulation. Besides, the disruption of plant NADPH oxidases, also known as respiratory burst oxidase homologs (RBOHs) including ROBHA, RBOHD, but mainly RBOHE, impaired root and shoot fresh weight and the root branching enhanced by the fungus in vitro. *RbohE* mutant plants displayed poor lateral root proliferation and lower superoxide levels than wild-type seedlings in both primary and lateral roots, indicating a role for this enzyme for *T. atroviride*–induced root branching. These data shed light on the roles of ROS as messengers for plant growth and root architectural changes during the plant-*Trichoderma* interaction.

Keywords *Trichoderma atroviride* · *Arabidopsis thaliana* · Reactive oxygen species · Plant biomass · Root development

Introduction

Plant roots are inhabited by a myriad of soil microbes due to the attraction exerted by sugars and other exudates (Nath et al. 2016). Some of these microbes, which mostly belong to bacteria and fungi, can colonize the rhizosphere or live inside plant tissues, resulting in enhanced host growth or improved protection against stress and diseases (Hassani et al. 2018; Pascale et al. 2020). Fungal species belonging to the *Trichoderma* genus effectively spread into the roots and behave as probiotic organisms that promote root branching and nutrient absorption, along with their widely recognized biocontrol properties (Villalobos-Escobedo et al. 2020; Esparza-Reynoso et al. 2021).

Studies with *T. atroviride-Arabidopsis* interaction indicated that root exudation of sucrose changes fungal metabolism, thus decreasing the expression of genes encoding degradative enzymes, whereas the release of auxins extends the branching capacity of roots for better soil exploration (Villalobos-Escobedo et al. 2020; Alfiky and Weisskopf 2021). During this chemical dialog, the production of ROS by NADPH oxidases in *T. atroviride* determines its ability to perceive plants and to assimilate simple forms of sugars (Villalobos-Escobedo et al. 2020). On the other hand, ROS production in the plant host increases by biotic and abiotic stresses and occurs as an early response to *Trichoderma* inoculation, activating afterward an antioxidant mechanism that confers tolerance to the oxidative stress imposed by pathogens or environmental factors (Choudhary et al. 2020; Nogueira-Lopez et al. 2018; Huang et al. 2019; Nawrocka et al. 2019; Alfiky and Weisskopf 2021).

In plants, the NADPH oxidases (NOXs) also known as respiratory burst oxidase homologs (RBOHs) are the main

Handling Editor: April H Hastwell

✉ José López-Bucio
jbucio@umich.mx

[1] Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B3, Ciudad Universitaria C. P, 58030 Morelia, Michoacán, Mexico

[2] Facultad de Químico Farmacobiología, Universidad Michoacana de San Nicolás de Hidalgo, C. P, 58240 Morelia, Michoacán, Mexico

enzymes that catalyze the apoplastic reduction of molecular oxygen (O_2) into the superoxide anion (O_2·–), which in turn is spontaneously or enzymatically dismutated into the more stable form hydrogen peroxide (H_2O_2) (Hu et al. 2020). RBOHs encompass a group of membrane-bound enzymes with homology to the mammalian phagocyte gp91phox (NOX2). These enzymes are directly activated in response to the rapid influx of Ca^{2+} or intracellular phosphorylation events by protein kinases (Chapman et al. 2019; Lee et al. 2020). The *Arabidopsis* genome encodes ten *Rboh* genes potentially involved in plant resilience toward environmental stress as well as developmental responses (Chapman et al. 2019). RBOHD and RBOHF play an important role in ROS production and are required for proper lateral root development (Otulak-Kozieł et al. 2020). The corresponding genes show comparable expression patterns in the developing lateral root primordia (Orman-Ligeza et al. 2016; Chapman et al. 2019), but their function and/or regulation as a response to probiotic fungi that enhance root branching has not been assessed.

In this report, we show that *Trichoderma atroviride* induces the accumulation of ROS at several stages of root development, which could be attributed to the signaling exerted by fungal acidification of the medium and to the release of its major volatile 6-pentyl-2H-pyran-2-one (6-PP). Consistently, comparison of the growth and biomass production in WT *Arabidopsis* seedlings and mutants affected in genes encoding ROBHA, RBOHD, and RBOHE enzymes indicates the importance of these RBOHs for root branching and biomass production elicited by the fungus. Detection of superoxide anion in *rbohE* mutant, which fails to mount an effective response to the fungal phytostimulation, further established the link between ROS levels with root branching patterns, and hence the whole capacity of plants for soil exploration.

Materials and methods

Plant material and growth conditions

A. thaliana ecotype Columbia (Col-0) was used as a wild-type (WT) plant throughout the study. The mutant lines *RbohA* (SALK_047391), *RbohD* (SALK_044865), and *RbohE* (SALK_030395) were obtained from the Salk Institute for Biological Studies (La Jolla, California, USA). Seeds were surface disinfected using 95% (v/v) ethanol and 20% (v/v) bleach for 5 and 7 min, respectively, followed by five washes with distilled, sterilized water. The seeds were stratified for two days at 4°C, and grown on Petri dishes containing 0.2× Murashige & Skoog (MS) basal salt mixture (M524, PhytoTech Labs), 0.6% (w/v) sucrose 1% (w/v) agar micropropagation grade (A111, PhytoTech Labs), and pH 7.0, 5.5, or 4.5. Petri dishes were placed vertically (at an angle of 65°) in a plant growth chamber (Percival AR-95L) at 22°C under continuous light conditions (300 µmol m^2 s^{-1}) and photoperiod (16 h of light/8 h of darkness).

Fungal growth and inoculum preparation

Trichoderma atroviride strain IMI206040 used to perform plant-fungus interaction assays was kindly provided by Dr. Alfredo Herrera-Estrella (CINVESTAV-Irapuato). Four days after germination, seedlings were inoculated with a 1×10^6 conidia at 5 cm from the root tip and incubated for additional four days to evaluate the plant responses to *Trichoderma*. The fungal growth trial and spore harvest were performed according to Pelagio-Flores et al. (2017).

6-PP treatments

Arabidopsis seedlings were germinated and grown in each Petri dish containing 0.2× MS medium supplemented with micromolar concentrations (0, 75, and 150 µM) of 6-PP (Sigma-Aldrich), prepared according to Garnica-Vergara et al. (2016). The seeds were sown to one side of the plate, placing 10 individuals in a row, and at least three plates were included for each treatment. Ten days after germination, the determination of the dose-response effect of 6-PP in plant growth related to ROS accumulation was performed.

Analysis of plant traits

The length of primary roots was measured with a ruler, while the lateral root length was measured using the IMAGEJ software (http://rsbweb.nih.gov/ij/). The quantitation of total lateral roots was determined by counting all mature roots that emerged from the primary root using a stereomicroscope (Leica MZ6). Lateral root density was scored as the lateral root number per centimeter of primary root, and was calculated by dividing the number of lateral roots by the primary root length for each seedling. Fresh weights of shoots or roots were determined using an analytical scale. Petri dish images were taken using a digital camera (Nikon D5600, Japan).

ROS visualization in roots

The production of intracellular ROS was assayed using the oxidation-sensitive fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA). H2DCF-DA is readily membrane-permeant and is trapped within cells by binding the chloromethyl group to cellular thiols. It becomes fluorescent when oxidized by hydrogen peroxide and downstream free radical products of hydrogen peroxide. *Arabidopsis* seedlings were incubated in 10 µM of H2DCF-DA

(Invitrogen™) in Trizma® hydrochloride buffer solution at 10 mM (pH 7.4) for 60 min at room temperature and in darkness. The roots were rinsed and mounted with fresh buffer solution on microscope slides for fluorescent imaging measurements using a confocal laser scanning microscope (Olympus FV1200). The 2′,7′-dichlorofluorescein (DCF) fluorescence was detected through excitation and emission wavelength of 485 nm and 500–535. Fluorescence from at least 8 treated seedlings was measured by calculating the green pixels in a determined area of each image using IMAGEJ software (http://rsbweb.nih.gov/ij/). The means of the relative fluorescence of each treatment were normalized according to the pixel values from the control condition.

Histochemical in situ detection of superoxide anion

Intracellular superoxide anion was detected using nitroblue-tetrazolium (NBT), which forms an insoluble dark blue formazan precipitate after reduction by superoxide. *Arabidopsis* seedlings co-cultivated with *Trichoderma* were incubated in 10 mM sodium phosphate buffer (pH 7.8) with 10 mM NaN_3 and 1 mg mL^{-1} NBT (Sigma-Aldrich) and kept at room temperature (26°C) and darkness for 5 min. The reaction was stopped by removing the NBT staining solution and washing roots twice in distilled water. The seedlings were mounted on glass slides to visualize intracellular color change through Nomarski's differential interference contrast microscopy. For each treatment, at least 8 plants were analyzed. Quantification of the NBT staining of roots was assessed by measuring the pixel intensity of comparable areas by ImageJ software. The means of the relative NBT staining were normalized in a similar way to fluorescence.

Data analysis

The data were analyzed through univariate and multivariate analyses followed by Tukey's post hoc tests using STATISTICA 10.0 program (Dell StatSoft, Austin, Texas, USA). All experiments were repeated three times. Different letters were used to indicate means that differ significantly ($P < 0.05$).

Results

T. atroviride increases ROS accumulation within *Arabidopsis* roots

Root colonization by *Trichoderma* triggers ROS production, which accounts for plant protection against pathogens (Saravanakumar et al. 2016; Chen et al. 2019). To visualize ROS in the root before physical contact between the plant and the fungus, we used the fluorescent cell-permeable probe H2DCF-DA and detected the corresponding fluorescence in root tissues through confocal microscopy. *Arabidopsis* seedlings were inoculated with *T. atroviride* 4 days after germination at 5 cm below the root tip and allowed to grow for 4 days. *Trichoderma*-mediated ROS accumulation increased in the cells surrounding lateral root primordia during their development (Fig. 1(a, b)) and emergence from the primary root (Fig. 1(c, d)), in primary root tips (Fig. 1(e, f)), and in mature lateral roots (Fig. 1(g, h)). Quantification of the fluorescence indicated that the surrounding layers of the primordia had around a 50% increase in ROS detection in inoculated seedlings, being much more evident in the primary root tip and mature lateral roots, which reached roughly 2.5 and 3-fold higher fluorescence, respectively, under fungal cocultivation when compared to axenically grown seedlings (Fig. 1(i–l)). These results clearly showed that *Trichoderma* triggers ROS accumulation on the root as an early response before root colonization.

Low pH induces ROS accumulation in roots

Two major factors from the fungus influence root architecture in *Arabidopsis*-*T. atroviride* interactions, acidification of the growth medium, and emission of 6-PP (Garnica-Vergara et al. 2016; Pelagio-Flores et al. 2017). To assess whether any of these factors may cause ROS overaccumulation within the root, we detected the ROS using H2DCF-DA and confocal microscopy on lateral root primordia and primary root tips of *Arabidopsis* seedlings grown on 0.2× MS agar medium adjusted to pH 4.5, 5.5, and 7.0. The more acidic pH (5.5 and 4.5) induced ROS accumulation in cell layers bordering the lateral root primordia (Fig. 2(a–c, g)), as well as at the meristematic zone of the primary root tip, including the stem cell niche and root cap (Fig. 2(d–f, h)). In mature lateral roots, acidity increased ROS accumulation, which was comparable to the pattern already observed in the primary root, suggesting that increases in ROS production occur in root branches (Fig. 3(a–d)). These data indicate that rhizosphere acidification by *T. atroviride* enhances ROS levels within the *Arabidopsis* root system.

The fungal volatile 6-pentyl-2H-pyran-2-one induces ROS accumulation in roots

Before direct contact with fungal hyphae, plants perceive the organic volatile compounds emitted by *Trichoderma atroviride* from which 6-PP is a major constituent (Garnica-Vergara et al. 2016; Carillo et al. 2020). Therefore, we hypothesized that 6-PP might be another factor for ROS accumulation in roots regarding its reported effects in both cell division and elongation (Garnica-Vergara et al. 2016). Supplementation of 75 and 150 µM 6-PP to the medium increased the detection of total ROS in primary root meristem and root elongation zones (Fig. 4(a–c)). Besides, quantification of

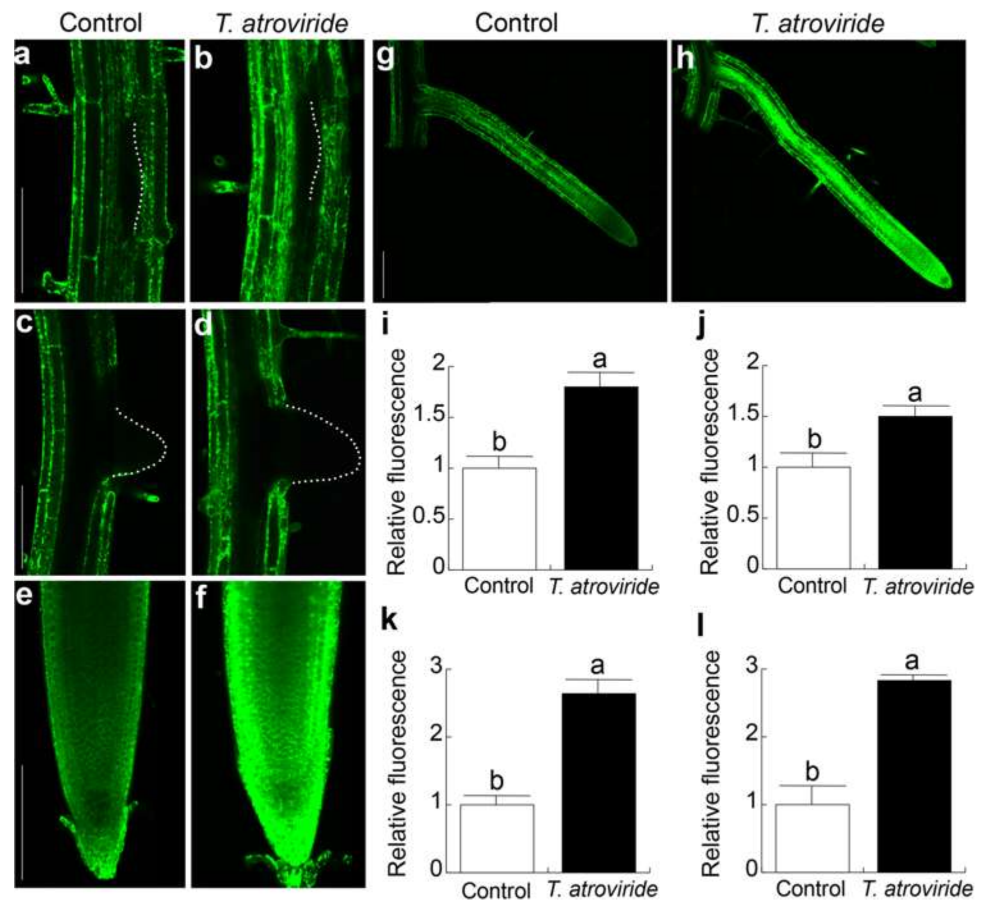


Fig. 1 Detection of total reactive oxygen species (ROS) in roots of *Arabidopsis* seedlings inoculated with *T. atroviride*. Intracellular ROS fluorescence using the probe H2DCF-DA and confocal microscopy. Representative micrographs of endogenous ROS levels around lateral root primordia (**a**, **b**), lateral roots leaving the parent root (**c**, **d**), primary root tips (**e**, **f**), and mature lateral roots (**g**, **h**). The graphs (**i**–**l**) show the mean fluorescence intensities for 8 independent seedlings that were imaged. Relative fluorescence quantitation around lateral root primordia (**i**), lateral roots leaving the parent root (**j**), primary root tips (**k**), and mature lateral roots (**l**). Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 µm. The experiment was repeated three times with comparable results

fluorescence confirms that 6-PP provokes ROS accumulation within the root meristem, which is dependent on the concentration of fungal compound supplied to the growth medium (Fig. 4(d)). Interestingly, in plants grown under 150 µM 6-PP, reduction in the width of the root tip and shortening of the meristematic and elongation zone were observed (Fig. 4(c)). Thus, we propose that the inhibitory effect of 150 µM of 6-PP in primary root growth is caused by ROS overproduction, affecting cell division and expansion processes.

T. atroviride induces superoxide anion production in roots

The above results showed that *Trichoderma atroviride* increases ROS production before root colonization, possibly due to the effect of the perception of volatiles. Since the superoxide anion accounts for the formation of all major ROS species, namely hydrogen peroxide, singlet oxygen, and hydroxyl radical, we next analyzed its distribution in roots of *T. atroviride* inoculated and non-inoculated seedlings using NBT, which forms a water-insoluble blue formazan precipitate upon reaction with superoxide. The accumulation of superoxide, detected by NBT staining, was observed at the tip of the primary and lateral roots of un-inoculated plants (Fig. 5(a–d)). Noteworthy, *Trichoderma*-inoculation led to a stronger NBT signal detection in the lateral roots, and the primary root, respectively (Fig. 5(e, f)). Thus, *T. atroviride* inoculation causes a superoxide-dependent burst that may act in early signaling events influencing root growth processes.

NADPH oxidases RBOHA, RBOHD, and RBOHE are required for *Trichoderma*-induced plant growth promotion

NADPH oxidases, also known as RBOHs in plants, are the main enzymes that catalyze the production of superoxide in the apoplast, which is rapidly dismutated to hydrogen peroxide (Hu et al. 2020). RBOH-dependent ROS production regulates a wide range of biological processes including plant development and stress responses. To investigate the role of NADPH oxidase-generated ROS in *Trichoderma*-plant interaction, we compared the biostimulant effect of *T. atroviride* in wild-type *Arabidopsis* seedlings (Col-0) and mutants defective in *RBOH* genes (*RbohA*, *RbohD*, and *RbohE*). The wild-type (WT) and mutant seedlings were grown for 4 days after germination and inoculated with *T. atroviride* at 5 cm from the root tip.

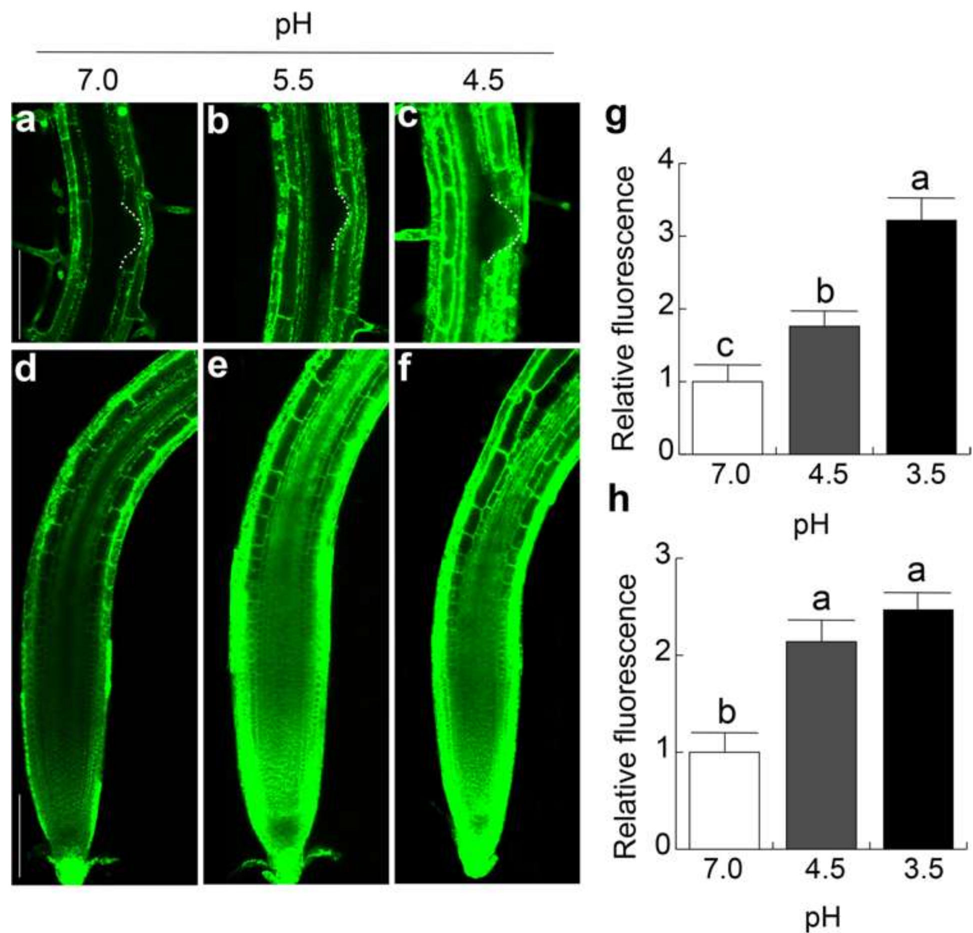


Fig. 2 Effect of acidic pH on ROS accumulation in *Arabidopsis* lateral root primordia and primary root meristems. *Arabidopsis* seedlings were germinated and grown for 8 days on agar plates containing MS 0.2× medium with pH adjusted to 7, 5.5, and 4.5. Representative micrographs of the detection of endogenous ROS levels around lateral root primordia revealed by H2DCF-DA (**a**–**c**), and primary root tips (**d**–**f**). The graphs show the means of relative fluorescence levels around lateral root primordia (**g**), and from the meristematic zone (**h**) of 8 seedlings ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 µm. The experiment was repeated three times with comparable results

After 4 days of co-cultivation in vitro, the WT seedlings inoculated with *T. atroviride* showed a clear phytostimulation when compared to un-inoculated plants, roughly reaching a 2- to 2.5-fold increase in shoot and total fresh weight, which significantly decreased in each *RbohA*, *RbohD*, and *RbohE* mutant (Fig. 6(a–j)). Consistently, the primary root growth was slightly affected by co-cultivation with the fungus, whereas the number and length of lateral roots increased by 4-fold and 3-fold, respectively, compared to axenically grown seedlings. This response correlated with root biomass accumulation (Fig. 7(a–d)). In contrast, the increased root branching and root biomass production elicited by *T. atroviride* diminished in the *Rboh* mutants. In particular, the *RbohE* mutant showed an impressive reduction of the lateral root number, lateral root length, and root fresh weight when compared to the WT in presence of *T. atroviride* (Fig. 7(b–d)). Taken together, these results suggest that RBOH-mediated ROS production determines the *Trichoderma*-induced lateral root formation and biomass production.

Loss of function of RBOHE compromises the ability of *T. atroviride* to increase superoxide accumulation in *Arabidopsis* roots

RBOH genes show an overlapping expression pattern in different sites of the root, and their corresponding enzymatic activity regulates primary root growth and lateral root formation (Mase and Tsukagoshi, 2021). To further evaluate the role of RBOH-dependent ROS synthesis in growth promotion by *T. atroviride* in *Arabidopsis*, we used NBT staining to detect superoxide anion in roots of wild-type plants and *rbohE* mutant, which was among the *rboh* mutants the less responsive to the *Trichoderma*-mediated plant growth promotion. We observed that fungal inoculation increased superoxide levels in the primary roots of wild-type seedlings (Fig. 8(a–b)), and the overall superoxide amount in the whole root was about 60% compared to non-inoculated plants (Fig. 8(e)). However, the superoxide levels in *rbohE* mutant roots were lesser than wild-type roots with or without *Trichoderma* presence (Fig. 8(c–d)).

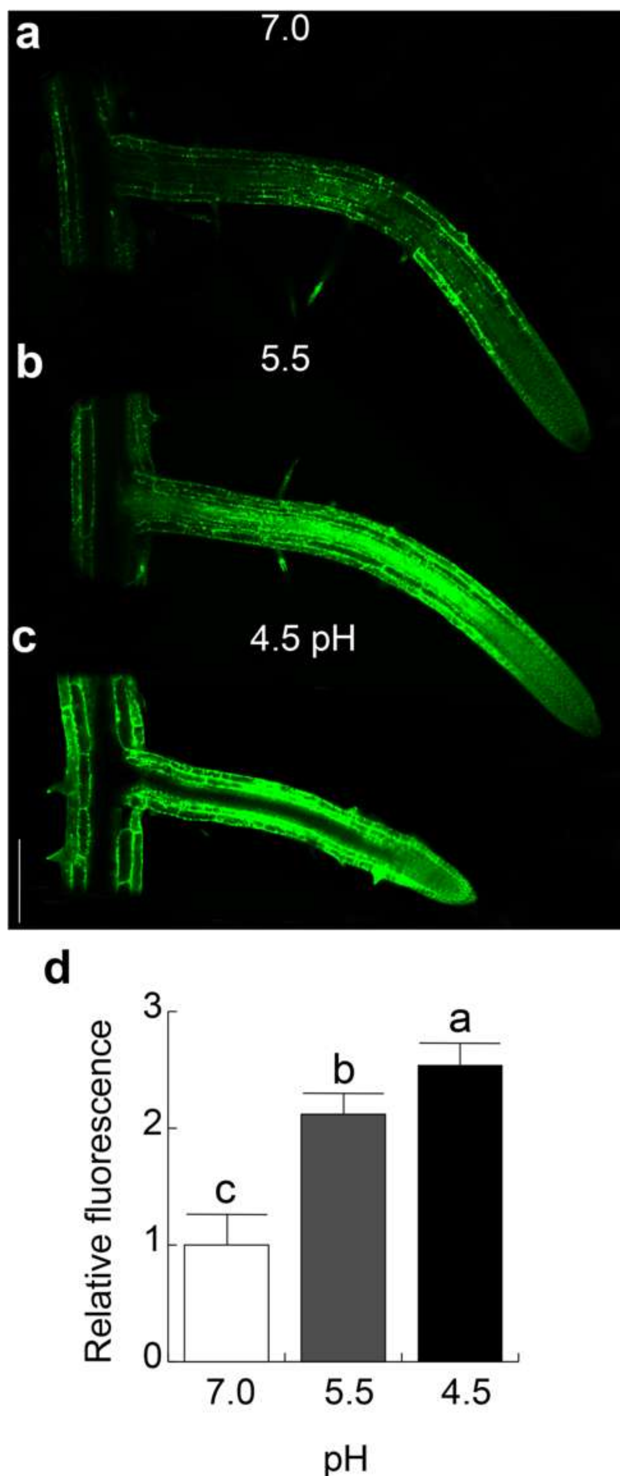


Fig. 3 Effect of acidic pH on ROS production in lateral root meristems. Representative micrographs of the detection of endogenous ROS levels in mature lateral roots (**a**–**d**). The graph show the means of relative fluorescence from meristematic zone for lateral roots at compable developmental stages, from 8 independent seedlings ± SD. Different letters indicate significant statistical differences ($P < 0.05$). Scale bar: 100 µm. The experiment was repeated three times with comparable results

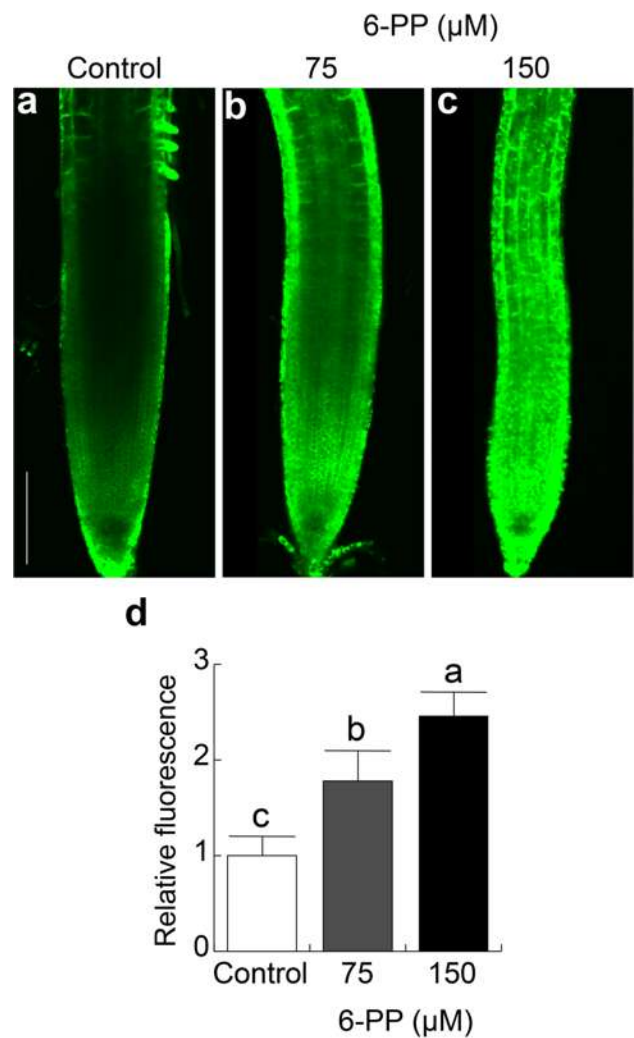


Fig. 4 6-PP enhances ROS accumulation in meristems of *Arabidopsis* primary roots. Seedlings were germinated and grown on 0.2× MS medium supplemented with 0, 75, and 150 µM 6-PP. Ten days after germination, detection of endogenous ROS levels was performed using H2DCF-DA. Representative micrographs show ROS accumulation in root meristems (**a**–**c**). The graph (**d**) represent the mean values of the relative fluorescence from 8 seedling root meristems ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 µm. The experiment was repeated three times with comparable results

Interestingly, *rbohE* mutants showed shorter meristem and elongation zones than WT roots due possibly to the imbalance of ROS accumulation (Fig. 8(f, g)). Regarding lateral roots, NBT detection unveiled lower levels of superoxide in lateral root tips of the *rbohE* mutant, which also was impaired in the promotion of root branching by *T. atroviride* (Fig. 9(a, b)). These results involve RBOHE for the *Trichoderma*-induced ROS production in roots and the configuration of root system architecture.

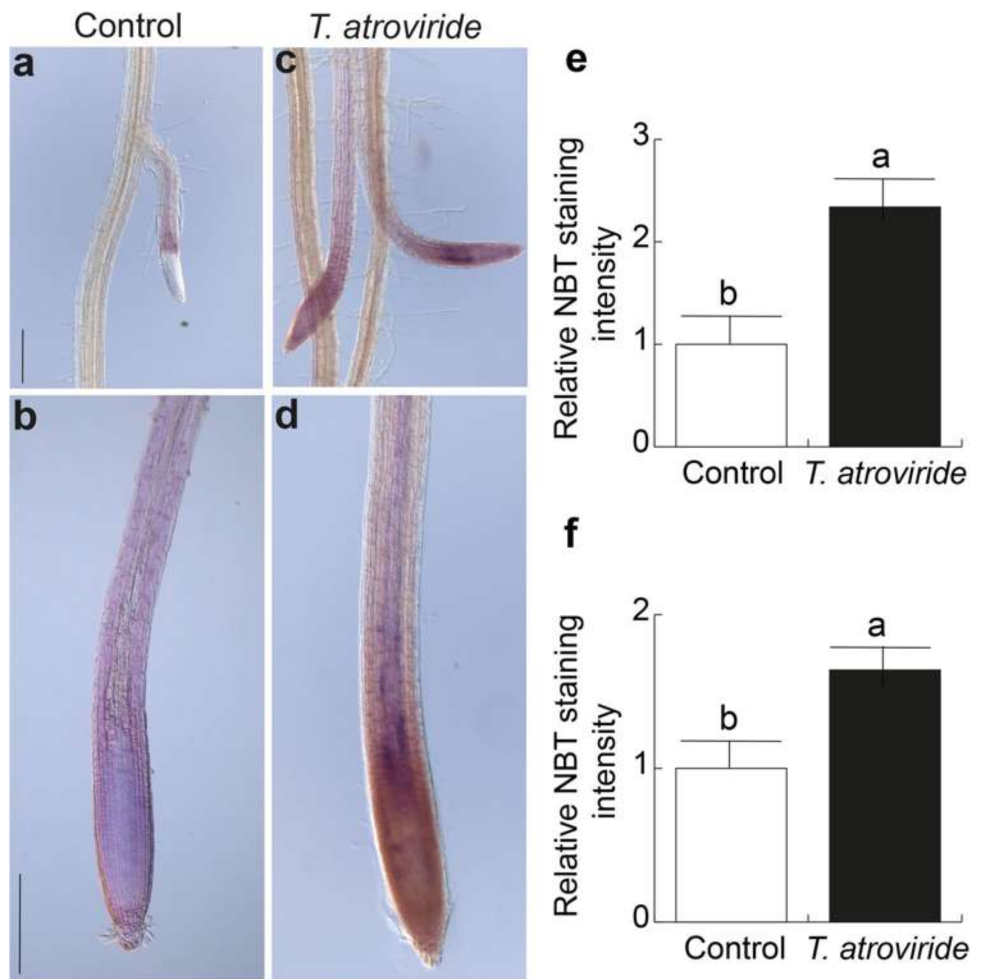


Fig. 5 *T. atroviride* induces the accumulation of superoxide anion in the root. Visualization of superoxide was recorded through NBT staining. *Arabidopsis* seedlings were co-cultured for 4 days with or without *T. atroviride*. Micrographs show lateral roots and primary roots of plants stained with NBT (**a**–**d**). The graphs illustrate differences in NBT staining intensity present in the elongation zone of lateral roots (**e**) and primary roots (**f**). Scale bar: 200 and 100 µm, respectively. The values shown represent the means for root tips imaged from 8 independent seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with comparable results

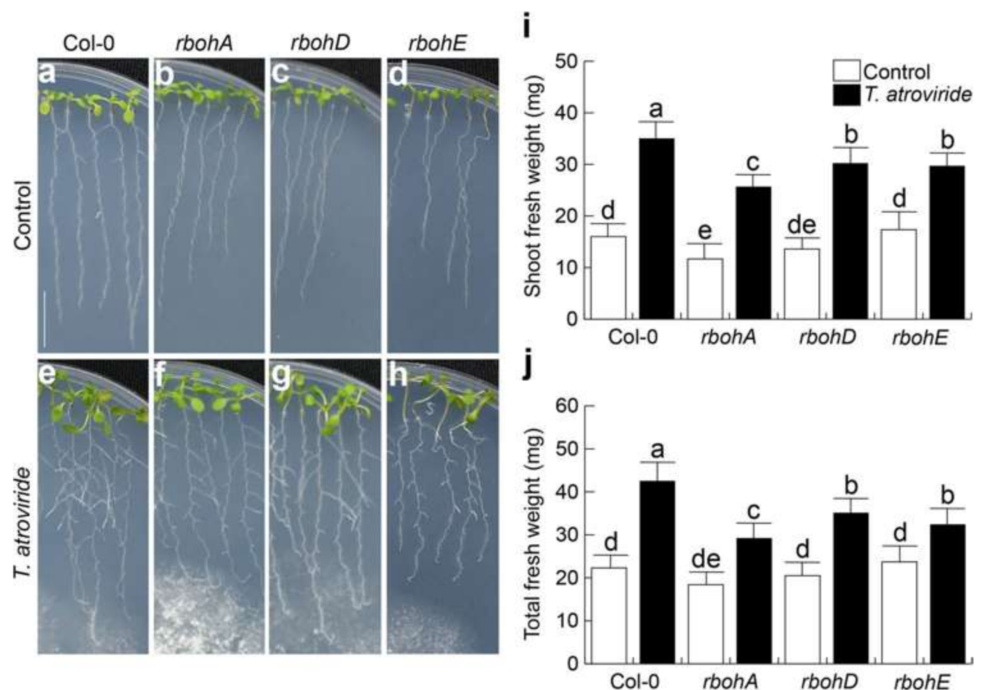


Fig. 6 Effect of *T. atroviride* on biomass production of *Arabidopsis* WT and *rboh* mutants. Four-day-old WT *Arabidopsis* seedlings and mutants lacking the functional isoforms of *RbohA*, *RbohD*, and *RbohE* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip. After 4 days of co-culture, representative photographs of seedlings co-cultivated with *T. atroviride* were taken (**a**–**h**), and shoot fresh weight (**i**), and total fresh weight (**j**) were recorded. Bars show the means ± SD, among three groups of ten plants each that were weighed on an analytical scale. Different letters indicate statistically significant differences ($P < 0.05$; $n = 15$). Scale bar: 1 cm. The experiment was repeated three times with comparable results

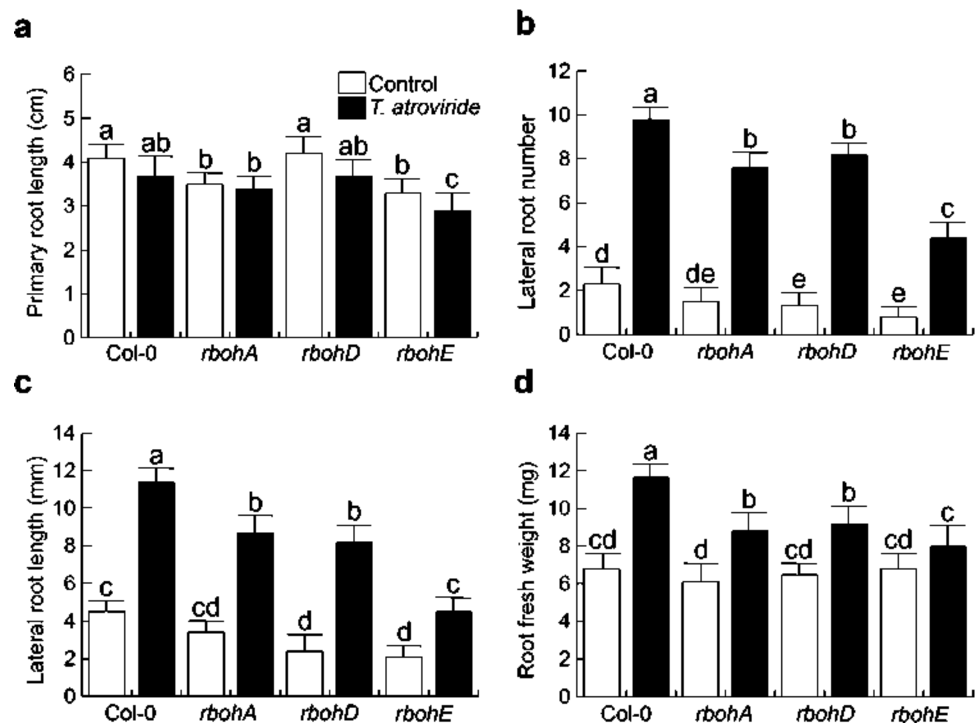


Fig. 7 Effect of *T. atroviride* on the root system architecture of *Arabidopsis* WT and *Rboh* mutants. Four-day-old WT *Arabidopsis* seedlings and mutants lacking functional isoforms of *RbohA*, *RbohD*, and *RbohE* enzymes were inoculated with *Trichoderma* at 5 cm from the root tip and allowed to grow for 4 additional days. Primary root length (**a**), lateral root number (**b**), lateral root length (**c**), and root fresh weight (**d**) were recorded. The values shown represent the means of 30 seedlings ± SD. Different letters indicate means that are statistically different ($P < 0.05$). The experiment was repeated three times with comparable results

Discussion

Trichoderma positively influences plant health and productivity by stimulating growth and development, and suppressing diseases caused by pathogens (Guzmán-Guzmán et al. 2019; Alfiky and Weisskopf 2021). The versatile mechanisms employed by these fungi to promote plant growth include synthesis of phytohormones (mainly IAA-related indoles), solubilization of soil nutrients, increased uptake and translocation of minerals, enhanced tolerance to abiotic stress, improved photosynthesis and sucrose metabolism, and production of secondary metabolites (Guzmán-Guzmán et al. 2019; Ramírez-Valdespino et al. 2019; Khan et al. 2020; Esparza-Reynoso et al. 2021; Harman et al. 2021; Vinale and Sivasithamparam 2020). All these functions rely upon a dedicated plant-fungus communication in which rhizosphere acidification and the emission of the unsaturated lactone 6-PP orchestrate root growth and branching (Garnica-Vergara et al. 2016; Pelagio-Flores et al. 2017; Estrada-Rivera et al. 2019; Guzmán-Guzmán et al. 2019).

In a recent report, Villalobos-Escobedo et al. (2020) showed the importance of ROS production by fungal NADPH oxidase for the mutual *Arabidopsis*-*T. atroviride* recognition, for which mutation of *NoxR* changed the saprophytic behavior toward the more effective usage of sugars secreted from roots as energetic sources, and this in turn led to a stronger plant defense response. ROS have been considered second messengers in signal transduction pathways; in particular, during plant-microbe interactions, the host-produced ROS includes the superoxide anion and H_2O_2, which accumulate at both colonized sites and distal parts (Contreras-Cornejo et al. 2011; Huang et al. 2019; Nawrocka et al. 2019; Xu et al. 2020; González-López et al. 2021). With this in mind, we visualized ROS in several parts of *Arabidopsis* roots prior to physical contact with a growing colony of *T. atroviride*. According to the H2DCF-DA fluorescence detection, the intracellular ROS levels were higher in the roots of plants inoculated with *T. atroviride* compared with non-inoculated plants, showing a higher ROS accumulation in cell layers bordering the lateral root primordia, as well as the root apex of primary and lateral roots.

ROS accumulation at the root tip could influence the balance between cell proliferation and elongation processes which are the hallmark of the indeterminate growth pattern of healthy roots. Our data are consistent with previous reports, in which exogenous application of H_2O_2 to *Arabidopsis* seedlings stimulates lateral root development (Su et al. 2016; Orman-Ligeza et al. 2016). H_2O_2 also affects the directional transport of auxin through changes in the expression of auxin carriers, mainly PIN transporters, a process also observed in plants exposed to *Trichoderma atroviride* or its main volatile 6-PP (Ivanchenko et al. 2013; Orman-Ligeza et al. 2016; Su et al. 2016; Velada et al. 2020).

The acidification generated by *Trichoderma* induces a redistribution of auxin within the root apex that originates a reorientation of the root growth, previously to growth cessation (Pelagio-Flores et al. 2017), and this deviation from the normal gravity-response also follows ROS generation

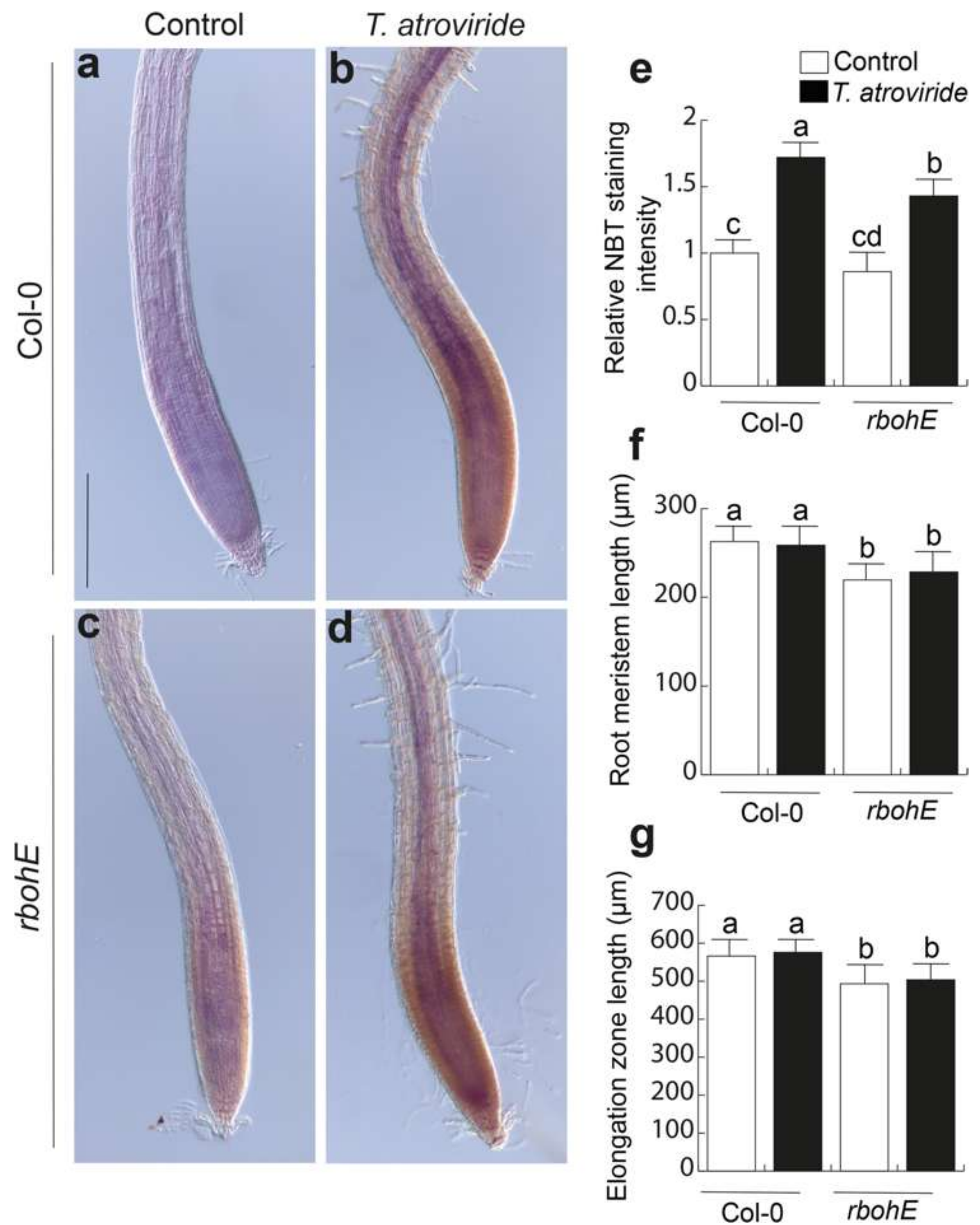


Fig. 8 Effect of *T. atroviride* in the RBOH-mediated superoxide anion accumulation in primary roots, root meristem length, and elongation zone length. NBT staining of the roots of wild-type and *rbohE* mutant roots grown axenically or inoculated with *Trichoderma* (**a**–**d**). Bars graphs show differences in NBT staining intensity in the meristem of the primary roots (**e**), measurements of meristem length (**f**) from the quiescent center to the start of the elongation zone, and elongation zone length (**g**). The values shown represent the means of 8 seedling roots ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 100 µm. The experiment was repeated three times with comparable results

(Eljebbawi et al. 2021). Therefore, we hypothesized that *T. atroviride* through acidification of the rhizosphere, and/or emission of 6-PP, may account for ROS overproduction. To test this hypothesis, we analyzed the impact of acidic pH on the architecture of the *Arabidopsis* root system. According to the results obtained, the plants grown at acidic pH (5.5 and 4.5) manifested stronger intracellular ROS accumulation in the whole root tissues and their lateral roots were shorter than those of plants grown in medium with pH 7.0. Such result fits well with previous reports, since exposure to low-pH stress causes root growth and developmental alterations that correlate with excessive accumulation of ROS, such as superoxide anion and H_2O_2 in root tips (Koyama et al. 2001; Zhang et al. 2015; Long et al. 2019; Graças et al. 2020). Lager et al. (2010) indicated that pH sensing by the plant triggers the regulation of gene expression resembling the transcriptional response provoked by auxin or pathogen defense signaling. They also assume that perception of environmental pH may act as an underlying signal to the cellular responses of auxin and pathogens. Under this assumption, we suggest that low pH-dependent accumulation of ROS functions as a downstream component in signal transduction during plant-*Trichoderma* recognition, although deciphering the molecular components of such signaling mechanisms is still pending.

The inhibitory effect of 6-PP on primary root growth at high concentrations was associated with an increased accumulation of ROS in root tips, which was missing in

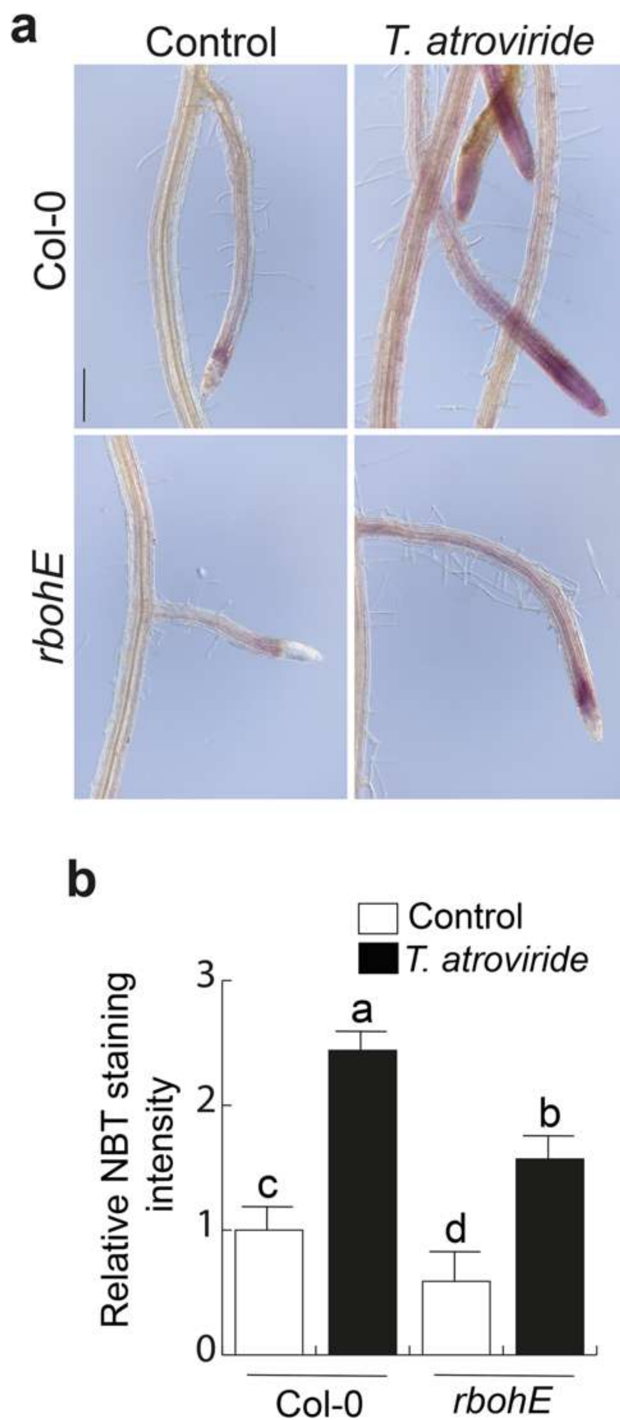


Fig. 9 Effect of *T. atroviride* in the RBOH-mediated superoxide anion accumulation in lateral roots. NBT staining of lateral roots of wild-type and *rbohE* mutants grown axenically or inoculated with *Trichoderma* (**a**). The graph shows differences in NBT staining intensity in lateral root tips (**b**). The values shown represent the means of 8 seedlings ± SD. Different letters indicate statistically significant differences ($P < 0.05$). Scale bar: 200 µm. The experiment was repeated three times with comparable results

Arabidopsis mutants defective on the gene encoding ETHYLENE INSENSITIVE 2 (EIN2), and this led to insensitivity to primary root growth stoppage (Garnica-Vergara et al. 2016). The emission of 6-PP largely varies according to environmental factors, growth media, presence of plants, etc. (Garnica-Vergara et al. 2016). In this study 0, 75, and 150 µM 6-PP were used as the two later concentrations show clear changes in the configuration of the *Arabidopsis* root architecture according to a previous report (Garnica-Vergara et al. 2016), and would help to correlate these changes with endogenous ROS detection. Interestingly, the inhibitory effect of 150 µM of 6-PP in primary root growth may be caused by ROS overproduction, affecting cell division and expansion processes.

Inhibition of root growth and superoxide anion accumulation in roots are typical effects of ethylene or its precursor ACC (Lv et al. 2018); thereby, we anticipated that 6-PP could regulate primary root elongation via ethylene-dependent ROS homeostasis. Intriguingly, EIN2 is required for the exacerbated oxidative stress and root growth repression caused by plant-pathogen effectors such as bacterial flagellin (flg22) and pyocyanin; however, loss-of-function of EIN2 enhanced the generation of ROS under salinity stress too, indicating the involvement of ethylene/ROS crosstalk in activation of both biotic and abiotic stress, clearly modulated in plants colonized with *Trichoderma* (Mersmann et al. 2010; Lin et al. 2013; Beck et al. 2014; Ortiz-Castro et al. 2014). Besides, *Trichoderma* triggers the specific accumulation of superoxide at the apex of lateral roots and inner tissues that form the stele. This vascular tissue-dominated accumulation of superoxide matches well with the gene expression of NADPH oxidase RBOHF, which can be induced by salinity or ACC treatment, suggesting the possible involvement of this RBOH isoform in *Trichoderma*-mediated oxidative signaling in roots (Jiang et al. 2012, 2013; Chapman et al. 2019). Recently, RBOH-mediated ROS production was involved in important root developmental processes, such as primary root elongation, and lateral root formation (Chapman et al. 2019).

Orman-Ligeza et al. (2016) reported the ROS generation by RBOH enzymes, which facilitates cell wall remodeling of overlying cell layers for the outgrowth and emergence of lateral root primordia. Rboh genes comprise a large, functionally redundant family, which makes very difficult to assign specific functions to particular members. Nevertheless, we decided to evaluate the *rbohA*, *rbohD*, and *rbohE* mutants because the expression patterns of the corresponding genes are in endodermis, cortex, and epidermal cells overlying lateral root primordia (Orman-Ligeza et al. 2016). Moreover, *RbohE* was selected for more detailed analysis because this mutant exhibited a semi-dwarf phenotype and showed less response to the promoting effects of *Trichoderma*. The wild-type (Col-0) seedlings and RBOH-deficient mutants *rbohA*, *rbohD*, and *rbohE* were inoculated with *T. atroviride* for

4 days and their growth and root developmental responses were compared to wild-type seedlings. *T. atroviride* promoted root and shoot biomass production and increased root branching in WT seedlings compared to non-inoculated plants; however, the mutations in *RBOH* genes slightly decreased the growth-promoting activity of *Trichoderma* and root branching. Interestingly, the *RbohE* mutant plants displayed poor lateral root proliferation and lower superoxide levels, indicating that the loss of RBOHE is critical for ROS generation during the plant-fungus interaction.

The evaluation of ROS production in *rbohE* mutant plant also demonstrated that ROS accumulation in lateral roots is mainly dependent on this isoform and this coincided with the specific pattern of *RbohE* expression in the cells overlying/surrounding the lateral root primordia and the mutant phenotype, which exhibits a delayed development of the primordia (Chapman et al. 2019; Eljebbawi et al. 2021). This is consistent with the phenotype of *rbohE* mutants, which exhibited a root meristem shorter than the wild-type due to decreased cell proliferation. On the other hand, RBOHA showed a comparable expression pattern to RBOHE within lateral root primordia, and the maturation zone of the primary root (stele and endodermis) and both were auxin-inducible, suggesting that RBOHA also participates in the initial stages of development of lateral roots (Orman-Ligeza et al. 2016; Chapman et al. 2019). *RBOHD* and *RBOHF* have been reported as regulators of lateral root formation (Otulak-Kozieł et al. 2020; Mase and Tsukagoshi, 2021), which indicates developmental stage-specific functions for each RBOH. In this sense, we cannot exclude possible redundant functions for RBOHA, RBOHD, and RBOHE for the root branching process being stimulated by *Trichoderma* and its metabolites. The possibility is open that their encoding genes could act as downstream target genes of the auxin-dependent growth programs and/or defense response signaling pathway elicited by *Trichoderma* in plants.

Conclusions

Taken together, the data presented in this work add a missing piece in the signal transduction events in the *Arabidopsis*-*T. atroviride* interaction. The notion that ROS are merely toxic molecules changed in recent times owing their function in modulating transcription factors and other regulatory proteins, which led the proposal of the term "oxidative signaling" for the ROS control of plant morphogenesis. Here, we described the dynamic changes in total ROS levels and superoxide anion at several stages of root development, which coincided with root growth and branching patterns stimulated by *T. atroviride*, its acidification of the rhizosphere and emission of its highly bioactive volatile 6-PP. Although the RBOH family includes many members, and functional redundancy may account for the dynamic ROS production in a tissue-specific manner and in response to abiotic or biotic stimuli, our work uncovered the important function of RBOHE for the phytostimulation and root architectural configuration driven by *Trichoderma* in *Arabidopsis*. Overall, the current data increase our knowledge into how plants interact with a fungal partner, widely applied in agriculture as a biocontrol agent and biostimulant microorganism.

Acknowledgements The authors thank Dr. León Francisco Ruíz Herrera for helping with image acquisition in laser confocal microscopy. SER and AAR are indebted to the Consejo Nacional de Ciencia y Tecnología (CONACyT) for doctoral and MSc fellowship, respectively.

Author contribution SER, AAR, and JLB designed and performed experiments and interpreted data; RPF and AAR provided technical support and analyzed data. SER and JLB wrote the manuscript. All authors revised and approved the submission.

Funding This work was supported by grants from SEP-CONACYT A1-S-34768.

Data availability The data and materials reported in this work are available upon contact with the corresponding author.

Declarations

Competing interests The authors declare no competing interests.

References

Alfiky A, Weisskopf L (2021) Deciphering *Trichoderma*–plant–pathogen interactions for better development of biocontrol applications. J Fungus 7:61. https://doi.org/10.3390/jof7010061

Beck M, Wyrsch I, Strutt J, Wimalasekera R, Webb A, Boller T, Robatzek S (2014) Expression patterns of flagellin sensing 2 map to bacterial entry sites in plant shoots and roots. J Exp Bot 65:6487–6498. https://doi.org/10.1093/jxb/eru366

Carillo P, Woo SL, Comite E, El-Nakhel C, Rouphael Y, Fusco GM, Borzacchiello A, Lanzuise S, Vinale F (2020) Application of *Trichoderma harzianum*, 6-pentyl-α-pyrone and plant biopolymer formulations modulate plant metabolism and fruit quality of plum tomatoes. Plants 9:771. https://doi.org/10.3390/plants9060771

Chapman JM, Muhlemann JK, Gayomba SR, Muday GK (2019) RBOH-dependent ROS synthesis and ROS scavenging by plant specialized metabolites to modulate plant development and stress responses. Chem Res Toxicol 32:370–396. https://doi.org/10.1021/acs.chemrestox.9b00028

Chen SC, Ren JJ, Zhao HJ, Wang XL, Wang TH, Jin SD, Li CY, Liu AR, Lin XM, Ahammed GJ (2019) *Trichoderma harzianum* improves defense against *Fusarium oxysporum* by regulating ROS and RNS metabolism, redox balance, and energy flow in cucumber roots. Phytopathology 109:972–982. https://doi.org/10.1094/PHYTO-09-18-0342-R

Choudhary A, Kumar A, Kaur N (2020) ROS and oxidative burst: roots in plant development. Plant Divers 42:33–43. https://doi.org/10.1016/j.pld.2019.10.002

Contreras-Cornejo HA, Macías-Rodríguez L, Beltrán-Peña E, Herrera-Estrella A, López-Bucio J (2011) *Trichoderma*-induced plant immunity likely involves both hormonal-and camalexin-dependent

mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungi *Botrytis cinerea*. Plant Signal Behav 6:1554–1563. https://doi.org/10.4161/psb.6.10.17443

Eljebbawi A, Guerrero YDCR, Dunand C, Estevez JM (2021) Highlighting reactive oxygen species as multitaskers in root development. iScience 24:101978. https://doi.org/10.1016/j.isci.2020.101978

Esparza-Reynoso S, Ruíz-Herrera LF, Pelagio-Flores R, Macías-Rodríguez LI, Martínez-Trujillo M, López-Coria M, Sánchez-Nieto S, Herrera-Estrella A, López-Bucio J (2021) *Trichoderma atroviride*-emitted volatiles improve growth of *Arabidopsis* seedlings through modulation of sucrose transport and metabolism. Plant Cell Environ 44:1961–1976. https://doi.org/10.1111/pce.14014

Estrada-Rivera M, Rebolledo-Prudencio OG, Pérez-Robles DA, Rocha-Medina MDC, González-López MDC, Casas-Flores S (2019) *Trichoderma* histone deacetylase HDA-2 modulates multiple responses in *Arabidopsis*. Plant Physiol 179:1343–1361. https://doi.org/10.1104/pp.18.01092

Garnica-Vergara A, Barrera-Ortiz S, Muñoz-Parra E, Raya-González J, Méndez-Bravo A, Macías-Rodríguez L, Ruiz-Herrera LF, López-Bucio J (2016) The volatile 6-pentyl-2H-pyran-2-one from *Trichoderma atroviride* regulates *Arabidopsis thaliana* root morphogenesis via auxin signaling and *ETHYLENE INSENSITIVE 2* functioning. New Phytol 209:1496–1512. https://doi.org/10.1111/nph.13725

González-López MDC, Jijón-Moreno S, Dautt-Castro M, Ovando-Vázquez C, Ziv T, Horwitz BA, Casas-Flores S (2021) Secretome analysis of *Arabidopsis–Trichoderma atroviride* interaction unveils new roles for the plant glutamate: glyoxylate aminotransferase GGAT1 in plant growth induced by the fungus and resistance against *Botrytis cinerea*. Int J Mol Sci 22:6804. https://doi.org/10.3390/ijms22136804

Graças JP, Ranocha P, Vitorello VA, Savelli B, Jamet E, Dunand C, Burlat V (2020) The class III peroxidase encoding gene *AtPrx62* positively and spatiotemporally regulates the low pH-induced cell death in *Arabidopsis thalian*a roots. Int J Mol Sci 21:7191. https://doi.org/10.3390/ijms21197191

Guzmán-Guzmán P, Porras-Troncoso MD, Olmedo-Monfil V, Herrera-Estrella A (2019) *Trichoderma* species: versatile plant symbionts. Phytopathology 109:6–16. https://doi.org/10.1094/PHYTO-07-18-0218-RVW

Harman GE, Doni F, Khadka RB, Uphoff N (2021) Endophytic strains of *Trichoderma* increase plants' photosynthetic capability. J Appl Microbiol 130:529–546. https://doi.org/10.1111/jam.14368

Hassani MA, Durán P, Hacquard S (2018) Microbial interactions within the plant holobiont. Microbiome 6:58. https://doi.org/10.1186/s40168-018-0445-0

Hu CH, Wang PQ, Zhang PP, Nie XM, Li BB, Tai L, Liu WT, Li WQ, Chen KM (2020) NADPH oxidases: the vital performers and center hubs during plant growth and signaling. Cells 9:437. https://doi.org/10.3390/cells9020437

Huang H, Ullah F, Zhou DX, Yi M, Zhao Y (2019) Mechanisms of ROS regulation of plant development and stress responses. Front Plant Sci 10:800. https://doi.org/10.3389/fpls.2019.00800

Ivanchenko MG, den Os D, Monshausen GB, Dubrovsky JG, Bednářová A, Krishnan N (2013) Auxin increases the hydrogen peroxide (H_2O_2) concentration in tomato (*Solanum lycopersicum*) root tips while inhibiting root growth. Ann Bot 112:1107–1116. https://doi.org/10.1093/aob/mct181

Jiang C, Belfield EJ, Cao Y, Smith JAC, Harberd NP (2013) An *Arabidopsis* soil-salinity–tolerance mutation confers ethylene-mediated enhancement of sodium/potassium homeostasis. Plant Cell 25:3535–3552. https://doi.org/10.1105/tpc.113.115659

Jiang C, Belfield EJ, Mithani A, Visscher A, Ragoussis J, Mott R, Smith JA, Harberd NP (2012) ROS-mediated vascular homeostatic control of root-to-shoot soil Na delivery in *Arabidopsis*. EMBO Rep 31:4359–4370. https://doi.org/10.1038/emboj.2012.273

Khan RAA, Najeeb S, Mao Z, Ling J, Yang Y, Li Y, Xie B (2020) Bioactive secondary metabolites from *Trichoderma spp.* against phytopathogenic bacteria and Root-knot nematode. Microorganisms 8:401. https://doi.org/10.3390/microorganisms8030401

Koyama H, Toda T, Hara T (2001) Brief exposure to low-pH stress causes irreversible damage to the growing root in *Arabidopsis thaliana*: pectin–Ca interaction may play an important role in proton rhizotoxicity. J Exp Bot 52:361–368. https://doi.org/10.1093/jexbot/52.355.361

Lager IDA, Andréasson O, Dunbar TL, Andreasson E, Escobar MA, Rasmusson AG (2010) Changes in external pH rapidly alter plant gene expression and modulate auxin and elicitor responses. Plant Cell Environ 33:1513–1528. https://doi.org/10.1111/j.1365-3040.2010.02161.x

Lee D, Lal NK, Lin ZJD, Ma S, Liu J, Castro B, Toruño T, Dinesh-Kumar SP, Coaker G (2020) Regulation of reactive oxygen species during plant immunity through phosphorylation and ubiquitination of RBOHD. Nat Commun 11:1–16. https://doi.org/10.1038/s41467-020-15601-5

Lin Y, Chen D, Paul M, Zu Y, Tang Z (2013) Loss-of-function mutation of EIN2 in *Arabidopsis* exaggerates oxidative stress induced by salinity. Acta Physiol Plant 35:1319–1328. https://doi.org/10.1007/s11738-012-1172-y

Long A, Huang WL, Qi YP, Yang LT, Lai NW, Guo JX, Chen LS (2019) Low pH effects on reactive oxygen species and methylglyoxal metabolisms in Citrus roots and leaves. BMC Plant Biol 19:1–17. https://doi.org/10.1186/s12870-019-2103-5

Lv B, Tian H, Zhang F, Liu J, Lu S, Bai M, Li C, Ding Z (2018) Brassinosteroids regulate root growth by controlling reactive oxygen species homeostasis and dual effect on ethylene synthesis in *Arabidopsis*. PLoS Genet 14:e1007144. https://doi.org/10.1371/journal.pgen.1007144

Mase K, Tsukagoshi H (2021) Reactive oxygen species link gene regulatory networks during *Arabidopsis* root development. Front Plant Sci 12:642. https://doi.org/10.3389/fpls.2021.660274

Mersmann S, Bourdais G, Rietz S, Robatzek S (2010) Ethylene signaling regulates accumulation of the FLS2 receptor and is required for the oxidative burst contributing to plant immunity. Plant Physiol 154:391–400. https://doi.org/10.1104/pp.110.154567

Nath M, Bhatt D, Prasad R, Gill SS, Anjum NA, Tuteja N (2016) Reactive oxygen species generation-scavenging and signaling during plant-arbuscular mycorrhizal and *Piriformospora indica* interaction under stress condition. Front Plant Sci 7:1574. https://doi.org/10.3389/fpls.2016.01574

Nawrocka J, Gromek A, Małolepsza U (2019) Nitric oxide as a beneficial signaling molecule in *Trichoderma atroviride TRS25*-induced systemic defense responses of cucumber plants against *Rhizoctonia solani*. Front Plant Sci 10:421. https://doi.org/10.3389/fpls.2019.00421

Nogueira-Lopez G, Greenwood DR, Middleditch M, Winefield C, Eaton C, Steyaert JM, Mendoza-Mendoza A (2018) The apoplastic secretome of *Trichoderma virens* during interaction with maize roots shows an inhibition of plant defence and scavenging oxidative stress secreted proteins. Front Plant Sci 5:09. https://doi.org/10.3389/fpls.2018.00409

Orman-Ligeza B, Parizot B, De Rycke R, Fernandez A, Himschoot E, Van Breusegem F, Bennett MJ, Périlleux C, Beeckman T, Draye X (2016) RBOH-mediated ROS production facilitates lateral root emergence in *Arabidopsis*. Development 143:3328–3339. https://doi.org/10.1242/dev.136465

Ortiz-Castro R, Pelagio-Flores R, Méndez-Bravo A, Ruiz-Herrera LF, Campos-García J, López-Bucio J (2014) Pyocyanin, a virulence factor produced by *Pseudomonas aeruginosa*, alters root development through reactive oxygen species and ethylene signaling in

Arabidopsis. Mol Plant Microbe Interact 27:364–378. https://doi.org/10.1094/MPMI-08-13-0219-R

Otulak-Kozieł K, Kozieł E, Bujarski JJ, Frankowska-Łukawska J, Torres MA (2020) Respiratory burst oxidase homologs RBOHD and RBOHF as key modulating components of response in turnip mosaic virus—*Arabidopsis thaliana* (L.) Heyhn System. Int J Mol Sci 21:8510. https://doi.org/10.3390/ijms21228510

Pascale A, Proietti S, Pantelides IS, Stringlis IA (2020) Modulation of the root microbiome by plant molecules: the basis for targeted disease suppression and plant growth promotion. Front Plant Sci 10:1741. https://doi.org/10.3389/fpls.2019.01741

Pelagio-Flores R, Esparza-Reynoso S, Garnica-Vergara A, López-Bucio J, Herrera-Estrella A (2017) *Trichoderma*-induced acidification is an early trigger for changes in *Arabidopsis* root growth and determines fungal phytostimulation. Front Plant Sci 8:822. https://doi.org/10.3389/fpls.2017.00822

Ramírez-Valdespino CA, Casas-Flores S, Olmedo-Monfil V (2019) *Trichoderma* as a model to study effector-like molecules. Front Microbiol 10:1030. https://doi.org/10.3389/fmicb.2019.01030

Saravanakumar K, Fan L, Fu K, Yu C, Wang M, Xia H, Sun J, Li Y, Chen J (2016) Cellulase from *Trichoderma harzianum* interacts with roots and triggers induced systemic resistance to foliar disease in maize. Sci Rep 6:1–18. https://doi.org/10.1038/srep35543

Su C, Liu L, Liu H, Ferguson BJ, Zou Y, Zhao Y, Wang T, Wang Y, Li X (2016) H2O2 regulates root system architecture by modulating the polar transport and redistribution of auxin. J Plant Biol 59:260–270. https://doi.org/10.1007/s12374-016-0052-1

Velada I, Cardoso H, Porfirio S, Peixe A (2020) Expression profile of PIN-formed auxin efflux carrier genes during IBA-induced in vitro adventitious rooting in *Olea europaea* L. Plants 9:185. https://doi.org/10.3390/plants9020185

Villalobos-Escobedo JM, Esparza-Reynoso S, Pelagio-Flores R, López-Ramírez F, Ruiz-Herrera LF, López-Bucio J, Herrera-Estrella A (2020) The fungal NADPH oxidase is an essential element for the molecular dialog between *Trichoderma* and *Arabidopsis*. Plant J 103:2178–2192. https://doi.org/10.1111/tpj.14891

Vinale F, Sivasithamparam K (2020) Beneficial effects of *Trichoderma* secondary metabolites on crops. Phytother Res 34:2835–2842. https://doi.org/10.1002/ptr.6728

Xu Y, Zhang J, Jiahui S, Haichao F, Ruifu Z, Qirong S (2020) Extracellular proteins of *Trichoderma guizhouense* elicit an immune response in maize (*Zea mays*) plants. Plant Soil 449:133–149. https://doi.org/10.1007/s11104-020-04435-1

Zhang YK, Zhu DF, Zhang YP, Chen HZ, Xiang J, Lin XQ (2015) Low pH-induced changes of antioxidant enzyme and ATPase activities in the roots of rice (*Oryza sativa L.*) seedlings. PloS one 10:e0116971. https://doi.org/10.1371/journal.pone.0116971

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.

Journal of Plant Growth Regulation

CLE14 peptide impairs root tip regeneration and callogenesis in Arabidopsis

--Manuscript Draft--

Manuscript Number:	
Full Title:	CLE14 peptide impairs root tip regeneration and callogenesis in Arabidopsis
Article Type:	Brief Communication
Funding Information:	Consejo Nacional de Ciencia y Tecnología (SEP-CONACYT A1-S-34768) — PhD José López-Bucio Consejo Nacional de Ciencia y Tecnología (FORDECYT-PRONACES/376120/2020) — Professor Javier Raya-González
Abstract:	CLE14 belongs into a family of plant secreted peptides that interact with leucine-rich repeat receptor-like kinase (LRR-RLK) receptors to orchestrate plant morphogenesis. Previous studies indicated that CLE14 plays an important role in cell division, phosphate homeostasis and senescence, but its specific involvement in cell fate determination and organogenesis remains largely unexplored. Here, through pharmacological, genetic and cell biology approaches, we show the critical roles for CLE14 in determining the balance between cell division and differentiation in root tip regeneration and callogenesis. Nanomolar concentrations of CLE14 or its overexpression in Arabidopsis represses primary root growth and triggers root branching and root hair formation. After resection of the primary root tip, pCLE14:GUS-GFP expression was located specifically at the cell layer adjacent to the cutting and at the outermost external cell layer of the root cap as the newly root cap formed. cle14 mutants had comparable root tip regeneration when compared to WT seedlings, whereas 35S:CLE14 seedlings fail to regenerate the missing root tip after resection. The de-differentiation of tissue into proliferative growth was analyzed in WT, cle14, and 35S:CLE14 stem explants grown in callus-inducing media. The results showed comparable callus-biomass production for WT and cle14, but a dramatically reduced callogenesis for 35S:CLE14 explants. Our data show that CLE14 acts as a “brake” for root tip regeneration as well as callus formation.
Corresponding Author:	Javier Raya-González, PhD Universidad Michoacana de San Nicolas de Hidalgo Morelia, Michoacán MEXICO
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	Universidad Michoacana de San Nicolas de Hidalgo
Corresponding Author's Secondary Institution:	
First Author:	Adrián Ávalos-Rangel
First Author Secondary Information:	
Order of Authors:	Adrián Ávalos-Rangel León Francisco Ruiz-Herrera Dolores Gutiérrez-Alanis Luis Herrera-Estrella Javier Raya-González, PhD José López-Bucio
Order of Authors Secondary Information:	
Author Comments:	
Suggested Reviewers:	Lewis L Feldman, PhD

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

	Berkeley City College ljfeldman@berkeley.edu He is expert in root biology, root meristem, and root cap.
	John Paul Délano Frier CINVESTAV IPN: Centro de Investigacion y de Estudios Avanzados del Instituto Politecnico Nacional john.delano@cinvestav.mx He is expert in small peptide signaling associated to biotic and abiotic stress.
	Javier Pozueta-Romero javier.pozueta@csic.es He is expert in plant hormonal signaling.
Keywords:	CLE14; secreted peptides; root architecture; cell division; regeneration

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Manuscript Click here to access/download;Manuscript;renamed_36cd8.docx

Click here to view linked References

Professor Jutta Ludwig-Mueller

Editor-in-chief

Journal of Plant Growth Regulation

Dear Prof. Ludwig-Mueller:

Please find enclosed our manuscript entitled “CLE14 peptide impairs root tip regeneration and callogenesis in Arabidopsis” by Ávalos-Rangel et al. that we would like you to consider for publication in *Journal of Plant Growth Regulation* as a brief communication.

After damage, plants activate cellular and molecular mechanisms to seal the wounds and/or regenerate the missing tissues via hormonal changes, including auxin and jasmonic acid signaling, which is critical for adaptation. Little is known about the genetic elements orchestrating these responses.

CLAVATA3 (CLE3)/Embryo Surrounding Region (ESR) (CLE) peptides encode for small peptides (12 or 13 amino acids residues in their functional form), which are involved in basic cellular processes as well as adaptation to biotic and abiotic stress. The Arabidopsis genome contains 32 CLE genes so the function of each CLE family member in plant development has just begun to be elucidated.

In our manuscript, we show that CLE14 is involved in cell damage and regenerative processes in the Arabidopsis root. Stem cell resection of primary root tip triggers CLE14 expression at the outermost external cell layer, and after root tip reconstruction, CLE14 expression returns to its basal domain, the external cell layer of the root cap. Analysis of CLE14 over expressor lines in root tip regeneration and callogenesis assays, suggests that CLE14 is a critical modulator of these processes.

We believe that these data may be of broad interest to plant biologists and deserves publication as a brief communication in JPGR.

We thank you in advance for your time and consideration.

Respectfully,

José López Bucio

Javier Raya-González

CLE14 peptide impairs root tip regeneration and callogenesis in Arabidopsis

Authors: Adrián Ávalos-Rangel[1], León Francisco Ruiz-Herrera[1], Dolores Gutiérrez-Alanis[2], Luis Herrera-Estrella[2,3], Javier Raya-González[4*], José López-Bucio[1*]

[1]Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo. Edificio B3, Ciudad Universitaria; 58030, Morelia, Michoacán, México.

[2]Institute of Genomics for Crop Abiotic Stress Tolerance (IGCAST), Department of Plant and Soil Science, Texas Tech University, 1006 Canton Ave #3135, Lubbock, TX 79409

[3]Unidad de Genómica Avanzada, Laboratorio Nacional de Genómica para la Biodiversidad, Centro de Investigación y de Estudios Avanzados del IPN, Campus Irapuato, Km. 9.6 Libramiento Norte Carretera Irapuato-León; 36821 Irapuato, Guanajuato, México.

[4]Facultad de Químico Farmacobiología, Universidad Michoacana de San Nicolás de Hidalgo, Avenida Tzintzunzan 173, Col. Matamoros, 58240, Morelia, Michoacán, México.

*Corresponding authors:

José López-Bucio

E-mail: jbucio@umich.mx

ORCID: 0000-0002-4849-5212

Javier Raya-González

E-mail: javier.raya@umich.mx

ORCID: 0000-0002-6685-5090

Running title: CLE14 represses root tip regeneration and callus formation

Abstract

CLE14 belongs into a family of plant secreted peptides that interact with leucine-rich repeat receptor-like kinase (LRR-RLK) receptors to orchestrate plant morphogenesis. Previous studies indicated that CLE14 plays an important role in cell division, phosphate homeostasis and senescence, but its specific involvement in cell fate determination and organogenesis remains largely unexplored. Here, through pharmacological, genetic and cell biology approaches, we show the critical roles for CLE14 in determining the balance between cell division and differentiation in root tip regeneration and callogenesis. Nanomolar concentrations of CLE14 or its overexpression in Arabidopsis represses primary root growth and triggers root branching and root hair formation. After resection of the primary root tip, *pCLE14:GUS-GFP* expression was located specifically at the cell layer adjacent to the cutting and at the outermost external cell layer of the root cap as the newly root cap formed. *cle14* mutants had comparable root tip regeneration when compared to WT seedlings, whereas *35S:CLE14* seedlings fail to regenerate the missing root tip after resection. The de-differentiation of tissue into proliferative growth was analyzed in WT, *cle14*, and *35S:CLE14* stem explants grown in callus-inducing media. The results showed comparable callus-biomass production for WT and *cle14*, but a dramatically reduced callogenesis for *35S:CLE14* explants. Our data show that CLE14 acts as a “brake” for root tip regeneration as well as callus formation.

Keywords: CLE14, secreted peptides, root architecture, cell division, regeneration.

Introduction

Plants as sessile organisms adapt to biotic and abiotic stressors through root regeneration and *de novo* organogenesis. Physical damage of leaves, stems and roots may occur upon attack by hervibores, pathogens and during soil exploration or exposure to pollutans, for which wound healing or tissue replacement orchestrate the adaptive response (Ikeuchi et al. 2016; Mathew and Prasad 2021). However the molecular players and mechanisms underlying cell fate decisions during organ regeneration and differentiation events remain obscure.

The CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) peptides comprise a large class of molecular ligands, able to bind to leucine-rich repeat receptor-like kinase (LRR-RLK) receptors (Cock and McCormick 2001; Sharma et al. 2003; Fletcher 2020; Willoughby and Nimchuk 2021). These interactions modulate critical processes during plant development, including meristematic activity (Hirakawa et al. 2021), nutritional root responses (Gutiérrez-Alanís et al. 2017), and senescense (Zhang et al. 2022). In particular, the CLE14 peptide has been reported as a repressor of root elongation and as inducer of root hair formation in Arabidopsis, rice and tomato (Hayashi et al. 2018; 2019) as well as in stoping root meristem activity under low phosphate conditions acting through the CLAVATA2/PEPR2 receptors (Gutiérrez-Alanís et al. 2017).

An important advancement towards understanding root regeneration was the finding that after root tip resection in Arabidopsis that eliminates the most distal part of the root tip, including the root cap and root meristem initials, cells withing surviving tissue transdifferentiate to develop a new quiescent center necessary to build the missing tissues (Heyman et al. 2013; 2016; Ruiz-Aguilar et al. 2020). This process involves changes in auxin gradients within the regenerating root tip as well as jasmonic acid and brassinosteroid signaling (Zhang et al. 2019; Zhou et al. 2019; Canher et al. 2020; Takahashi and Umeda 2022). Shoot regeneration from stem tissue involves de-differentiation of cells and the entrance into mitosis, a process promoted by auxin and cytokinins (Ikeuchi et al. 2016; Mathew and Prasad 2021). Complete plant regeneration from cuttings is an important tool for safe plant propagation, with an

impact in horticulture and floriculture (Ikeuchi et al. 2016). This process involves the formation of pluripotent cells derived from callus tissue, from which, new stems or roots can differentiate via management of the cytokinin-auxin balance (Ikeuchi et al. 2016; Mathew and Prasad 2021). Considering the key role of CLE-peptide signaling in cell division and differentiation and their tight link with abiotic stress, CLE14 peptide could play a role in promoting or antagonizing root tip regeneration or callogenesis.

In this report, pharmacological, genetic and cell biology approches were applied to uncover the role of CLE14 in Arabidopsis root architecture configuration, root tip regeneration and callogenesis.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana WT (ecotype Columbia-0, Col-0), the transgenic lines *pCLE14:GUS-GFP* and *35S::CLE14* (Gutierrez-Alanis et al. 2017) and the mutant line *cle14* (Yamaguchi et al. 2017) were used in this report. Seeds from each genotype were disinfected with 95% (v/v) ethanol for five minutes,20% (v/v) commercial bleach for seven minutes and washing five times with sterile distilled water. The seeds were stored at 4°C for 48 h, and plated on Murashige and Skoog (MS) medium 0.2X supplemented with 0.6% sucrose and 1% phytagar (commercial grade). Plates were placed into a Percival AR95L chamber at 21°C and 16 h light/8 h darkness, in a vertical position. The length of the primary root was measured with a ruler and an analytical balance was used to quantify the fresh weight of callus.

Callus induction

For callus induction, seeds were germinated and grown for 4 days in Gamborg's B5 medium with 2% sucrose, solidified with 0.8% phytagar and supplemented with 10 μM 1-naphthaleneacetic acid (NAA). Then, seedlings were transferred onto the callus-inducing media (CIM) according to Wang. et al. (2015), and analyzed after 21

d. Callogenesis capacity for each line was evaluated measuring the fresh weight of 15 explants. For the medium supplemented with the CLE14 peptide, we applied concentrations from 0.001 to 1 μM of the synthetic CLE14 peptide (purity of 98%), which was generated by GeneScript Biotech Corp.

Root cutting-assay and regeneration

Four-day-old Arabidopsis seedlings were used and root tips were cut by hand using a sterile scalpel and a stereomicroscope microscope (Leica MZ6) under axenic conditions. The cuts were made at quiescent center of the primary root tip. The analysis of root apical meristem (RAM) structure, cell viability and cell regeneration were performed every 24 h for 3-5 days post-cutting. For this, plant roots were stained with propidium iodide (PI) and placed on slides to be observed with a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan). The experiment included 45 seedlings for each genotype and was repeated at least three times.

PI staining and GFP detection

Arabidopsis seedlings were incubated in a 10 μg/ml PI solution (Sigma) for 1 min, then plant roots were placed on microscope slides, and observed in a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan). Specific wavelengths were used for IP (568 nm excitation; 585-610 nm emission) and GFP (500-523 emission; 488 nm excitation) and recorded separately when necessary to generate the final image.

Results and discussion

Nanomolar applications of CLE14 drive cell differentiation events within the Arabidopsis primary root

Previous studies have tested pharmacologically the bioactivity of CLE14 on root growth and root hair formation, which indicate contrasting facets of the peptide on

these root traits (Hayashi et al. 2018; 2019; Gutiérrez-Alanís et al. 2017). The repression of root growth and concomitantly, the loss of apical dominance of the main root axis could be reproduced in our experiments, in which 1 μM or lower concentrations of CLE14 repressed Arabidopsis primary root growth (Fig. 1a), which coincided with root branching (Fig. 1b), and the formation of root hairs at the root tip (Fig. 1c). Comparison of growth of wild-type (Col-0) seedlings and CLE14 overexpressing seedlings (*35S:CLE14*) side-by-side showed the short root phenotype of the transgenics (Fig. 1d, e). Our results are consistent with the report of Meng and Feldman (2010), which showed that CLE14 halts irreversibly Arabidopsis primary root growth through the reduction of both cell division in the meristem and *CyclinB1* expression, and with those of Hayashi et al. (2018) where CLE14 impaired root growth and promoted root hair formation. These previous and present data confirm the important role of CLE14 as an inhibitor of cell proliferation and as an inducer of differentiation, which may be shared by other members of the CLE family since the root meristem arrest has been evidenced for *CLE8*, *CLE12*, *CLE19* and *CLE22* overexpression lines (Fiers et al. 2005; Ito et al. 2006; Strabala et al. 2006; Jun et al. 2010).

CLE14 is expressed during Arabidopsis root tip regeneration

Plants as sessile organisms manifest a strong capacity to rebuilt the missing tissues after damage, via reversion of the differentiated status and acquiring pluripotent cell identity (Heyman et al. 2013; 2016; Ruiz-Aguilar et al. 2021; Takahashi et al. 2022). Resection of the Arabidopsis primary root has been used to characterize the regeneration potential, which after cutting with a scalpel at the quiescent center zone rebuilds the missing tissues through an activation of cell division (Sena et al. 2009). An analysis of *pCLE14:GUS-GFP* expression via propidium iodide staining and GFP fluorescence detection by confocal microscopy showed the green signal at the outermost external cell layer of the columella that remained comparable from the 4-7 days after germination (dag, Fig. 2a-d). Interestingly, after resection of the root tip, GFP expression was detected at the outermost external cell layer of the root (Fig.

2e, f). From 2-3 days after cutting a novel root tip with root cap was formed, and the *CLE14* expression was now restricted to the external cell layer of the cap comparable with intact roots (Fig. 2g-h).

The CLE14 expression pattern at the root cap was first visualized by Meng and Feldman (2011), and here we noted its specific induction in the cell layer immediately adjacent to the cutting site, where propidium iodide marks a red patch in damaged cells (Fig. 2e). Thus, CLE 14 could be involved either in the process of reconstruction and acquisition of cell fates as root tip regeneration proceeds or in wound healing after damage.

CLE14 represses root tip regeneration after resection

Wounding promotes a strong defensive reaction necessary to keep outside potentially dangerous microbes via sealing of the wound or alternatively, to drive the regeneration of the missing tissue to restart growth (Zhang et al. 2019; Zhou et al. 2019). Next, we compared the process of root tip regeneration after cutting in WT (Col-0) seedlings, *cle14* mutants and *35S:CLE14* overexpressors. Representative images at 1, 3 and 5 days after cutting (dac) showed that *cle14* mutants have comparable regeneration potential compared to the WT, whereas *35S:CLE14* seedlings fail to regenerate the missing root tip (Fig. 3a-l). In this sense, Zhang et al. (2019) demonstrated the induction of CLE14 by jasmonic acid, a hormone actin as a sentinel for both plant immunity and defense. Wounding elicits the rapid biosynthesis and signaling of jasmonic acid (Glauser et al. 2008; Koo et al. 2009), which may explain the expression pattern of *pCLE14:GUS-GFP* in border cells during root tip resection. This inducible expression may account for sealing the wound, since CLE14 overexpression interferes with the normal regeneration process as shown in Fig. 2.

CLE14 overexpression halts callus formation

Shoot regeneration from stem tissue involves de-differentiation of cells and the entrance into mitosis, a process promoted by auxin and cytokinins (Ikeuchi et al. 2013). Undifferentiated cellular masses can be obtained from dark-grown Arabidopsis shoot explants transferred to media enriched with auxins and cytokinins, which enabled us to compared the de-differentiation process and proliferation underlying callus formation. To determine the relationship between CLE14 and de-differentiation process, callus biomass was quantified 25 days after transfer of WT (Col-0), *cle14*, and *35S:CLE14* stem explants into callus-inducing media. The data show comparable callus-biomass for WT and *cle14*, but a dramatically reduced biomass for *35S:CLE14* explants (Fig. 4a). Images taken directly from the explants clearly showed the highly reduced callus in *35S:CLE14* explants (Fig. 4b), which can be explained by their overall inhibitory effect on mitosis. Despite its well-recognized utility in plant research and horticultural applications for plant asexual propagation, the molecular basis of callus formation has remained obscure. The correlation found in our work that the CLE14 peptide both represses cell division in root meristem and during root regeneration or callus formation reveals the commonalities in these processes.

The balance between cell division and differentiation is critical for adaptation to abiotic stress, noteworthy, in plants experiencing phosphate scarcity stress, the root meristem becomes exhausted and their cells differentiate producing root hairs through the action of CLE14 and their receptors CLV2 and PEPR2 (Gutierrez-Alanis et al. 2017). Ligand interaction down-regulates the master stem genes *short root* and *scarecrow*, which operate through the PIN/AUXIN pathway. The current report extends what is known into how CLE14 influences organogenesis by controlling the balance between cell division and differentiation. If CLE14 operates through CLV2-PEPR2 receptors and/or involving SHR-SCR module to regulate root tip regeneration and callus formation remains to be revealed.

Figure legends

Figure 1. CLE14 nanomolar concentrations halts Arabidopsis primary root growth, and promotes root branching and root hair formation at the root tip. (a) Effect of CLE14 application to the growth media on primary root growth. b) Phenotypes of Arabidopsis seedlings grown in medium lacking CLE14 (left) or supplemented with 1 μM CLE14 peptide (right). (c) Estructure of the primary root tip in control (left) and CLE14-treated seedlings (medium and right). Right image is presented at low magnification to show the proliferation of root hairs (white arrows). (d) Representative images and quantitation of root growth in WT and *35S:CLE14* seedlings into the same plate. Graphs show the mean and standard deviation from 12 seedlings analyzed. Different letters indicate means that differ statistically ($P<0.05$). The experiment was repeated three times with comparable results. Images of plates are representative from 4 independent plates. Confocal images were selected from six independent individuals. Scale bar= 1 cm (a, d); 50 μm (c).

Figure 2. CLE14 is expressed in border cells after root tip excision. Confocal images of Arabidopsis root tips expressing *pCLE14:GUS-GFP* stained with propidium iodide 4-7 days after germination (a-d), and the changes in GFP fluorescence during regeneration of the root tip (e-h). Images are representative from six independent individuals analyzed. The experiment was repeated three times with comparable results. Scale bar= 100 μm.

Figure 3. Primary root tip regeneration after excision in WT, *cle14* and *35S::CLE14* Arabidopsis seedlings. Confocal images of WT (Col-0), *cle14*, and *35S::CLE14* Arabidopsis root tips stained with propidium iodide at control condition and 1, 3 and 5 days post-cutting. Images are representative from six independent individuals analyzed. The experiment was repeated three times with comparable results. Scale bar= 100 μm.

Figure 4. Overexpression of CLE14 impairs callus formation. Fresh weight measurements of callus biomass produced in fifteen WT (Col-0), *cle14*, and *35S::CLE14* shoot explants grown in callus-inducing media (a). Images of calli-producing explants from WT (Col-0), *cle14*, and *35S::CLE14* seedlings. Images of explants are representative from 3 independent plates. Note that *35S::CLE14* fails to develop callus. Different letters indicate means that differ statiscally (P<0.05). The experiment was repeated three times with comparable results. Scale bar= 5 mm.

Author contributions

A.A.R., L.F.R.H., and J.R.G., performed the experiments. A.A.R., J.R.G., D.G.A., L.H.E., and J.L.B. analyzed the data. J.R.G., and J.L.B. designed the experiments. J.R.G., and J.L.B. wrote the manuscript. All authors approved the manuscript.

Acknowledgements and funding information

This project was found by grants from SEP-CONACYT A1-S-34768 and FORDECYT-PRONACES/376120/2020.

Competing interest statement

The authors declare no competing financial interest

References

Canher B, Heyman J, Savina M, Devendran A, Eekhout T, Vercauteren I, Prinsen E, Matosevich R, Xu J, Mironova V, De Veylder L (2020) Rocks in the auxin stream: Wound-induced auxin accumulation and *ERF115* expression synergistically drive stem cell regeneration. Proc Natl Acad Sci U S A 117(28):16667-16677. doi: 10.1073/pnas.2006620117.

Cock JM, McCormick S (2001) A large family of genes that share homology with CLAVATA3. Plant Physiol 126(3):939-942. doi: 10.1104/pp.126.3.939.

323 Fiers M, Golemiec E, Xu J, van der Geest L, Heidstra R, Stiekema W, Liu CM (2005) The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the root meristem in Arabidopsis through a CLAVATA2- dependent pathway. Plant Cell 17:2542–2553. doi: 10.1105/tpc.105.034009.

327 Fletcher JC (2020) Recent advances in Arabidopsis CLE peptide signaling. Trends Plant Sci 25:1005-1016. doi: 10.1016/j.tplants.2020.04.014.

329 Glauser G, Grata E, Dubugnon L, Rudaz S, Farmer EE, Wolfender JL (2008) Spatial and temporal dynamics of jasmonate synthesis and accumulation in Arabidopsis in response to wounding. J Biol Chem 283(24):16400-7. doi: 10.1074/jbc.M801760200.

333 Gutiérrez-Alanís D, Yong-Villalobos L, Jiménez-Sandoval P, Alatorre-Cobos F, Oropeza-Aburto A, Mora-Macías J, Sánchez-Rodríguez F, Cruz-Ramírez A, Herrera-Estrella L (2017) Phosphate starvation-dependent iron mobilization induces CLE14 expression to trigger root meristem differentiation through CLV2/PEPR2 signaling. Dev Cell 41:555-570.e3. doi: 10.1016/j.devcel.2017.05.009.

338 Hayashi N, Tetsumura T, Sawa S, Wada T, Tominaga-Wada R (2018) CLE14 peptide signaling in Arabidopsis root hair cell fate determination. Plant Biotechnol (Tokyo) 35(1):17-22. doi: 10.5511/plantbiotechnology.18.0122a.

341 Hayashi N, Rongkavilit N, Tetsumura T, Sawa S, Wada T, Tominaga-Wada R (2019) Effect of the CLE14 polypeptide on *GLABRA2* homolog gene expression in rice and tomato roots. Plant Biotechnol (Tokyo) 36(3):205-208. doi: 10.5511/plantbiotechnology.19.0725a.

345 Heyman J, Cools T, Vandenbussche F, Heyndrickx KS, Van Leene J, Vercauteren I, Vanderauwera S, Vandepoele K, De Jaeger G, Van Der Straeten D, De Veylder L (2013) ERF115 controls root quiescent center cell division and stem cell replenishment. Science 342(6160):860-3. doi: 10.1126/science.1240667.

349 Heyman J, Cools T, Canher B, Shavialenka S, Traas J, Vercauteren I, Van den Daele H, Persiau G, De Jaeger G, Sugimoto K, De Veylder L (2016) The

heterodimeric transcription factor complex ERF115-PAT1 grants regeneration competence. Nat Plants 2(11):16165. doi: 10.1038/nplants.2016.165.

Hirakawa Y (2021) CLAVATA3, a plant peptide controlling stem cell fate in the meristem. Peptides142:170579. doi: 10.1016/j.peptides.2021.170579.

Ikeuchi M, Sugimoto K, Iwase A (2013) Plant callus: mechanisms of induction and repression. Plant Cell 25(9):3159-3173. doi: 10.1105/tpc.113.116053.

Ikeuchi M, Ogawa Y, Iwase A, Sugimoto K (2016) Plant regeneration: cellular origins and molecular mechanisms. Development 143:1442-1451. doi: 10.1242/dev.134668

Ito Y, Nakanomyo I, Motose H, Iwamoto K, Sawa S, Dohmae N, Fukuda H (2006) Dodeca-CLE peptides as suppressors of plant stem cell differentiation. Science 313(5788):842-5. doi: 10.1126/science.1128436.

Jun J, Fiume E, Roeder AH, Meng L, Sharma VK, Osmont KS, Baker C, Ha CM, Meyerowitz EM, Feldman LJ, Fletcher JC (2010) Comprehensive analysis of CLE polypeptide signaling gene expression and overexpression activity in Arabidopsis. Plant Physiol 154(4):1721-36. doi: 10.1104/pp.110.163683.

Koo AJ, Gao X, Jones AD, Howe GA (2009) A rapid wound signal activates the systemic synthesis of bioactive jasmonates in Arabidopsis. Plant J 59(6):974-986. doi: 10.1111/j.1365-313X.2009.03924.x.

Mathew MM, Prasad K (2021) Model systems for regeneration: *Arabidopsis*. Development 148(6):dev195347. doi: 10.1242/dev.195347.

Meng L, Feldman LJ (2010) CLE14/CLE20 peptides may interact with CLAVATA2/CORYNE receptor-like kinases to irreversibly inhibit cell division in the root meristem of Arabidopsis. Planta 232(5):1061-74. doi: 10.1007/s00425-010-1236-4.

Meng L, Feldman LJ (2011) CLE genes may act in a variety of tissues/cells and involve other signaling cascades in addition to CLV3-WUS-like pathways. Plant Signal Behav 6(1):105-8. doi: 10.4161/psb.6.1.14186.

Ruiz-Aguilar B, Raya-González J, López-Bucio JS, Reyes de la Cruz H, Herrera-Estrella L, Ruiz-Herrera LF, Martínez-Trujillo M, López-Bucio J (2020) Mutation of

MEDIATOR 18 and chromate trigger twinning of the primary root meristem in Arabidopsis. Plant Cell Environ 43(8):1989-1999. doi: 10.1111/pce.13786.

Sena G, Wang X, Liu HY, Hofhuis H, Birnbaum KD (2009) Organ regeneration does not require a functional stem cell niche in plants. Nature. 26;457:1150-3. doi: 10.1038/nature07597.

Sharma VK, Ramirez J, Fletcher JC (2003) The Arabidopsis CLV3-like (CLE) genes are expressed in diverse tissues and encode secreted proteins. Plant Mol Biol 51(3):415-25. doi: 10.1023/a:1022038932376.

Strabala TJ, O'donnell PJ, Smit AM, Ampomah-Dwamena C, Martin EJ, Netzler N, Nieuwenhuizen NJ, Quinn BD, Foote HC, Hudson KR (2006) Gain-of-function phenotypes of many CLAVATA3/ESR genes, including four new family members, correlate with tandem variations in the conserved CLAVATA3/ESR domain. Plant Physiol 140(4):1331-44. doi: 10.1104/pp.105.075515.

Takahashi N, Umeda M (2022) Brassinosteroids are required for efficient root tip regeneration in *Arabidopsis*. Plant Biotechnol (Tokyo) 39(1):73-78. doi: 10.5511/plantbiotechnology.21.1103a.

Wang J, Zhang L, Guo S (2015) Arabidopsis leaf explant culture. Bio-protocol, 5(22), e1654-e1654. doi: 10.21769/BioProtoc.1654.

Willoughby AC, Nimchuk ZL (2021) WOX going on: CLE peptides in plant development. Curr Opin Plant Biol 63:102056. doi: 10.1016/j.pbi.2021.102056.

Yamaguchi YL, Ishida T, Yoshimura M, Imamura Y, Shimaoka C, Sawa S (2017) A collection of mutants for CLE-peptide-enconding genes in Arabidopsis generated by CRISPR/Cas-mediated gene targeting. Plant Cell Physiol 58(11):1848-1856. Doi.org/10.1093/pcp/pcx139.

Zhang G, Zhao F, Chen L, Pan Y, Sun L, Bao N, Zhang T, Cui CX, Qiu Z, Zhang Y, Yang L, Xu L (2019) Jasmonate-mediated wound signalling promotes plant regeneration. Nat Plants 5(5):491-497. doi: 10.1038/s41477-019-0408-x.

Zhang Z, Liu C, Li K, Li X, Xu M, Guo Y (2022) CLE14 functions as a "brake signal" to suppress age-dependent and stress-induced leaf senescence by promoting JUB1-mediated ROS scavenging in Arabidopsis. Mol Plant 15(1):179-188. doi: 10.1016/j.molp.2021.09.006.

Zhou W, Lozano-Torres JL, Blilou I, Zhang X, Zhai Q, Smant G, Li C, Scheres BA (2019) Jasmonate signaling network activates root stem cells and promotes regeneration. Cell 177(4):942-956.e14. doi: 10.1016/j.cell.2019.03.006.

Figure 1

Click here to access/download;Figure;Fig. 1.tif

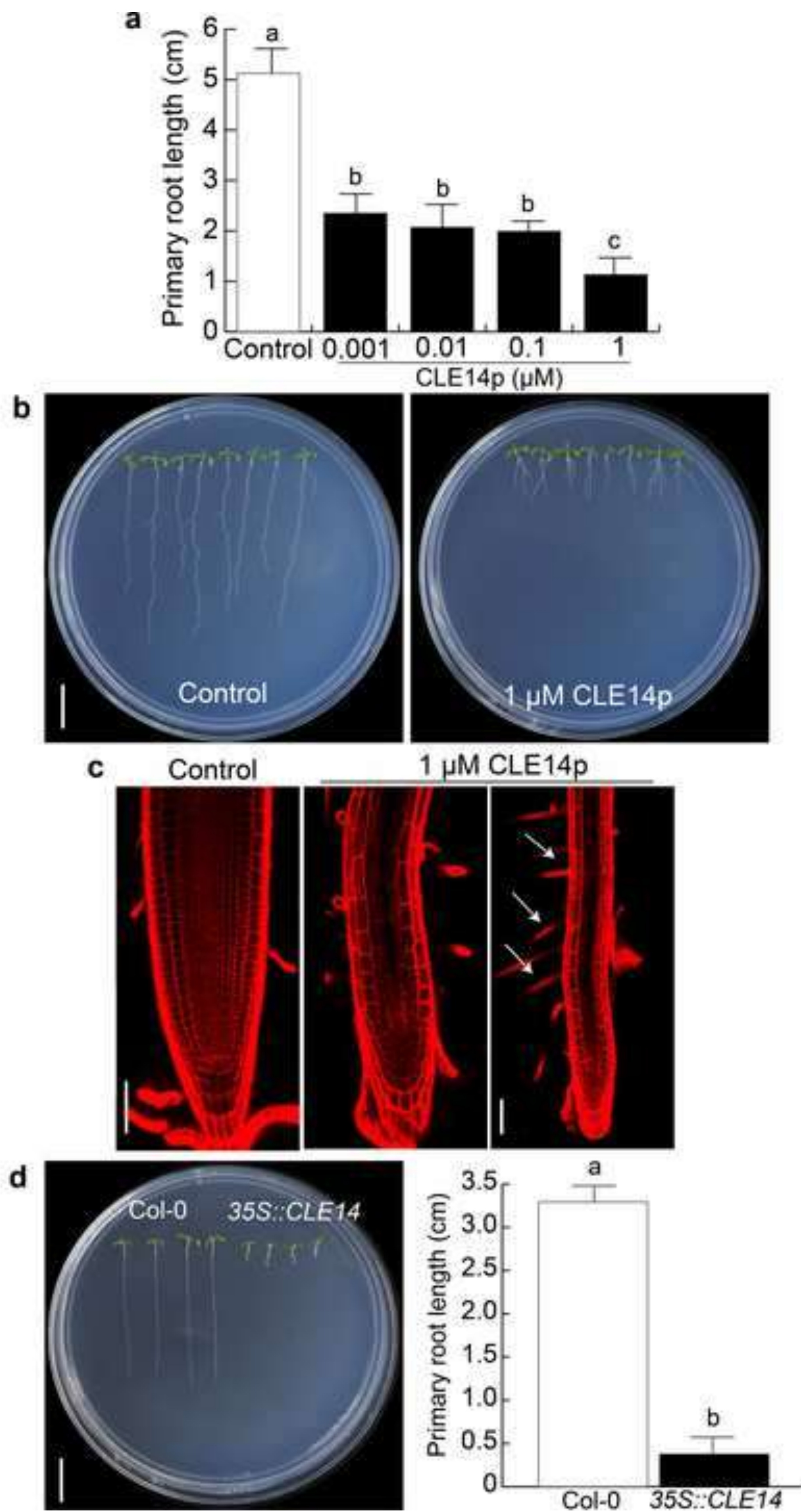


Figure 2

Click here to access/download;Figure;Fig. 2.tif

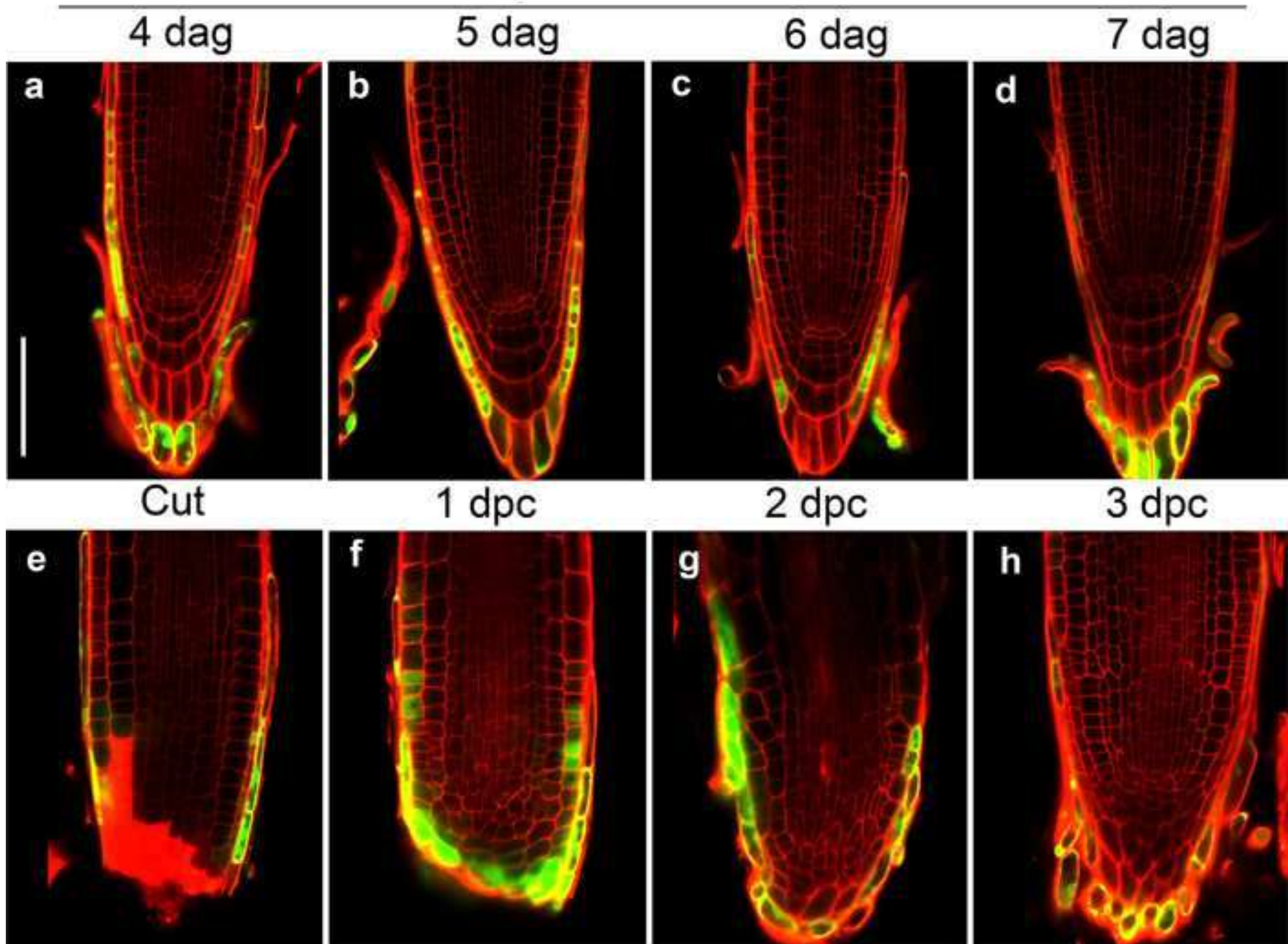
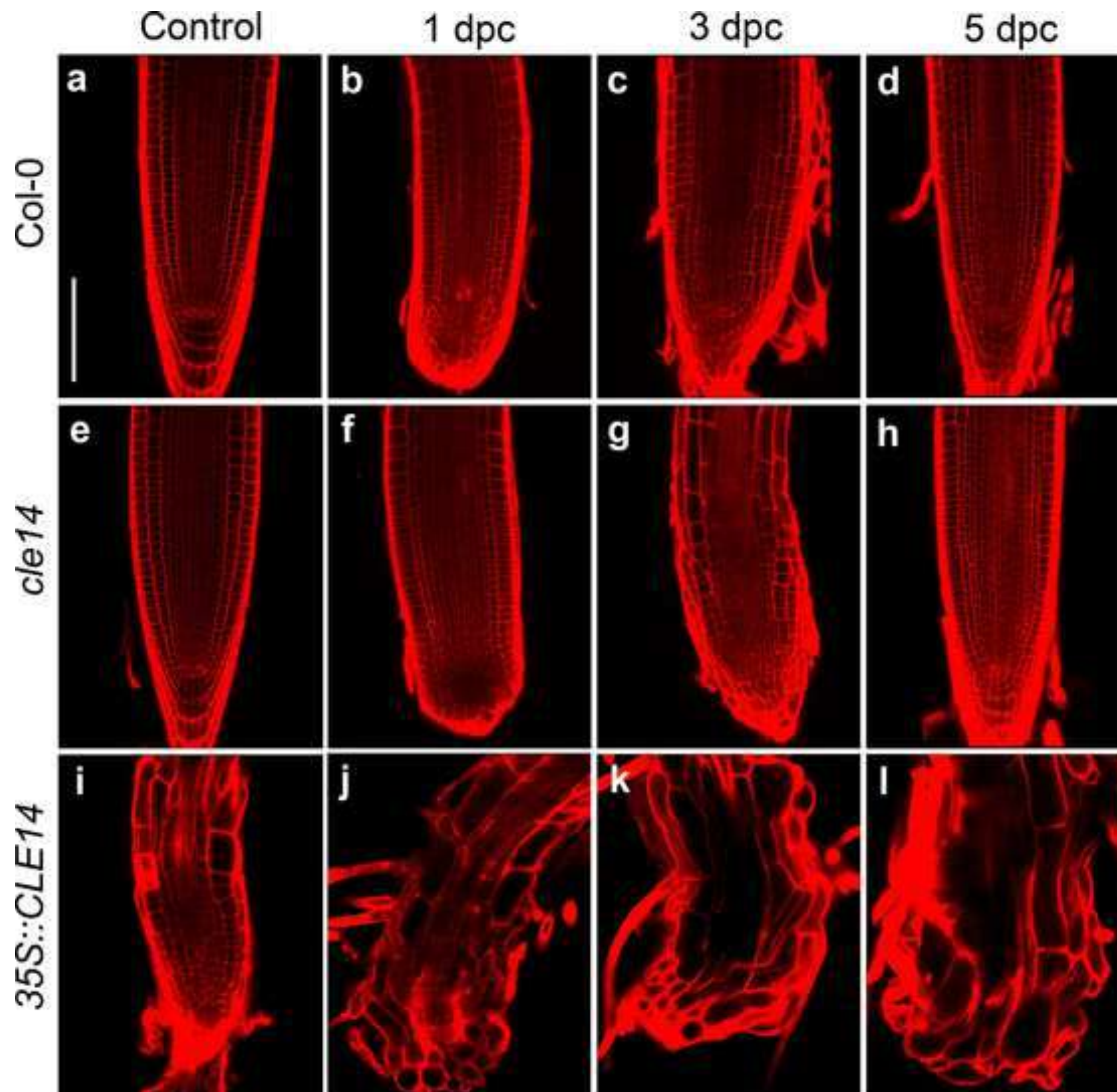


Figure 3

Click here to access/download;Figure;Fig. 3.tif



Click here to access/download;Figure;Fig. 4.tif