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“Señalización por luz en la muerte celular programada de células del meristemo de la raíz de *Arabidopsis”*

TESIS

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS EN BIOLOGIA EXPERIMENTAL

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RESUMEN

La luz actúa como una señal ambiental que estimula la organogénesis de las plantas a través de la activación de los fotorreceptores y de factores de transcripción. No obstante, la exposición de los tejidos a ciertas longitudes de onda, puede inducir estrés oxidativo que daña a los lípidos, las proteínas y el ADN, lo cual conlleva a la muerte celular programada o apoptosis, caracterizada por la eliminación selectiva de células a través de una degradación proteolítica.

En este proyecto, se estudió la participación de los fotorreceptores en la muerte celular inducida por agentes genotóxicos en el meristemo la raíz de *Arabidopsis*. La comparación del crecimiento y la estructura del meristemo de las plantas silvestres del ecotipo Columbia-0 (Col-0), así como las mutantes *phyA-211, phyB-9*, *cry1* y la doble mutante *phyA-211phyB-9* reveló una función critica del fitocromo PhyA en la tolerancia al estrés impuesto por la zeocina y la fleomicina.

La expresión de PhyA se localizó en el ápice de la raíz primaria, en concordancia con la expresión de genes involucrados en las respuestas al daño del ADN, incluyendo ERF115, PAT1, RAD51, MYB3R3 y CYCB1, y su mutación causa hipersensibilidad de la raíz primaria a genotóxicos. También se caracterizó la participación del factor SOG1, en cuyas mutantes no se expresan los genes antes mencionados, y su raíz primaria se diferencia ante el estrés genotóxico causado por la radiación gamma. Observamos que las mutantes *phyA-211* presentan una mayor sensibilidad al estrés genotóxico que las mutantes *sog1-1*. Adicionalmente, las mutantes de *phyA-211* tratadas con zeocina no afectaron la expresión del represor del ciclo celular MYB3R3 lo que sugiere que PhyA y SOG1 son requeridos para salvaguardar la integridad del ADN y evitar la segregación de mutaciones a la progenie celular. Estudios con el factor de transcripción HY5 revelaron que PhyA regula la viabilidad celular a través de un mecanismo independiente de la vía de señalización de la luz y la fotomorfogénesis. En conjunto, nuestros datos revelaron una función novedosa del fitocromo PhyA como un factor clave para la viabilidad celular, fundamental para la función de los meristemos y el crecimiento adecuado de las raíces.

Palabras clave: *Arabidopsis*, PhyA, fitocromos, muerte celular, meristemo de la raíz.

ABSTRACT

Light acts as an environmental cue that stimulates plant organogenesis through photoreceptor activation and intermediary transcription factors. However, it may cause oxidative stress in tissues exposed to high irradiation. DNA damage caused by oxidative stress or genotoxic agents can activate the so-called programmed cell death (PCD). In plants, multiple efforts have been made to elucidate how PCD is regulated. However, the molecular mechanisms underlying this process remain to be explored. Here, we characterized the function of photoreceptors on the damage caused by genotoxic agents in the *Arabidopsis* root meristem. Comparing root growth and meristem structure of wild-type (WT) Columbia-0 (Col-0), *phyA-211, phyB-9, cry1*, and the double mutant *phyA-211 phyB-9* we unveiled a critical function of PhyA during DNA-damaging stress tolerance. Our results revealed that genotoxic stress, imposed by zeocin and phleomycin, induced DNA fragmentation and PCD of the stem cell niche (SCN) and the subsequent exhaustion of primary and lateral root meristems of *phyA-211* and *phyA-211 phyB-9*, but not in *phyB-9* and *cry1* mutants. PhyA expression was localized in root tips, where it apparently influences genes related to DNA damage responses (DDRs), including ERF115, PAT1, RAD51, MYB3R3, and CYCB1.

Interestingly, the DDRs are canonically influenced by the SOG1 transcription factor since *sog1-1* mutants failed to induce the genes mentioned above. We observed that the *phyA-211* mutants have a higher sensitivity to genotoxic stress than the *sog1-1* mutants. Additionally, zeocin-treated *phyA-211* mutants did not affect the repressor of cell cycle progression MYB3R3, suggesting that PhyA and SOG1 are required for safeguarding the DNA integrity through preventing aberrant or mutation segregation to cell progeny. Moreover, analysis of the PhyA downstream component HY5 showed that PhyA regulates DDRs via an independent mechanism of the canonical light and photomorphogenesis signaling pathway. Taken together, our data revealed a novel role of phytochrome PhyA as a key driver for cell viability, critical for meristem function and proper root growth.

1. INTRODUCCIÓN

Las plantas son organismos sésiles sensibles al daño provocado por el estrés ambiental, incluyendo la infección con fitopatógenos, la exposición a toxinas o iones tóxicos y por la actividad de herbívoros que se alimentan de sus órganos. La exposición a tales factores vulnera la viabilidad de las células del meristemo del follaje y la raíz cada que duplican su material genético durante la división celular (Fulcher y Sablowski, 2009). La intensidad del estímulo puede causar daños en el ADN y la activación de la muerte celular programada.

Como respuesta al daño en el ADN inducido por el agobio ante factores bióticos y abióticos, se activan las vías de reparación dependientes de las proteínas cinasas ATAXIA-TELANGIECTASA MUTATED (ATM) y ATM-AND RAD3-RELATED (ATR), las cuales regulan la actividad del factor transcripcional SUPRESSOR OF GAMMA RESPONSE 1 (SOG1) (Maréchal y Zou, 2013). SOG1 es un regulador maestro análogo a p53 de animales que modula la expresión de cientos de genes que codifican para proteínas relacionadas con la reparación del ADN, la división celular, la endorreplicación y la muerte celular programada que son esenciales para controlar la tolerancia al estrés ambiental y evitar la acumulación o propagación de ADN aberrante a la progenie celular en cada ciclo mitótico (Adachi et al., 2011; Yoshiyama et al., 2009; Bourbousse et al., 2018; Ogita et al., 2018).

Algunos blancos moleculares río abajo de SOG1 incluyen a las proteínas necesarias para la reparación del ADN a través de la recombinación homologa (RH), como BREAST CANCER SUSCEPTIBILITY GENE1 (BRCA1) y RADIATION SENSITIVE51 (RAD51) (Ogita et al., 2018). El control de SOG1 sobre la división celular ocurre a través de la regulación de las proteínas SIAMESE-RELATED5/7 (SMR5/7), la cinasa WEE1 y los factores de transcripción ANAC044/085, los cuales inhiben la actividad de las cinasas CYCLIN-DEPENDENT KINASES (CDK) y promueven la acumulación del factor de transcripción MYB3R3 para el arresto del ciclo celular en las fases G2/M (De Schutter et al., 2007; Chen et al., 2017; Bourbousse et al., 2018; Ogita et al., 2018; Takahashi et al., 2019). Las plantas con la perdida de función de cualquier de estos genes presentan a una respuesta

ineficiente ante el daño, por lo que se consideran como genes esenciales que mantienen la integridad celular.

En nuestro grupo se caracterizó la función de la subunidad MED18 del complejo MEDIADOR como un componente molecular clave de la regeneración de órganos y la tolerancia a la muerte celular programada. Bajo condiciones normales de crecimiento y en respuestas a agentes que dañan el ADN, el meristemo radicular de las plantas mutantes *med18* mostraron una marcada susceptibilidad al daño en el nicho de células iniciales y de las células de la pro-vasculatura dentro del meristemo de la raíz, lo cual fue asociado con defectos en los sistemas de reparación del ADN (Raya-González et al., 2018). Interesantemente, la muerte celular en la mutante *med18* fue bloqueada en plantas etioladas, lo que sugiere que la exposición y/o la señalización por luz están involucradas en procesos de viabilidad e integridad celular, mediada por MED18 (McCormick, 2018; Raya-González et al., 2018).

Se ha descrito que la luz a través de la activación de los fotorreceptores regula una gran variedad de procesos del desarrollo en las plantas a lo largo del ciclo de vida. Los fotorreceptores vegetales incluyen a los criptocromos (CRY1, 2) para la percepción de la luz azul, los fitocromos (PhyA–PhyE) para la luz roja/roja lejana y al fotorreceptor UV-B RESISTANCE LOCUS8 (UVR8) para la luz ultravioleta B (Galvão y Fankhauser, 2015). La percepción de la luz promueve la estabilidad de diversos factores de transcripción, incluyendo ELONGATED HYPOCOTYL5 (HY5), un regulador central de la fotomorfogénesis, la asimilación de nutrientes, las respuestas adaptativas y el crecimiento de la raíz (Gangappa y Botto, 2016). No obstante, HY5 ha sido reportado como un inductor del estrés oxidativo durante la des-etiolación de las plantas controlada por los fotorreceptores de la luz roja (PhyB). Adicionalmente, estimula la expresión de genes reguladores de la muerte celular inducida por el estrés biótico y abiótico, como ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) (Chen et al., 2013; Chai et al., 2015).

El factor de transcripción ETHYLENE RESPONSE FACTOR115 (ERF115) estimula la división celular a través de la activación del factor MONOPTEROS/ERF5 (MP/ERF5) y la proteína CYCD6 (Kong et al., 2018; Zhou et al., 2019; Canher et al., 2020). Estudios de interacciones moleculares demostraron que ERF115 forma

dímeros con PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1) para estimular la regeneración celular en el meristemo de la raíz primaria (Heyman et al., 2016; Canher et al., 2020) . Interesantemente, PAT1 es un factor de transcripción que opera en la vía de señalización controlada por PhyA (Torres-Galea et al., 2013; Bolle et al., 2000). Estos datos sugieren que los fotorreceptores de luz roja y roja lejana (PhyA, B) pueden integrar las señales de luz con la muerte celular en el meristemo de la raíz. En el presente proyecto identificamos al fotorreceptor PhyA como un elemento novedoso que participa en la tolerancia al estrés causado por genotóxicos en la raíz primaria de *Arabidopsis*. A través del uso de estrategias farmacológicas, genéticas y fisiológicas comprobamos que el fotorreceptor PhyA protege a la raíz de la muerte celular mediante un mecanismo dependiente de la expresión de los genes *MYB3R3*, *RAD51* y *CYCB1*. Adicionalmente, encontramos que el fotorreceptor PhyA se localiza en la punta de la raíz, lo que sugiere que PhyA podría controlar de manera local las respuestas de la tolerancia al estrés que mantienen la integridad del ADN y previenen la diseminación de mutaciones o aberraciones genéticas a la progenie celular.

2. ANTECEDENTES

2.1. *Arabidopsis thaliana*

Arabidopsis thaliana es una planta dicotiledónea nativa de Europa y Asia que pertenece a la familia *Brassicaceae* y está relacionada con plantas de interés agrícola como el repollo (*Brassica pekinensis*) y las coles (*Brassica oleracea*).

Arabidopsis es usada ampliamente en el campo de la investigación científica; en parte, porque se adapta bien a las condiciones de crecimiento del laboratorio, su germinación *in-vitro* dura 2 días, tiene una alta tasa de autofertilización y quizás una de las características más importantes es el tamaño (0.05 mm) y la dormancia de las semillas, lo cual, las hace fácil de almacenar durante un largo periodo de tiempo (Woodward y Bartel, 2018). Su desarrollo comienza con la formación de la radícula, el hipocótilo y los cotiledones, estructuras primordiales del sistema radicular y el follaje (Sliwinska et al., 2009). Durante el crecimiento vegetativo, *Arabidopsis* desarrolla una roseta basal de donde surgen los tallos florales que alcanzan una altura de 30 cm. En la etapa de reproducción, de las flores hermafroditas emergen frutos alargados llamados silicuas que pueden contener de 30-50 semillas. Finalmente, alcanza su maduración y senescencia hasta las 6-8 semanas de edad (Krämer, 2015).

Los ecotipos Columbia (Col-0 – Col-8), Wassilewskija (Ws-0 – Ws-4) y *Landsberg erecta* (Ler-0 y Ler-1) han sido los fondos genéticos más populares debido a que se cuenta con numerosas líneas mutantes generadas mediante el uso de métodos tradicionales como el tratamiento con metanosulfonato de etilo e irradiación gamma (Maple y Møller, 2007). Incluso, pueden ser transformadas por la bacteria patógena *Agrobacterium tumefaciens,* la cual es capaz de integrar ADN de transferencia (T-ADN) al genoma para formar líneas mutantes y reporteras que proveen información sobre los patrones de expresión y los mecanismos moleculares de regulación genética (Hwang et al., 2017).

El genoma de *Arabidopsis* está compuesto por 5 cromosomas y 25,498 genes que codifican para 11,000 familias de proteínas (The Arabidopsis Genome, 2000). Análisis genéticos y moleculares han demostrado que la mayoría de las plantas, incluyendo *Arabidopsis*, contienen conjuntos similares de genes y proteínas

involucrados en las vías de señalización hormonales que controlan el crecimiento, la tolerancia al estrés y la defensa contra patógenos (Provart et al., 2016). Por lo tanto, el análisis de *Arabidopsis* nos permite acércanos al conocimiento general del funcionamiento intrínseco de otras especies vegetales de interés agrícola, ornamental y forestal.

2.1.1. La raíz de *Arabidopsis*

El sistema radicular de *Arabidopsis* está formado por una raíz primaria, raíces laterales y pelos radiculares. La formación de la raíz primaria comienza durante el desarrollo embrionario donde el cigoto obtenido por la fusión de los gametos masculinos y femeninos se divide asimétricamente para producir dos células: una célula apical que origina los cotiledones y otra célula basal más larga denominada hipófisis, la cual forma a la raíz (Scheres et al., 1994).

La raíz es una estructura cilíndrica conformada por varias capas celulares que surgen alrededor del centro quiescente mediante divisiones asimétricas del nicho de células iniciales. Los procesos celulares como la división, elongación y diferenciación provocan que en la raíz se distingan al menos cuatro zonas del desarrollo bien definidas: meristemo, meristemo basal, zona de elongación y la zona de diferenciación, esta última zona es importante debido a su potencial para producir raíces laterales (Fig. 1) (Barrada et al., 2015).

El meristemo se localiza en el ápice de la raíz y está constituido por el centro quiescente y el nicho de células iniciales. El centro quiescente se conforma por 4 células con muy poca capacidad de división pero que mantiene en su estado indiferenciado al nicho de células iniciales. Por su parte, el nicho de células iniciales consiste de la pro-vasculatura, el córtex/endodermis, la epidermis, la columnela y la cofia lateral. Las divisiones asimétricas del nicho dan forma a los tejidos que componen la raíz (Fig. 1) (Dolan et al., 1993; Dolan et al., 1994;).

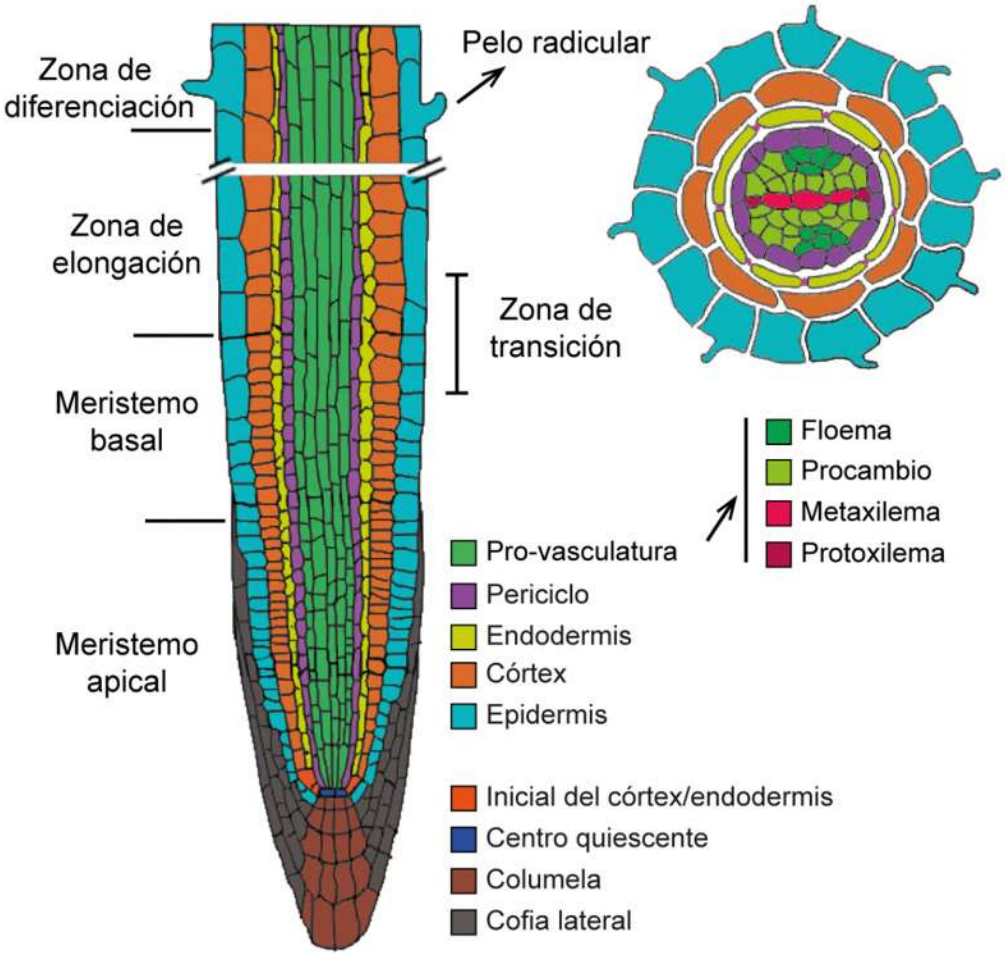

Figura 1. Organización celular de la raíz primaria de *Arabidopsis thaliana*. Esquema representativo del corte longitudinal (izquierdo) y transversal (derecho) de la raíz primaria que muestra la organización general de los tejidos en la zona del meristemo apical, el meristemo basal, la zona de elongación y la zona de diferenciación. Las divisiones asimétricas del nicho de células iniciales causan la formación de la columela, la cofia lateral, la pro-vasculatura o estela (integrada por el procambio, el floema, el metaxilema y protoxilema), el periciclo, la endodermis, el córtex y la epidermis (Modificado de De Smet et al., 2015).

Los pelos radiculares son estructuras delgadas y alargadas que surgen de las células epidérmicas en la zona de diferenciación de la raíz primaria y las raíces laterales (Fig. 1). Existen dos tipos de células epidérmicas, las cuales se diferencian de acuerdo a su posición: se conocen como tricoblastos a las células epidérmicas adyacentes a dos células corticales y atricoblastos a las células que están en contacto con una célula cortical. Bajo condiciones óptimas de crecimiento, los pelos radiculares solo surgen de los tricoblastos y su desarrollo se completa a través de una elongación muy rápida provocada por el incremento de la turgencia (Grierson

et al., 2014). Estas estructuras incrementan el contacto de la raíz con el suelo, implicando una mayor eficacia durante la captación de recursos (Jungk, 2001).

Las raíces laterales son órganos post-embrionarios formados a partir de células del pericicloo de la raíz primaria que incrementan la capacidad de las plantas para explorar el suelo (Casimiro et al., 2003). La células del pericicloo rodean al haz vascular compuesto por el floema y xilema; sin embargo, solo las células adyacentes al xilema mantienen actividad de iniciales y pueden especificarse como células fundadoras de las raíces laterales (Parizot et al., 2007). La especificación de las raíces laterales comienza entre el meristemo basal y la zona de elongación, mientras que en la zona de diferenciación se desarrollan los primordios mediante una serie de divisiones celulares y se establece *de novo* un centro quiescente y un nicho de células iniciales de las raíces laterales, cuya emergencia de la raíz parental procede a través de siete estadios primordiales (I-VII, Fig. 2) (Banda et al., 2019).

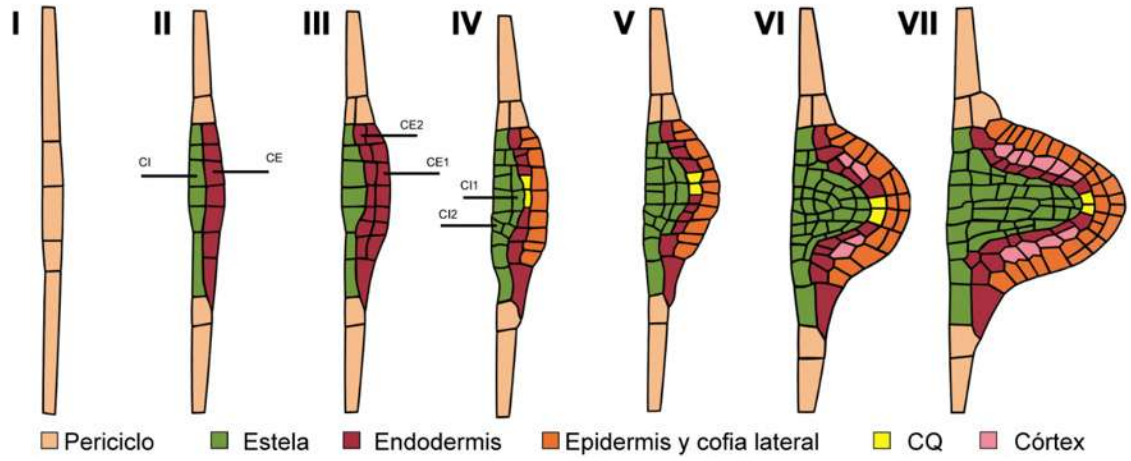

Figura 2. Etapas del desarrollo de las raíces laterales. Imagen representativa del desarrollo de los primordios de las raíces laterales a través de los siete estadios primordiales. El número corresponde con las capas que se forman por mitosis (Modificado de Torres-Martínez et al., 2019).

2.2. Programas de mantenimiento del crecimiento indeterminado de la raíz

La raíz es un órgano con un comportamiento altamente dinámico y adaptable debido a que tiene la capacidad de redirigir su crecimiento hacia el sustrato más óptimo mediante la alteración de la longitud y el diámetro de la raíz primaria, así como, la densidad, la longitud y el ángulo de crecimiento de las raíces laterales (López-Bucio et al., 2003; Gruber et al., 2013). Gran parte de esta capacidad se debe a la proliferación celular dentro del área meristemática, la cual se encuentra regulada por múltiples factores que inducen cambios en los ritmos de transición entre las fases que la componen.

2.2.1. Aspectos básicos de las divisiones en las plantas

En organismos multicelulares; incluyendo humanos, hongos y plantas, el ciclo celular se constituye por las fases Gap1 (G1), S, Gap2 (G2), mitosis (M) y citocinesis. En la fase S, el genoma de las células se duplica mediante la replicación del ADN promovida por las enzimas ADN polimerasas (Bryant, 2014). Por otra parte, la mitosis marca los pasos de segregación del genoma y el núcleo al pasar por la profase, metafase, anafase y telofase. La formación del huso mitótico es esencial para la mitosis debido a que interviene en el alineamiento, el apareamiento y la segregación de los cromosomas replicados (Bannigan et al., 2007; Herrmann et al., 2020). Finalmente, en la citocinesis el citoesqueleto y las vesículas de membrana promueven la separación del citoplasma y la fusión de las membranas para formar dos células hijas (Jürgens, 2005).

Las fases G1 y G2 funcionan como puntos de control que retrasan la progresión del ciclo celular hasta que las condiciones ambientales, energéticas y de proteínas sean las adecuadas para el crecimiento, la replicación y la mitosis (Jones et al., 2017). Alternativamente, las células pueden escapar del ciclo celular convencional y entrar a la fase G0 o a la endorreplicación. La fase G0 es una forma de arrestar al ciclo celular en la fase G1 durante un periodo de tiempo indefinido, manteniendo de esta forma a las células en reposo o quiescentes (Velappan et al., 2017), mientras que durante la endorreplicación las células incrementan los niveles de ploidía mediante la replicación el ADN sin entrar a la mitosis. En la raíz se ha relacionado

a la fase G0 y la endorreplicación con el desarrollo y la tolerancia al estrés (Heyman et al., 2013; Bhosale et al., 2018).

La progresión del ciclo celular depende de la actividad coordinada de las cinasas Ser/Thr CYCLIN-DEPENDENT KINASES (CDKs) y de las proteínas transitorias CYCLIN (CYC), las cuales interactúan y controlan la activación de las CDK. La quiescencia celular es regulada por las proteínas RETINOBLASTOMA RELATED1 (RBR1) que reprimen y secuestran a los factores de transcripción E2F (Desvoyes et al., 2005; Park et al., 2005; Wildwater et al., 2005). Los factores E2F son esenciales para el ciclo celular debido a que forman dímeros con las proteínas DIMERISATION PARTNERS (DP) y activan la expresión de genes asociados con la fase S y la replicación del ADN (Inzé y Veylder, 2006; Magyar et al., 2012). No obstante, la decisión de comenzar con un nuevo ciclo celular depende de las proteínas CYCD. *Arabidopsis* cuenta con siete miembros conservados de CYCD (CYCD1-7) que interactúan con las proteínas cinasas CDKA (Menges et al., 2007; Tank y Thaker, 2011). El heterodímero formado por CDKA-CYCD fosforila al represor RBR1, causando la liberación del factor E2F y la progresión de la fase G1 a S (Nakagami et al., 2002; Menges et al., 2006; Collins et al., 2015) (Fig. 3).

Las proteínas CYCA/B y las cinasas CDKA/B controlan la transición de la fase G2/M (De Veylder et al., 2007). Durante esta fase, las células evalúan la integridad de los cromosomas y activan mecanismos que reparan el daño y los errores en el ADN (Weimer et al., 2016). Además, se marcan las pautas para continuar con la mitosis o la endorreplicación, los cuales dependen de la acumulación de las proteínas CYCA, CYCB y CDKB (Boudolf et al., 2009). En este mismo sentido, se ha observado que la endorreplicación esta controlada por el complejo ANAPHASE PROMOTING COMPLEX/CYCLOSOME (APC/C), el cual es una ubiquitina E3 ligasa que promueve la degradación de las proteínas CYCA y CYCB en la fase G2 y M (profase) (Willems et al., 2020; Saleme et al., 2021) (Fig.3).

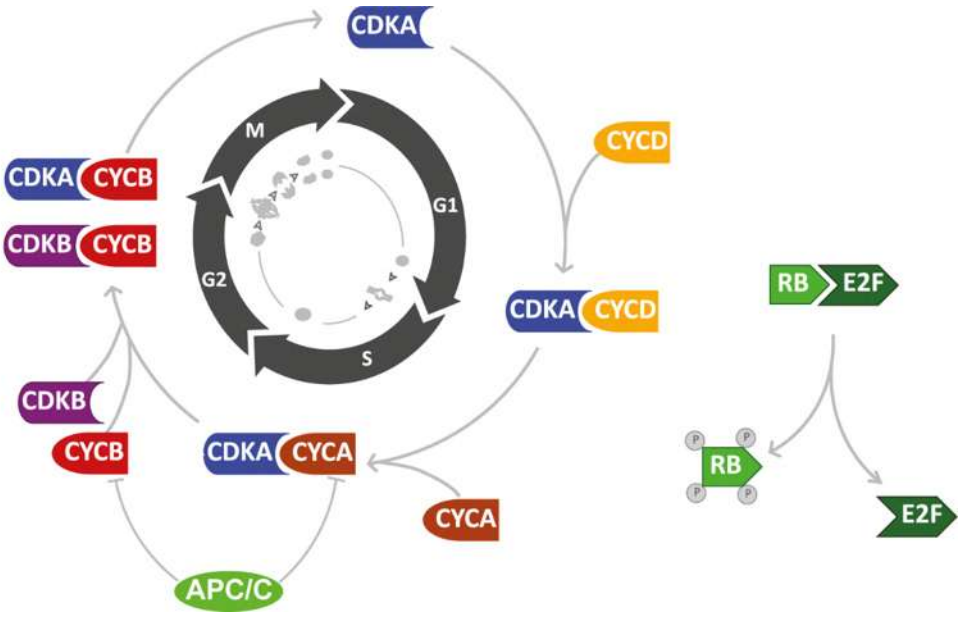

Figura 3. Representación del ciclo celular. La quiescencia o la fase G0 del ciclo celular la impone el secuestro del factor de transcripción E2F por el represor RBR. La fosforilación del represor RBR por el complejo CDKA-CYCD promueve el inicio del ciclo celular. las proteínas CYCB se asocian con las quinasas CDKB y CDKA para promover la transición entre las fase G2/M. Antagónicamente, el complejo ubiquitina E3 ligasa promueve el arresto del ciclo celular y la endorreplicación a través de la degradación proteasomal de CYCB y CYCA (Modificado de Scofield et al., 2014).

2.2.2. El mantenimiento del centro quiescente

Un factor importante para el funcionamiento del meristemo y el crecimiento de la raíz son los procesos que regulan la división celular y el mantenimiento del estado indiferenciado del centro quiescente y el nicho de células iniciales. El centro quiescente se integra por un grupo de cuatro células que se dividen en muy pocas ocasiones; sin embargo, se divide de forma asimétrica bajo condiciones especiales para auto-renovarse y para formar al nicho de células iniciales *de novo* (Cruz-Ramírez et al., 2013). El daño o el malfuncionamiento del centro quiescente puede causar la diferenciación del nicho de células iniciales y la pérdida del repositorio que sostiene el crecimiento indeterminado de la raíz, por lo que la principal función del centro quiescente es la de reprimir los factores hormonales y ambientales que causan la diferenciación celular (Sarkar et al., 2007).

El mantenimiento del centro quiescente se regula a través del factor de transcripción WUSHEL-RELATED HOMEOBOX5 (WOX5). El factor WOX5 inhibe

la división celular y retiene al centro quiescente en la fase G0 mediante la represión de las proteínas ciclina D3 (CYCD3;1 y CYCD3;3) (Forzani et al., 2014).

Los factores de transcripción SHORTROOT (SHR) y SCARECROW (SCR), de la familia de proteínas GRAS, también son importantes para el funcionamiento del meristemo. Estos factores forman el heterodímero de transcripción SHR-SCR que induce la expresión de WOX5 (Helariutta et al., 2000; Sarkar et al., 2007). SCR también interactúa con las proteínas SEUSS (SEU) y reclutan a la metiltransferasa SET DOMAIN GROUP4 (SDG4), la cual produce modificaciones epigenéticas que conducen a la expresión de WOX5 (Zhai et al., 2020). Adicionalmente, estudios moleculares mostraron que SCR interactúa con el represor RBR para suprimir la división asimétrica del centro quiescente (Wildwater et al., 2005; Cruz-Ramírez et al., 2013). En este sentido, los análisis de las líneas mutantes *scr* o *rbr* mostraron que el meristemo experimentó una acelerada división del centro quiescente. Por lo tanto, se sugiere que el módulo formado por SHR-SCR-RBR actúan rio arriba de WOX5 durante el control de la división (Cruz-Ramírez et al., 2013).

2.2.3. El estado indiferenciado del nicho de células iniciales

Experimentos de ablación con láser y las mutantes *wox5* mostraron que las células iniciales de la columela se diferenciaban rápidamente después de la alteración del centro quiescente (van den Berg et al., 1997; Sarkar et al., 2007;). Posteriormente, se demostró que el factor WOX5 en conjunto con los represores TOPLESS/TOPLESS-RELATED (TPL/TPR) y la HISTONA DESACETILASA19 (HDA19) reprimen la expresión transcripcional del factor CYCLING DOF4/ DNA-BINDING WITH ONE FINGER2.1 (CDF4/DOF2.1), el cual promueve la división y la diferenciación de las células iniciales de la columela (Pi et al., 2015). Interesantemente, se ha observado que CDF4/DOF2.1 también promueve la proliferación de la pro-vasculatura (Smet et al., 2019). Por lo tanto, WOX5 podría estar influyendo en el mantenimiento del nicho de células iniciales de la columela y la pro-vasculatura mediante la represión de CDF4/DOF2.1.

Las células de la columela eventualmente envían señales de retroalimentación para promover la diferenciación celular mediante la secreción del péptido señal CLAVATA3/ESR-RELATED40 (CLE40) y las proteínas cinasa ARABIDOPSIS

CRANKLY (ACR4) Y CLAVATA1 (CLV1). En este proceso, ACR4 fosforila a la subunidad catalítica PROTEIN PHOSPHATASE 2A-3 (PP2A-3) de la fosfatasa PP2A para inhibir la expresión de WOX5. De esta forma, CLE40 y WOX5 actúan de manera antagónica para mantener indiferenciadas a las células iniciales de la columela (Fig. 4) (Berckmans et al., 2019).

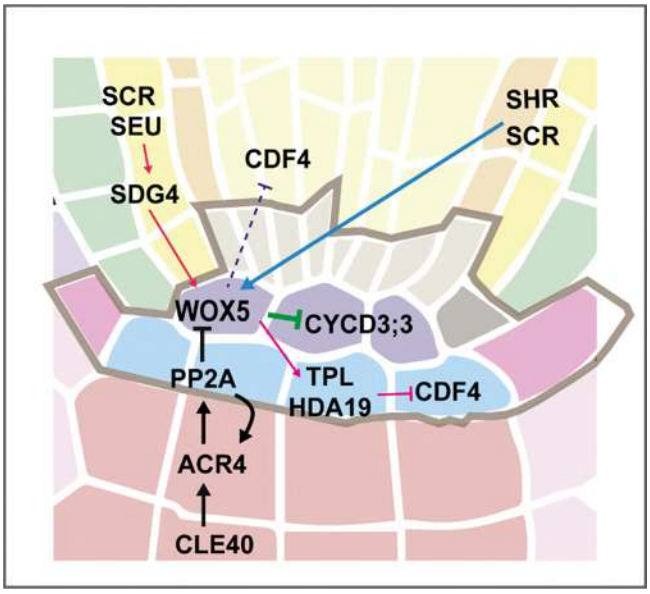

Figura 4. El mantenimiento indiferenciado del centro quiescente y el nicho de células iniciales. WOX5 es el regulador maestro del mantenimiento del estado indiferenciado del centro quiescente y el nicho de células iniciales en el meristemo de la raíz. SHR, SCR, SEU y SDG4 influyen positivamente sobre la expresión de WOX5 (color azul y rojo). WOX5 inhibe a las CYCD3;3 para reprimir la división del centro quiescente (color verde). WOX5 recluta a los represores TPL/TPLR-HDA19 para inhibir la división y diferenciación de las células iniciales de la columela mediada por CDF4 (color magenta). El péptido señal CLE40 promueve la diferenciación de las células de la columela a través de la inhibición de WOX5 por la quinasa ACR4 y la fosfatasa PP2A (color negro). La línea seccionada indica que WOX5 posiblemente regula la división y diferenciación de las células iniciales de la pro-vasculatura mediante la inhibición de CDF4 (Modificado de Choe y Lee, 2017).

Los factores de transcripción de la familia ETHYLENE RESPONSE FACTOR también controlan al centro quiescente. Específicamente, se ha observado que ERF115 induce la expresión de PHYTOSULFOKINE 5 (PSK5), un péptido con atributos de fitohormona que promueve el crecimiento de las plantas e induce la división del centro quiescente (Heyman et al., 2013). La proteína represora PROHIBITINA3 (PHB3) y las especies reactivas de oxígeno (ERO) regulan de forma antagónica la expresión de ERF115 en el meristemo, lo que indica que ERF115

integra las señales ambientales con la diferenciación de la raíz (Fig. 5) (Kong et al., 2018).

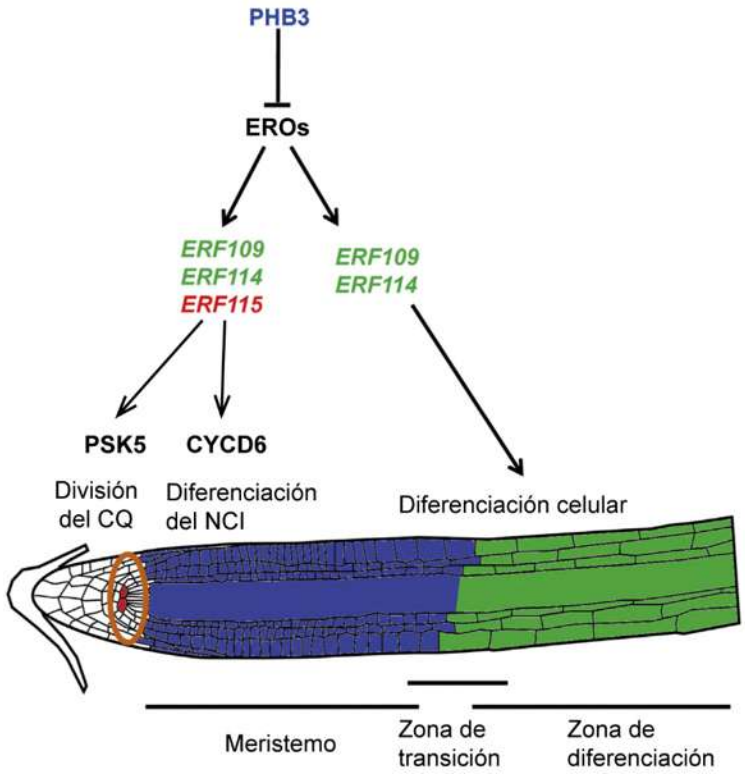

Figura 5. ERF115 promueve la división y diferenciación del nicho de células iniciales en la raíz de *Arabidopsis*. El módulo de señalización PHB3 y los factores de transcripción de la familia ETHYLELE RESPONSE FATOR (ERF109, ERF114 Y ERF115) coordinan las respuestas ambientales inducidas por la producción de ERO durante el mantenimiento indiferenciado del centro quiescente (QC) y el nicho de células iniciales (NCI) a través del control transcripcional de PSK5 y CYCD6 (Modificado de Kong et al., 2018).

2.3. El control de la división en el meristemo

En el meristemo de la raíz de *Arabidopsis* se producen dos tipos de división celular, las simétricas y las asimétricas. En la división simétrica se producen dos células hijas con la misma identidad, mientras que en la división asimétrica se forman dos células hijas con diferente identidad celular. Las células iniciales se dividen de forma asimétrica para generar una célula inicial que permanece adyacente al centro quiescente y una célula hija que se divide simétricamente hasta pasar por los procesos de elongación y diferenciación (Pillitteri et al., 2016).

2.3.1. Formación del córtex y la endodermis

Los tejidos del córtex y endodermis se forman tras completar dos divisiones asimétricas. Primero, las células iniciales del córtex/endodermis se dividen de forma asimétrica para mantener la reserva de células iniciales y para formar una célula hija con propiedades de inicial. Posteriormente, la célula hija se divide para generar a las células que componen al córtex y la endodermis. Estas divisiones asimétricas son dependientes de SHR y SCR debido a que ambas mutantes puntuales en *shr* o *scr* formaron solo una capa celular. El represor RETINOBLASTOMA-RELATED (RBR) suprimen la división asimétrica de las células iniciales del córtex/endodermis a través de su interacción con SCR. No obstante, la dimerización de SCR-SHR promueve la expresión de la proteína CYCD6;1, la cual se une a las proteínas quinasas CDKA y CDKB para estimular la división celular asimétrica (Fig. 6) (Sozzani et al., 2010; Weimer et al., 2012; Cruz-Ramírez et al., 2013).

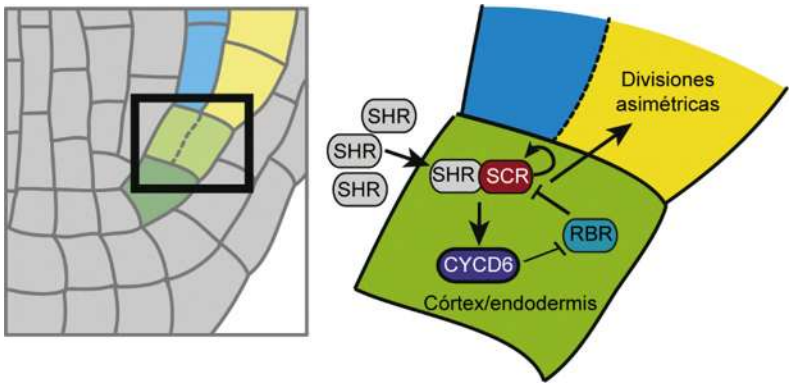

Figura 6. Control transcripcional de la división asimétrica de las células iniciales del córtex/endodermis. La proteína represora RBR previene la división asimétrica mediante el secuestro de SCR; sin embargo, la formación del complejo SHR-SCR activan la expresión de la proteína CYCD6 para promover la división a través la represión de RBR (Modificado de Drapek et al., 2017).

2.3.2. Formación del haz vascular

El haz vascular es la principal ruta de comunicación que transporta agua, nutrientes y moléculas de señalización a todas las partes de la planta, conectando principalmente al follaje con la raíz. El haz vascular se forma por las divisiones asimétricas del nicho de células iniciales de la pro-vasculatura. Algunos de los factores que promueve la división del haz vascular son ERF115, ERF114 y ERF109 a través de la activación de la proteína CYCD6;1 (Fig. 5) (Kong et al., 2018; Zhou et al., 2019). Las células iniciales de la pro-vasculatura se desarrollan durante la embriogénesis, en la etapa globular, después de la especificación de la hipófisis por acción del regulador maestro de la organogénesis MONOPTEROS (MP)/AUXIN RESPONSE FACTOR5 (ARF5) y sus genes blancos que codifican para los factores de transcripción tipo bHLH (basic helix-loop-helix), TARGET OF MONOPTEROS 5, 7 (TMO5, TMO7) y TMO5-LIKE 1, 2 y 3 (T5L1, 2 y 3) (Schlereth et al., 2010; De Rybel et al., 2013). Las células iniciales de la pro-vasculatura también son conocidas como células iniciales del procambio que se dividen de manera periclinal para formar las células precursoras de xilema (metaxilema y protoxilema), el floema, y el cambium. El factor TMO5 se expresa en las células iniciales de la pro-vasculatura y en las células precursoras de xilema. Se ha visto que interactúan con los factores de transcripción tipo bHLH LONESOME HIGHWAY (LHW) para promover la expresión del factor CDF4/DOF2.1, el cual estimula la división periclinal de las iniciales del procambium (De Rybel et al., 2013; Smet et al., 2019). Del mismo modo,

la formación del dímero TMO5-LHW promueve la expresión las proteínas LONELY GUY3, 4 (LOG3, LOG4) para incentivar el desarrollo de las células precursoras de xilema (Ohashi-Ito et al., 2013; De Rybel et al., 2014; Ohashi-Ito et al., 2014). Por lo tanto, los factores TMO5 y T5L son esenciales para promover la división de la pro-vasculatura y mantener el crecimiento indeterminado de la raíz. Por otra parte, la formación del floema dependen del factor SHR y sus genes blanco que codifican para NAC-REGULATED SEED MORPHOLOGY 1 (NARS1) y SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 2 (SND2) (Kim et al., 2020).

2.3.3. Formación de la columela

La formación de la columela está regulada por los factores de transcripción SOMBRERO (SMB) y FEZ de la familia NAC, los cuales funcionan de forma antagónica. Por un lado, el factor FEZ se expresan en las células iniciales de la columela para promover su división asimétrica. No obstante, SMB en conjunto con RBR inhiben la división a través de la represión de la actividad de FEZ (Willemsen et al., 2008; Bennett et al., 2014). La importancia de FEZ y SMB durante el desarrollo de la columela se comprobó a través de las mutantes *fez* y *smb,* que presentan menor y mayor número de capas de la columela, respectivamente, en comparación con las plantas WT.

2.4. Participación de la luz en la regulación del crecimiento de *Arabidopsis*

Organismos como las algas, las cianobacterias y las plantas recogen la luz solar y la transforman en ATP, NADPH y carbohidratos mediante la fotosíntesis (Johnson, 2016). La luz solar es un factor ambiental común que dirige el comportamiento y el crecimiento de la mayoría de los organismos (fotomorfogénesis). En contraste, la obscuridad conduce al crecimiento etiolado (escotomorfogénesis); en el cual, las plántulas experimentan una rápida elongación del hipocótilo, la formación de un gancho apical y el subdesarrollo de los cotiledones (Josse y Halliday, 2008). Paralelamente, la luz también puede inducir estrés oxidativo que perjudica la supervivencia de las plantas (de Jager et al., 2017).

2.4.1. Fotorreceptores vegetales

Las plantas emplean distintas proteínas fotorreceptoras para detectar las longitudes de onda que constituyen al espectro de la luz solar. Dentro de estos, se incluyen a los criptocromos (CRY1 y CRY2) de la luz azul (320 - 500n nm), los fitocromos (PhyA – PhyE) de la luz roja y roja lejana (600 - 750 nm) y al fotorreceptor UV-B RESISTANCE LOCUS8 (UVR8) de la luz ultravioleta B (Uv-B, 282 -320 nm) (Galvão y Fankhauser, 2015). Los criptocromos y los fitocromos son proteínas diméricas que permanecen inactivas en el citosol en ausencia de la luz y el fotorreceptor UVR8 existe como un monómero inactivo. Los fotorreceptores contienen un grupo cromóforo covalentemente unido a su estructura que sirve para detectar la luz. Los criptocromos poseen como grupo cromóforo a la coenzima flavina adenina dinucleótido (FAD); la cual, al ser estimulada por la luz azul inicia una serie de reacciones redox y de fosforilación que provocan un cambio estructural en los fotorreceptores, haciéndolos activos y desencadenando las señalizaciones dependientes de la luz (Legris et al., 2019). El grupo de fitocromos en *Arabidopsis* está compuesto de cinco proteínas fotorreceptoras de las cuales, solo PhyA detecta a la luz roja lejana mientras que PhyB – PhyE perciben a la luz roja.

Los fitocromos tienen como grupo cromóforo a una molécula tetrapirrolica lineal denominada como fitocromobilina. La detección de la luz roja y roja lejana por la fitocromobilina causa una serie de cambios conformacionales en los fitocromos, haciéndolos activos y disponibles para ser transportados hacia el núcleo por las proteínas FAR-RED ELONGATED HYPOCOTYL1 (FHY1) y FHY1-LIKE (FHL) (Burgie y Vierstra, 2014; Hoang et al., 2019). Los fotorreceptores UVR8 no contienen un grupo cromóforo bien definido, en su lugar, contienen varios residuos de triptófano que absorben la luz UV-B por medio de sus anillos aromáticos. Las proteínas REPRESSOR of UV-B PHOTOMORPHOGENESIS 1, 2 (RUP1, RUP2) se unen a los fotorreceptores UVR8 y facilitan la dimerización de los monómeros de UVR8 en respuesta a luz Uv-B (Fig. 7) (Yang et al., 2015).

En el núcleo, los fotorreceptores reprimen al regulador maestro de la fotomorfogénesis, la proteína ubiquitina E3 ligasa CONSTITUTIVE PHOTO-MORPHOGENESIS 1 (COP1) (Podolec y Ulm, 2018). Adicionalmente, los fotorreceptores también se asocian con los factores de transcripción PHYTOCHROME-INTERACTING FACTOR (PIFs) para modular su actividad. Un

ejemplo de esto es como el fotorreceptor PhyA fosforila a los factores PIF para promover su degradación y estimular el crecimiento fotomorfogenético en las plantas (Fig. 7) (Hoang et al., 2019).

La proteína COP1 funciona como un regulador central de la vía de señalización de la luz debido a que junto a SUPPRESOR OF PHYA-105 (SPA) y el complejo E3 ligasa CULLIN4-DAMAGED DNA BINDING PROTEIN 1 (CUL4-DDB1)-COP10-DE-ETIOLATED1 (DET1) promueven la proteólisis de múltiples factores de transcripción estimulados por la luz y de esta forma, reprimen las respuestas vegetales como la fotomorfogénesis, el fototropismo, la germinación, la desetiolación, la apertura de los estomas y el tiempo de floración (Ponnu and Hoecker, 2021). COP1 promueve la degradación proteasomal de los fotorreceptores en la oscuridad (Fig. 7) (Seo et al., 2004), en tanto que la actividad represora COP1/SPA estabiliza indirectamente a los factores PIF. Curiosamente, la proteína COP1 de *Arabidopsis* es homóloga de los animales y en ambos organismos regula los ciclos circadianos definidos por los tiempos de sueño-vigilia (Yi and Deng, 2005; Yu et al., 2008; Lee, 2019; Xu, 2020).

La activación de los fotorreceptores y la consecuente inhibición de COP1 y los factores PIFs incrementa la acumulación de múltiples factores de transcripción pertenecientes a las familias bZIP (basic leucine zipper), bHLH (basic helix-loop-helix), Myb (myeloblastosis), etc. Interesantemente, el factor de transcripción ELONGATED HYPOCOTYL5 (HY5) de la familia bZIP sobresale como un regulador maestro debido a que tiene un papel central en la mayoría de las respuestas estimuladas por la luz (Gangappa y Botto, 2016). Su mecanismo de acción involucra la asociación con otros factores de transcripción para estimular respuestas específicas. Por ejemplo, HY5 se asocia con HY5-HOMOLOG (HYH) para promover la fotomorfogénesis (Zhang et al., 2017). HY5 también interactúa con las proteínas B-BOX (BBX20-22) para inhibir la elongación del hipocótilo y promover la biosíntesis de antocianinas. Además el complejo HY5-BBXs estimula la desetiolación de las plantas, proceso dependiente de PhyA y PhyB (Bursch et al., 2020). Sumado a esto, los factores LONG HYPOCOTYL IN FAR-RED 1 (HFR1) de la familia bHLH y LONG AFTER FAR-RED LIGHT1 (LAF1) de la familia Myb pueden formar complejos con HY5 para estimular la fotomorfogénesis en respuesta a la luz roja lejana y a PhyA

(Jang et al., 2013). Los fotorreceptores PhyA y CRY1 estimulan la fotomorfogénesis a través de HFR1 (Duek y Fankhauser, 2003). Los fotorreceptores UVR8 estabilizan la formación del complejo HY5-BBXs (BBX20-22) durante la tolerancia a luz UV-B (Podolec et al., 2022). Además de promover la fotomorfogénesis, la luz azul y CRY1 estimulan a HY5 para modular la actividad de los fotorreceptores UVR8 y PhyB durante la tolerancia a la luz UV-B y en la germinación de las semillas (Stawska y Oracz, 2019; Tissot y Ulm, 2020).

2.4.2. Los fitocromos como reguladores del crecimiento de la raíz

La fotomorfogénesis de la raíz de *Arabidopsis* depende de la percepción de la luz roja a través de los fitocromos. Los fotorreceptores PhyA y PhyB se han visto involucrados en la regulación de la elongación y el gravitropismo de la raíz primaria en plantas etioladas (Correll y Kiss, 2005). PhyA probablemente controla la elongación de la raíz a través del control de la expresión de la acuoporina TIP2 (Uenishi et al., 2014). Se ha observado que los fitocromos podrían tener roles complementarios en el crecimiento de la raíz primaria y las raíces laterales, ya que en las mutantes *phyB,* el crecimiento de la raíz se reduce más que en las plantas silvestres expuestas a luz blanca, mientras que en las mutantes *phyA-211* decrece la raíz primaria solo cuando son expuestas a una baja intensidad lumínica (Ha et al., 2018; Kumari et al., 2019).

La emergencia de las raíces laterales y las raíces adventicias también disminuye en las mutante *phyA-211* y *phyB-9* expuestas a cantidades bajas de luz blanca (Kumari et al., 2019). Estos resultados indican que los fitocromos modulan el crecimiento de la raíz de manera dependiente de la afluencia de la luz. Los genes *PhyA* y *PhyB* se expresan en la punta de la raíz y en la vasculatura de la zona de diferenciación donde controlan el crecimiento debido a que la luz roja puede transmitirse de forma autónoma a través de la vasculatura y el suelo (Somers y Quail, 1995; Hall et al., 2001; Mo et al., 2015; Lee et al., 2016). De forma complementaria, se ha observado que los fitocromos promueven la acumulación del factor HY5 en el follaje y este a su vez, se transporta a través de la vasculatura hacia los primordios de las raíces laterales, regulando su crecimiento (Chen et al., 2016; van Gelderen et al., 2018). Fenotípicamente, las mutantes *hy5* son parecidas a las

plantas etioladas debido a la exagerada elongación del hipocótilo. No obstante, las mutantes *hy5* presentan una mayor densidad de las raíces laterales en comparación con las plantas silvestres. Posteriormente, se encontró que HY5 promueve la expresión de los reguladores negativos de la vía de señalización de las auxinas AUXIN RESISTANT2 (AXR2)/IAA17 y SOLITARY ROOT1 (SLR)/IAA14 (Cluis et al., 2004). La pérdida de función de *HY5* condujo a una desregulación de AXR2/IAA17 y SLR/IAA14, los cuales reprimen la emergencia de las raíces laterales, por lo que los fitocromos controlan el crecimiento de la raíz de *Arabidopsis* a través de la regulación transcripcional del factor HY5.

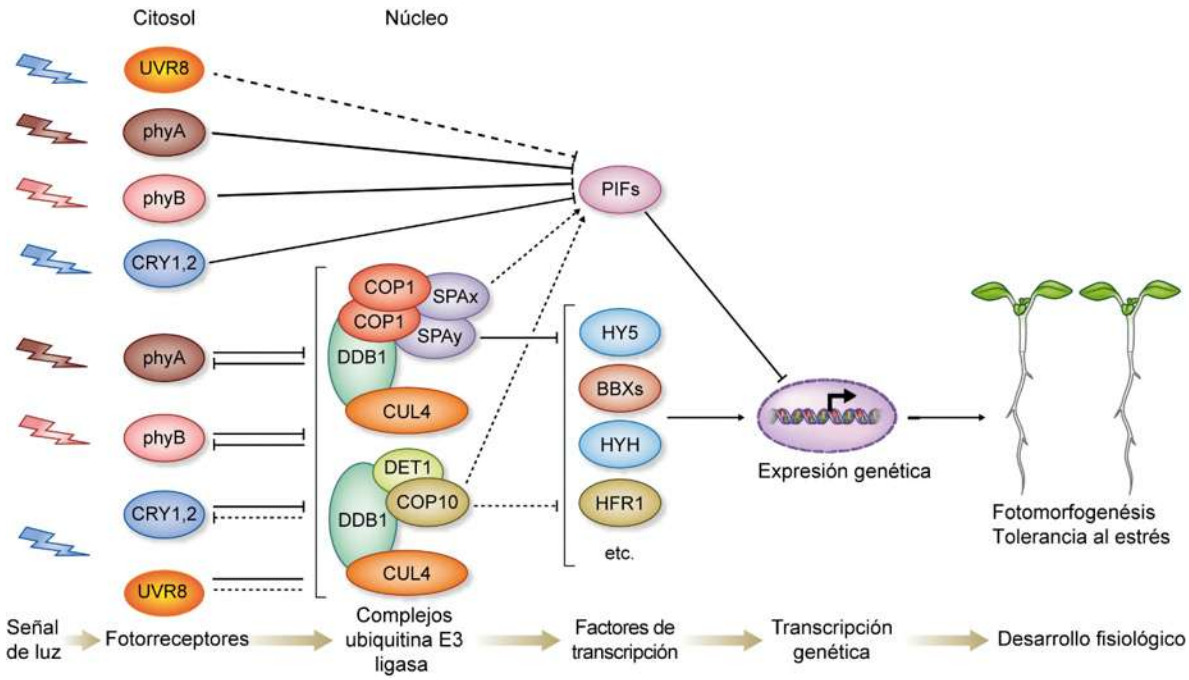

Figura 7. Vía de señalización estimulada por la luz en las plantas. Los fotorreceptores son proteínas citosólicas que tras ser activadas por la luz son transportadas hacia el núcleo donde modulan la represión de las proteínas COP1/SPA y PIFs. De esta manera estabilizan a los factores de transcripción HY5, BBXs, HYH, HFR1, etc., los cuales dirigen la expresión de genes asociados con la fotomorfogénesis, la tolerancia al estrés, etc. Las proteínas COP1/SPA se asocian con el complejo ubiquitina E3 ligasa DET1-COP10-DDB1-CUL4 para impulsar la degradación de los factores de transcripción mediante el proteasoma 26S (Modificado de Xu, 2020).

2.4.3. El papel de los fitocromos en el control del estrés

En condiciones naturales, las raíces de las plantas no se exponen a la luz ya que se encuentran enterradas en el suelo. Sin embargo, algunos factores ambientales y animales pueden remover la tierra lo que provoca la exposición de las raíces a la luz. La intensidad alta de luz puede inducir la generación de especies

reactivas de oxígeno (EROs) mediante un desacoplamiento de electrones en los fotosistemas I y II (PSI, II) durante la fotosíntesis. Las ERO son radicales libres muy reactivas e inestables que en altas concentraciones pueden imponer un daño oxidativo, lo que lleva a una reducción del crecimiento de las raíces o a la muerte celular. Los fitocromos son necesarios para regular la producción de las ERO cuando la raíz primaria de *Arabidopsis* se expone a la luz. Por ejemplo, en la raíz primaria de las mutantes *phyB* se incrementó significativamente la producción del peróxido de hidrógeno (Ha et al., 2018). Posteriormente se descubrió que el fotorreceptor PhyB estimula la biosíntesis del ácido abscísico (ABA) en el follaje y las señales derivadas viajan a través de la vasculatura hacia la raíz para promover la expresión de *AtPER1*, una peroxiredoxina antioxidante que median las reacciones de desintoxicación del peróxido de hidrogeno (Gil et al., 2018; Ha et al., 2018).

La desetiolación y las respuestas de evasión de las sombras son procesos regulados por los fitocromos de forma dependiente de la fluencia de luz. En presencia de la luz blanca, PhyB restringe la elongación del hipocótilo mediante la fosforilación de los factores PIF4/5 y a través de la estabilización del complejo formado por el represor AUX/IAA con los factores de transcripción AUXIN RESPONSE FACTOR (ARF). En cambio, durante la evasión de las sombras el fotorreceptor PhyA impide la expresión génica mediante la estabilización del complejo AUX/IAA (Xu et al., 2018; Yang et al., 2018; Ma y Li, 2019).

La desetiolación se describe como el proceso regulado por los fitocromos PhyA y PhyB en el cual las plantas pasan del crecimiento escotomorfogénico hacia el fotomorfogénico. Los factores PIF1/3 y HY5 actúan de forma antagónica para controlar la expresión de genes asociados a la producción de ERO. En la oscuridad, PIF1/3 reprimen la expresión génica; en cambio, durante la desetiolación el factor HY5, de forma dependiente de los fitocromos, interactúa con PIF1/3 para promover la expresión de los genes *APX2*, *ZAT10*, *SIB1*, *ERF4* y *NDB2* que codifican para enzimas que catalizan la producción de ERO (Chen et al., 2013). Una elevada exposición a luz roja promueve la acumulación HY5 y se estimula la expresión del gen *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), un regulador positivo de estrés oxidativo y la muerte celular (Chai et al., 2015).

2.5. Impacto del estrés ambiental sobre el crecimiento y la viabilidad de las plantas

2.5.1. Influencia del estrés abiótico

El estrés abiótico se manifiesta cuando las plantas perciben cambios en el ambiente como la temperatura y la radiación, así también en las propiedades del suelo como la disponibilidad de nutrientes, la sequía, la salinidad y la contaminación con metales pesados o metaloides (Hasanuzzaman et al., 2020).

Generalmente, las plantas responden al agobio ambiental mediante la producción de ERO, en las que se incluye al anión superóxido ($O_2^{\bullet -}$), el radical hidroxilo ($^{\bullet}OH$), el peróxido de hidrógeno (H_2O_2), el singlete de oxígeno ($^1O_2$), entre otros. Las ERO son producidos por el metabolismo oxidativo del O_2 durante la fotosíntesis y la cadena respiratoria dentro de los cloroplastos y la mitocondria, respectivamente. Además, las reacciones enzimáticas antioxidantes en el peroxisoma, el citoplasma, el retículo endoplásmico, la pared celular y el apoplasto también contribuyen a la generación de ERO (Hasanuzzaman et al., 2020). Parte de la importancia de las ERO reside en que el anión superóxido y el peróxido de hidrógeno son precursores del radical hidroxilo, el cual es la especie con mayor potencial de oxidación. Específicamente, el peróxido de hidrógeno resulta de la dismutación del anión superóxido, mientras que el radical hidroxilo se forma después de las reacciones de Fenton entre los iones ferroso (Fe^{2+}) y cuproso (Cu^{+}) con el peróxido de hidrógeno (Winterbourn, 2008), radical que puede difundirse a través de las acuoporinas de las membranas plasmáticas, estableciendo comunicaciones locales de célula a célula y de larga distancia (Fig. 8) (Bienert y Chaumont, 2014).

Las ERO son causantes del estrés oxidativo, pero también funcionan como moléculas de señalización o segundos mensajeros, lo cual depende de su potencial redox. Por lo tanto, los procesos que controlan su homeostasis son esenciales para mantener la integridad celular y el desarrollo en los organismos. El mantenimiento de la homeostasis es regulado por enzimas antioxidantes como la superoxido dismutasa, SOD; catalasas, CAT; ascorbato peroxidasas, APX; NADPH oxidasas,

glicolato deshidrogenasa y antioxidantes no enzimáticos de bajo peso molecular como ácido ascórbico, ASA; glutatión, GSH; flavonoides, y oxilipinas, que catalizan la transformación del anión superóxido al peróxido de hidrógeno y al agua. Sin embargo, las mismas reacciones enzimáticas pueden incrementar la producción de ERO cuando el sistema antioxidante es superado por el estrés ambiental (Fig. 8) (Carocho y Ferreira, 2013).

Los procesos fisiológicos en los cuales intervienen las ERO se manifiestan durante la tolerancia al estrés (Choudhary et al., 2020); la fotosíntesis (Exposito-Rodriguez et al., 2017); la apertura de los estomas (Qi et al., 2017), la defensa contra patógenos (Fones y Preston, 2012) y el ciclo celular (Diaz-Vivancos et al., 2010). Asimismo, la modulación de varias respuestas morfológicas en la raíz primaria de *Arabidopsis* como la expansión celular (Guillou et al., 2022), la formación de los pelos radiculares (Mangano et al., 2018), la división y la diferenciación del centro quiescente y el nicho de células iniciales, a través del módulo de señalización ERF115-PSK5 (Kong et al., 2018).

Durante el estrés oxidativo, las ERO provocan un daño generalizado sobre las células y activan los mecanismos de destrucción celular, conocidos como muerte celular programada y la necrosis. Los blancos principales de las ERO son la membrana plasmática y las moléculas de ADN. El daño a la membrana plasmática ocurre por la peroxidación de los lípidos, la oxidación de los residuos amino de las proteínas y por el incremento en la concentración intracelular del calcio (Ca^{2+}) (Sweetlove y Møller, 2009; Görlach et al., 2015; Gaschler y Stockwell, 2017). De manera general, el estrés oxidativo incrementa la permeabilidad de las membranas plasmáticas provocando la fuga de moléculas que activan la muerte celular como el citocromo C (Cyt-C) mitocondrial (Chen et al., 2003). Además, la oxidación de los aminoácidos puede incrementar la formación de proteínas aberrantes o mal plegadas provocando el estrés en el retículo endoplásmico (RE) y la muerte celular (Howell, 2013). El daño al ADN ocurre en múltiples niveles, desde el daño especifico a las bases nitrogenadas y las desoxirribosas hasta la abstracción de los nucleótidos, la formación de aductos y la fragmentación de una o las dos cadenas del ADN (Cadet y Wagner, 2013).

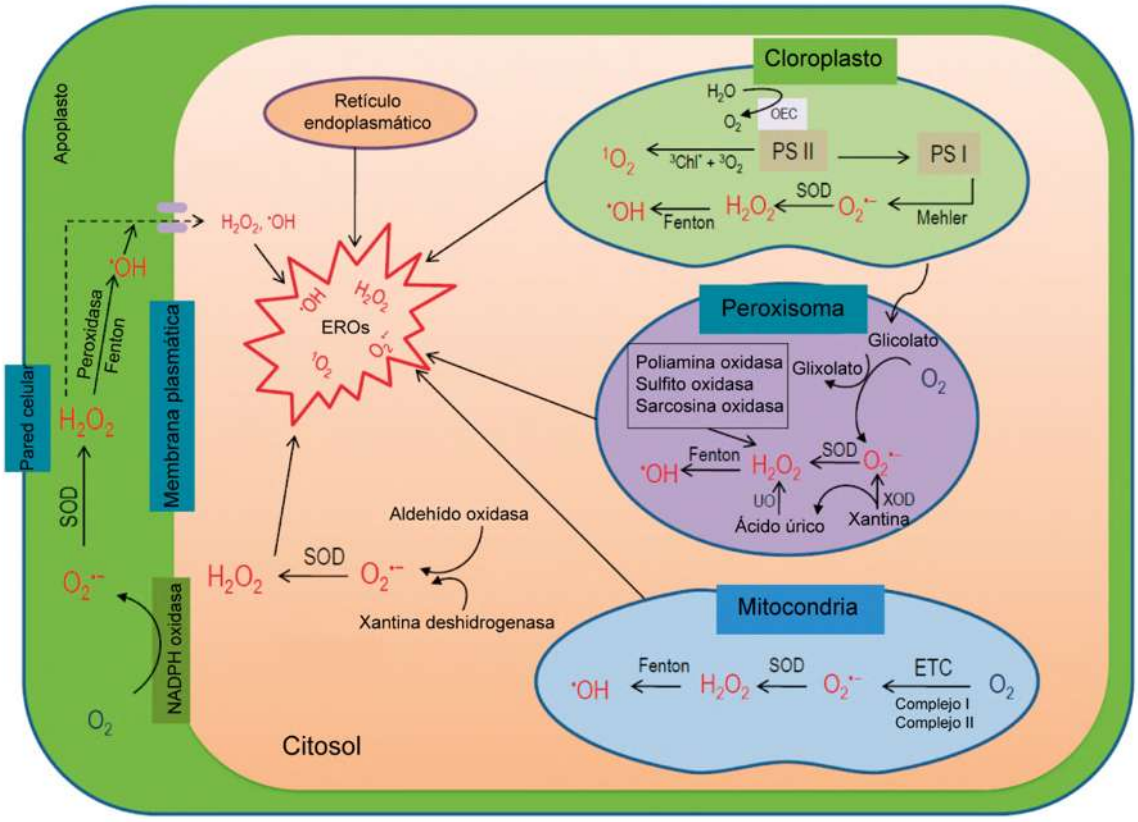

Figura 8. Producción de especies reactivas de oxígeno (ERO). El cloroplasto y la mitocondria son la principal fuente de biosíntesis de ERO por el desacoplamiento de electrones entre el PSI y PSII el complejo I y II durante la fotosíntesis y la cadena transportadora de electrones. Múltiples reacciones enzimáticas llevadas a cabo por SOD, NADPH oxidasas, glicolato deshidrogenasa, las reacciones Fenton en el citosol, el peroxisoma, el retículo endoplásmico, la membrana plasmática y el apoplasto catalizan la formación de ERO a partir del O_2 y $O_2^{\bullet-}$ (Modificado de Hasanuzzaman et al., 2020).

2.5.2. Influencia del estrés biótico

Las plantas establecen relaciones de competencia, comensalismo, mutualismo y parasitismo con una amplia variedad de microorganismos. Las interacciones mas comunes son el comensalismo y el mutualismo, donde uno o las dos especies se benefician de su interacción sin perjudicarse (Wu et al., 2009). Durante el parasitismo, por el contrario, los microorganismos actúan como fitopatogénos que entran a los tejidos vegetales, se alimentan de ellos y causan enfermedades (Mansfield et al., 2012). Los fitopatogénos pueden entrar al interior de las plantas a través de heridas o aperturas naturales como los estomas, como en el caso de

Pseudomonas syringae, la cual infecta una amplia variedad de especies, provocando desde manchas necróticas hasta la muerte del hospedero (Melotto et al., 2006; Sugio y Hogenhout, 2012). La entrada de los fitopatogénos también es asistida por enzimas extracelulares que degradan la pared celular y proporcionan una ruta hacia al apoplasto y el citoplasma de las células vegetales (Toth et al., 2003; Lee et al., 2014;). Una vez dentro de las plantas, los fitopatogénos secretan fitotoxinas y proteínas efectoras que suprimen la inmunidad o alteran respuestas fisiológicas a través de cambios en los niveles hormonales (Mendoza-Vázquez et al., 2019). Por ejemplo, la bacteria *Pseudomonas aeruginosa* secreta a la fitotoxina piocianina como un factor de virulencia que perjudica el crecimiento de la raíz de *Arabidopsis* a través de la producción de las ERO y la modulación de la vía de señalización del etileno (Ortiz-Castro et al., 2014).

Algunas bacterias, hongos y oomicetos pueden comportarse como biótrofos o necrótrofos. Los biótrofos dependen de las células vivas, mientras que los necrótrofos causan la muerte de los tejidos para obtener su alimento. Las plantas reaccionan ante la infección por un patógeno biótrofo mediante la estimulación de mecanismos de defensa denominados como el sistema inmune vegetal. La respuesta hipersensible (RHS) forma parte del sistema inmune y en este, las plantas promueven una muerte localizada en el sitio de infección con el objetivo de evitar la proliferación y la diseminación de los fitopatogénos (Bashir et al., 2013). Sorprendentemente, diversos microorganismos necrotróficos sacan provecho de esta característica para inducir la muerte celular y, de este modo, beneficiarse de los nutrientes de los tejidos vegetales muertos (Laluk y Mengiste, 2010; Dickman y de Figueiredo, 2013). Entonces, esto nos indica que la muerte celular es una característica común de la resistencia y la sensibilidad a los microorganismos biótrofos y necrótrofos. Independientemente del estímulo que lo origine, el daño representa un considerable gasto energético debido a la activación de diversas vías de señalización dependientes del ATP (Jose et al., 2020). Este gasto energético puede repercutir en otras actividades como los programas del crecimiento y el desarrollo, y en ultimas instancias acelerar incluso la senescencia y la muerte de las plantas (Azad et al., 2008).

2.6. Estudios comparativos de la muerte celular programada en las células animales y vegetales

En los organismos eucariontes existen tres tipos principales de muerte celular que difieren en su morfología y en sus mecanismos moleculares de activación y/o propagación de la señal: la apoptosis, la autofagia y la necrosis. La necrosis se caracteriza por la lisis de la membrana plasmática y por la filtración de los desechos citoplasmáticos al espacio extracelular (Dickman y de Figueiredo, 2013). La necrosis ocurre de manera caótica y no se necesita del uso de energía para activarla o de vías de señalización, por lo que los organismos no poseen un control genético sobre estos eventos (Dickman y de Figueiredo, 2013). La apoptosis, por otra parte, describe al tipo I de la muerte celular por el cual los mamíferos descomponen de manera controlada y selectiva a las células innecesarias o disfuncionales que representan un riesgo, por ejemplo las células cancerígenas o tumorales (Lowe y Lin, 2000). La autofagia normalmente describe a un proceso de supervivencia celular que se activa bajo estrés oxidativo, estrés metabólico y estrés biótico, es decir se presenta cuando las células tienen deficiencia de energía, aminoácidos y nutrientes; cuando el estrés oxidativo causa daño en los orgánulos y el contenido citoplasmático o cuando las células han sido infectadas por patógenos o por la presencia de fitotoxinas. Sin embargo, la autofagia excesiva puede conducir a la apoptosis mediante mecanismos de señalización modulados por las proteínas B-CELL LYMPHOMA-2, (BCL-2), proteasas, el factor de transcripción p53, entre otros (Flannagan et al., 2012; Su et al., 2013; Chang et al., 2022).

En las células vegetales no existe una apoptosis o autofagia verdadera. En su lugar es posible encontrar una muerte celular análoga a la apoptosis y una mediada por la vacuola, análoga a la autofagia (Fig. 9). El proceso apoptótico implica la condensación de la cromatina (picnosis), la fragmentación del ADN, la permeabilización de la mitocondria, la liberación del Cyt-C al citoplasma, la producción de ERO y la degradación celular asociada a cisteína proteasas análogas a las caspasas, llamadas metacaspasas. Sin embargo, las células vegetales cuentan con una pared celular que impide la formación de cuerpos apoptóticos y carece de un sistema fagocitario mediado por células móviles como fagocitos y macrófagos, clásicos de la apoptosis (Fig. 9a) (Erwig y Henson, 2008; Dickman et

al., 2017). La muerte mediada por la vacuola, por su parte, ha sido comparada con la autofagia animal. La autofagia se define por la formación de estructuras de doble membrana llamadas autofagosomas que envuelven materiales intracelulares para su eliminación por medio de la degradación autolisosomal (Flannagan et al., 2012). Las células vegetales también carecen de un autolisosoma, así que en su lugar usan a las enzimas de las vacuolas líticas para degradar a los materiales intracelulares y promover la muerte celular. Este proceso es dependiente de la cisteína proteasa VASCULAR PROCESSING ENZIME (VPE), la cual cataliza la maduración y activación de las hidrolasas vacuolares encargadas de la proteólisis celular, como las aspartato y cisteína proteasas. Morfológicamente, comienza con la expansión vacuolar por la fusión de las vesículas citoplasmáticas y finaliza con el rompimiento de la vacuola, lo cual permite la liberación de las hidrolasas acidas que rápidamente digieren a los orgánulos celulares y los residuos citoplasmáticos (Fig. 9c) (Hara-Nishimura y Hatsugai, 2011). Curiosamente, la enzima VPE es un análogo funcional de la proteasa ejecutora de la apoptosis, CAS-1 (Kinoshita et al., 1995), por lo que, esta correlación sugiere que la degradación celular mediada por la vacuola podría estar relacionada con la autofagia en los mamíferos (Dickman et al., 2017).

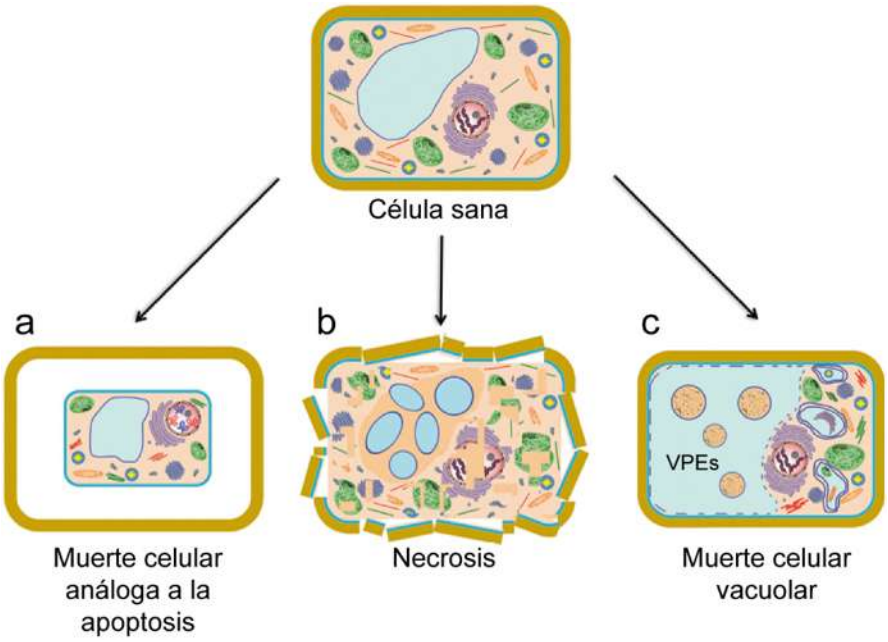

Figura 9. Características morfológicas de la muerte celular en las plantas. a) La muerte celular análoga a la apoptosis. b) Necrosis. c) La muerte celular vacuolar (Modificado de Floyd et al., 2015).

2.7. Mecanismos moleculares que controlan la muerte celular programada de las células vegetales

Se reconocen al menos dos mecanismos por los cuales las células estimulan la muerte celular en los animales y plantas: la vía intrínseca y extrínseca. Existe una relación muy estrecha entre los dos mecanismos. Por una parte, podemos encontrar que las vías de señalización intrínseca y extrínseca comparten algunos elementos de señalización, los cuales finalizan con la activación de las caspasas o proteínas análogas a las caspasas (por ejemplo, las metacaspasas), cisteína proteasas que degradan a múltiples proteínas y que causan la fragmentación del ADN.

2.7.1. La vía intrínseca

La vía intrínseca ocurre por la permeabilización de la membrana de la mitocondria y la liberación del Cyt-C al citoplasma. El núcleo de señalización de la vía intrínseca en la apoptosis está integrada por miembros de la familia de proteínas B-CELL LYMPHOMA-2 (BCL-2) y las caspasas. Las proteínas BCL-2 incluyen a tres subgrupos: i) proteínas efectoras proapotóticas BCL-2 (pBCL), BAX y BAK, que forman poros en la membrana mitocondrial externa para liberar el Cyt-C al

citoplasma; ii) proteínas BH3-only (BIM, BID, BAD Y NOXA) que activan a las proteínas pBCL en respuesta al estrés oxidativo (ERO), al Ca^{2+} y al daño al ADN; y iii) proteínas antiapoptóticas BCL-2 (aBCL), BCL2, BCL-XL y MCL1, las cuales se unen y neutralizan a las proteínas pBCL y BH3-only (Tait y Green, 2010). El Cyt-C junto a la proteína APOPTOTIC PEPTIDASE ACTIVATING FACTOR1 (APAF1) y ATP forman al apoptosoma, una plataforma de activación de la procaspasa iniciadora CAS-9, encargada de iniciar la cascada de escisiones que promueven la apoptosis a través de las caspasas ejecutoras CAS-3, 6 y 7 (Fig. 10a) (Li et al., 1997).

El estrés oxidativo y la acumulación intracelular del Ca^{2+} provocan la despolarización de la membrana de la mitocondria y la liberación del Cyt-C. Aunque las plantas carecen de un apoptosoma, se ha observado a través de ensayos de complementación que el Cyt-C de *Arabidopsis* interactúa con cisteína proteasas como RESPONSIVE TO DEHYDRATION21 (RP21). Además, en el mismo estudio se encontró que Cyt-C se asocia con NUCLEOSOME ASSEMBLY PROTEIN1 (NRP1), una proteína esencial en el mantenimiento de la integridad del ADN (Fig. 10b) (Martínez-Fábregas et al., 2013).

Hasta la fecha no se han identificado genes homólogos de las caspasas y del grupo de proteínas BCL-2 en las plantas. Sin embargo, algunos reportes sugieren mecanismos similares a los dirigidos por las proteínas BCL-2 en la vía intrínseca de la apoptosis. Esta hipótesis se basa en los hallazgos de Ishikawa et al., (2011), en los cuales se detalla que el genoma de *Arabidopsis* contiene al gen *BAX INHIBITOR-1* (*BI-1)* que codifica para la proteína inhibidora de BAX. Por lo tanto, es probable que las plantas contengan un análogo funcional de esta proteína proapotótica. Además, en líneas transformadas de arroz y *Arabidopsis* se observó que los genes de animales *BAX* y *BCL2* se expresan en membrana de la mitocondria y que ambos genes regulan la muerte celular de forma dependiente de la concentración de ERO (Deng et al., 2011; Yoshinaga et al., 2005). Por un lado, la expresión de BAX incrementó la producción de ERO lo que condujo a la muerte celular (Yoshinaga et al., 2005). En cambio, la sobreexpresión de *BCL2* disminuyó los niveles de Ca^{2+} intracelulares, la producción de ERO y suprimió la expresión de

los genes *OsVPE2* y *OsVPE3*, los cuales promueven la muerte celular (Deng et al., 2011; Kim et al., 2014).

2.7.2. La vía extrínseca

La activación de la vía extrínseca en los animales comienza con la interacción de los receptores RM con sus ligandos (L) específicos en la superficie celular (FAS-L, TNF-L). En los animales, la estimulación de los RM, como TUMOR NECROSIS FACTOR1 (TNFR1), TNF-RELATED APOPTOSIS-INDUCING LIGAND RECEPTOR 1/2 (TRAILR1/2) y FAS activan diferentes proteínas para promover la apoptosis. En una de ellas, reclutan a la proteasa CAS-8 mediante el dominio especifico FAS-ASSOCIATED PROTEIN WITH DEATH DOMAIN (FADD) para escindir a la proteína pro-apotótica BID en tBID, el cual posteriormente se transloca a la membrana interna de la mitocondria para activar a las proteínas BAX y BAK (Brentnall et al., 2013). Adicionalmente, los RM también activan a las proteínas cinasas Ser/Thr MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) para regular el proceso apoptótico (Farley et al., 2006; Tran et al., 2001). Las cinasas MAPK tiene una función dual que depende del estímulo. Por una parte, se ha observado que C-JUN N-TERMINAL KINASE1 y 2 (JNK1, 2) fosforilan a BID para impedir la apoptosis (Prakasam et al., 2014). En cambio, cuando ocurre un daño en el ADN, EXTRACELLULAR SIGNAL-REGULATED KINASE 1 y 2 (ERK1, 2) fosforilan al factor de transcripción p53 cuyo gen blanco es BAX, por lo que la activación de p53 concluye con la liberación del Cyt-C (Fig. 10a) (Persons et al., 2000).

En las plantas se conoce relativamente poco sobre el proceso de señalización de la vía extrínseca en comparación con los mamíferos. No obstante, diversos estudios han señalado que las proteínas MAPK estimulan la muerte mediante la activación de las proteínas codificadas por los genes R, con función de receptores transmembranales. Las proteínas R reconocen a los factores de virulencia de los fitopatogénos, que incluyen a las proteínas efectoras de avirulencia (Avr) específicas y fitotoxinas no específicas del hospedero denominadas como patrones moleculares asociados a los microbios o a los patógenos (PMAMs o PMAPs) (Jose et al., 2020; Petit-Houdenot y Fudal, 2017). Las proteínas R pertenecen al subgrupo de proteínas LRR (leucine-rich repeat) de la familia de proteínas cinasas Ser/Thr

RLK (receptor like kinases), análogas a las proteínas RLK de los mamíferos (Jose et al., 2020). Tras la percepción de su ligando, las proteínas R inician una cascada de fosforilación que involucra a otras proteínas cinasas RLK citoplasmáticas (RLKC), los cuales, a su vez, tienen como blancos moleculares directos a importantes reguladores como las proteínas MAPKs y las enzimas NADPH oxidasas/RBOH (respiratory burst oxidase homolog) encargadas de la producción de las ERO (Jose et al., 2020; Liang y Zhou, 2018; Marcec y Tanaka, 2022).

Las cinasas MAPK promueven la expresión de genes asociados con la defensa contra patógenos en respuesta al ácido jasmónico, como *VEGETATIVE STORAGE PROTEIN1* (*VSP1*) (Menke et al., 2004). Sin embargo, en este caso particular, son unas promotoras muy activas de la muerte celular cuando el estrés supera el umbral de tolerancia mínimo (Ren et al., 2002). Se ha observado, por ejemplo, que la MPK6 fosforila y activa a las enzimas cisteína proteasas, análogas de las caspasas, VPE y γVPE, necesarias para el desmantelamiento del ADN y la progresión hacia muerte celular mediada por la vacuola (Li et al., 2012; Menke et al., 2004). Además, las proteínas R o RLK fosforilan a las enzimas RBOH para incrementar la concentración de Ca^{2+} intracelular y la producción de ERO, las cuales provocan la permeabilización de la membrana mitocondrial y la liberación del Cyt-C al citoplasma (Fig. 10b). Al mismo tiempo, las ERO dañan al ADN y provocan la activación del factor de transcripción análogo a p53, SUPPRESOR OF GAMMA RESPONSE1 (SOG1) el cual promueve la MCP a través de las proteínas quinasas OXIDATIVE SIGNAL INDUCIBLE 1 (OXI1) y MPK6/3 (Hendrix et al., 2020; Ogita et al., 2018).

2.7.3. La fragmentación del ADN mediado por las proteasas de las plantas

Las plantas no poseen caspasas verdaderas, en su lugar contienen cisteína proteasas nombradas como metacaspasas y VPEs, las cuales son análogas a las caspasas. La actividad catalítica de las caspasas se basa en la hidrolisis de los enlaces peptídicos a través de los residuos del ácido aspártico. En contraste, las metacaspasas prefieren los residuos de arginina o lisina, mientras que las enzimas VPEs escinden a las proteínas a través de los residuos de asparagina (Rantong y Gunawardena, 2015). De manera similar a las caspasas, las metacaspasas y las

VPEs dirigen su actividad catalítica mediante una cascada de escisiones que comienza con las proteasas iniciadoras y termina con las proteasas ejecutoras (Rantong y Gunawardena, 2015). Las metacaspasas están involucradas en diversos procesos biológicos, como en el desarrollo de la vasculatura, la producción de semillas, la fotosíntesis y la muerte celular programada (Valandro et al., 2020). El genoma de *Arabidopsis* codifica para nueve metacaspasas, AtMC1 – AtMC9. De las cuáles, se ha observado que las proteasas AtMC1, AtMC2 y AtMC8 modulan la apoptosis activada por el estrés biótico (*Pseudomonas syringae*) y por el estrés abiótico (luz UV-C y el peróxido de hidrógeno) (He et al., 2008; Coll et al., 2010; Watanabe y Lam, 2011). La proteasa AtMC3/CAS-3-LIKE participa en la muerte celular mediada por MPK6 e inducida por la intoxicación por cadmio y la exposición a la luz UV-C (Zhang et al., 2009; Ye et al., 2013). En los animales, CAS-3 ha sido identificada como una proteasa ejecutora que promueve la fragmentación del ADN al reprimir la actividad del inhibidor de la endonucleasa CASPASE ACTIVATED DNASE (CAD). Además, escinden e inhiben a las proteínas POLYADP-RIBOSE POLYMERASE1 (PARP1) y MEDIATOR OF DNA DAMAGE CHECKPOINT PROTEIN1 (MDC1), necesarias para el reconocimiento del daño en el ADN y el reclutamiento de proteínas de reparación (Larsen et al., 2010; Solier y Pommier, 2011; Mashimo et al., 2021).

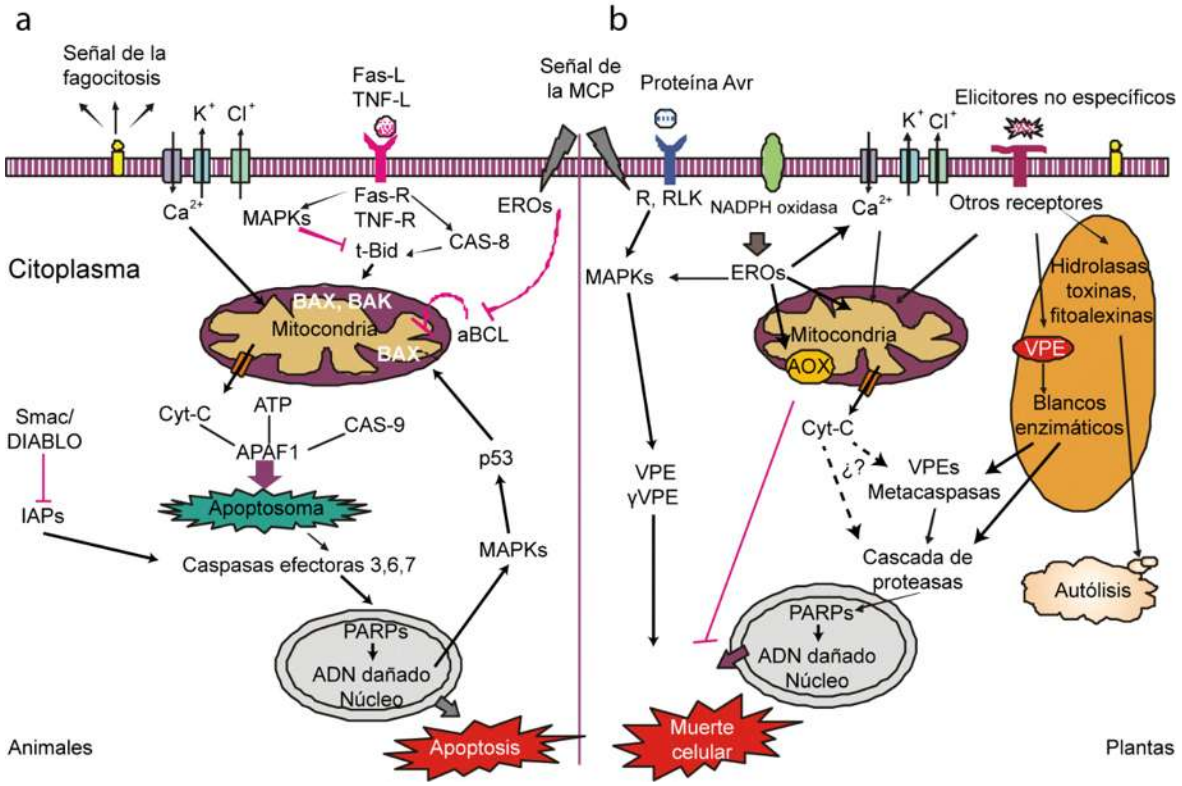

Figura 10. Diagrama simplificado de la muerte celular en animales y plantas. a) En la vía intrínseca y extrínseca de la apoptosis, las ERO, el Ca^{2+} y la unión de los ligandos Fas-L/TNF-L a los receptores Fas-R/TNF-R eventualmente convergen en la inhibición de las proteínas antiapoptóticas BCL-2, la activación de las proteínas proapotóticas BAX y ABK, en la permeabilización de la membrana mitocondrial y en la liberación del Cyt-C. En el citoplasma, Cyt-C junto con APAF1, el ATP y CAS9 forman al apoptosoma, una plataforma de activación de las caspasas ejecutoras CAS-3, 6 y 7. Las proteínas Smac/DIABLO potencian la actividad del apoptosoma mediante el antagonismo del inhibidor de las caspasas INHIBITOR OF APOPTOSIS (IAP). Ante el daño al ADN, las MAPKs promueven la apoptosis a través de p53. **b)** De forma análoga a los animales, las ERO y el Ca^{2+} despolarizan la membrana mitocondrial y liberan al Cyt-C. Las plantas carecen del apoptosoma pero se ha observado que Cyt-C se asocia con cisteína proteasas. La RHS detonada por las fitotóxinas y las proteínas R promueven la muerte celular a través de las quinasas MAPKs y las cisteínas proteasas VPEs. El agobio ambiental es un detonante la muerte celular a través de receptores y elicitores desconocidos (Modificado de Collazo et al., 2006).

2.8. La muerte celular programada en la raíz de *Arabidopsis*

2.8.1. Antibióticos y/o anticancerígenos genotóxicos que promueven la muerte celular programada mediante el daño inducido al ADN

La acumulación excesiva de las ERO y las fitotoxinas producidas por fitopatogénos pueden desencadenar la muerte localizada en organismos multicelulares, incluyendo a las plantas y los animales. En la mayoría de los casos, como resultado al daño de las membranas plasmáticas, el estrés del retículo

endoplásmico y el daño al material genético. Interesantemente, se ha descrito que los antibióticos funcionan de la misma manera para suprimir el crecimiento microbiano (Chandra y Kumar, 2017; Pancu et al., 2021).

Algunos antibióticos son proapotóticos, por lo que su utilización se ha ampliado al terreno de los tratamientos quimioterapéuticos contra el cáncer (Gao et al., 2020). El mecanismo de acción de los antibióticos anticancerígenos generalmente comprende: i) la fragmentación del ADN; ii) la inhibición de enzimas que participan en la recombinación de cromosomas, la replicación y la transcripción; iii) la producción de ERO; iv) y el aumento en la actividad de diversas proteínas que regulan la apoptosis animal, incluyendo al factor de transcripción p53, las MAPK quinasas, proteínas proapotóticas BAX, BAK y las caspasas (Dwyer et al., 2012; Gao et al., 2020; Persons et al., 2000). La hidroxiurea (HU) o hidroxicarbamida, por ejemplo, se incrusta a la enzima que contiene hierro, ribonucleótido reductasa (RNR), para reprimir la síntesis de los desoxirribonucleótidos y por lo tanto la síntesis del ADN, causando el estrés replicativo. La HU también puede provocar la fragmentación del ADN mediante el aumento en la producción de ERO debido a las reacciones de Fenton con los iones Fe^{2+} y otros metales (Musiałek y Rybaczek, 2021).

Los antibióticos de la familia de la bleomicina (bleomicina, fleomicina y zeocina) son potentes moléculas genotóxicas sintetizadas por actinobacterias rizosféricas del género *Streptomyces* que causan la ruptura de una cadena (RUC) o la rotura de las dos cadenas (RDC) del ADN y estimulan la apoptosis en los animales y bacterias (Hwang et al., 2005; Tsukuda y Miyazaki, 2013). De la misma manera, la bleomicina y la zeocina inducen la muerte en las células vegetales (Fulcher y Sablowski, 2009). Se ha reportado, gracias a la tinción vital con yoduro de propidio (YP), que la zeocina promueve la muerte de células mitóticamente activas, mientras que el centro quiescente, el cual se divide muy poco, permanece morfológicamente intacto (Fig. 11a) (Fulcher y Sablowski, 2009; Timilsina et al., 2019).

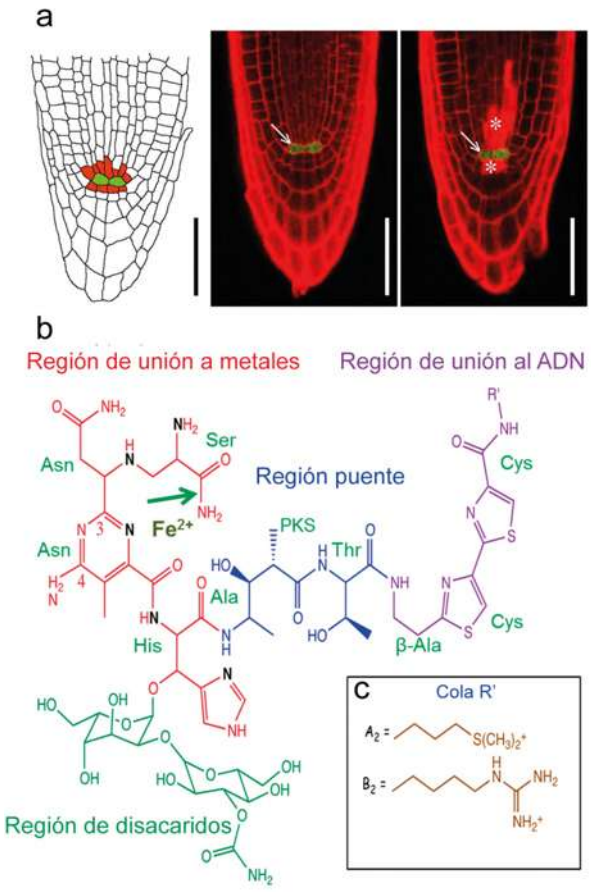

Figura 11. Los antibióticos bleomicina/zeocina inducen la muerte celular en el meristemo de la raíz de *Arabidopsis.* a) Imagen representativa de la muerte causada por la zeocina en el meristemo, observable por un parche rojo y la integridad del centro quiescente se distingue por la expresión de *WOX5* mediante el uso del gen reportero *GFP* (verde fluorescente). b) Estructura química de los antibióticos del grupo de la bleomicina. A2 = bleomicina, B2 = zeocina (Modificado de Fulcher y Sablowski, 2009; Murray et al., 2018).

Los antibióticos de la familia de la bleomicina son macromoléculas con un mecanismo de acción comparable, aunque presentan algunas diferencias estructurales. Ambas están compuestas por cuatro regiones: la región de disacáridos, una región de unión a metales, una región puente y una región de unión al ADN (Fig. 11b). A través de la región de unión al ADN es posible distinguir a la bleomicina de la zeocina: la cola formada por el sulfuro de dimetilo corresponde a la bleomicina, mientras que la guanidina forma parte de la zeocina (Fig. 11c). Estos antibióticos son capaces de intercalarse en el ADN mediante la interacción con los residuos de adenina, guanina o citosina para posteriormente escindirlo mediante el ataque de la pirimidina (Py). Para inducir el daño en el ADN, la bleomicina primero

captura al Fe^{2+} y al O_2 a través de su región de unión a los metales y pasa a considerarse como una bleomicina activada (Ble-Fe^{2+}-O_2). Después, la bleomicina activada atrae un átomo de H^+ de la desoxirribosa de la timina y como resultado se obtiene una radical intermediario C4'. A partir de este punto, la reacción se separa en dos vías, las cuales dependen de la disponibilidad del O_2. En presencia del O_2, la bleomicina activada escinde a los enlaces fosfodiéster y forma a los extremos 3'-fosfoglicolato y 5'-fosfato causando la fragmentación del ADN (RUC o RDC). En ausencia del O_2, el radical intermediario C4' se convierte en un sitio abásico 4'-oxidado (Fig. 12) (Murray et al., 2018).

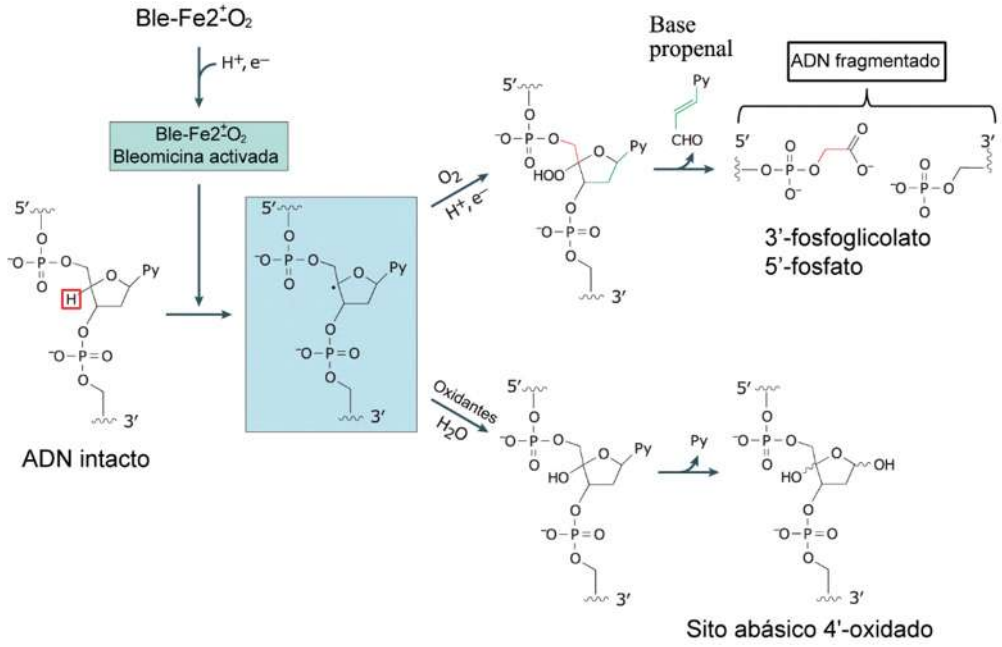

Figura 12. La bleomicina induce la fragmentación del ADN. La forma activada de la bleomicina (Ble-Fe^{2+}-O_2) extrae un átomo de H del C4 de la pirimidina (Py). En presencia del oxígeno, la bleomicina fragmenta los enlaces fosfodiéster de la Py arrojando como productos una base propenal, y los fragmentos 3'-fosfoglicolato y 5'-fosfato. En condiciones anóxicas, el radical intermediario C4' pierde la base de Py, presentado un sitio abásico 4'-oxidado (Modificado de Murray et al., 2018).

2.8.2. Mantenimiento de la integridad celular en respuesta al daño del ADN

Para subsanar las fracturas del ADN, las plantas y los animales desarrollaron una serie de mecanismos conocidos en su conjunto como respuestas al daño del ADN (RADN), los cuales activan las vías de reparación del ADN y detienen el ciclo celular hasta que la reparación sea completada. En caso de que el ADN no pueda ser reparado, las RADN resultan en la activación de la muerte celular para evitar la

transmisión de mutaciones o ADN aberrante a la siguiente generación de células derivadas del ciclo celular (Maréchal y Zou, 2013).

Los mecanismos de activación de las RADN están muy conservados en los organismos eucarióticos. En los animales, las RADN dependen de la activación de las proteínas cinasas ATAXIA-TELANGIECTASIA MUTATED (ATM) y ATM AND RAD3-RELATED (ATR). ATM reconoce a las RDC pero ATR detecta las RUC y el estrés replicativo (Maréchal y Zou, 2013). Las proteínas homologas de ATM y ATR de los animales y levaduras fueron identificadas en *Arabidopsis* mediante el análisis de similitud de las secuencias (Garcia et al., 2000; Culligan et al., 2004). Al igual que en los animales, las cinasas ATM y ATR de *Arabidopsis* tienen una función distinta pero aditiva debido a que las mutantes *atm* y *atr* fueron hipersensibles a genotóxicos, mientras que solo la mutante *atr* fue hipersensible al estrés replicativo causado por la hidroxiurea (Culligan et al., 2006; Sweeney et al., 2009). Ambas cinasas fosforilan y activan al factor de transcripción SOG1, el cual funciona como un regulador maestro análogo a p53 de los animales (Yoshiyama et al., 2009; Yoshiyama, 2016).

El factor SOG1 fue caracterizado a partir de la observación que las mutantes *sog1-1* no detienen el ciclo celular en la fase G2/M en respuesta al daño provocado por la radiación gamma y la zeocina en las hojas y la raíz (Preuss y Britt, 2003; Yoshiyama et al., 2017). Este fenómeno provoca que la raíz de las mutantes *sog1-1* muestren una aparente resistencia al daño provocado por los genotóxicos en tiempos cortos de exposición. No obstante, las mutantes *sog1-1* son incapaces de reparar el daño celular por lo que terminan diferenciándose. En la figura 13 se muestra el meristemo de la raíz primaria de *Arabidopsis* WT y las mutantes *sog1-1*; en la cual, las plantas fueron expuestas por 1.5 h a radiación gamma para después evaluar la capacidad regenerativa de la raíz. A diferencia de las plantas normales, las mutantes *sog1-1* mostraron un avanzado estado de diferenciación de la raíz primaria desde los 3-7 d después de la exposición, evidenciando que las mutantes son incapaces de regular las RADN, las cuales aseguran la supervivencia y estabilidad de las células (Fig. 13) (Johnson et al., 2017).

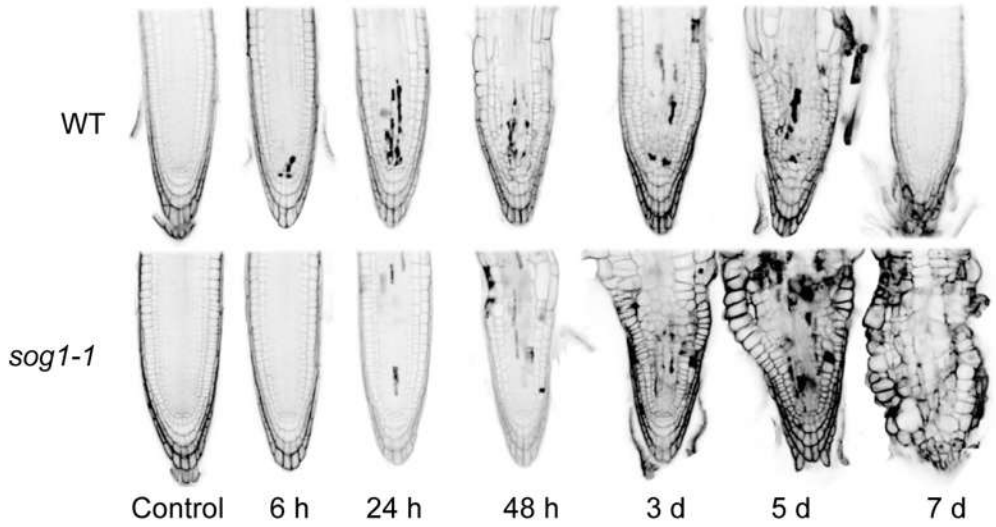

Figura 13. La perdida de función del gen *SOG1* compromete las respuestas de recuperación de la raíz. Se evaluó la recuperación de la raíz primaria de las plantas de *Arabidopsis* WT y las mutantes *sog1-1* expuestas a radiación gamma por 1.5 h. Observe en la figura que la raíz de plantas silvestres presenta parches negros, indicativos de la muerte celular en el meristemo a partir de las 6 h después de la exposición, pero se recupera satisfactoriamente a los 7d. En contraste, las mutantes *sog1-1* mostraron una aparente resistencia al daño durante 48 h después de la exposición, pero su raíz termina por diferenciarse a los 7d (Modificado de Johnson et al., 2017).

La regulación transcripcional de SOG1 sobre la división involucra a la inducción de la expresión de genes que arrestan el ciclo celular y promueven la endorreplicación. Los genes que codifican para las proteínas SIAMESE-RELATED5/7 (SMR5/7), los factores de transcripción ANAC044/085 y la proteína cinasa WEE1 son blancos moleculares de SOG1 que inhiben la actividad de las quinasas CDKB1/2 y promueven la acumulación de los factores de transcripción MYB3R3 para reprimir la progresión del ciclo celular (Fig. 14) (De Schutter et al., 2007; Chen et al., 2017; Bourbousse et al., 2018; Ogita et al., 2018; Takahashi et al., 2019). Por su parte, el factor de transcripción MYB3R3 se expresa en el meristemo de la raíz de *Arabidopsis* en respuesta al daño provocado por la zeocina (Fig. 15) para reprimir la transcripción de los genes *CYCB1;2* y *KNOLLE*; los cuáles, son necesarios para la transición de la fase G2/M y la citocinesis (Haga et al., 2007; Haga et al., 2011; Kobayashi et al., 2015).

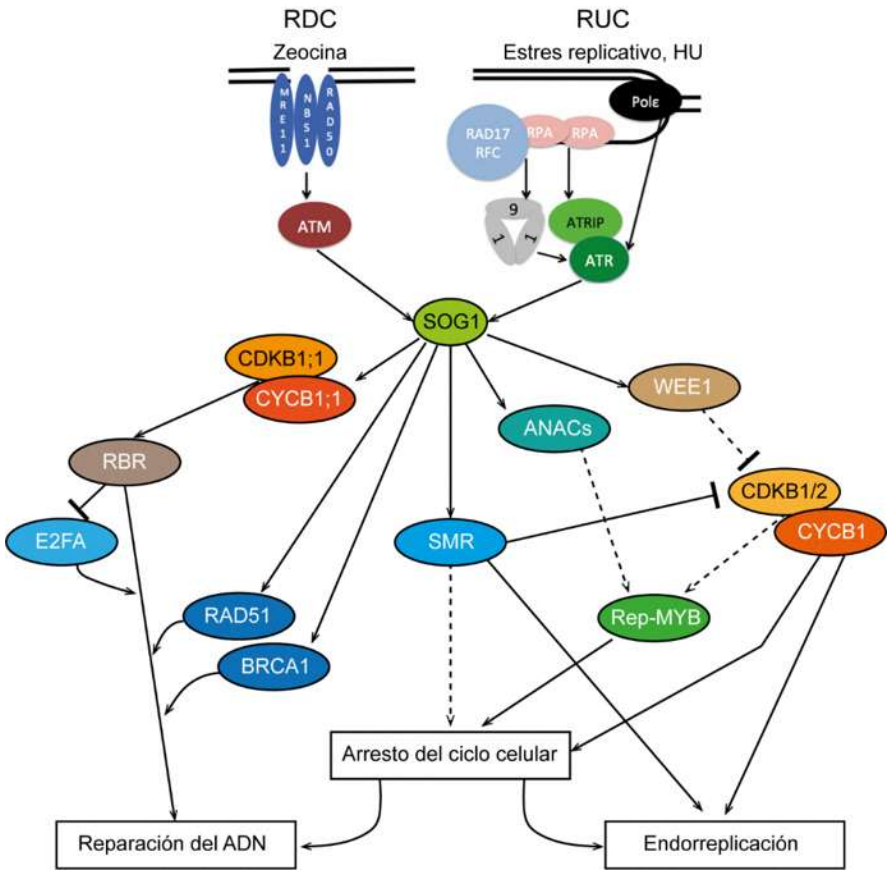

Figura 14. Mecanismos activados por el daño al ADN. La actividad de las cinasas ATM y ATR es asistida por diferentes complejos proteicos encargados de la detección primaria del daño en el ADN. En animales y plantas, el complejo MRN formado por las proteínas MEIOTIC RECOMBINATION 11 (MRE11), RADIATION SENSITIVE 50 (RAD50) y NIJMEGEN BREAKAGE SYNDROME 1 (NBS1) rápidamente reconocen las RDC y reclutan a la cinasa ATM para controlar la RADN. Por otra parte, las proteínas REPLICATION PROTEIN-A (RPA) detectan las RUC, recubren los extremos del ADN rotos y reclutan a los complejos RAD17-REPLICATION FACTOR C (RAD17-RFC), 9-1-1 (RAD9-RAD1-HUS1) y las proteínas ATRIP para activar a las cinasas ATR. Ambas cinasas fosforilan al regulador central SOG1; el cual, promueve el arresto del ciclo celular mediante el control transcripcional de las proteínas SMR, WEE1, ANACs y Rep-MYB, inhibidoras de las CDKB1/2 y CYCB1;2. Simultáneamente, SOG1 estimula la reparación del ADN mediante la regulación de CYCB1;1 del ciclo celular y las proteínas recombinantes RAD51 y BRCA1 (Modificado de Gentric et al., 2021; Nisa et al., 2019).

Alternativamente, se ha observado que SOG1 promueve la expresión de CYCB1;1, la cual en conjunto CYCB1;1-CDKB1;1 reprime a la proteína RBR y activan a las proteínas RADIATION SENSITIVE 51 y BREAST CANCER GENE1 (BRCA1) (Fig. 14) (Adachi et al., 2011; Da Ines et al., 2013; Weimer et al., 2016). RAD51 y BRCA1 son proteínas homólogas de animales que promueven la reparación del ADN mediante la recombinación homologa (RH); en la cual, los

segmentos fragmentados de la doble hélice con una secuencia similar o idéntica son intercambiados o recombinados (Da Ines et al., 2013; Ogita et al., 2018). Este mecanismo de reparación se considera libre de errores debido a que la recombinación permite la recuperación de los segmentos dañados mediante la actividad de las enzimas ADN polimerasas (Li y Heyer, 2008).

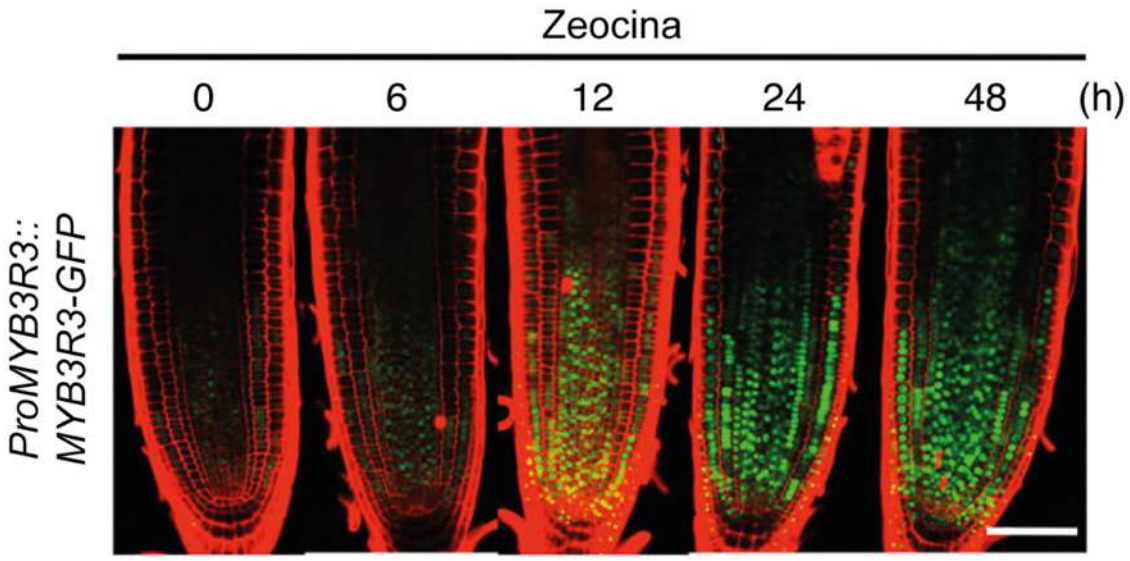

Figura 15. Expresión de MYB3R3 en el meristemo de la raíz en respuesta a zeocina. El factor transcripcional MYB3R3 es una proteína nuclear presente en las células del meristemo. La expresión de *MYB3R3* reportada por la proteína verde fluorescente (GFP) es inducible por el daño ocasionado por la zeocina en todos los tiempo analizados (Modificado de Chen et al., 2017).

2.9. La luz y el complejo MEDIADOR coordinan la tolerancia al estrés y el mantenimiento de la integridad celular en el meristemo de la raíz

2.9.1. La subunidad MED18 del complejo MEDIADOR regula la muerte celular en respuesta a la luz

El complejo multimérico MEDIADOR (MED, ~30 subunidades) es un co-activador de la transcripción genética que promueve la interacción de los factores transcripción y la enzima RNA pol II con la secuencia promotora de los genes previo a la síntesis del ARN mensajero (Dolan y Chapple, 2016). Estos componentes de la maquinaria transcripcional aportan estabilidad y participan en el mantenimiento de la viabilidad celular en el meristemo. En *Arabidopsis*, la perdida de función del factor TFIIF y de las subunidades RPB1 o NRPB2 de la RNA pol II provocan la muerte de las células de la pro-vasculatura y la diferenciación celular en el meristemo radicular (Babiychuk et al., 2017; Zhang et al., 2018; Raya-González et al., 2022). La subunidad MED18 forma parte del módulo “cabeza” del complejo MED. Previo al trabajo de Raya-González y col. (2018), MED18 estaba relacionada con el control del tiempo de floración, la germinación de semillas, la tolerancia a la infección por hongos necrotróficos (*Boytritis cinerea* y *Alternaria brassicola*) y al estrés salino (Zheng et al., 2013; Lai et al., 2014; Zhu et al., 2017).

Raya-González y col. (2018) reportaron que MED18 es esencial para mantener la integridad celular en el meristemo de la raíz (Fig. 16). Para demostrarlo, tiñeron con yoduro de propidio la raíz primaria del ecotipo silvestre de *Arabidopsis* Col-0 y las mutantes del gen *MED18*. Posteriormente, registraron la estructura del meristemo por microscopía confocal y midieron el área de la muerte celular, así como el área de las células de la epidermis, córtex y endodermis durante una cinética de tiempo de 1, 3, 5 y 7 días después de la germinación (ddg) bajo condiciones de luz y temperatura estándar (22 ˚C, fotoperiodo de 16 h luz / 8 h oscuridad, intensidad de luz > 200 µmol m^{-2} s^{-1}). Las mutantes *med18-1* presentaron muerte espontanea de las células de la pro-vasculatura en la zona del meristemo (Fig. 16a). Interesantemente, el área de muerte celular se incrementó en función de la edad de las plantas (Fig. 16b). Además, el daño celular incrementó el diámetro de la raíz (Fig. 16a-c). Para finalizar, los autores aplicaron el ensayo TUNEL

(terminal deoxynucleotide transferase-mediated dUTP nick-end labeling), con el cual corroboraron que el daño observado en las mutantes *med18-1* fue una consecuencia del daño en el ADN (Fig. 16a). De hecho, este fenotipo se intensificó cuando las mutantes fueron expuestas al cisplatino, un antibiótico/anticancerígeno que induce la fragmentación del ADN. El fenotipo de las mutantes *med18-1* se correlacionó con una disminución en la expresión de los genes *PARP1*, *WEE1*, *RAD51*, *XRI* y *BRCA1,* asociados con la reparación del ADN y el arresto del ciclo celular. Por lo tanto, se concluyó que la subunidad MED18 es fundamental para la reactivación de las RADN en la raíz de *Arabidopsis*.

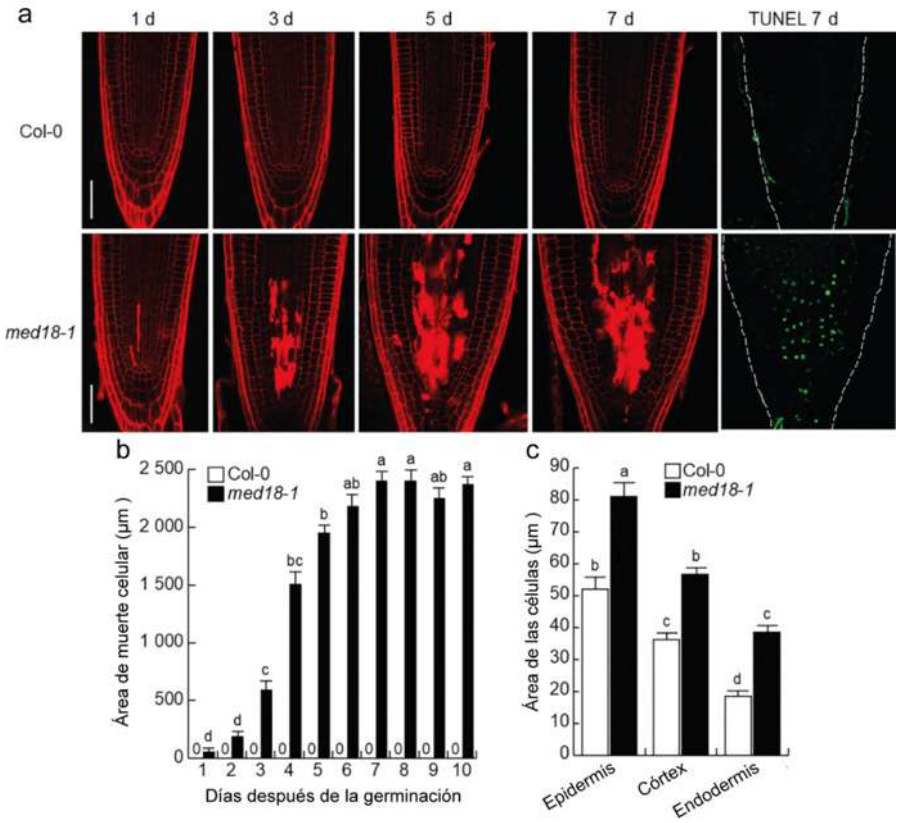

Figura 16. La pérdida de función del gen *MED18* induce la muerte celular en el meristemo de la raíz primaria de *Arabidopsis.* a) Micrografías representativas obtenidas con el microscopio confocal del meristemo de la raíz primaria de *Arabidopsis* WT y las mutantes *med18-1* teñidas con YP. b) Área de la muerte celular en el meristemo de WT y *med18-1*. c) Área de las células de la epidermis, córtex y endodermis de las plantas WT y *med18-1* (Modificado de Raya-González et al., 2018).

Interesantemente, la muerte observada en la pro-vasculatura de la raíz de las mutantes *med18-1* disminuyó en condiciones de crecimiento en la oscuridad (Fig.

17), indicando que la exposición a la luz desencadena el daño al material genético en células altamente proliferativas (McCormick, 2018; Raya-González et al., 2018).

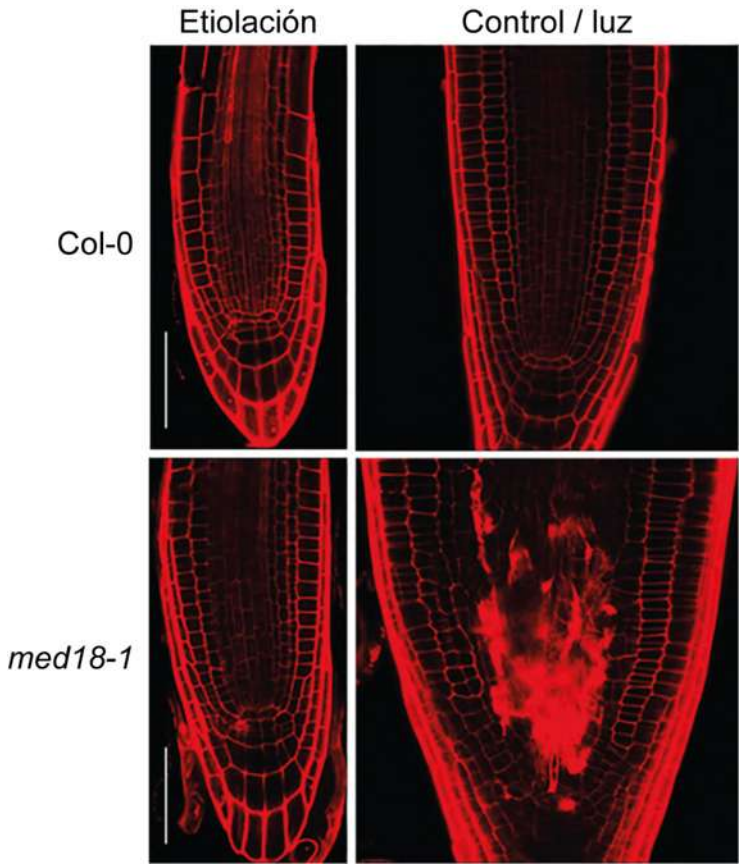

Figura 17. La luz induce la muerte celular en las mutantes *med18-1*. Micrografías representativas obtenidas con microscopia confocal del meristemo de la raíz primaria de las plantas WT y las mutantes *med18-1* crecidas en la oscuridad y con un fotoperiodo normal de crecimiento (Modificado de Raya-González et al., 2018).

2.9.2. Participación de los fitocromos en el control de la regeneración celular en el meristemo de la raíz

Las plantas manifiestan un programa de regeneración altamente flexible que les permite reparar, renovar o reemplazar células, tejidos y órganos lesionados o mutilados por daño mecánico o por compuestos genotóxicos y citotóxicos. La regeneración vegetal involucra la reparación de los tejidos dentro de los meristemos del follaje y la raíz, la embriogénesis somática y a la organogénesis *de novo*. En la embriogénesis somática las células se desdiferencian en células iniciales embrionarias a partir de los cuales se desarrolla un embrión y crece un organismo

completamente nuevo. En la organogénesis *de novo* las células no sufren un periodo de desdiferenciación y se manifiesta por la formación de brotes axilares, raíces laterales y raíces adventicias cuando los meristemos del follaje y la raíz no se pueden reparar (Ikeuchi et al., 2016).

El factor ERF115 es un regulador central en las respuestas de reparación de los tejidos dañados en el meristemo de la raíz. Inicialmente fue identificado como un factor de transcripción que controla la expresión del péptido PSK5 para promover la división del centro quiescente, por lo que su función principalmente se atribuía al mantenimiento del meristemo (Heyman et al., 2013). Sin embargo, Heyman y col. (2016) notaron que el daño ocasionado por estrés mecánico, laser y genotóxicos inducían una rápida expresión transcripcional de *ERF115* en el área adyacente al área de muerte celular (Fig. 18) (Heyman et al., 2016). Más tarde se demostró que ERF115 interactúa con el complejo RBR-SCR y controla la activación del regulador maestro de la organogénesis MP/ERF5 para estimular la regeneración de las células en respuesta al estrés ambiental (Zhou et al., 2019; Canher et al., 2020). Estudios de interacciones moleculares con ERF115 condujeron a la identificación del factor PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1) perteneciente a la familia SCARECROW (GRAS). La expresión de *PAT1* se localiza cerca del área de muerte celular, principalmente en la columela y complementa la actividad de ERF115 mediante la formación de dímeros (Fig. 18) (Heyman et al., 2016). Posteriores análisis con las líneas mutantes *erf115* y *pat1* demostraron que carecen de la capacidad de regeneración lo que permitió concluir que el complejo ERF115-PAT1 es crucial para la recuperación del meristemo de la raíz en respuesta a un agobio ambiental (Heyman et al., 2016; Canher et al., 2020). PAT1 es un factor de transcripción que opera río abajo de la vía de señalización controlada por PhyA (Bolle et al., 2000; Torres-Galea et al., 2013), lo que sugiere que PhyA podría estar involucrado en las respuestas a la muerte celular en la raíz de *Arabidopsis*.

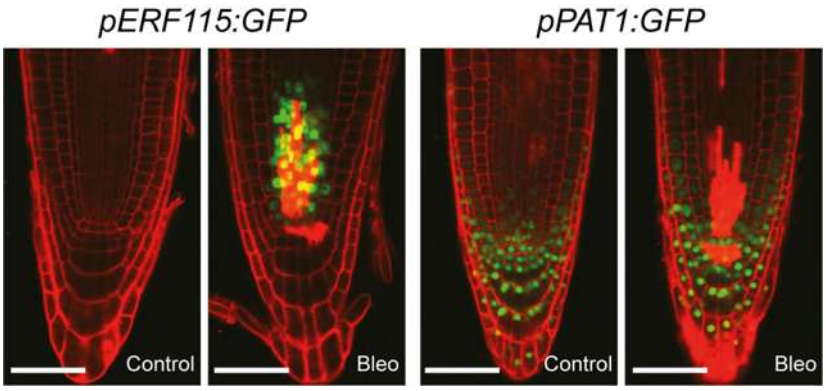

Figura 18. La muerte celular promueve la expresión de los factores transcripcionales ERF115 y PAT1 en el meristemo de la raíz primaria de *Arabidopsis.* El antibiótico genotóxico bleomicina induce la expresión del gen reportero *pERF115:GFP* en la pro-vasculatura. El reportero *pPAT1:GFP* presenta un dominio de expresión que involucra a la columela y al nicho de células iniciales. La inducción de la muerte por la bleomicina potencia la expresión de ambos genes reporteros en células intactas adyacentes al área dañada del meristemo de la raíz. El dímero ERF115-PAT son complementarios y promueven el reemplazo de las células dañadas a partir de la división de poblaciones celulares preexistentes (Modificado de Heyman et al., 2016).

3. JUSTIFICACIÓN

La subunidad MED18 del complejo mediador define a un represor de la muerte celular programada en el meristemo de la raíz, proceso que es sensible a la percepción de la luz en diferentes etapas del desarrollo de la planta. Evidencias recientes han propuesto a los fotorreceptores de la luz roja y roja lejana como elementos de la señalización de luz que controlan la tolerancia de *Arabidopsis* a la muerte celular programada promovida por el estrés biótico y abiótico. Esta propuesta busca esclarecer la participación de los elementos de la señalización por luz, su transporte de larga distancia del follaje a la raíz y su relevancia en la viabilidad de las células en el meristemo de la raíz de *Arabidopsis*.

4. HIPÓTESIS

La señalización por luz a través de los fitocromos regula el proceso de muerte celular programada y la diferenciación celular en el meristemo de la raíz de *Arabidopsis.*

5. OBJETIVOS

5.1. OBJETIVO GENERAL

Caracterizar a los elementos de la señalización por luz en la viabilidad de las células meristemáticas de la raíz de *Arabidopsis*.

5.2. OBJETIVOS PARTICULARES

- Determinar la participación de los fotorreceptores y los elementos de señalización por luz en el mantenimiento de la viabilidad de células meristemáticas y el crecimiento de la raíz en respuesta a compuestos genotóxicos.

- Evaluar la participación de los fotorreceptores en la regeneración y las respuestas al daño del ADN en el meristemo de la raíz.

- Caracterizar la participación de la luz, los fotorreceptores y moléculas de señalización en la transducción de la señal a larga distancia del follaje a la raíz durante el mantenimiento del meristemo de la raíz.

6. RESULTADOS

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PHYTOCHROME A controls the DNA damage response and cell death tolerance within the *Arabidopsis* root meristem

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ABSTRACT

Environmental stress, toxins, metals and UV light may damage the DNA, and plant cells mount the so-called DNA damage response to avoid the passing of mutations into dividing cells, particularly in meristems. Currently, the molecular mechanisms underlying this process remain to be explored. Here, we analyzed the role of photoreceptors on the restriction of root growth imposed by genotoxic agents and its relationship with cell viability and performance of meristems. Comparison of root growth of *Arabidopsis* WT, *phyA-211*, *phyB-9*, *cry1* and double *phyA-211phyB-9* mutants unveiled a critical role for phytochrome A (PhyA) in protecting root meristems from cell death caused by DNA damaging stress and the subsequent exhaustion of primary and lateral root meristems. PhyA was located on primary root tips, where it apparently influences genes related to the repair of DNA including *ERF115* and *RAD51* in plants exposed to genotoxics. Interestingly, *phyA-211* mutants treated with zeocin failed to induce the expression of the repressor of cell cycle progression *MYB3R3*, which correlated with the *CycB1* expression in root meristems, suggesting that PhyA is required for safeguarding the DNA integrity and mutation segregation to the progeny. Moreover, analysis of the PhyA downstream component HY5 showed that PhyA regulates DNA damage responses via an independent mechanism of the canonical light and photomorphogenesis signaling pathway. Together, our data revealed a new role of PhyA as a key player for cell division, stem cell niche maintenance and DNA damage responses, which are critical for the function of meristems and proper root growth.

Keywords: *Arabidopsis* root, stem cell niche, DNA damage, photoreceptors, programmed cell death, phytochrome A.

INTRODUCTION

Plants as sessile organisms are constantly exposed to biotic and abiotic stress, which affect proper growth and development. Undifferentiated cells located inner within the root tip, known as the stem cell niche (SCN), control root organogenesis and indeterminate growth. Because of their constant mitotic activity, the SCN and daughter cells are highly susceptible to environmental hazards that cause DNA damage, which can activate programmed cell death (PCD) and thus avoid the transmission of mutations to dividing cells (Ubogoeva et al. 2021).

The SCN undergoes PCD after extended exposure to genotoxic agents that induce DNA breaks, such as salt stress, UV light, and genotoxic compounds including antibiotics and drugs (Fulcher and Sablowski 2009; Timilsina et al. 2019). To cope with DNA breaks, an array of cellular responses are initiated upon activation of ATAXIA-TELANGIECTASA MUTATED (ATM) and ATM-AND RAD3-RELATED (ATR) sensor kinases, where ATM recognizes double-strand breaks (DSB) and ATR senses single-strand breaks (SSB) and stalled replication forks (Maréchal and Zou 2013). Both ATM and ATR kinases regulate the activity of the master regulator SUPPRESSOR OF GAMMA RESPONSE1 (SOG1), a NAC-type transcription factor that controls the expression of hundreds of genes related to the DNA repair mechanism, cell division, endoreduplication, and PCD (Yoshiyama et al. 2009; Adachi et al. 2011; Bourbousse et al. 2018; Ogita et al. 2018). These responses are highly coordinated and together are referred as DNA damage responses (DDR).

Upon DNA breaks, SOG1 activates the expression of DNA repair genes, such as *BREAST CANCER SUSCEPTIBILITY GENE1* (*BRCA1*) and *RADIATION SENSITIVE51* (*RAD51*), two components of the homologous recombination system involved in the DSBs repair (Ogita et al. 2018). To allow DNA repair, SOG1 regulates the expression of genes that arrest the cell cycle, such as *SIAMESE-RELATED 5,* and *7* (*SMR5; SMR7*), which inhibit CYCLIN-DEPENDENT KINASES (CDK), and promote the accumulation of MYB3R3 and MYB3R5, two transcriptional repressors of the transition from G2 to M phase (Adachi et al. 2011; Chen et al. 2017; Ogita et al. 2018; Takahashi et al. 2019).

Recently, we described the function of the MEDIATOR18 (MED18) subunit of the Mediator complex as a key component of the PCD process in *Arabidopsis* (Raya-Gonzalez et al. 2018). *med18* mutants manifest a short root phenotype caused by

cell death within the root meristem, associated to defects in the DNA repair systems (Raya-Gonzalez et al. 2018). Interestingly, cell death was abolished in plants grown in darkness, but appeared after shoot exposure to light indicating a long shoot-to-root signaling that triggers PCD in *med18* root meristems (Raya-Gonzalez et al. 2018).

Plants sense light by at least four types of photoreceptors, including cryptochromes (CRY1, 2), phototropins (PHOT1, 2), phytochromes (PHYA-E), and *UV-B resistance locus 8* (UVR8) (Galvão and Fankhauser 2015). Cryptochromes and phototropins detect blue/ultraviolet A (UV-A) wavelengths, whereas UVR8 perceives UV-B light. The *Arabidopsis* genome encodes five types of phytochromes where PhyA perceives far-red light and PhyB-PhyE are responsible for red light sensing (Mathews 2010). The perception of light promotes the accumulation of several intermediary transcription factors, among which ELONGATED HYPOCOTYL5 (HY5) acts downstream of photoreceptors to drive photomorphogenesis, nutrient assimilation, and stress adaptive responses (Gangappa and Botto 2016). Noteworthy, HY5 promotes PCD during plant de-etiolation process dependent of PhyB signaling in shoots (Chen et al. 2013; Chai et al. 2015).

PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1) is a novel component of root cell viability mechanisms related to SCARECROW, and both are members of the GRAS family of transcription factors that operate downstream of the PhyA signaling pathway (Bolle et al. 2000; Torres-Galea et al. 2013). PAT1 directly interacts with ETHYLENE RESPONSE FACTOR115 (ERF115) to replenish cell populations after damage within the *Arabidopsis* root meristem (Heyman et al. 2013; Heyman et al. 2016). These data suggest that red and far-red photoreceptors (PhyA, B) may integrate light signals with the PCD response.

Here, comparison of root growth of *Arabidopsis* WT seedlings and mutants affected in several photoreceptors enabled identification of PhyA as an essential component in safeguarding cell viability in *Arabidopsis* root meristems. Our results show that low concentrations of DNA damaging agents, such as zeocin and phleomycin, trigger PCD within root meristems and cause earlier cell differentiation in *phyA-211* root tips, which is accompanied by isodiametric cell growth, regeneration-related gene expression, and ultimately halt root growth. Besides, we found that PhyA regulates

MYB3R3 and RAD51 expression in response to DNA damaging stress, suggesting that PhyA acts to protect the SCN and support cell viability and meristem functioning. These data uncover an unexpected function of a photoreceptor in root viability and adaptation to mutagenic stressors.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana seedlings of the wild type (WT) Columbia-0 (Col-0), the *phyA-211* (Reed et al. 1994), *phyB-9* (Reed et al. 1993), *phyA-211phyB-9*, *cry1* (Bruggemann et al. 1996), *hy5* (SALK_096651C), and *sog1* (CS822355) mutant alleles and the transgenic line *35S:PhyA:YFP* (Bauer et al. 2004) were used in this study. The genetic constructs *pERF115:GFP* (Heyman et al. 2016), *pPAT1:GFP* (Heyman et al. 2016), *pRAD51::RAD51-GFP* (Da Ines et al. 2013), *proMYB3R3:MYB3R3:GFP* (Chen et al. 2017) and *CycB1:uidA* (Colón-Carmona et al. 1999) were introduced into *phyA-211* mutants via manual outcrossing. Seeds from each genotype were disinfected with 500 µL ethanol 96% (v/v) for 5 minutes and 500 µL commercial bleach 20% (v/v) for 5 minutes into Eppendorf tubes. The seeds were washed five consecutive times with sterilized distilled water and stored at 4 °C for 48 h to synchronize germination.

The seeds were germinated and grown on Petri plates containing agar-solidified 0.2x MS medium, which contained 0.09% (w/v) of commercial Murashige and Skoog basal salts mixture purchased from Sigma-Aldrich (catalog M5524), 0.6% (w/v) of sucrose, and 1% (w/v) of phytagar purchased from Phytotechnology Laboratories (catalog A111). Petri plates were placed in a vertical position into a plant growth chamber Percival AR-95L having a light intensity of 300 µmol m^{-2} s^{-1}, a photoperiod of 16 h light / 8 h darkness, and a temperature of 22 °C. Zeocin solution (100 mg/mL) was purchased from Invitrogen. Phleomycin and hydroxyurea were purchased from Sigma-Aldrich and dissolved in sterilized distilled water or dimethyl sulfoxide prior the application into the media.

Analysis of primary root growth

Homozygous seedlings from each genotype were germinated and grown on agar-solidified 0.2x MS medium for 4 days and then transferred to agar-solidified 0.2x MS medium supplemented with different concentrations of zeocin, phleomycin or hydroxyurea, where the control condition contained only the solvent. The primary root growth was measured from a point marked over the plate at transfer to the root tip of seedlings attained 6 days later.

Root tip excision and regeneration

The primary root tip of 4-day-old seedlings grown on 0.2x MS medium was excised with a sterile scalpel using a stereoscopic microscope (Leica model EZ4D) in a laminar flow cabinet. Petri plates were sealed and the root regeneration was examined after 6 days of excision. The regenerated root tips were registered using a stereoscopic microscope (Leica DMZ6) at the 3.2x objective.

GUS staining and histochemical analysis

Arabidopsis thaliana seedlings harboring the *CycB1:uidA* construct were immersed in β-glucuronidase (GUS) buffer at 36 °C overnight. The GUS buffer contained 1 mg/mL of 5-bromo-4-chloro-3-indolyl β-D-glucuronide, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, E.D.T.A. 10 mM, 0.1 % (v/v) Triton™ X-100, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$; pH adjusted to 7 with KOH. Seedlings were clarified with the solution 1 (HCl 2% v/v and CH_3OH 20%) for 60 minutes at 62 °C and solution 2 (NaOH 7% w/v and CH_3CHOH 60% v/v) for 30 min at room temperature. Consecutive steps 30 min each with 10 %, 20 %, and 40 % of CH_3CH_2OH were applied to finish the clearing. The cleared seedlings were stored with glycerol 50 % (v/v) at 4 °C. Nomarski optics using a Leica DM5000B microscope was coupled to a digital camera (Leica DFC450C) to take representative micrographs where the blue color of GUS indicates the gene expression.

Propidium iodide (PI) staining and fluorescence detection

Cell viability and GFP or YFP fluorescent proteins were evaluated 12 h, 24 h, 48 h, and 72 h after genotoxic exposure in the primary root tip of *Arabidopsis*. Samples

were placed on a microscope slide, immersed in 80 µL of PI and covered with a coverslip. Each sample was registered with a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan) configured for PI (excitation peak = 568 nm, emission = 585 - 610 nm) and GFP (excitation = 488 nm, emission = 505 - 550 nm) fluorescence. The two images were merged to produce a final image.

Root measurements and statistical analysis

The primary root was measured with a ruler from the transfer point to the root tip, and the length was normalized to 100 % in the control condition. Root meristem length, cell death area, expression area, and the relative fluorescence of reporter genes were measured with the Image J software (https://imagej.nih.gov/ij/). Relative fluorescence was examined from green pixels for a determined area of the root meristem, and the data obtained from WT in the control condition was normalized to 1. Data were analyzed with the Statistica 12 program (TIBCO Data Science-Statistica) applying univariate and multivariate Tukey's and chi-square test, and the means that statistically differ were marked with letters or asterisks ($p \leq 0.01 - 0.05$).

RESULTS

PhyA activity is required for DNA damage responses within *Arabidopsis* root meristems

A recent study showed that shoot exposition to light triggers cell death of root meristem cells in *Arabidopsis med18* mutants, inhibiting primary root growth (Raya-González et al. 2018). To determine whether blue, red and far-red light photoreceptors could be involved in meristem cell viability under genotoxic stress, WT (Col-0) seedlings, and the *phyA-211*, *phyB-9*, double *phyA-211phyB-9*, and *cry1* mutants were germinated and grown on 0.2x MS medium for 4 days, and then transferred for 6 days to the same media or media supplemented with 0.25, 5, 7.5, and 10 µM zeocin, a genotoxic agent that induces DNA-double strand breaks (DSBs). Zeocin concentrations higher that 5 µM reduced the primary root length in all *Arabidopsis* genotypes tested (Fig. 1a-b). WT, *phyB-9*, and *cry1* seedlings

showed 20% reduction of growth at 5 μM zeocin (Fig. 1a-b). However, the primary roots of *phyA-211* and *phyA-211phyB-9* single and double mutants, respectively, were reduced by 70-80% from the lowest zeocin concentration (2.5 μM) applied, which indicates a hypersensitive response to the effects of the genotoxic that is dependent of PhyA (Fig. 1 a-b). These observations were replicated using *phyA-211* mutants grown on media supplemented with phleomycin, another genotoxic compound that induces DSBs (Fig. S1). However, in response to replication-blocking agents, such as hydroxyurea, *phyA-211* mutants showed a comparable response to WT seedlings on primary root length (Fig. S1), suggesting that PhyA is specifically involved in DNA damage responses induced by DSBs. To determinate whether PhyA protection against genotoxic stress involves a light-dependent pathway, we assessed the role of HY5 as a possible PhyA downstream component in response to zeocin. The phenotypes and quantitative data shown in Fig. S2 indicated that the primary root growth of *hy5* mutants was comparable to that of the WT in response to zeocin (Fig. S2). Together, these data indicate that the photoreceptor PhyA acts as a cell protector against genotoxic stress through HY5 independent mechanisms in *Arabidopsis* root.

PhyA is essential for stem cell niche maintenance of root meristems in response to genotoxic stress

Radiomimetic drugs (phleomycin or zeocin) may cause the permanent arrest of root growth and cell death in the SCN (Fulcher and Sablowski 2009). To determine whether the hypersensitive response shown by *phyA-211* and *phyA-211phyB-9* mutants on root development could be associated to effects on cell integrity and/or alteration of root structure, we analyzed WT, *phyA-211*, *phyB-9*, and *phyA-211phyB-9* seedlings grown under normal conditions and exposed for 6 days to 5 μM zeocin. Then, primary roots were stained with the vital marker propidium iodide (PI), which freely penetrates damaged cells and causes the formation of red patches, as visualized by confocal microscopy (Truernit and Haseloff 2008).

Under standard growth control conditions, we did not find cell death symptoms, and the root tip structures were comparable among the genotypes (Fig. 2a). In contrast,

5 µM zeocin triggered a full differentiation program in primary root tips of *phyA-211* and *phyA-211phyB-9* mutants evidenced by the formation of root hairs close to the root tip as well as isodiametric growth of meristem cells prior to the collapse of the root tip (Fig. 2a). These results were also observed in lateral root tips of *phyA-211* and *phyA-211phyB-9* seedlings, indicating that the role of PhyA on DNA damage responses already occurs in both primary and lateral root meristems.

PhyA dysfunction increases the root sensitivity to genotoxic drugs through modulating the cell cycle

To more in detail investigate the effects of genotoxic agents on cell viability and root tip morphology, we analyzed the progress of cell death and its influence on root meristem size in WT, *phyA-211*, and *phyA-211phyB-9* seedlings. *Arabidopsis* plants were germinated and grown for 4 days on 0.2x MS media, and then transferred to the same media or media supplemented with 5 µM zeocin and exposed for 24 h, 48 h, and 72 h. In response to zeocin, we did not find a significant effect on cell viability or meristem size in WT and *phyB-9* seedlings (Fig. 3 a-c). However, confocal microscopy images and quantitative analysis revealed that upon 24 h of zeocin exposure, *phyA-211* and *phyA-211phyB-9* root meristems manifested cell death symptoms in the SCN and provasculature cells, observed as red patches, as well as a strong reduction in the size of meristems (Fig. 3b-c).

DNA damage induced by genotoxic agents or specific gene mutations affects mitotic activity in the root meristem through cell cycle-related gene expression (Raya-González et al. 2018; Raya-González et al. 2022). To assess if root tip exhaustion in *phyA-211* as a response to zeocin could be associated to defects on cell cycle progression in the root meristem, we analyzed the expression of *CycB1* in WT and *phyA-211* introducing the *CycB1;1:uidA* gene construct into *phyA-211* homozygous mutants via outcrossing. WT and *phyA-211* seedlings harboring *CycB1:uidA* were exposed to zeocin for 24 h, 48 h, and 72 h. Under standard growth conditions, *CycB1;1* expression in *phyA-211* roots was comparable to that of WT seedlings, indicating that *PhyA* mutation does not compromise cell cycle activity. However, *CycB1;1* expression was strongly induced in WT and *phyA-211* seedlings exposed

to zeocin from 24 h. We also noted that *CycB1;1* activity was mainly localized in the SCN, the region of root meristem where cell death is induced by zeocin. Importantly, at 72 h after zeocin exposure, *CycB1;1* was drastically reduced in *phyA-211* mutants, which correlates with the advancement of cell differentiation at the root tip (Fig. S3). These data suggest that cell death in combination with cell cycle arrest in the *phyA-211* root meristem after exposure to zeocin, ultimately leads to the exhaustion of the meristem and unveils a critical function of PhyA for SCN maintenance and cell viability under DNA-damaging stress conditions.

PhyA loss-of-function does not disrupt root regeneration in *Arabidopsis*

Cell damage induces the expression of ETHYLENE RESPONSE FACTOR115 (ERF115) and PHYTOCHROME A SIGNAL TRANSDUCTION1 (PAT1) transcription factors within the root meristem, which activates the cell regeneration process to replace damaged cells with new cells originated from pre-existing neighbors (Heyman et al. 2013; Heyman et al. 2016). To determine if *phyA-211* mutants may be oversensitive to genotoxic agents because they are unable to activate cell regeneration-related gene expression and to replace damaged cells, the genetic constructs *pERF115:GFP* and *pPAT1:GFP* were transferred into *phyA-211* mutants and their expression assessed via confocal microscopy. WT seedlings and *phyA-211* mutants carrying *pERF115:GFP* and *pPAT1:GFP* were germinated and grown for 4 days on MS 0.2X medium, and then transferred for 48 h to MS 0.2X medium supplemented with 5 µM zeocin, which induces cell death in *phyA-211* root meristem (Fig. 3a). In medium devoid of zeocin, we did not observe *ERF115* expression either in the WT or *phyA-211* root meristems, whereas *PAT1* expression was observed in the SCN, columella, and the lateral root cap of both genotypes (Fig. 4a, c). Zeocin did not induce *ERF115* and *PAT1* expression in WT seedlings (Fig. 4a, c). In contrast, we observed a strong induction on *ERF115* and *PAT1* expression in *phyA-211* root meristems, which co-localized with the cell damage zone and neighbor cells (Fig. 4a, c). This observation was validated with quantitative analysis of GFP fluorescence (Fig. 4b, d). These results indicate that PhyA loss-of-function did not affect *ERF115* and *PAT1* expression.

Cell regeneration assays were performed, in which the *Arabidopsis* root tip was excised and allowed to regenerate (Sena et al. 2009; Raya-González et al. 2022). We compared the capacity of WT and *phyA-211* seedlings to rebuild a new functional root meristem after root tip excision. For this, primary root tips of WT and *phyA-211* plants were excised in a very narrow area between quiescent center (QC) and the basal meristem, and five days after excision, root tip recovery was analyzed and quantified (Fig. S4). We found that *phyA-211* mutants and WT plants were able to regenerate a new functional meristem (Fig. S4), indicating that root tip regeneration does not require PhyA. Thus, our data suggest that the increased sensitivity to DNA damage after zeocin exposure in *phyA-211* mutant could be associated to defects in DNA repair mechanisms and/or cell cycle check points.

PhyA is involved in DNA repair mechanism in *Arabidopsis* root meristems

DNA repair mechanisms are activated after SSB or DSB stress via SOG1, which controls the expression of genes for the homologous recombination repair system, including RADIATION SENSITIVE 51 (RAD51) (Ogita et al. 2018). In *Arabidopsis* roots, RAD51 is expressed in the cytosol, but migrates to nuclear foci of dividing cells when plants are exposed to stress conditions that compromise the DNA integrity (Da Ines et al. 2013; Weimer et al. 2016). To investigate if PhyA could be involved in DNA repair mechanisms, we used the translational construct *pRAD51::RAD51-GFP*, in which we could monitor the protein levels of RAD51 by confocal microscopy. 4-day-old WT and *phyA-211* seedlings carrying the *pRAD51::RAD51-GFP* gene construct were grown on 0.2x MS medium, and then transferred to the same medium supplemented or not with 5 µM zeocin and exposed for 12 h, 24 h, and 48 h. In medium without zeocin, RAD51 level in *phyA-211* meristems was comparable to that of WT seedlings, which indicates that PhyA mutation does not compromise the basal expression of *RAD51* under standard growth conditions. However, as previously reported, RAD51 was induced up 3-fold after exposure to zeocin (Fig. 5a-b). Interestingly, RAD51 levels were up 5-fold in the *phyA-211* root meristem at 24 h of zeocin treatment, where occurs cell death. At 48 h after zeocin exposure, RAD51 levels were drastically reduced and spread to

the distal meristem, including columella cells. This result indicates that PhyA regulates RAD51 expression and localization in the root meristem in response to zeocin-induced DSBs.

PhyA regulates MYB3R3 accumulation in the root meristem to prevent cell death

In plants exposed to DNA damaging stress, cell cycle arrest is activated at the G2/M checkpoint to keep DNA integrity. This prevents the prevalence of mutations and/or the segregation of aberrant chromosomes into daughter cells. Recently, the MYB3R transcription factor family was found to act as activators (Act-Myb) or repressors (Rep-Myb) of cell cycle progression via direct regulation of G2/M-specific genes (Haga et al. 2007; Haga et al. 2011; Kobayashi et al. 2015). Hence, to determine whether PhyA could be involved in cell cycle progression in response to zeocin, we analyzed the levels of MYB3R3, by using *pMYB3R3::MYB3R3-GFP* translational fusion, which was introduced into *phyA-211* mutant via outcrossing and visualized by confocal microscopy. WT and *phyA-211* seedlings were germinated and grown for 4 days on 0.2x MS medium and then transferred to the same medium supplemented with 5 μM zeocin and exposed for 48 h. As previously reported, MYB3R3 is expressed in the root meristems under standard growth conditions, and its expression increased after zeocin treatment in WT seedlings (Fig. 6), suggesting that MYB3R3 restricted cell cycle progression. Interestingly, we found that under standard growth conditions, MYB3R3 levels are lightly compromised in *phyA-211* mutant, and it fails to induce MYB3R3 expression in response to zeocin (Fig. 6). Together, these data suggest that PhyA controls cell cycle progression through regulation of MYB3R3 in root meristem, which may prevent genetic aberrations in plants exposed to DNA damaging stress.

Zeocin affects PhyA expression in *Arabidopsis* root tips

The PhyA photoreceptor is expressed throughout the shoot of *Arabidopsis*, whereas in the root, *PhyA* expression is found in the vascular tissues and the tip of primary

and lateral roots, suggesting that PhyA not only transmit light-dependent inputs to leaf tissue, but also could acts as a regulator of root development (Somers and Quail 1995; Hall et al. 2001; Salisbury et al. 2007). To further investigate whether zeocin-induced cell death affects PhyA accumulation, we assessed the expression of the *35S:PhyA:YFP* gene construct in the primary root tip of four-day-old seedlings exposed to 5 μM zeocin for 12 h, 24 h, and 48 h. In control conditions, we observed that PhyA accumulates mainly in vasculature cells, lateral root cap, and weakly in SCN (Fig. 7a). Interestingly, zeocin altered PhyA accumulation, by reducing its levels in lateral root cap and SCN, but increasing it in the vascular tissue in a time-dependent manner (Fig. 7b, c). The fluorescence analysis revealed that PhyA protein mainly accumulates in vascular cells of plants exposed by 48 h to zeocin, which could be associated with the reduction of root meristem and isodiametric cell growth, and together led to root tip differentiation. These data indicate that genotoxic stress affects PhyA accumulation patterns on the *Arabidopsis* root tip.

DISCUSSION

Light signaling control plant growth and development through the activity of distinct photoreceptors, which trigger different adaptive responses to specific wavelengths, including de-etiolation shade avoidance, gravitropism and phototropism (Correll et al. 2003; Su et al. 2015, Tepperman et al. 2006; Yang et al. 2018). Here, we showed that PhyA is required for stem cell niche maintenance of root meristems in response to DNA damaging agents. By using single and double mutants affected in red/far-red, and blue light photoreceptors and downstream components, we found that *phyA-211* and *phyA-211phyB-9* mutants, but not *phyB-9* or *hy5* single mutants had an increased sensitivity to DNA damage agents on root development, indicating that PhyA acts as a cell protector for DNA integrity under genotoxic stress through a light signaling-independent mechanism.

PhyA activity has been characterized in the shoot on the light-related process, whereas its role in root development is less known. Previous reports point to a role of PhyA in both primary root growth and lateral root formation, because its endogenous expression patterns (Somers and Quail 1995; Hall et al. 2001; Salisbury

et al. 2007; van Gelderen et al. 2018; Kumari et al. 2019). This indicates that PhyA can be found in the shoot, but also in root tissues, suggesting that PhyA could have additional roles in plant development. By using *35S::PhyA-GFP* gene construct, we found that under standard growth conditions, *PhyA* expression can be localized in the lateral root cap (LRC), whereas in response to zeocin, *PhyA* was repressed in LRC and induced in vasculature tissues, indicating that zeocin changes PhyA detection within the *Arabidopsis* root tip. Recently, several light-signaling components, including PhyA, were related to phosphate deficiency responses through PHOSPHATE STARVATION RESPONSE1 (PHR1) transcription factor (Liu et al. 2017), suggesting their possible involvement in response to multiple abiotic stresses. This also opens the possibility that PhyA may be part of a low-Pi adaptive mechanism.

Light can act as a stress factor via increasing ROS accumulation and triggering PCD in *Arabidopsis* leaves. In plants, stress tolerance to light largely depends on the blue, red/far-red, and UV-B photoreceptor activation and downstream components, such as the transcriptional factor ELONGATED HYPOCOTYL 5 (HY5). Interestingly, HY5 can act as a regulator that promotes or restrain PCD in response to several factors, including light stimuli and plant growth conditions. Under UV-B irradiation or during plant de-etiolation, HY5 activates the expression of ROS-responsive genes and genes encoding for R2R3-MYB transcription factors, which control the accumulation of photo-protective compounds and DNA repair proteins (Stracke et al. 2010; Chen et al. 2013; Job et al. 2022). However, under our experimental conditions, we found that HY5 activity is not involved in cell damage induced by genotoxic agents, suggesting that PhyA protective effect in the root tip is independent of HY5 activity. Thus, PhyA probably regulates cell death tolerance independently of the light signaling pathway in the root of *Arabidopsis*.

Plant exposure to DNA damaging agents, triggers single or double strand breaks (SSB; DSB), which leads to activation of ATM and ATR kinases, respectively, which act via phosphorylation to control SOG1 activity (Sjogren et al. 2015; Yoshiyama et al. 2017; Yoshiyama and Kimura 2018). Upon genotoxic challenge, such as ionizing radiation (IR), SOG1 loss-of-function leads to the arrest of root growth, because cells fail to undergo cell division in root meristems (Johnson et al. 2017). Under our

experimental conditions, comparing the effects of zeocin on *phyA-211* and *sog1* mutants in root growth and root tip structure, we found that primary root growth of *sog1* was arrested 4 d after zeocin exposure, whereas in *phyA-211* already by 2 d after treatment, greater reduction of growth could be observed (Fig. S5). This highlights the high sensitivity of *phyA-211* to genotoxic stress and its critical function for safeguarding DNA integrity.

DNA damage leads to cell cycle arrest and DNA repair before mitosis resumption (Gentric et al. 2021). Nevertheless, previous reports have shown that *CYCB1;1*, a G2/M-specific gene, is significantly induced by genotoxic agents (Culligan et al. 2006; Yoshiyama et al. 2009; Adachi et al. 2011). These observations could be reproduced in our experimental conditions in WT seedlings. However, in *phyA-211* mutant, *CycB1;1* expression was strongly induced at short exposure times to zeocin, but was reduced at later times. In fact, *CycB1* expression was associated with the cell damage zone in *phyA-211* mutant exposed to zeocin, accompanied with defects on root morphology and structure, as well as the formation of root hairs close to the root tip, suggesting that DNA damage-induced cell death on root meristem activates PhyA-dependent exhaustion of the meristem through promotion of differentiation events. CYCB1;1, which is activated by SOG1, together with CDKB1;1 (CDKB1;1-CYCB1;1 complex) phosphorylates RAD51 to promote homologous recombination (HR)-dependent DNA repair mechanisms, and CYCB1;1 and CDKB1;1 loss-of-function fail to recruit RAD51 to DNA damaged cells (Weimer et al. 2016). Here, we found that *CYCB1;1* and *RAD51* expression co-localized with the cell damage in *Arabidopsis* root meristem, which implies that *phyA-211* mutant does not have compromised DNA repair mechanisms mediated by CYCB1;1 and RAD51.

In *Arabidopsis*, MYB3R3 is expressed in nuclei of cells within the root meristem, where it directly suppresses the transcription of genes needed for mitosis progression such as *CYCB1;2* and *KNOLLE* in response to zeocin-induced DNA strand breaks (Chen et al. 2017). We found that MYB3R3 in root meristem is compromised in *phyA-211* mutants when exposed to zeocin, which could explain the increased sensitivity of *phyA-211* seedlings. Other reports have shown that PhyA physically interacts with several members of the MYB3R3 family, including MYB30 and LONG AFTER FAR-RED LIGHT1 (LAF1) (Ballesteros et al. 2001; Yan et al.

2020). Such interaction might be relevant for cell cycle progression and safeguarding DNA integrity in response to genotoxic agents.

SOG1 is target of ATM and ATR kinases, and the status of SOG phosphorylation is critical for response to DDRs, including transcriptional regulation, cell cycle progression, DNA integrity and PCD (Yoshiyama et al. 2014). Considering that PhyA has kinase activity, and that *phyA-211* mutant shows higher sensitivity to zeocin than *sog1* mutant, it would not be surprising that PhyA via phosphorylation regulating SOG1 activity in response to DNA damage in plants.

Here, by using physiological, pharmacological, genetic, and microscopy strategies, we have shown that (i) *PhyA* mutation compromise stem cell niche maintenance in the root meristems after exposition to genotoxic compounds; (ii) DNA damage-induced programed cell death in root meristem activates cell differentiation process, which trigger root tip exhaustion via a PhyA-dependent mechanism; (iii) PhyA accumulation in the root tips is regulated by treatment with genotoxic agents; (iv) cell damage induced by genotoxic compounds is HY5 independent; (v) PhyA controls cell cycle progression through regulation of MYB3R3 expression in root tips. Collectively, our data point to PhyA as a new key player involved in safeguarding DNA integrity and stem cell niche maintenance in response to DNA damaging stress.

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FIGURES

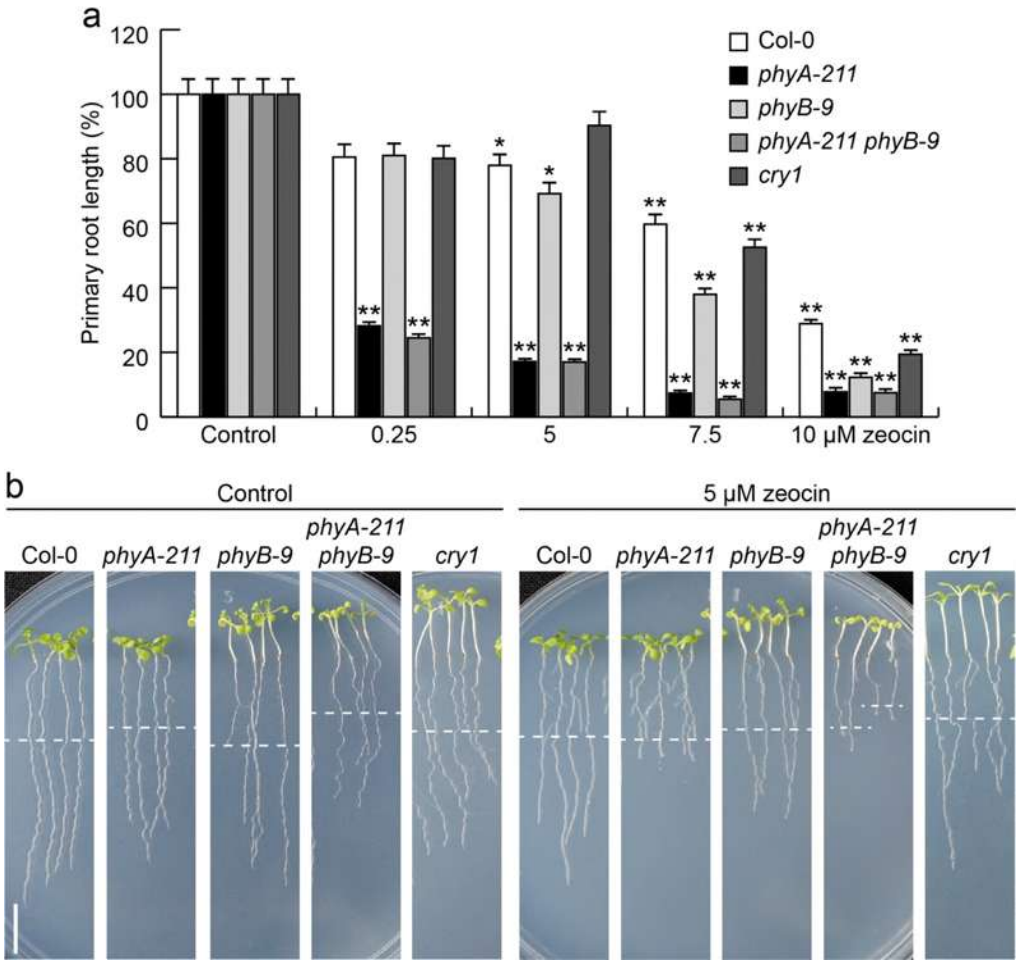

Figure 1. Effect of zeocin on the primary root growth of *Arabidopsis* blue and red/far-red photoreceptor mutants. Four-day-old *Arabidopsis* WT (ecotype Col-0) seedlings and the *phyA-211*, *phyB-9*, *phyA-211 phyB-9*, and *cry1* single and double mutants were transferred for 6 days to 0.2X MS medium supplemented with increasing zeocin concentrations. (a) The primary root length of either WT seedlings or photoreceptor mutants was normalized to 100% in the control condition. (b) Representative photographs of WT seedlings and the photoreceptor mutants 6 days after transference (DAT) to 0.2X MS medium in control and 5 µM zeocin treatments. Data were analyzed with the X^2 test and presented as mean ± SE from 15 seedlings. Values marked with (*) or (**) are significantly different from control condition plants at $p \leq 0.05$ or $p \leq 0.01$, respectively. The white dash lines indicate the start point of primary root after to transfer. The white scale bar represents 1 cm. The experiment was repeated three times with similar results.

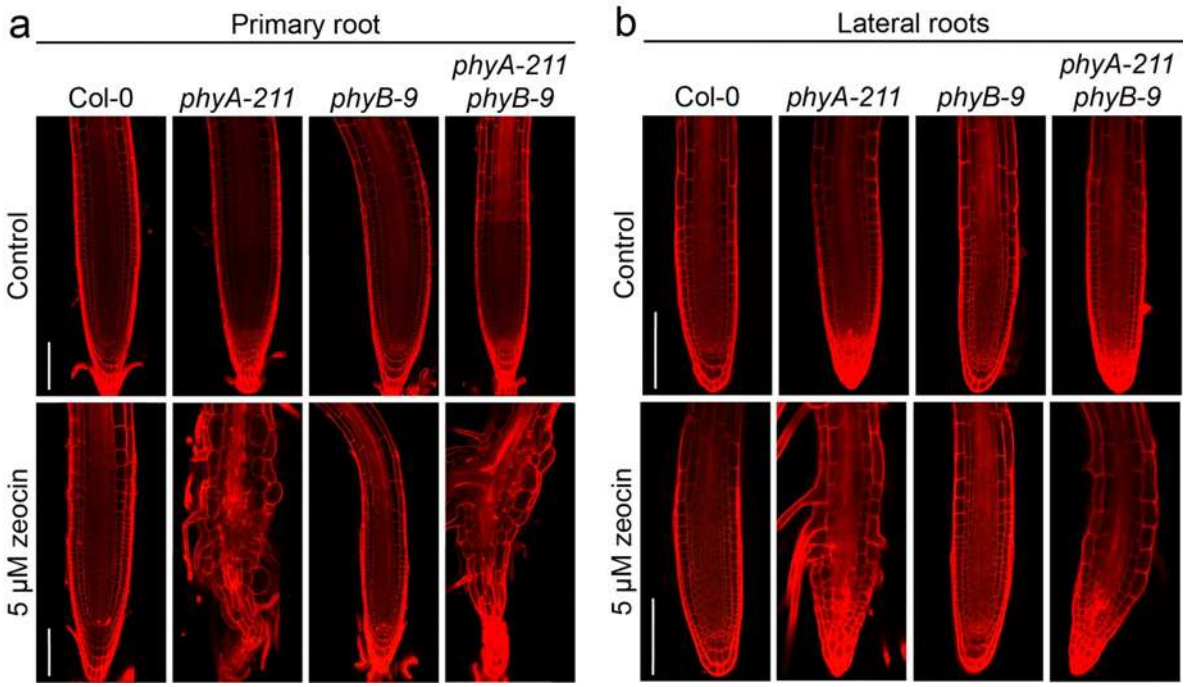

Figure 2. Effect of zeocin on primary and lateral root tips in WT plants and photoreceptor mutants. *Arabidopsis thaliana* WT (Col-0) and *phyA-211, phyB-9,* and *phyA-211phyB-9* seedlings grown for 4 days on agar-solidified 0.2X MS medium, then were transferred to agar-solidified 0.2X MS medium supplemented with 5 µM zeocin. Ten days after transfer, primary (a) and lateral (b) root tips were stained with propidium iodide and analyzed by confocal microscopy. The white scale bar represents 100 µm. The experiment was repeated three times with comparable results.

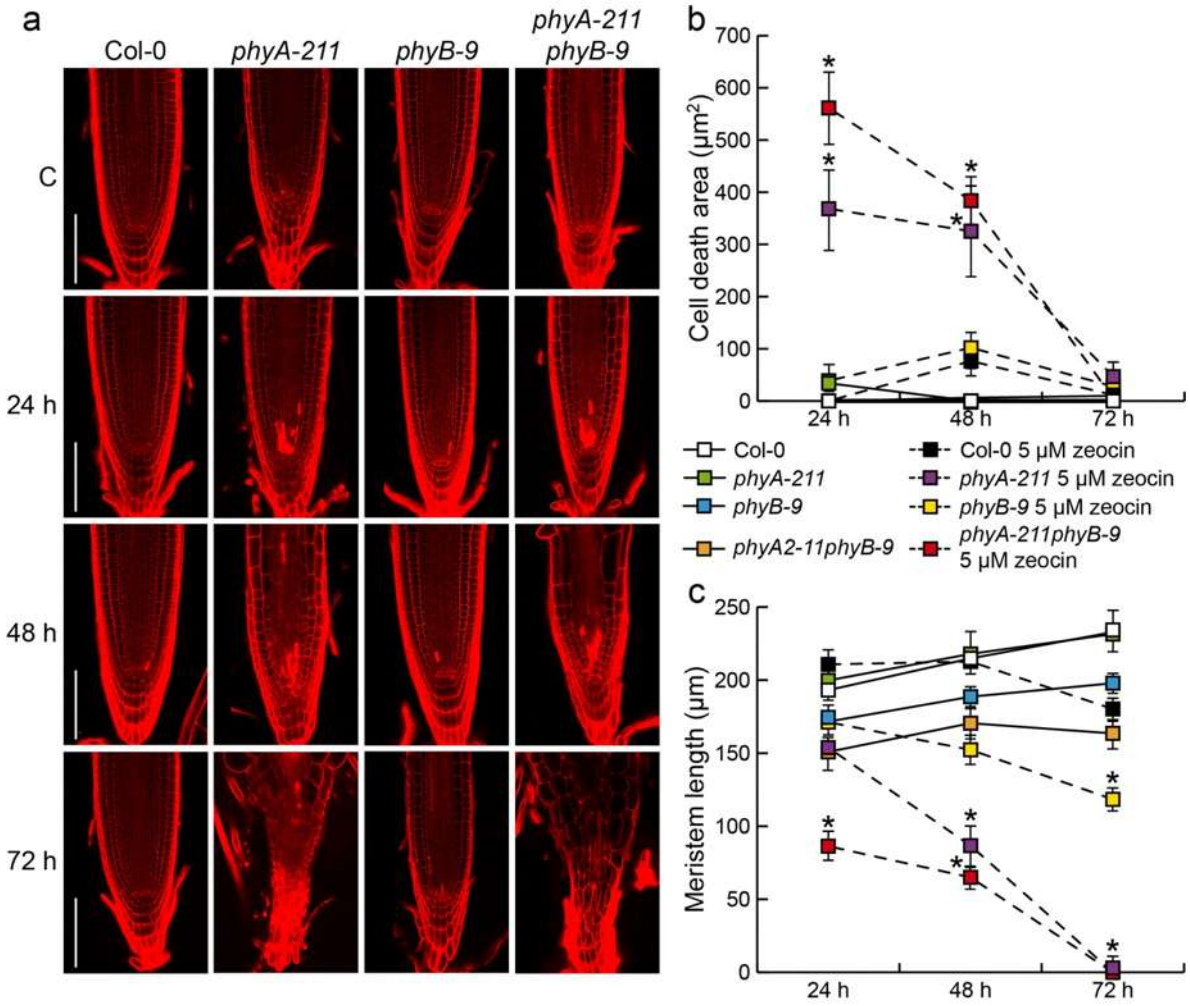

Figure 3. Time course of zeocin effects on primary root meristem in WT and photoreceptor mutants in *Arabidopsis*. Four-day-old WT (Col-0), *phyA-211*, *phyA-211phyB-9 Arabidopsis* seedlings were exposed to zeocin 5 μM for 24 h, 48 h, and 72 h, and stained with propidium iodide to be visualized by confocal microscopy. (a) Representative micrographs of the root meristems after zeocin exposure at the indicated times. (b) Cell death area in WT and *phyA-211*, *phyA-211phyB-9* root meristems after zeocin exposure. (c) Meristem length in WT and *phyA-211*, *phyA-211 phyB-9* root meristems after zeocin exposure. Values are presented as mean ± SE from 15 seedlings. Data were analyzed with the factorial ANOVA and Tukey's post-hoc test with a $p \leq 0.05$. Values marked with (*) denote significant differences between WT and mutant plants in the control and zeocin treatments. The experiment was repeated three times with comparable results.

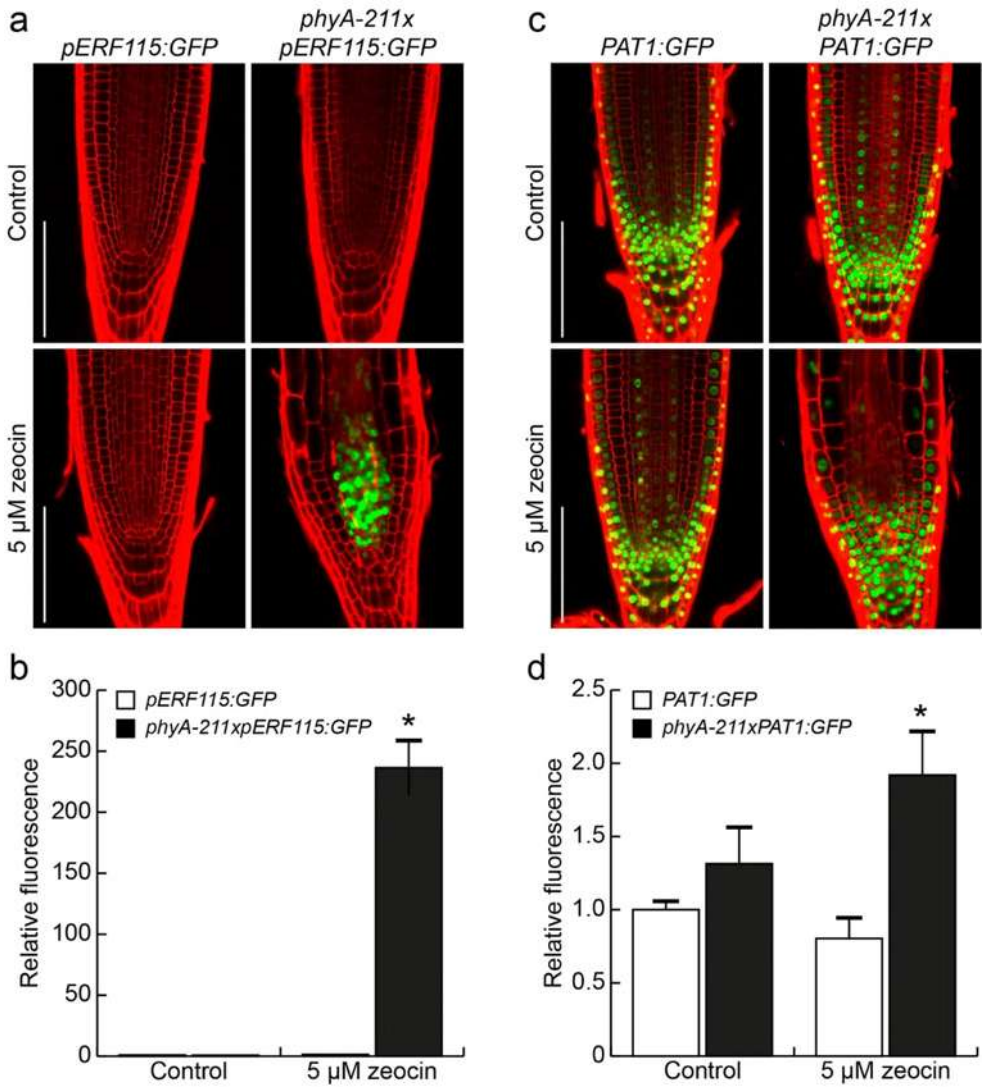

Figure 4. Expression of cell regeneration-related genes *ER115* and *PAT1* in response to zeocin in WT and *phyA-211* root meristems. *Arabidopsis* WT (Col-0) and the *phyA-211* mutant seedlings harboring *pERF115:GFP* and *pPAT1:GFP* gene constructs were exposed to 5 µM zeocin for 48 h. Representative micrographs and relative fluorescence (a, b) from *pERF115:GFP* and (c, d) *pPAT1:GFP* expression in the root meristem of WT and *phyA-211* seedlings stained with propidium iodide and analyzed by confocal microscopy. Note that induction of *ERF115* and *PAT1* expression colocalize with cell death zone in the *phyA-211* mutant after zeocin exposure. The factorial ANOVA and Tukey's post-hoc test determined the significant differences (*) at a $p \leq 0.05$ from 15 seedlings. The white scale bar inside (a) and (c) panels represents 100 µm. The experiment was repeated twice with similar results.

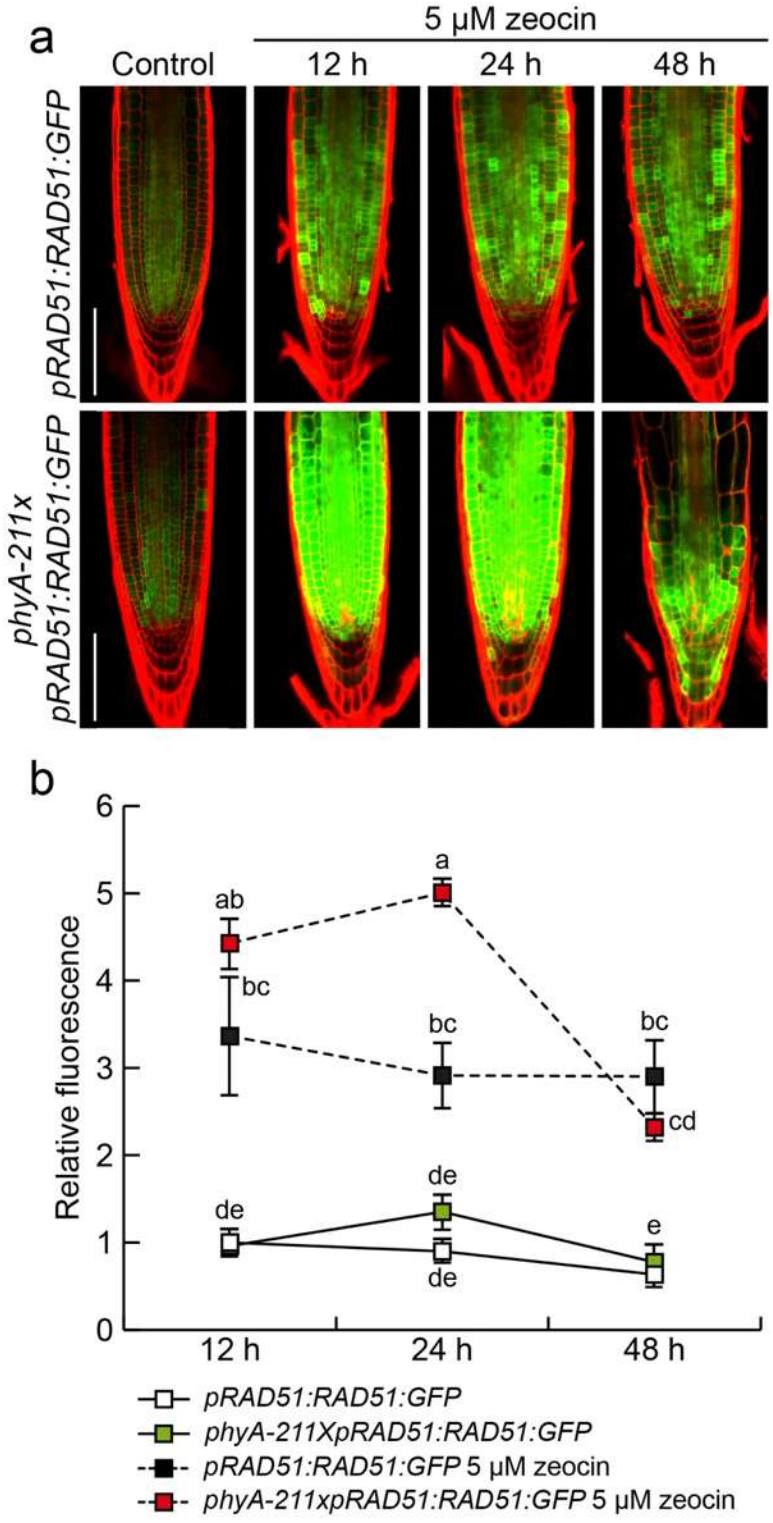
a
5 µM zeocin
Control
12 h
24 h
48 h
pRAD51:RAD51:GFP
phyA-211x
pRAD51:RAD51:GFP
b
Relative fluorescence
0
1
2
3
4
5
6
ab
a
bc
bc
bc
cd
de
de
de
e
12 h
24 h
48 h
pRAD51:RAD51:GFP
phyA-211XpRAD51:RAD51:GFP
pRAD51:RAD51:GFP 5 µM zeocin
phyA-211xpRAD51:RAD51:GFP 5 µM zeocin

Figure 5. Zeocin-induced cell death promotes RAD51 overexpression in the root of *phyA-211* mutant. Four-day-old *Arabidopsis* WT (Col-0) and *phyA-211* seedlings harboring the gene construct *pRAD51:RAD51:GFP* were transferred to agar-solidified 0.2X MS media supplemented with zeocin 5 μM for 12 h, 24 h, and 48 h. After zeocin exposure, primary root was stained with propidium iodide and analyzed by confocal microscopy. (a) Representative micrographs showing the expression pattern of *RAD51* in the root tip of WT and *phyA-211* seedlings. (b) Relative fluorescence. Note that zeocin exposure induces RAD51 overproduction in *phyA-211* root meristem. The white scale bar represents 100 μm. The factorial ANOVA and Tukey's post-hoc test determined the statistical significances at a $p \leq 0.05$ from 15 seedlings. Letters indicate significant differences. The experiment was repeated two times with similar results.

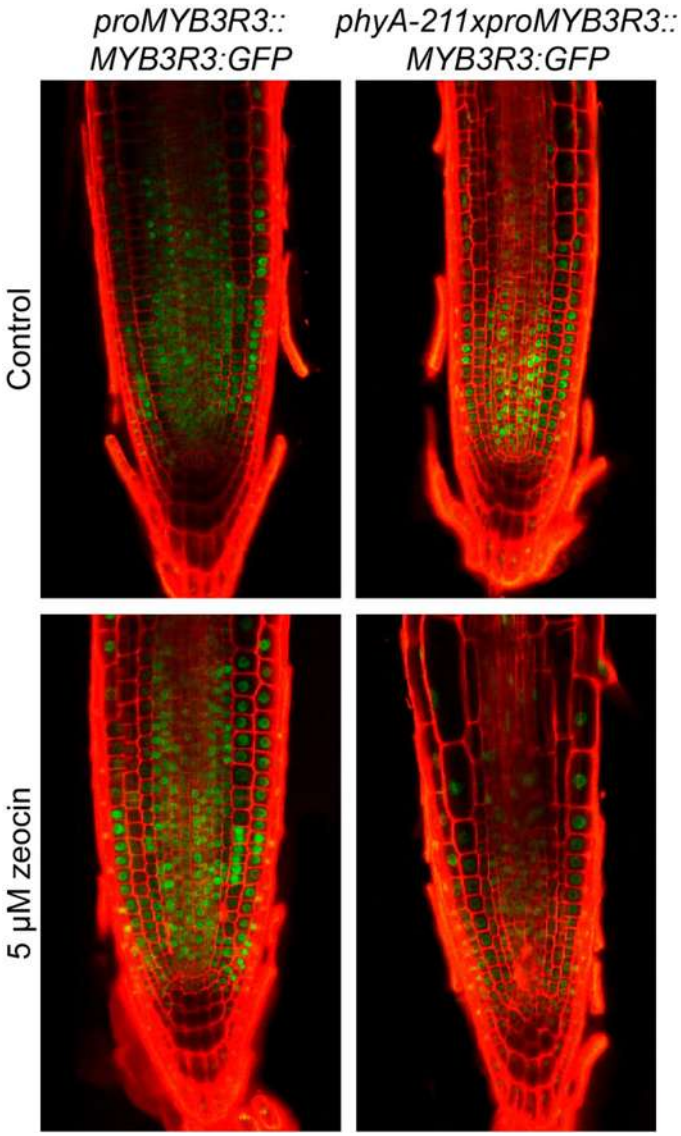

Figure 6. PhyA is required for MYB3R3 expression in the root meristem in response to genotoxic effect. Four-day-old *Arabidopsis* WT and *phyA-211* harboring the gene construction *pMYB3R3::MYB3R3:GFP* were transferred to MS 0.2X media supplemented with zeocin 5 µM and 48 h after exposition, MYB3R3 accumulation was analyzed by confocal microscopy. Note that under zeocin exposure, MYB3R3 expression is compromised in *phyA-211* mutant. The white scale bar represents 100 µm. The experiment was repeated twice with similar results.

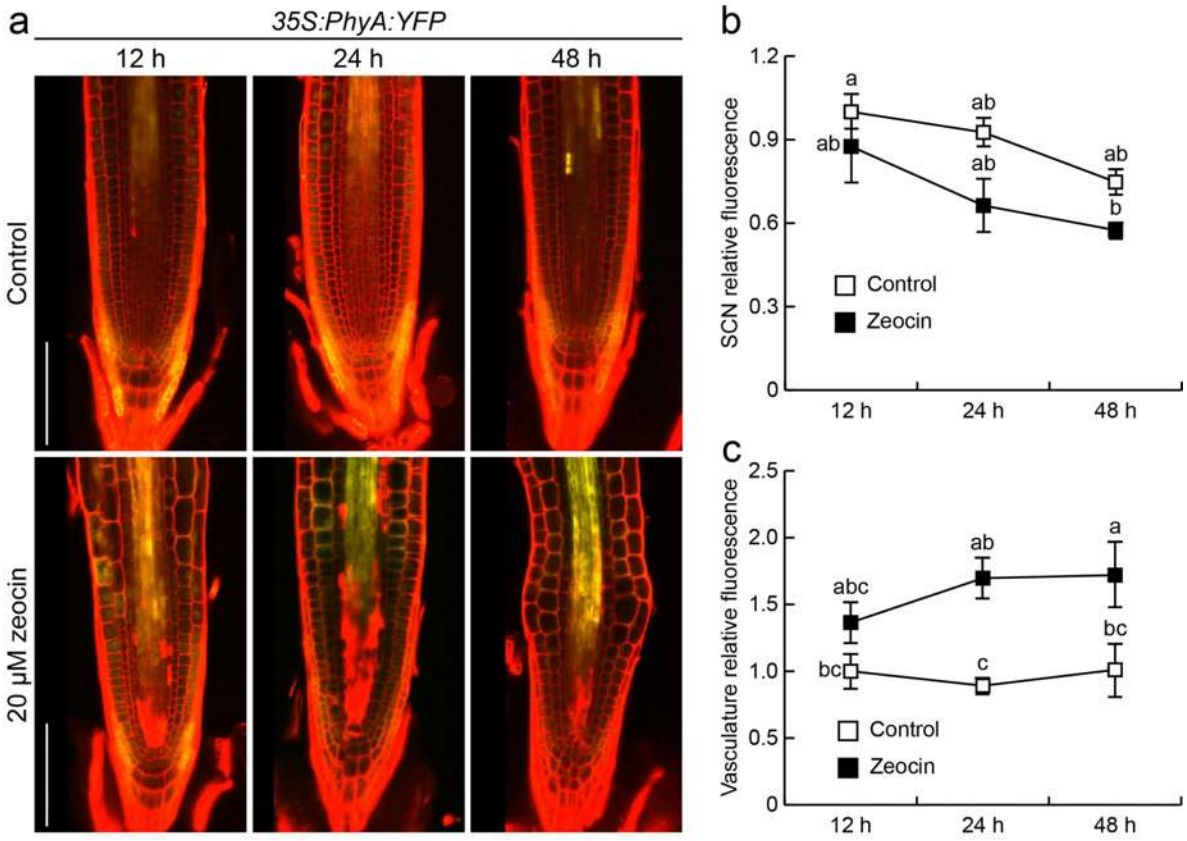

Figure 7. Effect of zeocin on PhyA accumulation in *Arabidopsis* root tips. *Arabidopsis* seedlings harboring the gene construction *35S::PhyA:YFP* were germinated and grown for 4 days on MS 0.2X, then were transferred to MS media supplemented with zeocin 20 µM and exposed with 12 h, 24 h, and 48 h. (a) Representative micrographs of *Arabidopsis* root tips. Relative fluorescence of *PhyA:YFP* in the stem cell niche (b) and vasculature (c) zones. Note that zeocin affects PhyA localization in root tips. The white scale bar represents 100 µm. The factorial ANOVA and Tukey's post-hoc test determined the statistical significances at a $p \leq 0.05$ from 15 seedlings. Letters indicate significant differences. The experiment was repeated two times with similar results.

7. DISCUSIÓN Y CONCLUSIONES

Recientemente se han identificado elementos de señalización en la regulación transcripcional que participa en el mantenimiento de la integridad celular (Babiychuk et al., 2017; Zhang et al., 2018; Raya-González et al., 2022). En *Arabidopsis*, la perdida de función del gen *MED18* codujo a la muerte celular de las células pro-vasculares dentro del meristemo de la raíz primaria. Sin embargo, la muerte celular no se presentó en las mutantes *med18* crecidas en la obscuridad, pero posteriormente fue restablecida en presencia de la luz. Por lo tanto, los autores concluyeron que la luz induce la fragmentación del ADN y promueve la muerte de las células meristemáticas en la raíz de *med18* (Raya-González et al., 2018).

La luz es un factor ambiental que altera el crecimiento y el desarrollo de las plantas. Las proteínas fotorreceptoras específicas de la luz roja/roja lejana (PhyA, PhyB), la luz azul (CRY1, CRY2) y la luz ultravioleta (UVR8) transducen la señal por luz en respuestas vegetales que impactan en la fotomorfogénesis, la reproducción, la germinación, etc., (Ponnu and Hoecker, 2021). Interesantemente, diversos análisis genéticos y moleculares han demostrado que los fotorreceptores de la luz roja y roja lejana controlan algunas respuestas de adaptación, incluyendo la des-etiolación, la evasión de sombra, el fototropismo y el gravitropismo (Correll et al., 2003; Su et al., 2015; Tepperman et al., 2006; Yang et al., 2018; Ma and Li, 2019). Se ha observado que un exceso de luz roja/roja lejana puede causar el estrés oxidativo y la muerte celular en el follaje durante la desetiolación mediada por PhyB (Chai et al., 2015; Chen et al., 2013). Además, recientemente se reportó que la luz roja restringe la respuesta hipersensible (RHS) durante el proceso de infección de la bacteria *Pseudomonas syringae* pv. tomato DC 3000 en hojas de tabaco (*Nicotiana benthamiana*) (Moyano et al., 2020).

La información anterior sugiere que la luz roja y roja lejana coordina las respuestas adaptativas con el mantenimiento de la integridad celular a través de la activación de los fitocromos PhyA y PhyB. Así que, optamos por caracterizar la participación de los fotorreceptores PhyA, PhyB y CRY1 durante el mantenimiento de la integridad celular en el meristemo de la raíz de *Arabidopsis* en respuesta a agentes genotóxicos que dañan el ADN y que causan estrés replicativo. Mediante

la experimentación con plantas mutantes afectadas en los fotorreceptores de luz roja/roja lejana y azul; encontramos que *phyA-211* y la doble mutante *phyA-211 phyB-9* presentaron una marcada hipersensibilidad al daño del ADN provocado por la exposición a agentes genotóxicos, como la zeocina y la fleomicina, pero no por el estrés replicativo provocado por la hidroxiurea (HU). El daño causado por la zeocina se asoció con la muerte de las células iniciales pro-vasculares, el agotamiento del meristemo, el colapso del haz vascular y la diferenciación temprana de la raíz primaria y las raíces laterales. El mismo fenotipo fue observado en las mutantes *tmo5* y *t5l* de los genes que controlan el crecimiento indeterminado de la raíz a través de las divisiones periclinales de la pro-vasculatura (De Rybel et al., 2013). Por lo tanto, estos resultados sugieren que el fotorreceptor PhyA regula el crecimiento indeterminado de la raíz a través de la protección del ADN y el mantenimiento de la integridad de las células iniciales de la pro-vasculatura.

El papel de PhyA ha sido ampliamente caracterizado en el follaje. No obstante, la función que desempeña en la raíz es pobremente conocida. Lo que sabemos es que PhyA regula la elongación de la raíz a través de la expresión de la acuoporina TIP2 y la emergencia de las raíces laterales laterales/adventicias (Uenishi et al., 2014; Kumari et al., 2019). Estas características se correlacionan con el dominio de expresión de PhyA, el cual se observó en la punta de la raíz y la vasculatura de la zona de diferenciación (Somers and Quail, 1995; Hall et al., 2001). Estos datos sugieren que PhyA podría tener funciones locales relacionadas con el crecimiento y el desarrollo de la raíz. A través del análisis de la construcción genética *35S:PhyA:YFP,* mostramos que *PhyA* se expresa predominantemente en la cofia lateral de la raíz en condiciones basales; sin embargo, la zeocina reduce su expresión en esta zona y la incrementa en el haz vascular, indicando que la expresión de *PhyA* en la punta de la raíz es regulada en función del daño acumulado.

El factor de transcripción HY5 es un intermediario de la vía de señalización de luz que funciona rio abajo de todos los fotorreceptores. De este factor dependen la fotomorfogénesis y las respuestas adaptativas al estrés ambiental impuesto por la radiación UV-B (Tissot and Ulm, 2020; Podolec et al., 2022). HY5 es un elemento móvil que comunica al follaje con la raíz; es decir, transduce las señales de luz

percibidas en el follaje hacia la raíz para activar las respuestas de captación de nutrientes y del desarrollo (Chen et al., 2016; van Gelderen et al., 2018). Interesantemente, algunos reportes señalan que HY5 interactúa con PhyB y los factores PIFs durante las respuestas de desetiolación para promover la muerte celular programada en el follaje de *Arabidopsis* (Chen et al., 2013; Chai et al., 2015). Por lo tanto, procedimos a evaluar el crecimiento de la raíz primaria de las mutantes *hy5* crecidas en tratamientos con agentes genotóxicos y encontramos que el factor HY5 no está involucrado en el mantenimiento de la viabilidad celular en el meristemo de la raíz de *Arabidopsis*.

La zeocina es un antibiótico genotóxico que induce la fragmentación del ADN produciendo rupturas de una o las dos cadenas. Las cinasas ATM y ATR detectan los daños en el ADN y fosforilan al factor de transcripción SOG1 de la familia NAC (NAM-ATAF-CUC) para controlar su actividad (Yoshiyama et al., 2009; Yoshiyama, 2016). Resultados previos mostraron que las plantas con pérdida de función de *SOG1* son incapaces de tolerar el daño genotóxico inducido por la radiación gamma y la zeocina en las hojas y la raíz (Preuss and Britt, 2003; Johnson et al., 2017; Yoshiyama et al., 2017). En el presente trabajo comparamos la sensibilidad de las mutantes *phyA-211* y *sog1* en una cinética de 2, 4 y 6 días de exposición a la zeocina. Notamos que el crecimiento de la raíz primaria de las mutantes *sog1* fue arrestada a los 4 días de exposición, mientras en *phyA-211* el arresto del crecimiento ocurrió a los 2 días. Este resultado denota la importancia biológica que tiene PhyA sobre la tolerancia al estrés genotóxico, la cual puede ser mayor que el de SOG1.

La activación de la cascada de señalización ATM-ATR-SOG1 por el daño en el ADN conduce al arresto del ciclo celular, el cual ocurre a través de la inhibición de proteínas específicas de la fase G2/M (Gentric et al., 2021). No obstante, resultados previos mostraron que los agentes genotóxicos promueven la expresión de *CYCB1* (Culligan et al., 2006; Yoshiyama et al., 2009; Adachi et al., 2011). En este trabajo, analizamos la expresión de CYCB1 mediante el uso de la construcción genética *CYCB1::uidA* en el meristemo de la raíz primaria de las plantas Col-0 y las mutantes *phyA-211* durante un periodo de 24, 48 y 72 h de exposición a la zeocina. Nuestros resultados mostraron que la zeocina incrementó significativamente la expresión de

CYCB1 en el meristemo de la raíz de las plantas Col-0. En las mutantes *phyA-211*, la zeocina incrementó expresión de *CYCB1* a las 48 h del tratamiento; sin embargo, la expresión decreció a las 72h de exposición. Interesantemente, la expresión de *CYCB1* se colocalizó en el área de muerte celular observada en las mutantes *phyA-211* y su expresión disminuyó en función del tiempo de exposición, el daño acumulado y el estado de diferenciación de la raíz, lo que sugiere que PhyA desempeña una función critica en el mantenimiento de los niveles de expresión de *CYCB1* ante el estrés genotóxico. Podemos concluir que la perdida de función del gen *PhyA* y el estrés genotóxico conducen a la muerte de las células pro-vasculares y al agotamiento de meristemo debido a la desregulación del ciclo celular dependiente de CYCB1.

La percepción del daño en el ADN provoca que SOG1 promueva la activación de la proteína CYCB1, la cual junto con CDKB1;1 (CYCB1;1-CDKB1;1) fosforilan a la recombinasa RAD51 para promover el mecanismo de reparación del ADN a través de la recombinación homóloga (HR) (Da Ines et al., 2013; Weimer et al., 2016; Ogita et al., 2018). Dado que la mutante presentó una respuesta alterada en la expresión de *CYCB1* tras la inducción del daño. Decidimos analizar la expresión de *RAD51* utilizando la construcción traduccional *RAD51:RAD51:GFP* en el meristemo de la raíz en la mutante *phyA-211* expuesta a la zeocina. Los análisis revelaron que *RAD51* presentó un patrón de expresión similar al de *CYCB1* en tiempo cortos y extensos de exposición a la zeocina. Los resultados reportados por Weimer y col. (2016) demuestran que las plantas con la perdida de función de *CYCB1* son incapaces de reclutar a RAD51 en el sitio del daño. Nuestros datos muestran que la expresión de *RAD51* y *CYCB1* colocalizan en el sitio del daño dentro del meristemo de las mutantes, sugiriendo que los mecanismos de reparación del ADN dependientes de RAD51 y CYCB1 se encuentran intactos en las mutantes *phyA-211*.

El arresto del ciclo celular regulado por SOG1 ocurre a través de la activación de la familia de los factores de transcripción Myb (myeloblastosis), incluyendo a los represores MYB3R3 y MYB3R5 (Rep-Myb). En *Arabidopsis*, MYB3R3 se expresa en los núcleos de las células dentro del meristemo de la raíz, donde directamente suprime la transcripción de los genes necesarios para la progresión de la mitosis y

la citocinesis, como CYCB1;2, CYCB1:4 y KNOLLE (Chen et al., 2017). De acuerdo con lo reportado por Haga y col. (2011), existen al menos 180 genes que están regulados por los factores Myb, por lo que su identificación resultó ser vital para entender las respuestas dirigidas por las vías canónicas de la señalización de ATM-ATR-SOG1 durante la regulación de la muerte celular y la prevención de la transmisión de mutaciones o ADN aberrante a células hijas derivadas de la división en los meristemos del follaje y la raíz. Interesantemente, encontramos que la expresión de *MYB3R3,* mediante el uso de la construcción traduccional *proMYB3R3::MYB3R3:GFP,* se reduce en el meristemo de la raíz de las mutantes *phyA-211* expuestas a la zeocina. Además, la expresión reducida de MYB3R3 correlacionó con el nivel de expresión de *CYCB1:uidA* a las 48 h del tratamiento, lo que demuestra que PhyA regula positivamente la expresión de *MYB3R3* para reprimir la división celular en respuesta a la fragmentación del ADN. Reportes previos demostraron que el complejo CDKB1/2-CYCB1 fosforilan a los factores MYB3R3 para reprimir su actividad (Chen et al., 2017). En cambio, los factores de transcripción ANAC044/085 de la familia NAC directamente promueven la acumulación de MYB3R3 para reprimir el ciclo celular en respuesta al estrés genotóxico (Takahashi et al., 2019). Tomando en cuenta que PhyA es una cinasa y la actividad de los factores SOG1, CDKB1/2-CYCB1 y ANAC044/085 dependen directamente e indirectamente de su estatus de fosforilación, PhyA podría regular a estos elementos y garantizar una respuesta eficiente ante los diferentes estresores que comprometen la integridad del ADN.

8. REFERENCIAS

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9. ADDENDA

Mendoza-Vázquez, M. A., Espinoza-Madrigal, R. M., Muñoz-Parra, E., Flores-García, A., **Huerta-Venegas, P. I**., Ruiz-Herrera, L. F., Martínez-Pacheco, M., & López-Bucio, J. (2019). Growth and development of *Arabidopsis thaliana* seedlings in interaction with fungi isolated from stem-end rot of avocado fruits. *Archives Of Phytopathology and Plant Protection.* 52:681-697.

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ABSTRACT

The influence of *Phomopsis viticola*, *Diaporthe phaseolorum* and *Pseudofusicoccum stromaticum* isolated from avocado stem-end rot on growth of *Arabidopsis* seedlings was investigated. We found responses that are specific for each fungus, whereas *P. viticola* and *D. phaseolorum* strongly repressed root growth, *P. stromaticum* induced growth and branching, and enhanced shoot biomass production. Detailed microscopy and structural analyses revealed that the fungi did not affect cell division in root meristems but rather influenced cell elongation and differentiation, and these responses were related to changes in auxin responsiveness. *P. stromaticum* strongly acidifies the growth medium and this correlated with induction of the jasmonic acid responsive gene construct *LOX2:GUS* in leaves, whereas *P. viticola* and *D. phaseolorum* showed much reduced acidification properties. Taken together, our results show that fungal isolates from the stem-end of avocado fruits interact with *Arabidopsis* plants in highly diverse and contrasting manners influencing growth, patterning and defence.

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1. Introduction

The plant disease stem-end rot of avocado fruits is caused by several fungi. It is characterised by a dark brown to black coloration halo, which further produces dark streaking of the water-conducting tissues, damages the edible mesocarp and reduces quality and economic value post-harvest. *Neofusicoccum*, *Phomopsis*, *Diaporthe* and *Colletotrichum* have been related to stem-end rot (Twizeyimana et al. 2013; Guarnaccia et al.

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2016). These fungi may be present on the avocado tree and grow into the stem of the fruit before harvest to develop stem-end rot upon tissue colonisation, afterwards the fungal pathogens proliferate taking up nutrients, which leads to the appearance of disease symptoms such as necrosis and black coloration of mesocarp, which reduces commercial acceptance (Hartill and Everett 2002).

In response to pathogen challenge, plants establish structural, biochemical and molecular responses to defend themselves, including cell wall reinforcement as well as production of protective metabolites such as phytoalexins that are toxic to pathogens and/or activate the so-called hypersensitive response. This later consists of the appearance of small necrotic patches around the infection site that restrict pathogen spread (Lam et al. 2001). In addition, an increased expression of pathogenesis-related (PR) proteins and defensins (PDFs) reinforces plant immunity (Narasimhan et al. 2001). The plant hormones jasmonic acid (JA), ethylene (ET), and salicylic acid (SA) are the main defence regulators that orchestrate signal transduction pathways involving both nitric oxide (NO) and reactive oxygen species (ROS) as second messengers for regulation of genes encoding inducible defence proteins (Bolwell 1999; Robert-Seilaniantz et al. 2011).

Fungi are metabolically diverse and this enables their adaptation to diverse habitats. Success of pathogens in colonising plants depends on the release of toxic proteins and secondary metabolites collectively called "effectors", which can kill cells, suppress immunity or alter physiological responses via hormonal regulation (Kazan and Lyons 2014; Presti et al. 2015; Chanclud and Morel 2016; Patkar and Naqvi 2017). Moreover, several fungi change pH as consequence of metabolic activities or produce plant hormones such as cytokinins or auxins that are perceived by their hosts and affect growth, development or defence via modulating mitogen activated protein kinase (MAPK) signalling (Contreras-Cornejo et al. 2009; Contreras-Cornejo et al. 2015; Pelagio-Flores et al. 2017; Spallek et al. 2018). Thus, fungal metabolites, including effectors, are critical players in cross-kingdom relationships between plants and fungi.

Arabidopsis thaliana is a good model for plant-pathogen studies since more than three decades of research led to an extensive collection of mutants and overexpressing lines for genes related to hormone homeostasis. Moreover, an increasing number of lines are available, which express defence-inducible promoters fused to reporter genes encoding fluorescent proteins or enzymes that can be used to investigate in a tissue specific level the effects of selected fungal isolates, such as those related to stem-end rot disease (O'Connell et al. 2004; Andargie and Li 2016). Recently, *in vitro* growth systems have been developed to

investigate the early steps in the plant-fungus communication and recognition, allowing identification of auxin, JA and ET as critical components in the molecular dialogue established (Contreras-Cornejo et al. 2009; Splivallo et al. 2009; Casarrubia et al. 2016; Cordovez et al. 2017).

To enhance resistance of crops to pathogens, a better understanding of plant hormone homeostasis as well as the subsequent metabolic readjustments during the interaction are required. Although avocado stem-end rot is a tremendous problem in several countries (Twizeyimana et al. 2013; Guarnaccia et al. 2016), the mechanism is scarcely investigated. Advances in this field may help develop breeding strategies to decrease disease severity and improve post-harvest performance and quality.

Here, *in vitro* experiments were performed to test the effects of *Phomopsis viticola*, *Diaporthe phaseolorum* and *Pseudofusicoccum stromaticum*, isolated from stem-end avocado fruits in growth, development and defence-related gene expression in *Arabidopsis* seedlings. The data show a complex network of interactions, by which each fungus differentially modifies root architecture and cell patterning likely influencing auxin signalling. Moreover, we investigated the acidification capacity and induction of jasmonic acid-related *LOX2:GUS* in leaves following root exposure to all three fungal species, which showed the correlation between acidification of the medium and plant defence response in interaction with *P. stromaticum*.

2. Materials and methods

2.1. Plant material and growth conditions

A. thaliana (Col-0) ecotype and transgenic plants that express the mitotic cyclin *CycB1,1:GFP* (Weimer et al. 2016), expansin protein *EXP7:GUS* (Cho and Cosgrove 2002), auxin-inducible construct *DR5:GFP* (Ottenschläger et al. 2003) and jasmonic acid-inducible *LOX2:GUS* (Jensen et al. 2002) were used in this study. Seeds were first disinfected with ethanol (95%) for 5 min and bleach (20%) containing sodium hypochlorite (NaOCl) as bioactive ingredient for 7 min, washed five times with distilled water, and stratified for 2 d at 4 °C. Seeds were sown on agar plates (9 cm diameter) supplied with 0.2X Murashige and Skoog medium (MS basal salts mixture, M524; PhytoTechnology), 0.6% sucrose (Sucrose: Ultrapure, MB Grade, 21938; USB Corporation) and 1% Agar (Agar, Micropropagation Grade, A111; PhytoTechnology) at pH 7. The suggested formulation is 4.3 g/L of salts for 1x medium; we used 0.9 g/L, which we consider and refer to as 0.2X MS. MES was included in the medium and the pH was adjusted to 7.0. The plates were included into a

Percival AR95L growth chamber, with 6 h light:8h darkness, 200 µmol m/s light and 22 °C of temperature. Seedlings were photographed using a Nikon SMZ1500 stereomicroscope equipped with a digital camera (Nikon SIGHT DS-Fi1c, Nikon Corporation, Tokyo, Japan).

2.2. Fungal strains and culture conditions

P. viticola 5480, *D. phaseolorum* 5185, and *P. stromaticum* were isolated from pericarp of avocado fruits cv. "Hass" collected in the main avocado-producing area at the Michoacán state in México. The isolates were phenotypically and molecularly characterised and the disease symptoms were clearly demonstrated upon infection of fruits in vitro with *P. viticola*, suggesting it as a stem-end rot causal agent in avocado. Although it remains to be determined if *D. phaseolorum* and *P. stromaticum* also cause fruit decay symptoms, the fact that they were isolated from symptomatic fruits suggest that they might be related to the disease.

Fungal inoculum was propagated on potato dextrose agar (PDA; Difco), at 28 °C. One cm fragment of hyphae was placed at 4 cm from *A. thaliana* primary root tips germinated and grown for 5 days on agar plates containing 0.2X MS medium. Each plate, which included five *A. thaliana* seedlings were arranged in a completely randomised design and the experiment included at least three plates per treatment. After 4 d of co-cultivation, plant growth was determined. For acidification experiments the fungi were inoculated on plates containing 0.2X MS medium supplemented with bromophenol blue (0.006%) and analysed 4 d every 24 h.

2.3. Propidium iodide staining and GFP detection

A. thaliana seedlings expressing the GFP protein were grown axenically or in co-cultivation with stem end fungi, then taken up from the medium and included in microscope slides and covered by propidium iodide (PI, 20 µM) solution. GFP detection was done in a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan), with a 568-nm wavelength argon laser for excitation, and an emission window of 585–610 nm for propidium iodide and GFP fluorescence (488 nm excitation/505–550 nm emission, 514 nm excitation/527 nm emission, and 532 nm excitation/588 nm emission, respectively). Ten independent seedlings were analysed, and representative images were selected for figure construction.

2.4. Histochemical analysis of GUS expression

Seedlings expressing GUS protein were first grown for 5 d on the agar plates supplemented with MS 0.2X salts and the fungal inoculum was placed at two cm from the root tip and co-cultivated for a 4 d period. GUS analyses were done via incubation of seedlings in 0.1% X-Gluc (5-bromo-4-chlorium-3-indolyl-D-glucuronide) in phosphate buffer (NaH_2PO_4 and Na_2HPO_4, 0.1 M; pH 7), 10 mM EDTA, 0.1% (v/v) Triton X-100 with 2 mM potassium ferrocyanide and 2 mM potassium ferricyanide for 12 h at 37 °C. Plants were cleared and fixed using the procedure by Malamy and Benfey (1997). The analysis included 15 transgenic seedlings and representative photographs showing the blue colour indicative of GUS expression were taken.

2.5. Analysis of plant growth

The growth of the primary root was measured using a ruler. Lateral roots were counted from the tip to the root/stem transition in primary roots. Root hair images were taken using a digital camera (Nikon D3300 Osaka, Japan). Data were analysed using the STATISTICA 10 software (Stat Soft, Inc. 2011). Univariate and multivariate analyses with a Tukey's post hoc test helped to establish significant differences among the means. Different letters indicate means that statistically differ ($P \leq .05$).

3. Results

3.1. Effects of stem-end-related fungi on growth of Arabidopsis seedlings

Previous results showed that avocado fruits harvested from Michoacán state, México orchards showing stem-end rot symptoms were colonised with the fungi *P. viticola*, *D. phaseolorum* and *P. stromaticum* (Figure 1). To understand the effects that each fungus has on *A. thaliana* seedlings, biomass production and root growth patterns were analysed after 4 days of fungal co-cultivation in close proximity to the primary root. Shoot fresh weight, primary root length and lateral root number were quantified for 15 seedlings per treatment. While *P. viticola* and *D. phaseolorum* did not significantly affect shoot biomass, *P. stromaticum* almost duplicated the fresh weight, which correlated with a 5-five-fold increase in lateral root number (Figure 2(a–d)). In contrast, *P. viticola* and *D. phaseolorum* strongly repressed primary root growth (Figure 2(b,d)). These data show the dynamic responses of *Arabidopsis* to stem-end-related fungi, and the very contrasting influence of *P. viticola* and *P. stromaticum* on plant growth and development.

3.2. *Stem-end-related fungi* P. viticola *and* D. phaseolorum *increase root hair length*

Since *P. viticola* and *D. phaseolorum* strongly repressed primary root growth (Figure 2(b)), it was of interest to evaluate if the root

Figure 1. Avocado stem-end rot. Representative images of avocado fruits developing the disease symptoms as ripening proceeds. The symptoms shown arise following infection with *P. viticola*.

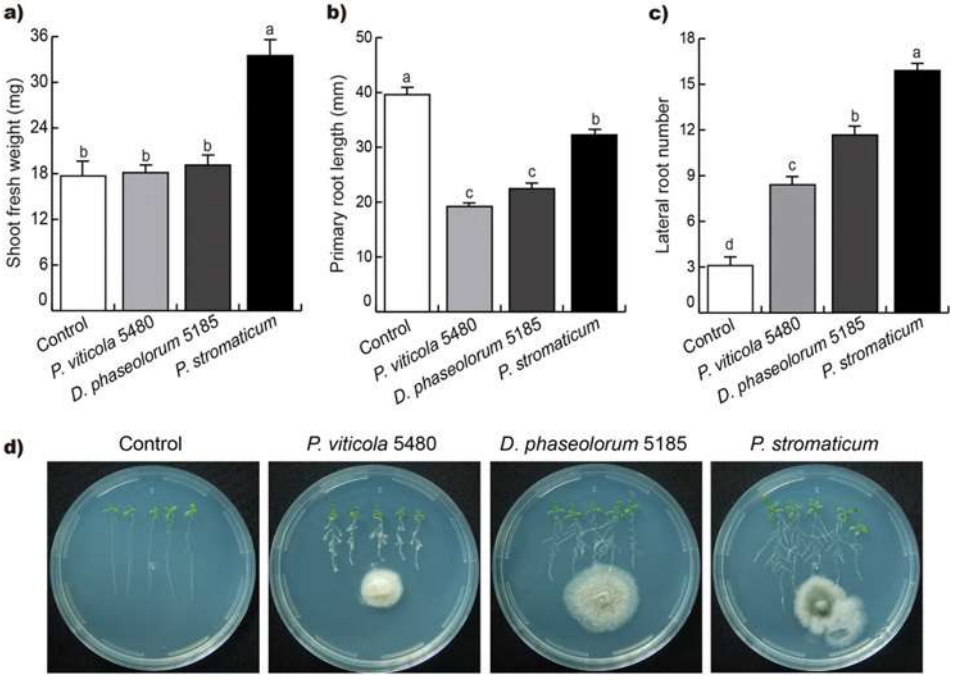

Figure 2. Effects of stem-end-related fungi co-cultivation on *Arabidopsis* shoot biomass and root architecture. (a) Shoot fresh weight; (b) primary root length; (c) lateral root number; and (d) representative photographs of *Arabidopsis* seedlings co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum*. *Arabidopsis* seedlings were germinated and grown for 5 d on the surface of agar plates containing MS 0.2X medium and then inoculated with fungal mycelium at two cm from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P < .05$), $n = 15$. Graph bars show the means ± SE. The experiment was repeated three times with similar results.

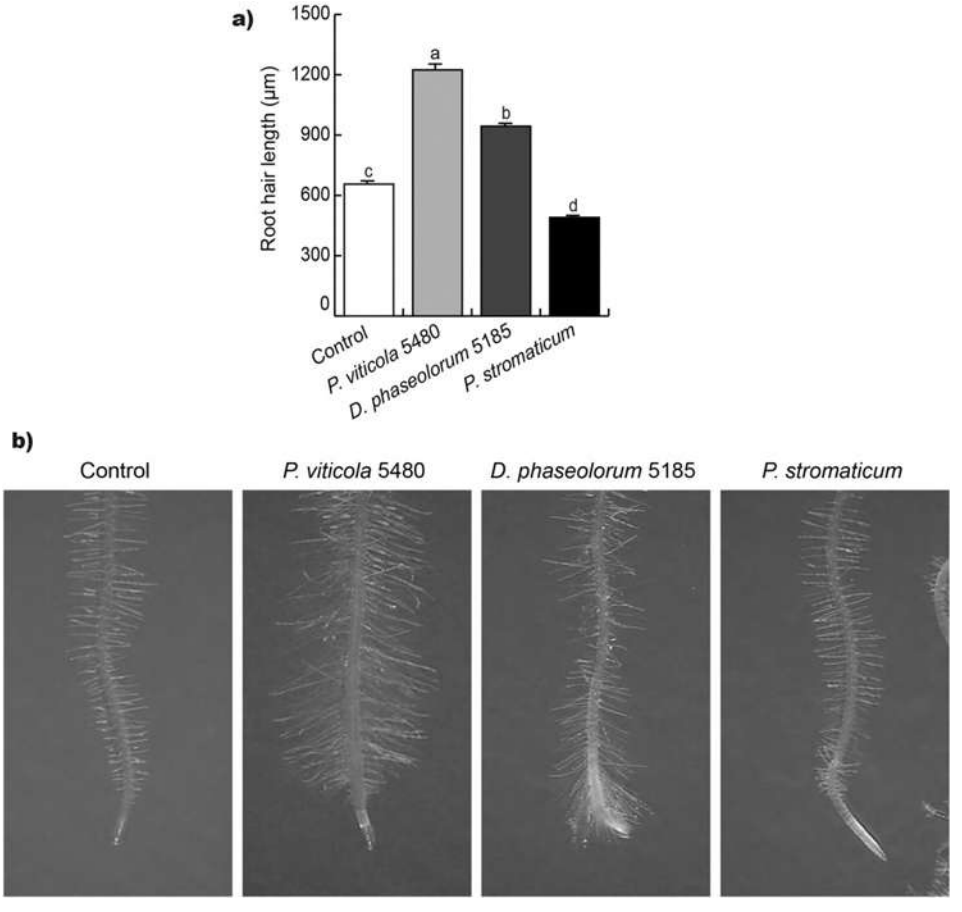

Figure 3. Effects of stem-end-related fungi co-cultivation on *Arabidopsis* root hair differentiation. (a) Root hair length and (b) representative photographs of root hairs developed on *Arabidopsis* primary roots grown under axenic conditions or co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum*. *Arabidopsis* seedlings were germinated and grown for 5 d on the surface of agar plates containing MS 0.2X medium and then inoculated with fungal mycelium at two cm from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P < .05$). Graph bars show the means ± SE. The experiment was repeated three times with similar results.

architectural adjustments elicited by these fungi, could influence epidermal cell differentiation. Root epidermal cells differentiate into root hairs, which are important for uptake of water and nutrients. We measured the length of 100 root hairs from primary roots of 15 independent *Arabidopsis* seedlings grown in axenic condition or co-cultivated with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. From all three fungi examined, *P. viticola* and *D. phaseolorum* could increase the length of root hairs, whereas *P. stromaticum* had the opposite effect, decreasing root hair growth (Figure 3(a,b)). These data show that root hairs develop as

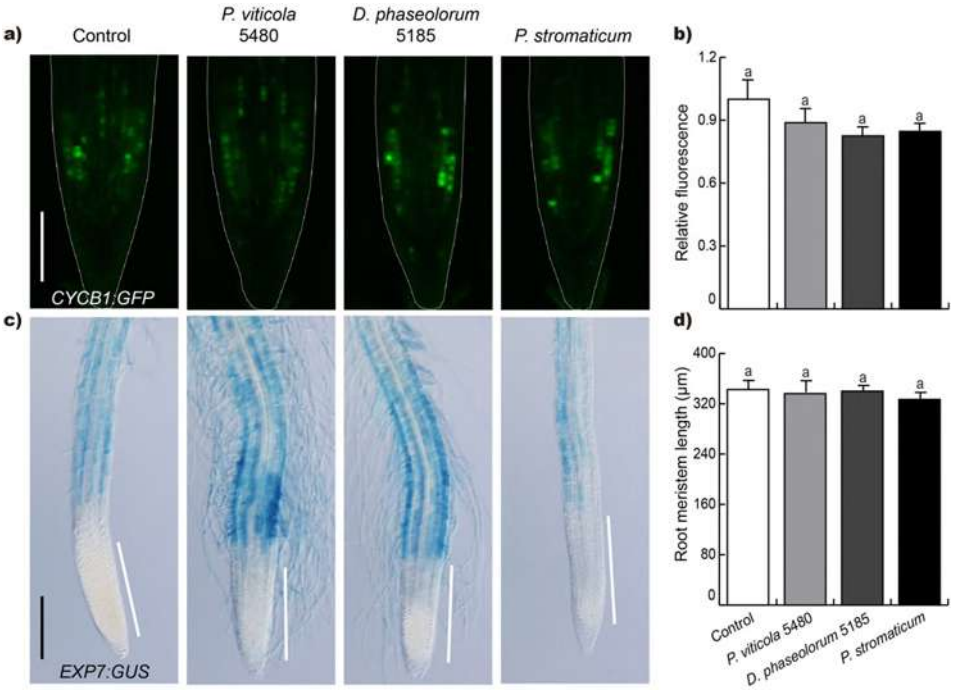

Figure 4. Effects of stem-end-related fungi co-cultivation on root cell division and differentiation. (a) Confocal microscopy images of expression of cell division marker *CycB1:GFP*; (b) cuantification of GFP fluorescence within primary root meristems; (c) expression of the cell differentiation marker *EXP7:GUS*; and (d) Length of the primary root meristem. Five-day-old *Arabidopsis* seedlings expressing the different genetic constructs were co-cultivated with *P. viticola 5480, D. phaseolorum 5185,* and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings and data represent the means of 15 seedlings. Scale bars = 100 µm. These analyses were performed twice with similar results. Note the increased expression of *EXP7:GUS* at the root hair forming zone of plants co-cultivated with *P. viticola* 5480 and *D. phaseolorum* 5185, which correlated with prolific growth of the cells.

primary root growth decreases and it occurs as a response to specific stem-end-related fungi.

3.3. P. viticola *and* D. phaseolorum *induce the expression of cell differentiation gene* EXP7:GUS

The expression of cell division and differentiation markers *CycB1,1:GFP* (Weimer et al. 2016) and *EXP7:GUS* (Cho and Cosgrove 2002), respectively, was assessed by growing plants under axenic conditions or in co-cultivation with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. Interestingly, no changes in *CycB1,1:GFP* already occur in meristems of primary roots from seedlings co-cultivated with the fungi (Figure 4(a,b)). However, these analyses revealed an increased expression of *EXP7:GUS* at the root hair forming zone of plants co-cultivated with *P.*

viticola and *D. phaseolorum* (Figure 4(c)), which correlated with prolific growth of these epidermal cells. Consistently with expression of CycB1,1:GFP, no significant changes were found in the length of the primary root meristem in axenic controls or seedlings inoculated with the fungi (Figure 4(d)). These data suggest that the inhibition of root growth is unlikely due to the production of toxins or virulence factors, since the root meristems remains viable (Figure 4(c), white lines) and instead it may be due to readjustments in cell growth and differentiation programs.

3.4. Effects of stem-end-related fungi on acidification of the growth medium

Stem-end pathogens can inhabit the unripe end or fruit cuticle, remain quiescent until ripening, and then proliferate. These fungal pathogens secrete organic acids or ammonia, which modify the host pH environment (Alkan et al. 2013; Prusky et al. 2016). Moreover, recent data demonstrated that fungi interacting with plants, such as *T. atroviride* induces acidification, which re-configurates *Arabidopsis* root architecture and this trait determines fungal phytostimulation (Pelagio-Flores et al. 2017). To assess whether *P. viticola*, *D. phaseolorum* or *P. stromaticum* could modify pH of the medium, equal amounts of fungal mycelia in MS 0.2X medium supplemented with bromophenol blue, which is used as a pH indicator. *P. stromaticum* strongly acidifies the growth medium, a property shared by *T. atroviride*. The growth repressing fungi *P. viticola* and *D. phaseolorum* showed much reduced acidification properties (Figure 5). Therefore, the amount of media acidification by stem-end-related fungi is species specific.

3.5. Effects of P. viticola *and* D. phaseolorum *on plant growth in buffered media*

To determine if the negative effects of *P. viticola* and *D. phaseolorum* on primary roots of *Arabidopsis* seedlings could be related to acidification, we evaluated the plant-fungal interactions in pH-buffered media. Under these conditions, *P. viticola* and *D. phaseolorum* did not increase shoot biomass production but strongly promoted lateral root formation (Figure 6(a,c)). Indeed, the growth repressing effects of these fungi on primary roots could not be attributed to acidification since both fungal isolates still repressed growth in pH-buffered media (Figure 6(b)). We conclude that acidification of the medium by stem-

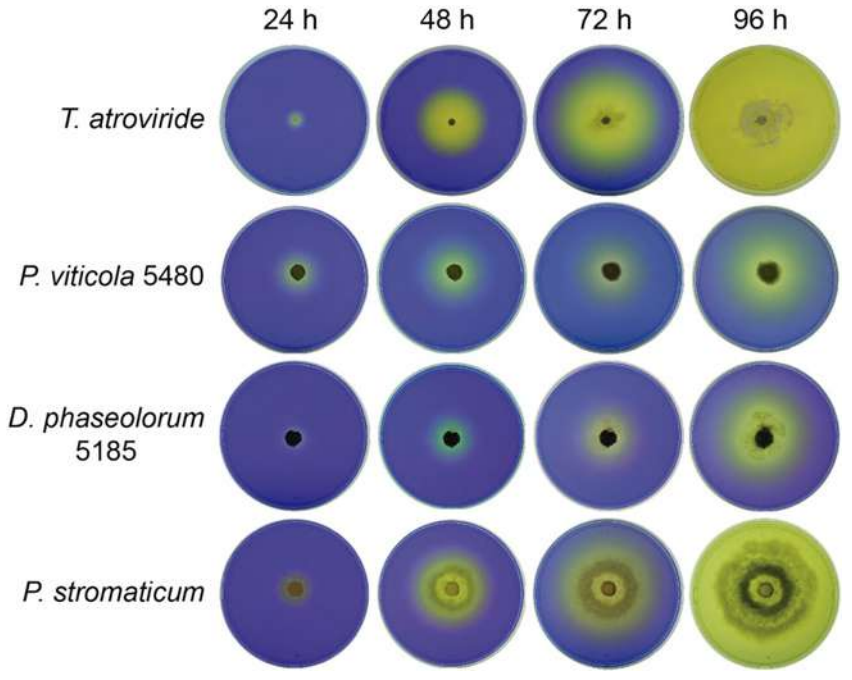

Figure 5. Effect of stem-end-related fungi and *T. atroviride* on acidification of plant growth medium. Stem-end-related fungi *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum,* and the plant symbiont *T. atroviride* were grown for the indicated times on 0.2x MS medium supplemented with bromophenol blue pH indicator. Photographs show representative images of plates in which yellow color indicates the degree of substrate acidification. Note the similar acidification effects of *T. atroviride* and *P. stromaticum*.

end fungi is unlikely related to their pathogenic or growth repressing effects on plants.

3.6. P. viticola *and* D. phaseolorum *induce local auxin response in primary roots*

The results that *P. viticola* and *D. phaseolorum* strongly repress root growth while inducing root hair development suggest the release of bioactive metabolite (s) into the growth medium, thus triggering the observed responses. Several fungi produce auxins (Contreras-Cornejo et al. 2009). Therefore, we analysed the changes in auxin-inducible gene marker *DR5:GFP* (Ottenschläger et al. 2003) in *Arabidopsis* primary roots in interaction with stem-end fungi. In axenically grown seedlings, auxins are distributed within the primary root tip in a narrow domain, which mostly extends following interaction with *P. viticola* and *D. phaseolorum* (Figure 7(a,b)). This increase in auxin response, at least in part, may explain the reduction of primary root growth and induction of root hairs caused by these fungi.

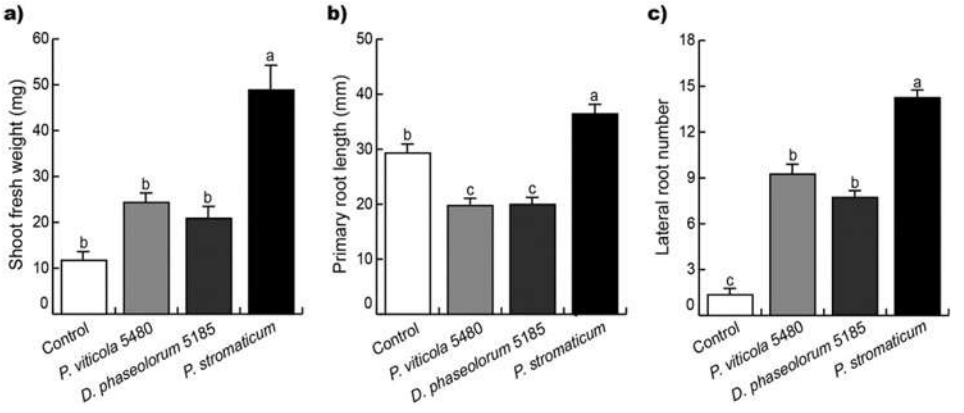

Figure 6. Effects of fungal co-cultivation on *Arabidopsis* shoot biomass and root development under buffered medium. (a) Shoot fresh weight, (b) primary root length, and (c) lateral root number. Fifteen *Arabidopsis* seedlings were germinated and grown on 0.2x MS medium buffered with MES 0.12%, after 5 days fungal mycelium was inoculated at 2 cm distance from the root tip and grown for four additional days. Different letters are used to indicate means that differ significantly ($P<.05$). Error bars represents SE. The experiment was repeated three times with similar results.

3.7. Stem-end related fungi induce the jasmonic acid responsive gene LOX2:GUS

Several phytopathogens influence growth and defence responses via jasmonic acid synthesis and signal transduction (Guo et al. 2018). Therefore, we asked if the avocado stem-end fungi could modulate jasmonic acid-inducible gene expression by testing the effect of *Arabidopsis* co-cultivation with *P. viticola*, *D. phaseolorum* and *P. stromaticum* on the expression of the jasmonic acid-responsive gene marker *LOX2:GUS*. *Arabidopsis* seedlings expressing *LOX2:GUS* (Jensen et al. 2002) were co-cultivated with each fungus and after four days in interaction, plants were assessed for GUS activity and cleared to show gene expression in cotyledons and leaves, which is manifested in blue color. Interestingly, an enhanced GUS expression could be observed in petioles of cotyledons and in leaves of seedlings co-cultivated with *P. stromaticum* (Figure 8), which indicates that this fungus activates defense responses via jasmonic acid signalling.

4. Discussion

Stem end rot pathogens colonise climacteric fruits such as avocado, mango, as well as citrus fruits. In several countries avocado postharvest losses have been mainly attributed to fruit decay caused by consortia of latent fungi (Twizeyimana et al. 2013; Guarnaccia et al. 2016; Montealegre et al. 2016; Fuentes-Aragón et al. 2018). Many of these

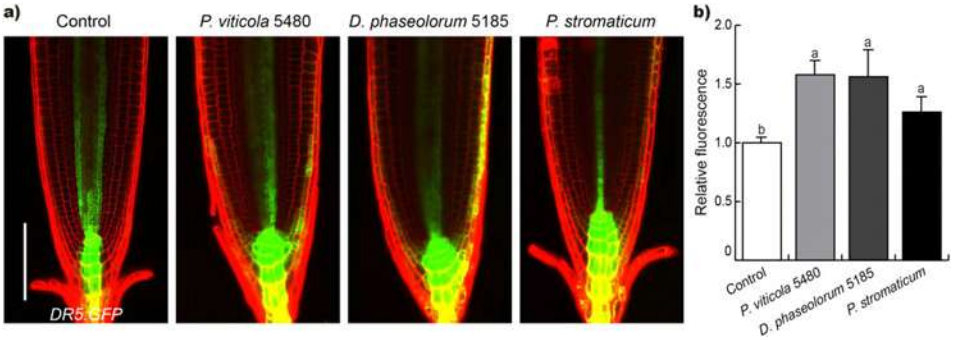

Figure 7. Effects of stem-end-related fungi co-cultivation on auxin-inducible gene expression in primary roots. (a) Confocal microscopy images of expression of auxin-inducible *DR5:GFP* gene construct and (b) relative GFP fluorescence at the root tip. Five-day-old *Arabidopsis* seedlings expressing the construct were co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings and data were quantified for 15 seedlings. Scale bars = 100 μm. These analyses were performed twice with similar results. Note the increased expression at the columella region of plants co-cultivated with all three fungal species.

fungi are natural endophytic inhabitants of stem tissue and have been identified prior to inflorescence emergence (Johnson et al. 1992).

Although during pre-harvest the use of fungicides is a common practice to control stem end rot, mesocarp damage on fruits has still been observed postharvest in several countries. Many fungal species have been isolated from symptomatic fruits and some of them have been proven to be causal agents of avocado stem end rot, including *Pestalotiopsis clavispora*, *Neofusicoccum australe, Neofusicoccum parvum, Neofusicoccum luteum, Colletotrichum fructicola, Colletotrichum gloeosporioides* and *Phomopsis* sp. (Valencia et al. 2011; Twizeyimana et al. 2013; Guarnaccia et al. 2016; Montealegre et al. 2016; Fuentes-Aragón et al. 2018). Considering the lack of details into the biology of the causative agents, and that several fungi may act in consortia, very little is known about the early responses of a plant host in terms of development and defence responses that are activated during infection with stem-end rot pathogens.

The aim of this study was to characterise the effects of three fungal isolates, involved in this symptomatology and their possible relationship with development and defence in *A. thaliana,* a model plant amenable to genetic and molecular analyses. Every fungal species behaved differentially in their interaction with *Arabidopsis* seedlings. *P. viticola* strongly repressed primary root growth, which may be explained by the production of phytohormones, secondary metabolites and/or virulence factors, which accumulate in the growth medium as the fungal colony proliferates. Unexpectedly, *P. stromaticum* enhanced biomass production and

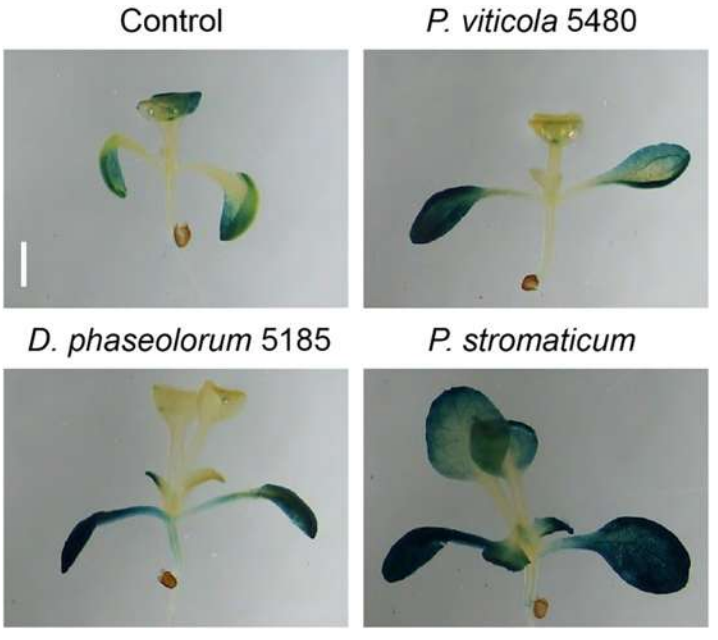

Figure 8. Effects of stem-end-related fungi co-cultivation on jasmonic acid-inducible gene expression in *Arabidopsis* shoots. Representative photographs showing expression of jasmonic acid inducible *LOX2:GUS* gene marker. Five-day-old *Arabidopsis* seedlings expressing the construct were grown under axenic conditions or co-cultivated with *P. viticola* 5480, *D. phaseolorum* 5185, and *P. stromaticum* and analysed 4 days later. Photographs show representative images of at least 10 seedlings. These analyses were performed twice with similar results. Note the differential growth of leaves and the increased expression in shoots of plants co-cultivated with *P. stromaticum*.

promoted lateral root formation and growth, similar to the effects reported for plant symbionts such as *T. atroviride* (Contreras-Cornejo et al. 2009). These data were certainly unexpected, but reveal the metabolic diversity among stem-end rot fungi, and it remains to be tested if *P. stromaticum* is a causal agent of avocado stem end.

The inhibition of primary root growth in plants co-cultivated with *P. viticola* and *D. phaseolorum* correlated with the differentiation and elongation of root hairs, which are epidermal cells specialised in water and nutrient acquisition. Similar root architectural adjustments have been reported for *Arabidopsis* seedlings exposed to environmental stress or in response to nutrient deprivation, for which the primary roots stop growing, lose their meristems and activate root hair growth (Sánchez-Calderón et al. 2005; Ruiz-Herrera et al. 2015). One possible explanation is that fungal metabolites are perceived at the root cap, a specialised sensory structure of the root, which transmits information to the cell division and elongation zones, changes growth and patterning to redirect growth to sites far away from the pathogen influence. In consonance

with the strong induction of root hair growth, *P. viticola* and *D. phaseolorum* enhanced expression of the cell differentiation marker *EXP7:GUS*. Previously, the expression of the *Arabidopsis EXP7* gene was tightly correlated to root hair initiation (Cho and Cosgrove 2002), thus, the positive regulation of this gene in the plant-fungi interactions helped us to establish how developmental, hormonal, and environmental factors orchestrate root growth and epidermal cell differentiation.

The metabolic diversity of stem-end rot fungi was also manifested when we compared their acidification capacity. Here, we found that both *P. stromaticum* and the plant beneficial fungus *T. atroviride* strongly acidified the medium, whereas *P. viticola* and *D. phaseolorum* showed much reduced acidification properties. These differences might depend on H^+-ATPase activity because treatment with the inhibitor sodium orthovanadate (Na_3VO_4), reduced acidification (Pelagio-Flores et al. 2017). The highly contrasting acidification capacities of all three stem-end rot fungi, indicates that pH lowering is unlikely linked to their developmental effects in *Arabidopsis*, but might play a role in the decay of avocado fruit when present as consortia.

Auxins repress root growth and concomitantly induce both lateral root and root hair formation (Du and Scheres 2018). Moreover, these compounds act as triggering factors for *EXP7* gene expression in *Arabidopsis* (Cho and Cosgrove 2002). Our analysis of expression of *DR5:GFP* in *Arabidopsis* primary roots in interaction with stem-end fungi showed that *P. viticola* and *D. phaseolorum* increase the auxin response at the very root tip, which suggest that at least in part, local activation of the auxin signalling pathway may account for primary root inhibition and the induction of root hairs as a response to plant co-cultivation with the fungi. We cannot exclude the possibility that the fungi already produce auxins, auxin precursors or auxin signal mimics, with bioactive functions in the plant host.

The coordination of growth and defence occurs as a response to availability of resources, or under pathogen challenge, and requires prioritisation towards the stimuli encountered. Jasmonic acid is an important mediator of trade-offs and has profound implications for adaptation and success of plants. Indeed, it has been recently involved in the regulation of root system architecture (Raya-González et al. 2012). To determine possible interactions between defence and growth signalling pathways elicited by stem-end rot fungi, the expression of the jasmonic acid-responsive gene marker *LOX2:GUS* was assessed in *Arabidopsis* seedlings co-cultivated with *P. viticola*, *D. phaseolorum* and *P. stromaticum*. Consistently with the activation of defence, an increased expression of this marker was observed throughout the shoot of seedlings, regardless

the fungal species co-cultivated in the vicinity of the root. These data show that stem-end fungi systemically activate defence responses in leaves via jasmonic acid signalling concomitantly with the growth and patterning changes elicited in roots.

In summary, the data presented here points to an extraordinary versatility in the manner by which stem-end rot pathogens interact with *Arabidopsis* seedlings. Understanding the molecular basis by which fungal metabolites balance growth–defence trade-offs in fruits as well as in model plants should provide a foundation to manage fungal-plant communication through chemical means, and for development of breeding strategies to protect avocado trees from stem-end rot or decrease the severity of symptoms in other climacteric fruits.

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Disclosure statement

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ORIGINAL ARTICLE

ALTERED MERISTEM PROGRAM 1 promotes growth and biomass accumulation influencing guard cell aperture and photosynthetic efficiency in Arabidopsis

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Abstract

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a putative glutamate-carboxypeptidase important for plant growth and development. In this study, by comparing the growth of Arabidopsis wild-type, *amp1–10* and *amp1–13* mutants, and *AMP1-GFP/OX2*- and *AMP1-GFP/OX7*-overexpressing seedlings in vitro and in soil, we uncover the role of AMP1 in biomass accumulation in Arabidopsis. *AMP1*-overexpressing plants had longer primary roots and increased lateral root number and density than the WT, which correlated with improved root, shoot, and total biomass accumulation. *AMP1*-overexpressing seedlings had an enhanced rate of growth of primary roots, and accordingly, sucrose supplementation restored primary root growth and promoted lateral root formation in *amp1* mutants, while reproductive development, fruit size, and seed content were also modified according to disruption or overexpression of *AMP1*. We further found that AMP1 plays an important role for stomatal development, guard cell functioning, and carbon assimilation. These data help explain the pleiotropic functions of AMP1 in both root and shoot system development, possibly acting in a sugar-dependent manner for regulation of root architecture, biomass accumulation, and seed production.

Keywords Stomata · Sugars · Plant biomass · Root architecture · Carbon fixation

Introduction

The coordination of growth among the diverse plant organs is critical for adaptation to the environment and supports productivity. Despite the knowledge gained in the past two decades on the regulation of cell division and elongation, two cellular processes critical for sustained growth, only a very few genes and proteins have been found to orchestrate overall plant biomass production (Demura and Ye, 2010).

Plant biomass accumulation relies on carbon fixation via photosynthesis, which occurs in the green plant organs, mainly mature leaves, where sucrose is produced and exported to non-photosynthetic tissues such as stems and roots for growth (Roldán et al. 1999; Puig et al. 2012; Dimitrov and Tax, 2018). CO_2 fixation may lead to increases in the sugar pool that upon demand or according to day/night fluctuations may be used to produce starch, a storing carbohydrate, and a carbon resource (Graf et al. 2010; Graf and Smith, 2011; Azoulay-Shemer et al. 2018). Growth and development from germination to senescence is coordinated by sugar availability, metabolism, and energetic signaling. Defects in these processes impair growth, reduce shoot and root apical dominance, and affect flowering and seed production (Kircher and Schopfer, 2012; Yang et al. 2013; Yu et al. 2013; Mason et al. 2014; Chen et al. 2015).

CO_2 uptake occurs via specialized leaf epidermal structures called stomata, whose guard cells open or close depending

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upon environmental conditions to allow gas exchange (Brodribb and McAdam, 2011). Stomatal activity tightly depends on the stress hormone abscisic acid (ABA), which cross-talks with sugar biosynthesis and/or metabolism (Roelfsema and Prins, 1995; Cheng et al., 2002; Yang et al. 2017; Joshi-Saha et al. 2011; Kang et al. 2018). For instance, *glucose insensitive 6* (*gin6*) and *sugar insensitive 5* (*sis5*) Arabidopsis mutants are defective in genes allelic to *aba deficient 3* (*aba3*) and *aba insensitive 4* (*abi4*), respectively (Arenas-Huertero et al. 2000; Leon and Sheen, 2003).

ALTERED MERISTEM PROGRAM 1 (AMP1) encodes a putative glutamate carboxypeptidase, an integral membrane protein associated with endoplasmic reticulum (Helliwell et al. 2001). AMP1 has been implicated in different growth and developmental processes including dormancy, germination, flowering time, shoot and root growth, and seed production (Chaudhury et al. 1993; Vidaurre et al. 2007; Griffiths et al. 2011; Huang et al., 2015; Kong et al. 2015; López-García et al. 2016). This protein negatively regulates the HD-ZIP III transcription factors implicated in vascular tissue differentiation (Li et al. 2013; Müller et al. 2016) and affects the translation rate of miRNA targets (Li et al. 2013); however its specific biochemical role is unclear and also to which extent the phenotypic changes are caused by this misexpression of miRNA targets.

The pleiotropic phenotype of *amp1* mutants has been explained by alterations in hormonal homeostasis, mainly involving ABA and cytokinins (Chin-Atkins et al. 1996; Shi et al., 2013a, b; Yao et al. 2014). Regarding root growth, a link between AMP1 and ABA has been established since *amp1* mutants are hypersensitive to growth repressing effects of ABA on primary roots, and conversely AMP1-overexpression confers ABA resistance. Despite the described ABA-related function of AMP1, any possible relation with sugar metabolism is unknown. In this work, we uncover the critical function of this protein for overall plant biomass and seed production, stomatal aperture, and carbon fixation.

Materials and methods

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0), the mutant lines *amp1–10* (SALK_021406) and *amp1–13* (SALK_22988), and the transgenic lines *AMP1-GFP/OX2* and *AMP1-GFP/OX7* (Shi et al. 2013b) were used for the experiments. Seeds were surface sterilized with ethanol 96% and sodium hypochlorite 20% for 5 and 7 min, respectively, and then washed five times with 1-ml sterilized distilled water and kept 2 days at 4 °C. The seeds were plated on 0.2x solidified MS medium containing basal salts (Murashige and Skoog Basal Salts Mixture, Sigma-Aldrich, St Louis MO), 1% agar (Phytagar, Gibco-BRL), and 0, 0.6, or 4.8% sucrose (Sigma-Aldrich, St Louis MO) and placed into a plant growth chamber (Percival AR-95 L) at 22 °C with a photoperiod of 16 h light/8 h darkness under light intensity of 105 μmol/m-2/s-1.

Growth analysis

Arabidopsis root systems were analyzed 6 days after germination (DAG) with a stereoscopic microscope (Leica MZ6). For soil experiments, plants at 10 DAG were transferred to pots and placed into a plant growth chamber to examine weekly overall growth, developmental transitions, and biomass accumulation, until plant life cycle completion. The siliques and seeds were collected and measured using a Leica MZ6 microscope.

Stomatal analysis

Stomata number and aperture were assessed in 10 DAG seedlings grown on 0.2x MS medium, supplemented with the solvent or with 1 μM ABA overnight. Images were acquired using a confocal microscope (Olympus FV1200). The stomatal aperture was measured in the ImageJ program.

Propidium iodide staining

For fluorescent staining with propidium iodide (PI), plants were transferred from the growth medium to 10 mg mL^{-1} of PI solution for 1 min. Seedlings were rinsed with deionized water and mounted on microscope slides. The same sample was recorded separately at wavelengths specific to both PI fluorescence with a 568-nm excitation line and emission window of 585–610 nm and GFP emission with 500–523 nm emission filter (488-nm excitation line), using a confocal microscope (Olympus FV1200). Finally, the three images were merged.

Detection of starch

For starch detection, plants were cleared and fixed with 0.24 N HCl in 20% methanol (v/v) and incubated for 60 min at 62 °C. The HCl solution was substituted by 7% NaOH (w/v) in 60% ethanol (v/v) for 20 min at room temperature. Then, plants were dehydrated with ethanol treatments at 40, 20, and 10% (v/v) for a 24-h period each and immersed in concentrated Lugol solution 1 min, washed with deionized water, and mounted on microscope slides. The samples were analyzed and photographed using a Leica DM500B microscope.

Preparation of CO_2 traps

Seeds of WT, *amp1*, and *OX7* lines were germinated in MS 0.2x medium and transferred 2 DAG to divided Petri plates. In each side of the plate, the agar solidified 0.2x MS medium was supplied either with 0.6% sucrose or 0.1 M $Ba(OH)_2$ solution. The primary root length was analyzed 10 DAG.

Results

AMP1 overexpression improves growth and biomass production of Arabidopsis seedlings in vitro

In a previous report, *amp1* mutant seedlings were found to produce short primary roots, which correlated with an altered response to ABA (López-García et al. 2016). To further clarify the phenotypical alterations arising upon AMP1 dysfunction, the growth and development of WT Arabidopsis (Col-0), the single mutant *amp1–10* (SALK_021406), and the transgenic line *AMP1-GFP/OX7* (Shi et al. 2013b) were compared in experiments in which seedlings from all three genotypes were grown side by side over the surface of Petri plates containing agar solidified 0.2x Murashige and Skoog (MS) medium. Consistently with a positive role of AMP1 in root growth, *AMP1*-overexpressing plants (*OX7*) had longer primary roots than the WT and *amp1* mutants and had increased lateral root number and density (Fig. 1a–c). A similar trend occurred when the root system architecture was compared among the WT and an additional mutant allele, namely, *amp1–13* and overexpression line *AMP1-GFP/OX2* (Supplementary Fig. S1). Root and shoot fresh weight determinations indicated that *OX7* seedlings grow faster and accumulate more shoot (Fig. 1d), root (Fig. 1e), and total biomass (Fig. 1f) than the WT and *amp1* mutants. These results indicate that AMP1 is critical for plant biomass accumulation.

AMP1 overexpression enhances root growth rate

The root length effects in the seedling stage are quite intriguing. However, in this context, it was helpful to resolve, to which extent the effect is driven by an altered germination behavior of the used lines, since *amp1* is ABA hypersensitive and *OX7* has been shown to be ABA resistant (Shi et al., 2013a, b). To clarify if the root growth effect might be to a significant extent mediated by altered timing of germination, we next determined the actual growth rate of the root, once it is fully emerged after germination. WT, *amp1–10*, and *AMP1-GFP/OX7* seeds were germinated, and at time of radicle protrusion, the primary root growth was measured daily during 8 days. The data show that *amp1–10* and *AMP1-GFP/OX7* lines have an opposite behavior, with reduced or enhanced growth rate, respectively, when compared to the WT (Supplementary Fig. S2). These data indicate that AMP1 plays a critical role in determining growth of the primary root.

AMP1 mutation or overexpression critically influences biomass accumulation in soil

AMP1-overexpressing seedlings develop a more robust root system that could efficiently take up water and nutrients and concomitantly may account to overall productivity. To test this possibility, the growth and development of the WT, *amp1–10*, and *AMP1-GFP/OX7* lines were analyzed in soil until life cycle completion. When compared to the WT, *amp1* mutants show decreased rosette and leaf sizes, flowered earlier, and had decreased apical dominance. On the other hand, *OX7* plants displayed an increased plant height, rosette, and leaf sizes (Fig. 2a–e). Reproductive development was also modified according to the AMP1 status, with the corresponding mutants producing shorter siliques with a reduced seed number compared to the WT, while *OX7* plants produced bigger fruits with much more seeds than the WT and heavier seeds (Fig. 3a–d). Thus, AMP1 regulates overall vegetative and reproductive development, which improves seed production.

Sucrose supplementation restores primary root growth and promotes lateral root formation in *amp1* mutants

The reduced growth caused by loss-of-function of *AMP1* is similar to recently identified *MEDIATOR med12* and *med13* mutant phenotypes, which could be rescued by sugar supplementation (Raya-González et al. 2017). To test if sugars could support more growth in WT, *amp1*, and *OX7* seedling, sucrose was applied to agar drops, where the aerial tissues were placed and primary root growth and lateral root formation assessed later on. Primary root growth was induced from 1.2 to 4.8% sucrose in the WT, this induction being higher in *OX7* seedlings, which attained the maximum response from all three genotypes analyzed (Fig. 4a). Noteworthy, in *amp1* mutants, as the sugar levels increase, primary root growth and lateral root formation reached similar values to the WT (Fig. 4a–d). These data could be confirmed in experiments that analyzed primary root growth and lateral root formation in WT, *amp1–13,* and *AMP1-GFP/OX2*, where sucrose supplementation normalized root growth of *amp1–13* (Supplementary Fig. S3). These results suggest a novel function AMP1 in a sugar-dependent pathway for regulation of root architecture and biomass accumulation.

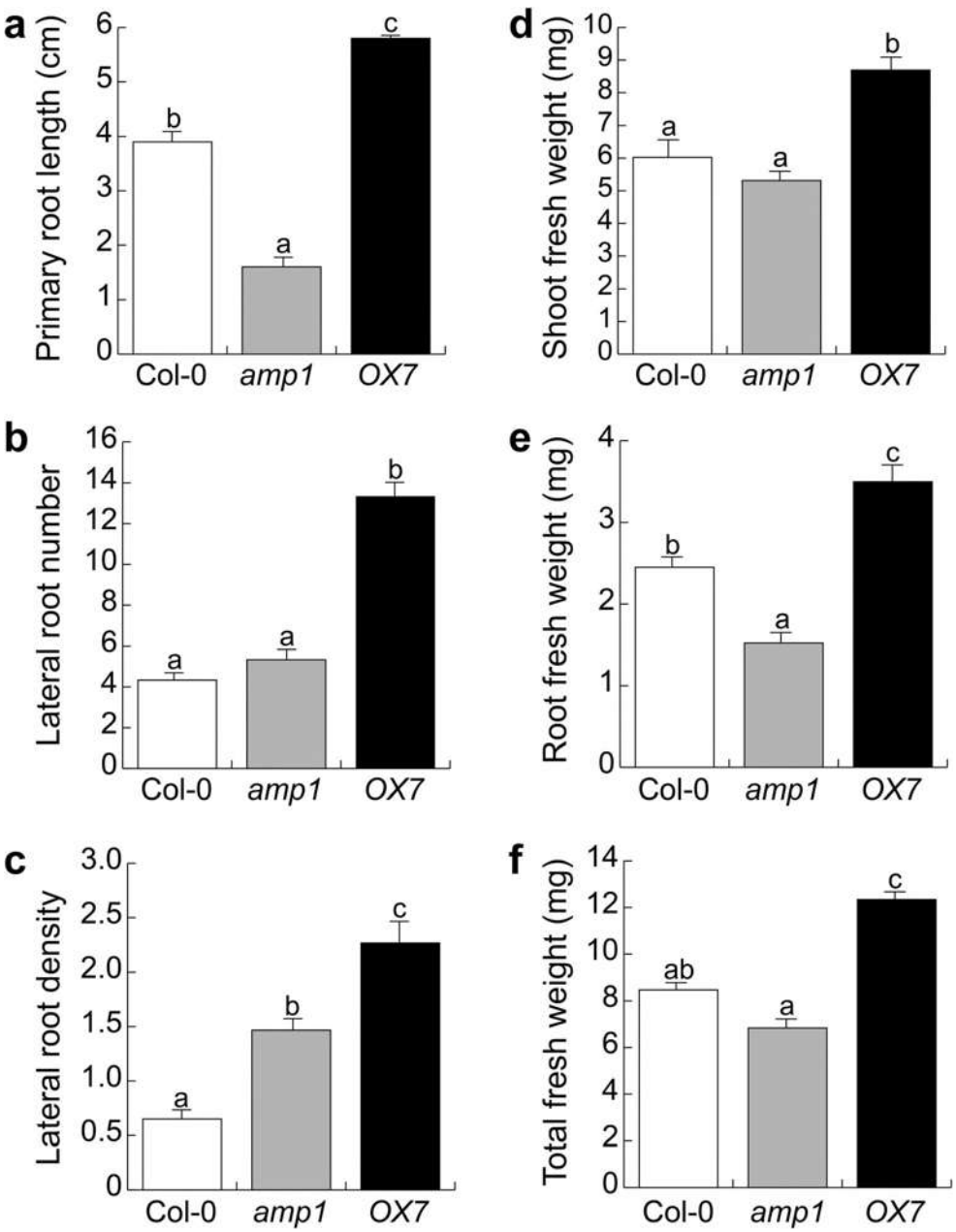

Fig. 1 AMP1 influences root growth and biomass-related traits. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were grown in vitro for 6 days and root architecture and biomass analyzed. (a) Primary root length, (b) lateral root number, (c) lateral root density, (d–f) shoot, root, and total plant fresh weight. Error bars represent SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

AMP1 is required for stomata development and mediates guard cell aperture in an ABA-dependent manner

To analyze a possible role of AMP1 in guard cells and stomata dynamics, we next quantified stomata and measured guard cell aperture with or without ABA in WT, *amp1*, and *OX7* seedlings. The number of stomata in *amp1* was lower than in the WT, while in *OX7*, it was increased (Fig. 5a). The stomatal aperture in *amp1* mutants is similar to the WT in control conditions, and ABA induces its closure in both plant genotypes. Interestingly, *OX7* seedlings had an increased guard cell aperture in control conditions irrespective of ABA treatments (Fig. 5b–c). Additionally, starch content increases in guard cells in *amp1* mutants and diminished in guard cells of *OX7* line when compared to the WT in control conditions, whereas ABA treatment induced starch accumulation in all three genotypes (Fig. 6). These data suggest that the stomatal development required AMP1, and at the same time, this gene mediates guard cell aperture and starch accumulation in an ABA-dependent manner.

AMP1 modulates CO_2 uptake

The altered number of stomata and aperture dynamics suggests that photosynthetic activity could be modulated by

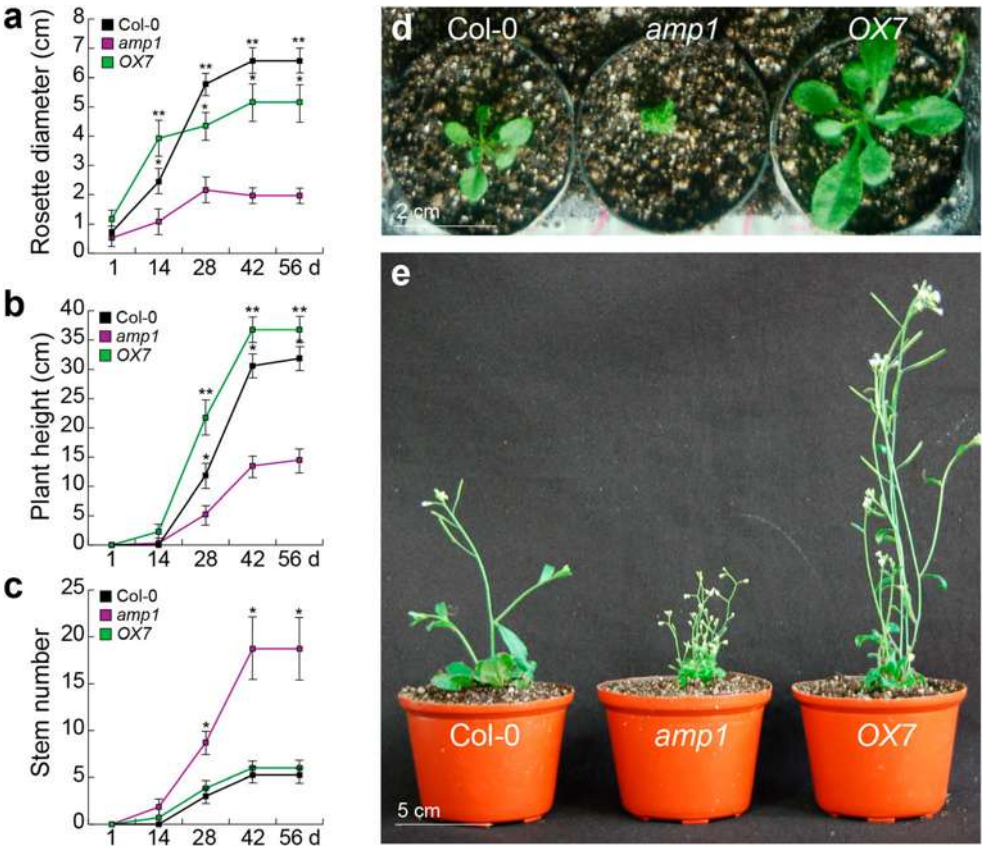

Fig. 2 AMP1 affects rosette size, stem number, and plant height. Arabidopsis wild-type, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium for 10 days and then transferred to soil pots to analyze growth and development during their life cycle. (a) Rosette size, (b) plant height, (c) stem number. Representative images of rosettes (d) and plants at reproductive stage (28 days) (e) showing the differences in soil phenotypes between the three lines. Error bars represent SD from 20 seedlings analyzed, and stars indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

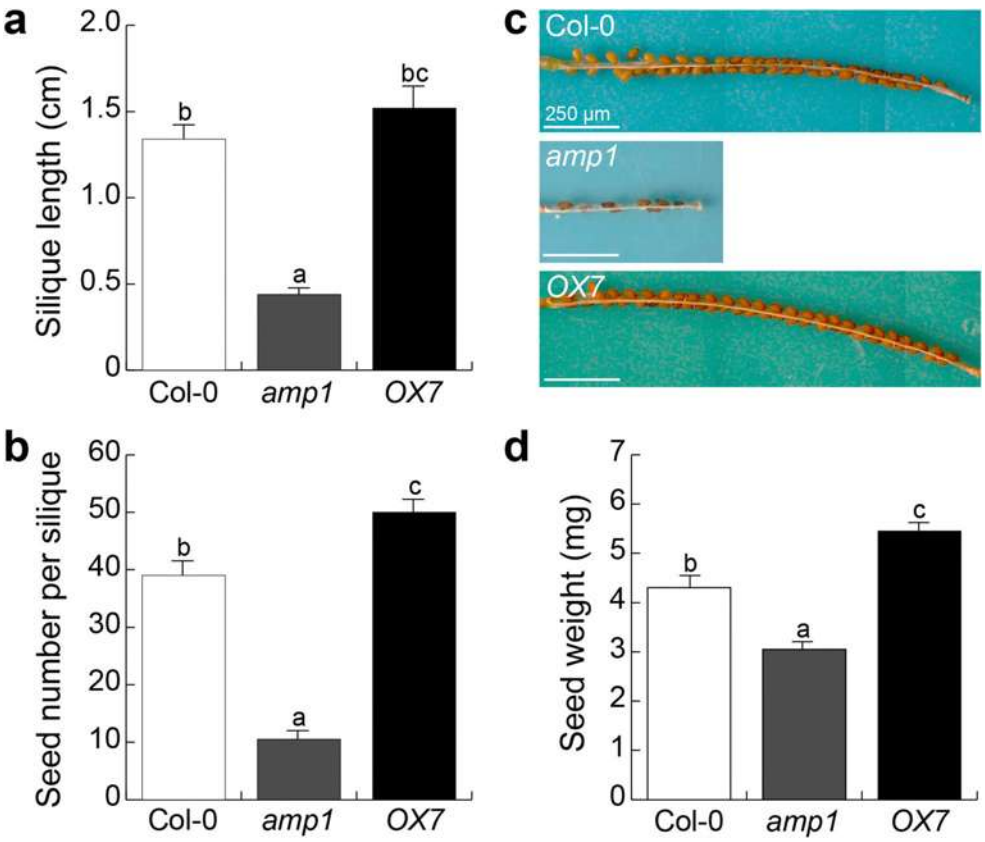

Fig. 3 AMP1 regulates seed number and weight. Siliques of WT, *amp1–10*, and *OX7* lines were collected and dissected at maturity to analyze seed content. (a) Silique length. (b) Seed number per silique. (c) Representative images of opened mature siliques. (d) Seed weight. Error bars represent SD from 10 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$

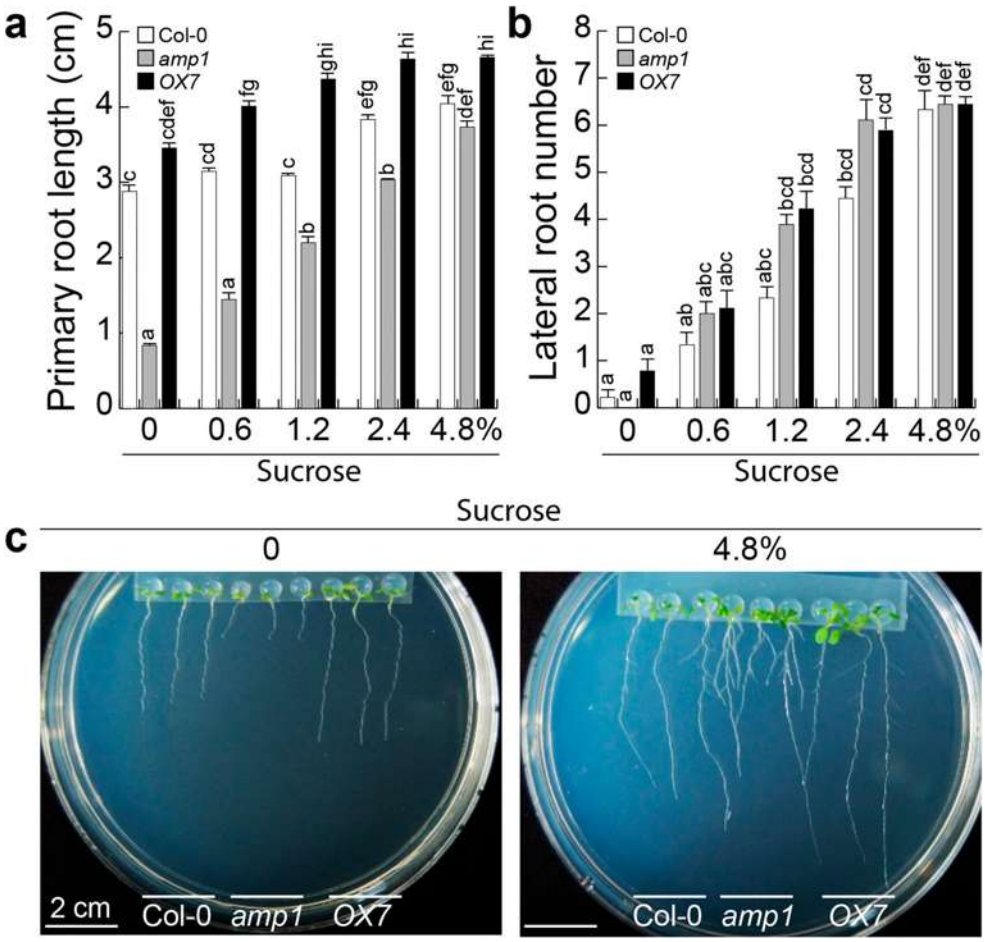

Fig. 4 Sucrose supplements restore normal growth in *amp1* primary roots. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium and 2 days after germination were transferred to fresh medium with the shoot placed over a drop of medium enriched with 0, 0.6, 1.2, 2.4, and 4.8% sucrose, and 4 days later, the primary root length (a) and lateral root number were analyzed (b). Representative images of seedlings grown side by side at 0 and 4.8% sucrose treatments are shown (c). Error bars represent SD from 36 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

AMP1. To examine the importance of CO_2 assimilation in the phenotype conferred by AMP1 modulation to plants, experiments with the CO_2 scavenger $Ba(OH)_2$ were performed in medium lacking sucrose or supplemented with 0.6% sucrose. Photosynthesis is determinant for root growth; thus, the primary root growth of WT, *amp1*, and *OX7* seedlings was compared under these conditions. Our results showed that primary root growth of plants from all three lines diminished with $Ba(OH)_2$ in medium without sucrose, with the greater inhibition observed in *OX7* seedlings (Fig. 7). In WT or *OX7* plants grown in 0.6% sucrose, $Ba(OH)_2$ inhibited the primary root in a similar fashion observed in plants grown without sucrose. These data evidenced the requirement of CO_2 to support the enhanced root growth in AMP1-overexpressing seedlings.

Discussion

Plants integrate the energetic status in root and shoot systems to optimize growth and development. Sugars are translocated from the shoot to the root for active growth, and conversely roots provide mineral nutrients and water for optimal reproduction. Our detailed comparison of several traits in WT, *amp1*, and *OX7* seedlings suggested the critical role of AMP1 in overall plant fitness.

Following germination, the primary root adapts the plant to the substrate, explores the environment, and branches via de novo organogenesis, typically through formation of lateral roots. Both primary root growth and branching were promoted in *OX7* seedlings, and this correlates with more root and shoot biomass being accumulated. In response to nutrient deficiency, toxic pollutants, heavy metals, and/or biotic and abiotic stress, the primary root growth halts and instead the formation of lateral roots are stimulated, and this architectural readjustment is believed to help plants to avoid the stress imposed (Gaedeke et al. 2001; López-Bucio et al. 2002; Giehl et al. 2014; Verbon and Liberman, 2016; Veerappa et al. 2019). Noteworthy, only a very few papers have reported an increased branching potential without compromising growth of the main root axis as it certainly occurs in *OX7* seedlings. Thus, the positive correlation in root growth traits with superior plant biomass production was certainly expected and might be relevant toward future agricultural applications.

Photosynthesis occurs in mature leaves, also known as source organs that supply sugars to demanding or sink tissues, principally young leaves, flowers, and roots (Zakhartsev et al. 2016). The optimal sink to source

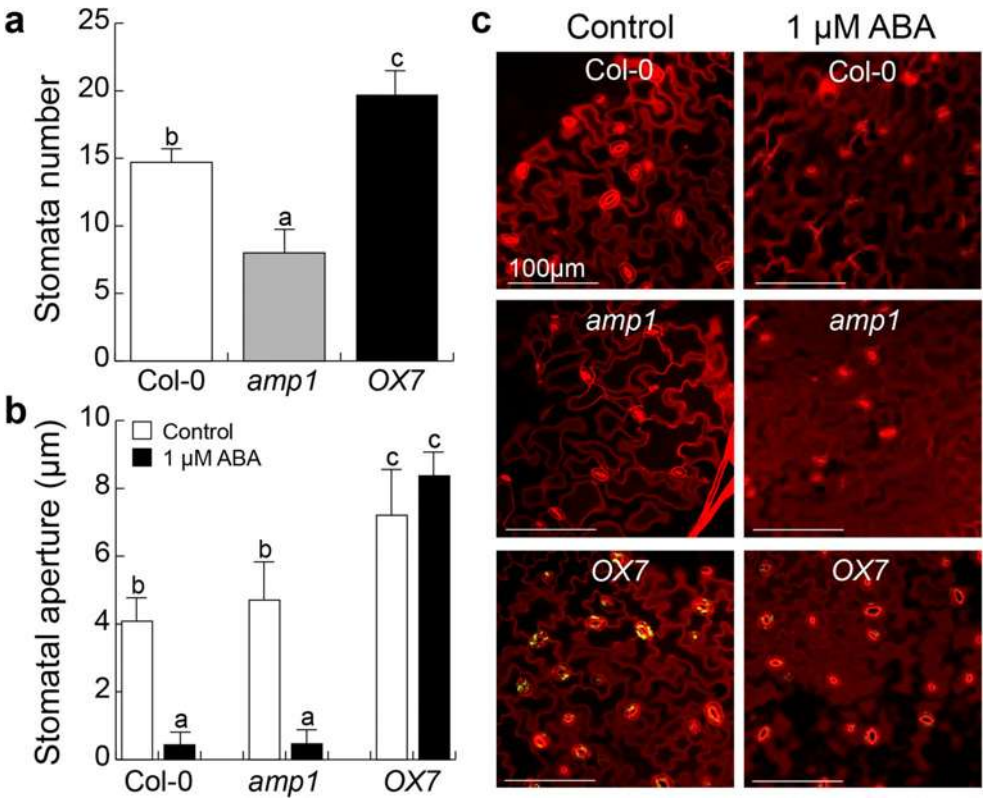

Fig. 5 AMP1 determines stomatal functioning. Stomata number and guard cell aperture was analyzed in WT, *amp1*, and *AMP1-GFP/OX7* seedlings under standard growth conditions and in response to 1 μM ABA in 0.2x liquid medium overnight. (a) Stomata number. (b) Stomatal aperture with or without ABA supplementation. (c) Representative images of stomata in Arabidopsis leaves from plants treated or not with ABA. Error bars represent SD from 3 fields and 30 stomata analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

relationships may help improve root growth and plant reproduction, two traits markedly affected in *amp1* mutants and improved in *OX7* overexpressing seedlings. Leaf and root growth is determined by genetic factors that drive cell proliferation and expansion, which rely upon an adequate supply of energy (Cookson et al. 2007; Hauben et al. 2009; Gonzalez et al., 2012). A correlation in the function of AMP1 with gibberellins (GA's) is possible, since the overexpressing lines of *GIBBERELLIN 20-OXIDASE 1* (*GA20OX1*) produced bigger leaves and blossom earlier (Huang et al. 1998; Gonzalez et al. 2010). Indeed, *amp1* was resistant to exogenous GA's application (Saibo et al., 2007) but oversensitive to ABA (López-García et al. 2016); such hormonal antagonism between ABA and GA's signaling may determine the pleiotropic functions of AMP1.

In soil both *amp1* and *OX7* lines had an early flowering phenotype, but at later times (28 days), rosette diameter of the *OX7* line decreased compared to the WT. This may be explained because stem height and number of stems increased with time, in particular stem number exacerbates after day 28 in the *OX7* line; thus, it is tempting to speculate that a change in biomass partitioning from rosette leaves occurred when stems develop their own leaves. Noteworthy, *amp1* mutants produced small and infertile flowers that yielded short siliques, in contrast to *OX7* plants that had bigger fruits with more seeds. This correlation may be explained because larger fruit contains either more or bigger seeds, and this was the case for *OX7* seedlings. *AMP1* mutation shortens the fruit length and the plant height, showing the opposite correlation between fruit length and seed content when compared to the WT and *OX7* line. Thus, AMP1 protein plays a fundamental role in fruit and seed harvest.

Through detailed analysis of *AMP1* mutation and overexpression in Arabidopsis, we unraveled its possible function in sugar production/metabolism. Plants with contrasting AMP1 levels had alterations in stomatal number and aperture, and this may lead to an increased or decreased gas exchange in the leaves, including CO_2 which is the main substrate for photosynthesis. Indeed, either *amp1* mutant plants or the *OX7* overexpressors had abnormal and contrasting amounts of starch in guard cells, supporting the idea that starch formation is modulated by AMP1. On the other hand, AMP1 prevented starch accumulation in guard cells in an ABA-dependent manner. The role of starch in guard cells and its relation with ABA signaling is a debatable topic; during starch synthesis, the stomata are closed in response to ABA (Santelia et al. 2011), but the opposite also occurs when starch is degraded to sugars and malate, since both act as osmotically active solutes and contribute to stomatal opening (Comparot-Moss et al. 2010; Fulton et al. 2008; Kötting et al. 2010; Graf et al. 2010). From our data, no correlation could be observed between stomatal aperture and starch accumulation in guard cells.

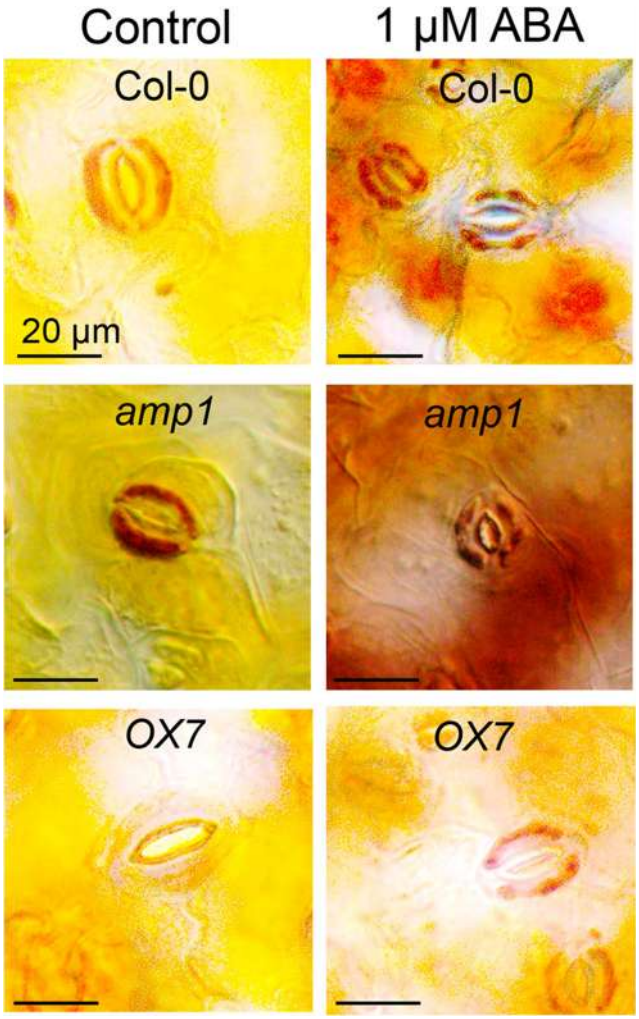

Fig. 6 AMP1 influences starch accumulation in guard cells. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium, and leaves were stained with Lugol to detect starch in guard cells in response to ABA treatment. Representative images from three independent fields on single leaves are presented, and at least six independent seedlings per line and treatment were analyzed

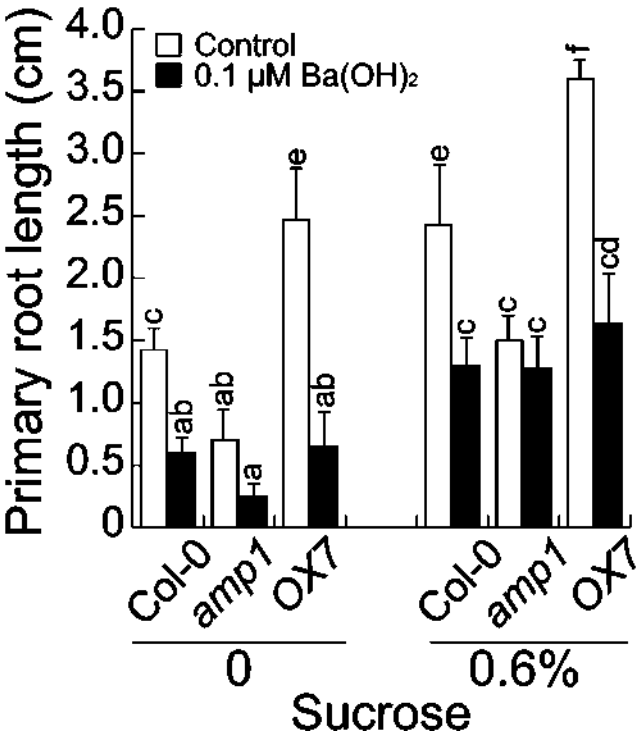

Fig. 7 Enhanced primary root growth of *OX7* seedlings is dependent on CO_2 uptake. Arabidopsis WT, *amp1–10* mutants, and *AMP1-GFP/OX7*-overexpressing seedlings were germinated and grown in MS 0.2x medium with or without sucrose in presence or absence of $Ba(OH)_2$ which acts as a CO_2 trap. The *OX7* primary root was the longest in 0 and 0.6% of sucrose in the presence of CO_2. However, when CO_2 is trapped by $Ba(OH)_2$ the primary root length is similar between all three genotypes. Error bars represent SD from 20 seedlings analyzed. Different letters indicate statistical differences at $P < 0.05$. The experiment was repeated three times with similar results

Sucrose supplements rescued the *amp1*-stunted root phenotype and in the WT and *OX7* seedlings still induced overall plant growth. This suggests the participation of AMP1 in the carbon assimilation pathway and/or metabolism. In support of this possibility, the use of CO_2 traps clearly decreased the growth potential of all three lines, namely, the WT, *amp1* mutants, and *OX7* overexpressors. Perhaps AMP1 drives sucrose biosynthesis via CO_2 uptake and the improvement of photosynthesis, resulting not only in accelerated growth and development but also in more biomass being accumulated, which is a highly desirable trait in agriculture.

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Mild High Concentrations of Boric Acid Repress Leaf Formation and Primary Root Growth in *Arabidopsis* Seedlings While Showing Anti-apoptotic Effects in a Mutant with Compromised Cell Viability

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Abstract

Boron (B) has been considered either as a nutrient or as a toxic non-essential element for plants and considerable debate arose recently regarding its functions and mechanisms of action. To gain further detail on the roles of boron in growth, development, and cell viability, *Arabidopsis* wild-type and *mediator18* mutants, these later showing genetically fixed cell death in the pro-vasculature of roots, were germinated and grown side by side in agar-solidified plates with a standard nutrient solution supplemented with increasing concentrations (0.25–8 mM) of boric acid (BA). In the WT and *med18-1* mutants, BA exerted a dose-dependent inhibition of leaf formation and primary root growth, but did not significantly promote root branching in the WT or *med18-1* mutants, these later manifesting an enhanced lateral root formation capacity under a wide range of BA concentrations. Although the overall effect of BA in roots was growth repressing, no signs of cell death in meristems of primary roots of WT seedlings could be appreciated, instead, it appears to diminish the cell death manifested in pro-vasculature of *med18* mutants, which correlates with reduced expression of the ERF115 transcription factor, which is induced in inner tissues upon damage, and recovery of auxin-inducible gene expression within the root tip. Irrespective of the overall growth-repressing effects in the shoot and root systems, it seems clear that important protective functions are orchestrated by BA, which supports cell viability in a mutant with spontaneous tissue damage.

Keywords *Arabidopsis* · Boric acid · Cell death · Root meristems · Lateral roots

Introduction

Boron (B) has been considered a critical nutrient for plants, which is taken up by roots in the form of boric acid (BA). Most soils are inherently defective on available BA, and despite decades of research focused to understand how B deficiency influences growth and productivity in several crop and horticultural species, it is still unclear how low BA stress shapes growth, development, and reproduction (González-Fontes and Fujiwara 2020; Matthes et al. 2020; Pereira et al. 2021).

In recent times, it has been a matter of debate whether BA acts as an essential element for plants or if it rather exerts unspecific functions on cellular processes owing its toxic effects (Lewis 2019, 2020; Wimmer et al. 2020). Regarding this point, it appears that boron supports growth and morphogenesis at low doses, and it is widely accepted its physiological function in cross-linking of pectin at the RG-II region (Kutschera and Niklas 2017). The essentiality of boron is well supported by this fact, but has deleterious effects when supplemented at concentrations above a threshold level (Aquea et al. 2012; Matthes and Torres-Ruiz 2016). Thus, it is important to perform in deep research to clarify the cellular and molecular mechanisms underlying BA regulation of plant development and optimize agriculture.

Plant organogenesis rely on the coordination among cell division, elongation, and differentiation, which are tightly linked to perception of light, temperature, nutrients, and pollutants among many other factors (Sarkar et al. 2007; Hong et al. 2017; Feraru et al. 2019). Cell division proceeds through mitosis in root meristems, where nutrient deficiency/

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toxicity strongly influences the cell cycle to drive or arrest proliferation (Sjogren et al. 2015; Wei et al. 2021). Roots are continuously exposed to natural chemical factories, namely their microbiomes, in such a manner that many fungal and bacterial species release antibiotics, secondary metabolites, and quorum-sensing compounds that are sensed by the root tips to reinforce or avoid cross-kingdom interactions (Ortiz-Castro et al. 2011, 2014, 2020; Garnica-Vergara et al. 2016). Meristems are highly sensitive to damage caused by genotoxic stressors, such as radiation, metals, and antibiotics (Furukawa et al. 2010; Sjogren et al. 2015; Hu et al. 2016). Indeed, the effects of low BA on plant biomass have been linked to poor meristem bioactivity since its deficit impairs mitosis (Matthes and Torres-Ruiz 2016; Poza-Viejo et al. 2018) whereas its toxicity induces an abscisic acid (ABA) response, cell wall modifications, and repression of water transporters (Aquea et al. 2012). Thus, excess boron triggers a hydric stress response that underlies root growth inhibition, likely causing DNA damage and apoptosis of meristem cells (Sakamoto et al. 2018). An impaired root functioning directly halts the growth and developmental transitions that are critical for both plant health and productivity.

The genes and proteins involved in root meristem viability have just started to be identified. In *Arabidopsis* mutants defective on the MEDIATOR 18 subunit (*med18*) or in a large subunit of the RNA polymerase II (*rpb1*) the death of pro-vasculature cells correlates with an enhanced auxin response (Raya-González et al. 2018; Zhang et al. 2018). Moreover, in a mutant showing determinate growth of primary and lateral roots termed *halted primary root1* (*hpr1*), differentiation of the meristem correlates with the formation of an enhanced auxin maximum around damaged sites (Raya-González et al. 2019). Cell death/regeneration appears to be two sides of the same coin, since in *med18* mutants a strong regeneration capacity is manifested by chromate, an environmental pollutant and DNA stressor, which triggers twinning of the primary root meristem (Ruiz-Aguilar et al. 2020). Exacerbated auxin accumulation within the root tip of the *med18* lines has been explained because the death of pro-vascular cells interferes with polar auxin transport, thus leading auxin to accumulate in endodermis where it triggers periclinal divisions to regenerate damaged tissues via ERF115 transcription factor (Canher et al. 2020; Hoermayer et al. 2020). All this recent information suggests that auxin orchestrates root growth through the balance between cell death and regeneration.

It is noteworthy that under particular situations, boron acts to protect root meristems from environmental stress probably acting as an antioxidant or through directly modulating metabolism (González-Fontes and Fujiwara 2020; Matthes et al. 2020; Pereira et al. 2021). In plants exposed to aluminum (Al) toxic ions, the deleterious effects on cell division and quiescent center (QC) differentiation were abolished by boron treatment (Li et al. 2018). Furthermore, via increasing the degree of methyl esterification of pectin in the root apex, BA improves cell wall plasticity and this may account to better Al tolerance (Riaz et al. 2018). This opens the possibility that BA could play protective roles through a mechanism still to be identified.

In this report, we take advantage of the genetically fixed cell death phenotype in root pro-vasculature cells of *med18* mutants to assess whether BA treatment could exacerbate or decrease tissue damage within the primary root tip. Through comparison of overall seedling growth, leaf formation, root development, and cell viability of WT and *med18-1 Arabidopsis* seedlings germinated and grown side by side in medium supplemented with a broad range of BA concentrations, our work uncovers a critical function of boron in leaf formation and primary root growth and to improve the viability of pro-vasculature cells, through influencing ERF115 and auxin-inducible gene expression in *med18-1* root tips, which unveils a beneficial role of BA application to recover damaged, cell proliferative tissues.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana seedlings of the Columbia-0 (Col-0) ecotype, and *MEDIATOR 18* mutant allele *med18-1* were analyzed in this study. The reporter genes *ERF115*:*GFP* (Heyman et al. 2013) and *DR5*:*GFP* were introduced into *med18-1* mutants via outcrossing (Raya-González et al. 2018). Homozygous seeds of each genotype were disinfected in 1.5 ml plastic tubes applying ethanol 95% (v/v) for 7 min and bleach 20% (v/v) for 5 min. The bleach was discarded and then 1 ml of sterilized distilled water was supplemented and the seeds were gently washed, and this process was repeated five times. The seeds were finally immersed in 0.5 ml sterilized distilled water and placed into a refrigerator (4 °C) for 3 days to promote and synchronize germination.

After cold treatment, six seeds of each Col-0 and *med18-1* genotypes were placed in a row in one of the sides of the Petri plate containing 0.2 × MS medium (Murashige and Skoog 1962) and allowed to grow placing the plates in a vertical position into a Percival AR95L *Arabidopsis* growth cabinet. The MS medium corresponds to a commercial formulation (Murashige and Skoog basal salts mixture; catalog M5524) sold by Sigma-Aldrich (St. Louis, MO). 4.3 g of the salts are recommended to prepare 1l of solution at 1 × concentration, thus 0.9 g were dissolved in 1l of distilled water for the 0.2 × MS final concentration. Boric acid was purchased from Sigma-Aldrich and dissolved in sterilized distilled water prior to application in final concentrations of 0, 0.25, 0.5, 1, 2, 4, and 8 mM into the media. As a solidifying agent, we supplemented

Phytagar (micropropagation grade, Phytotechnology, Shawnee Mission, KS, USA). Each experiment included at least four Petri plates, with six seedlings of each genotype ($n=24$) that were accommodated in a fully random design into the plant growth cabinet with a photoperiod of 16 h of light, 8 h of darkness, light intensity of 100 µmol m^2 s^{-1}, and temperature of 22 °C. Each experiment was repeated at least three times.

Plant Growth Analysis and Statistics

The overall growth and phenotypes of seedlings were visually recorded and images of representative Petri dishes were taken with a digital camera (Nikon D50, Nikon, Japan). The phenotypes of the shoots and leaf count were registered using a stereomicroscope (Leica DMZ6, Leica, Germany) at the 0.63× objective. The length of the main root axis was measured using a rule. The number of lateral roots indicates the mature branches that emerged from the primary root and that are observable at the 0.63× objective in the stereomicroscope. The data were statistically analyzed in the SPSS 10 program (SPSS, Chicago). Univariate and multivariate analyses were applied with a Tukey's post hoc test and the means that statistically differ were indicated with different letters ($p<0.05$).

Propidium Iodide Staining and Detection of the Green Fluorescent Protein (GFP)

Arabidopsis WT and *med18-1* seedlings harboring either *ERF115*:*GFP* or *DR5*:*GFP* were germinated and grown on the plates with the different BA treatments and at 7 days after germination were transferred into a solution (10 mg/ml) of propidium iodide for 1 min. The seedlings were washed using sterilized distilled water and then transferred into microscope slides with 0.3 ml 50% glycerol. These samples were analyzed for green fluorescent protein detection basically following the guidelines by Hanson and Köhler (2001) and imaged at wavelengths of 568-nm excitation line and emission window of 585–610 nm for propidium iodide and 500- to 523-nm emission filter (488-nm excitation line) for GFP fluorescence using a confocal microscope (Zeiss META 270). The independent photos obtained were merged to produce the final image.

Results

Boric Acid has Either Promoting or Repressing Effect in Growth of the Primary Roots of Wild Type and *med18-1* Mutants

The recent identification of mutants with genetically compromised cell viability in root meristems, which are highly sensitive to genotoxic stressors, offers the possibility to directly test whether BA could exert beneficial or deleterious influences to *Arabidopsis* seedlings and to gather further information into its mechanisms of action on plant cells. In vitro experiments were performed to compare growth and development of WT seedlings (Col-0 ecotype) and *med18-1* mutants germinated and grown side by side on agar plates supplemented with 0, 0.25, 0.5, 1, 2, 4, and 8 mM BA. Overall seedling inspection confirmed that *med18-1* seedlings had reduced growth of primary roots in medium without BA when compared to WT seedlings (Fig. 1a). Noteworthy, application of 0.5 and 1 mM BA restored primary root growth of the mutants making them comparable to the WT (Fig. 1b, c), whereas 2 mM or greater BA concentrations exerted a drastic inhibition of root growth in both the WT and *med18-1* mutants (Fig. 1d). Noteworthy, the rescue of the defective growth of the primary roots in the mutants and the growth-repressing effects by BA occurred in a very narrow range of concentrations, from 1 to 2 mM BA.

Boric Acid Represses Leaf Formation in WT and *med18-1* Mutants

A clear phenotypic change manifested by *med18-1* mutants grown in vitro is an elongated hypocotyl. Thus, we were interested to define if early shoot growth could be modulated by BA through visually comparing WT and *med18-1* seedlings 10 days after germination (d.a.g.). In contrast to primary roots, BA did not promote stem elongation in the WT or in *med18-1* line (Fig. 2a). The number of true leaves was counted 5 days later to allow the developmental program to proceed with the help of a dissecting microscope (Fig. 2b), roughly three pairs of leaves were already developed in control conditions or in 0.25 mM BA treatment, whereas only half the number was counted in the highest concentration tested (8 mM). These data clearly indicated that BA represses leaf formation in WT and *med18-1* mutants in a dose-dependent manner (Fig. 2b). Thus, BA exerts differential and dynamic effects in shoot and root growth.

WT and *med18-1* Mutants Differ in Their Sensitivity to BA Application in *Arabidopsis* Primary Roots

Given the very interesting effect observed during the recovery of root growth in *med18-1* mutants by low amounts of BA, we now focused on primary root growth to define how BA could compensate the genetic defect that compromises root cell viability. First, side-by-side growth of a total of 24 WT and *med18-1* seedlings distributed in four independent Petri plates and determination of their primary root lengths at 10 d.a.g. demonstrated that although BA did not strongly promote root growth in the mutants, the percentage of growth with regard to the minus BA treatment indicates that their growth is somewhat recovered (Fig. 3a, b). These measurements also indicated that BA strongly repressed

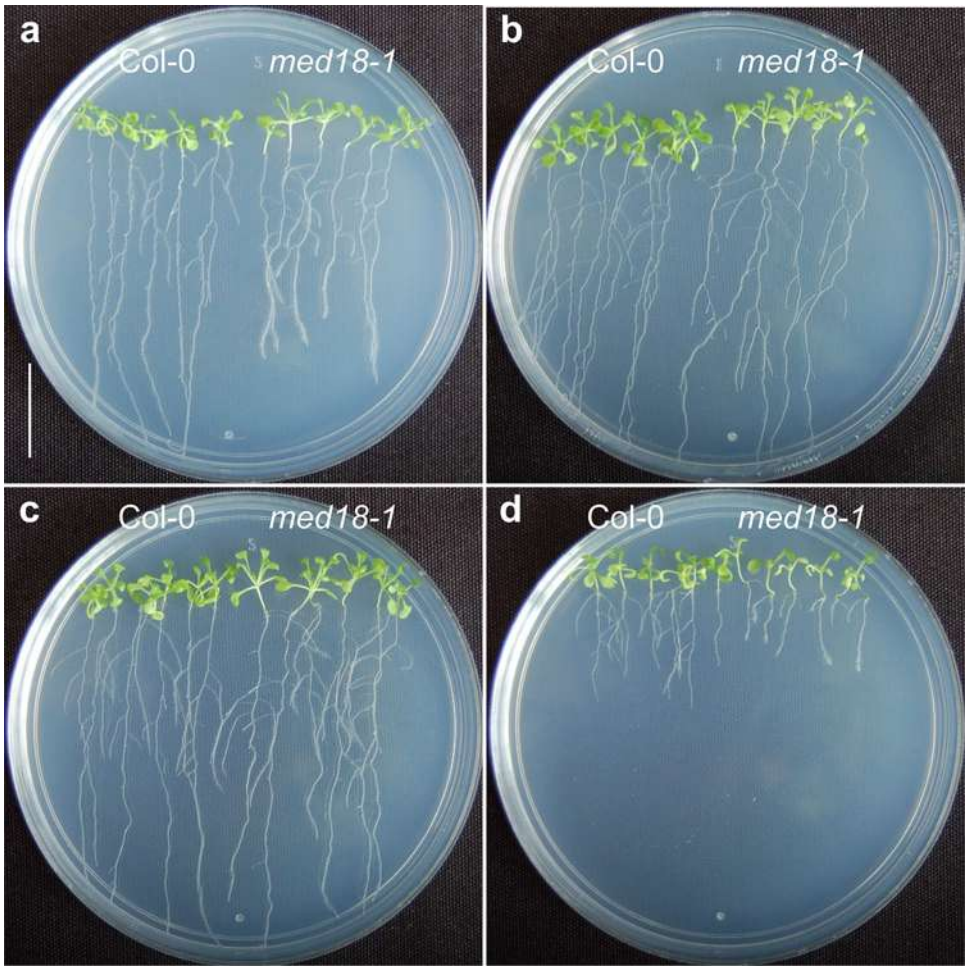

Fig. 1 Effects of boric acid (BA) on growth and development of *Arabidopsis* WT and *med18-1* seedlings.(**a**–**d**) Representative photographs of agar plates where *Arabidopsis* WT Columbia-0 (Col-0) were grown side by side with *med18-1* mutants for 15 days on agar-solidified 0.2 × MS medium in the control condition or 0.5, 1, and 2 mM of BA, respectively. Scale bar: 1 cm

root growth in a comparable manner in the WT and *med18-1* mutants at 2 mM or greater concentrations (Fig. 3a, b). The fact that mild supplementation of BA rescued the root growth inhibition of *med18-1* seedlings to some extent supports that not all boric acid concentrations are toxic for plants.

Boric Acid Did Not Enhance Root Branching in WT or *med18-1* Mutants

Root branching is a very important agricultural trait owing to its roles for soil exploration and nutrient and water uptake. By counting the total lateral roots already formed along the primary root of 10 d.a.g. seedlings visualized under a stereoscopic microscope, it was evident the increased lateral root formation capacity of the *med18-1* mutants, which developed almost twice the number of lateral roots of WT plants at concentrations of 0, 0.25, 0.5, and 1 mM BA (Fig. 4a, b). Concentrations of 4 and 8 mM BA further decreased the lateral root number in WT and *med18-1* mutants (Fig. 4b). Since the overall lateral root numbers tightly depends on the length of the primary root and this later trait is strongly repressed by increasing BA in the media, we next determined whether the number of lateral roots over the length of the primary root, also termed lateral root density, changes in these experiments. Our results indicate a really modest, if any, increase in lateral root density in the WT from 0 to 4 mM BA, reaching comparable density to *med18-1* seedlings at 4 mM BA, but diminishing in both genotypes upon 8 mM BA supplementation (Fig. 4c). These data indicate that BA did not promote root branching in *Arabidopsis*, implying that not all developmental traits are under its influence.

Boric Acid Shows an Anti-apoptotic Effect in *med18-1* Mutants and Reduces *ERF115* Expression

Low doses of BA somewhat recovered primary root growth of *med18-1* mutants and this may be related to the already described cell death phenotype. To assess whether BA supplementation could affect the integrity of root pro-vasculature cells, WT and *med18-1* seedlings harboring the *ERF115*:*GFP* gene construct were germinated and grown for 7 days on agar-solidified 0.2 × MS medium supplemented

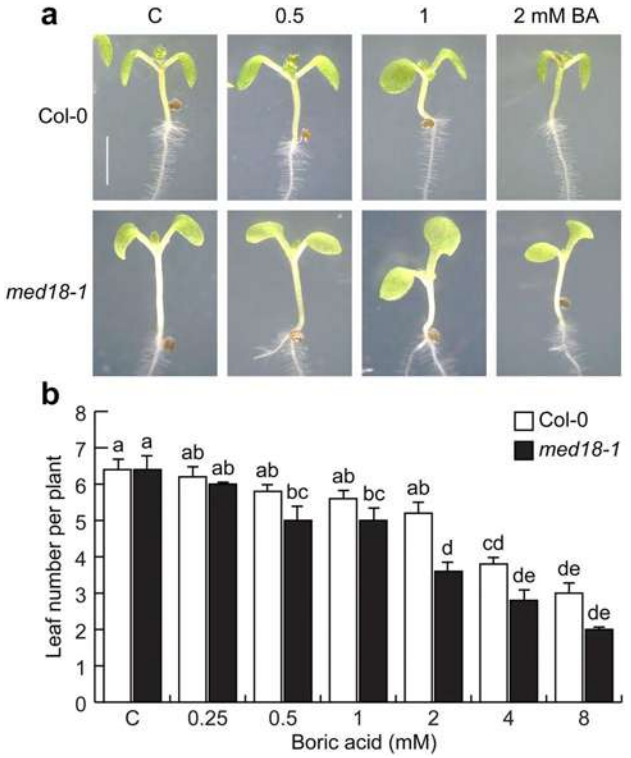

Fig. 2 Effects of boric acid on shoot development and leaf number of WT and *med18-1* mutants. **a** Representative photographs of 10-d-old Col-0 and *med18-1* shoots grown on agar-solidified 0.2×MS medium supplemented with increasing BA concentrations. **b** Leaf number per plant 15 days after germination. Bars represent means + SE from 24 seedlings analyzed. The data were analyzed with the ANOVA Tukey test at a $p < 0.05$, and the letters indicate means statistically different. The experiment was repeated three times with similar results. Scale bar: 1 mm

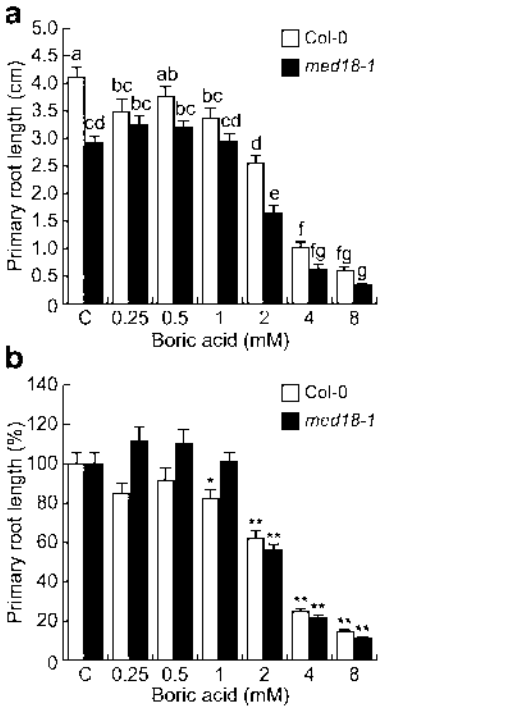

Fig. 3 Effect of boric acid in the primary root growth of WT and *med18-1 Arabidopsis* seedlings. *Arabidopsis* WT (Col-0) and *med18-1* mutants were grown for 10 days on agar-solidified 0.2×MS medium supplemented with BA. **a** The primary root length (cm) was analyzed with the ANOVA Tukey test at $p < 0.05$, and the statistically different means were represented with letters. **b** Primary root length (%) was analyzed with a chi-square test, and asterisks indicate significant differences at a $p < 0.05$ (*) and a $p < 0.01$ (**) compared with the control condition, respectively. Bars represent means ± SE from 24 seedlings. The experiment was repeated three times with similar results

or not with 1, 1.5, 2, 2.5, and 3 mM BA, and *ERF115*:*GFP* expression was examined in roots stained with propidium iodide (PI) using a confocal microscope. Representative images of at least 24 seedlings analyzed show that the structure of the primary root tip in both the WT and *med18-1* mutants becomes thinner than those of seedlings grown without BA (Fig. 5). BA further decreased *ERF115*:*GFP* expression around the pro-vasculature of *med18-1* mutants (Fig. 5), indicating that it conferred protection against the apoptotic program already reported for *med18-1* seedlings.

Boric Acid Normalizes Auxin Responsive Gene Expression in *med18* Primary Root Tips

The cellular damage genetically imposed by the loss of function of *med18* can be easily visualized via vital staining with the vital dye propidium iodide (PI). The cell death of pro-vasculature cells disturbs normal auxin transport, which can be assessed in vivo through the green fluorescence of cells conferred by *DR5*:*GFP* expression (Raya-González et al. 2018; Canher et al. 2020). To more in deep determine if BA could affect cell viability in the WT or if it could diminish the cellular lesion in pro-vasculature of *med18-1* mutants, the *DR5*:*GFP* expression was examined in WT and *med18-1* primary roots stained with PI. In medium without BA, a red patch indicative of cell death could be observed immediately upstream of the green florescence area at the very root tip covered by the *DR5* marker in *med18-1* mutants, but not in the WT. The expression of the marker was higher in the mutant roots at all concentrations of BA assayed, and interestingly, the red zone comprising the damage area decreased upon BA application and the *DR5*:*GFP* stream normally present in the WT at all BA concentrations throughout the meristem could be also visualized in *med18-1* mutants, which indicate the restoration of auxin response (Fig. 6). Thus, BA decreases cell death of pro-vasculature cells imposed by the loss of *med18* and restores normal auxin response within the root meristem.

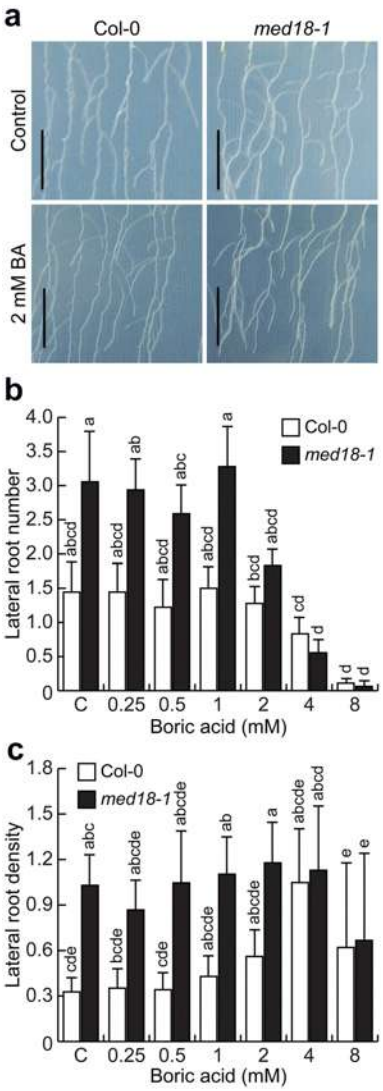

Fig. 4 Effects of boric acid on lateral root number and density of WT and *med18-1* seedlings. *Arabidopsis* WT (Col-0) and *med18-1* seedlings were grown for 10 days on agar-solidified 0.2×MS medium supplemented with BA. **a** Representative images of lateral roots. **b** Lateral root number. **c** Lateral root density. Bars represent means ± SE from 24 seedlings. The data were analyzed with the ANOVA Tukey test at a $p < 0.05$, and letters indicate means statistically different. The experiment was repeated three times with similar results

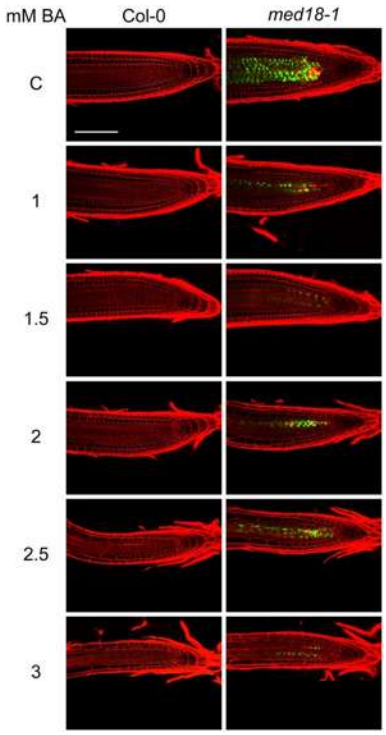

Fig. 5 Effects of boric acid on root tip structure and expression of *ERF115*:*GFP*. *Arabidopsis* WT (Col-0) and *med18-1* mutants harboring the *ERF115*:*GFP* gene construct were grown 7 d on agar-solidified 0.2×MS medium supplemented with 1–3 mM BA, and *ERF115*:*GFP* expression was examined in roots stained with propidium iodide by confocal microscopy. Scale bar: 100 µm

Discussion

Boron is a metalloid present in the soil in small amounts, but under certain conditions it may reach higher levels that affect plant growth and development. Therefore, plant adaptations have been evolved to deal with B deficiency or toxicity (Landi et al. 2019; Matthes et al. 2020). B is an important bio-element with reported antioxidant, anti-apoptotic, and anti-lipid peroxidation properties in most organisms and with mitochondria stabilizing properties in animals (Park et al. 2005; Routray and Ali 2019; Bouchareb et al. 2020; Ayhanci et al. 2020; Xiong et al. 2021), but in plants the notion that it acts as a nutrient has been challenged (Lewis 2019, 2020; Wimmer et al. 2020). This makes necessary additional research efforts to clarify its potential to strengthen productivity of crops at low or mild concentrations, since high levels are toxic and deleterious for plant growth. In this report, we took advantage of the recently identified *Arabidopsis* mutant *med18-1* that shows genetically fixed cell death in pro-vasculature cells within the root apical meristem to assess whether boric acid could exacerbate the damage or protect from the apoptotic program.

Raya-González et al. (2018) and Ruiz-Aguilar et al. (2020) indicated that the cell death in root meristems of seedlings defective on two different alleles of *MED18* exacerbated with time and this caused slow growth and root bifurcation, particularly upon light or pollutant exposition and accordingly, no damage could be observed in WT plants of the same age or in *MED18* seedlings grown in darkness. Following the report by Sakamoto et al. (2018), who showed that high B induces DNA double-strand breaks that trigger apoptosis of root meristem cells, we actually hypothesized that the *med18* mutants could be oversensitive to BA

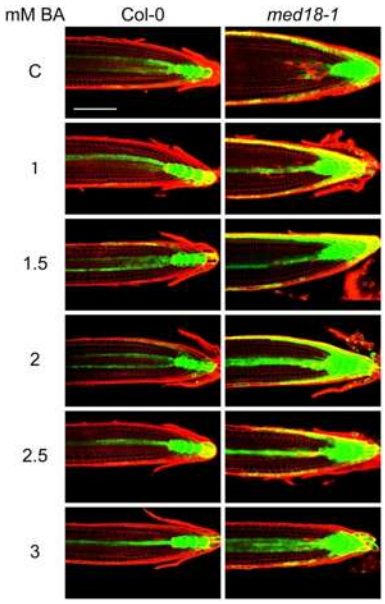

Fig. 6 Effect of boric acid on cell death and *DR5:GFP* expression in the meristem of WT and *med18-1* roots. The *DR5:GFP* expression was examined in roots stained with propidium iodide by confocal microscopy in 7-day-old Col-0 and *med18-1* seedlings grown on agar-solidified 0.2×MS medium supplemented with 1–3 mM BA. Scale bar: 100 µm

in a manner comparable to the *boron hypersensitive* (*heb*) mutants that manifest drastic root growth inhibition under mild or high-B (0.1–1 mM) concentrations. Our assumption was based on the fact that the *med18-1* mutants have compromised adaptation to genotoxic stress (Raya-González et al. 2018). Certainly, this was not the case and whereas root growth of the wild-type upon supplementation of doses lower than 1 mM BA did not much affect root elongation, the growth retardation of *med18-1* mutants observed in standard growth medium could be diminished by 0.5 mM and 1 mM BA.

Boron levels may increase in the soil through weathering, volcanic, and geothermal processes and under these circumstances plants activate physiological, biochemical, and molecular mechanisms to protect not only DNA from excision (Sakamoto et al. 2018), but also the impairment of cell division interference with primary metabolism as well as protein conformation and biosynthesis, which are major targets of B toxicity (Landi et al. 2019). Different studies have documented the phytotoxic effects of boron in plants, for instance, zucchini (*Cucurbita pepo* L.) and cucumber (*Cucumis sativus* L.) seedlings grown in an artificial substrate (soil-peat moss) irrigated with 0.2, 10, and 20 mg/l of boron manifested leaf necrotic burns, reduced leaf growth, and poor biomass accumulation. The toxicity symptoms correlated with decreased photosynthesis, low stomatal conductance, and impaired transpiration as well as induction of the antioxidant systems, which indicate the overall negative impact for plant–water relations (Landi et al. 2013). To test if BA could exert some influence in shoot development in *Arabidopsis* seedlings, we phenotypically assessed shoot development at 10 days after germination (d.a.g.), an early time, and quantified leaf formation later on (15 d.a.g.) where three pairs of leaves were already developed in the WT under control conditions. The 0.25 mM BA did not affect the leaf number, but increasing concentrations diminished leaf formation in a dose-dependent manner such that only half the number could be recorded at 8 mM BA in both the WT and *med18-1* mutants. In this regard, Papadakis et al. (2004) reported that increasing B in shoot tissues decreases leaf thickness, chloroplast size, and carbohydrate content in citrus plants, which may help explain the repression of leaf formation recorded in our experiments. The comparable leaf formation defect exerted by BA in the WT and *med18-1* mutants indicates that the mechanisms of toxicity/tolerance in shoots and roots differ. In these later organs the transition to repressing effects in *med18-1* mutants occurred in a very narrow interval, from 1 to 2 mM, an interesting trend already reported in animals (Park et al. 2005).

B plays important roles in phytohormone signaling and influences the mechanical properties of cell walls. It has been proposed that B could orchestrate critical functions during the evolutionary development of lignified tissues and therefore affects the root systems of vascular plants (Kutschera and Niklas 2017). Cell lignification is very important for cell plate formation during cell division programs that occur not only in root meristems but also during the formation of the lateral root primordia and this process is regulated by ethylene (Huang et al. 2013). The reconfiguration of the architecture of the root system is basically a post-embryonic process that integrates a myriad of biotic and abiotic stimuli. Most studies regarding B effects have focused on the growth of primary roots and less is known about the responses of lateral roots. Boron deficiency impairs the cell division and elongation processes (Cohen and Lepper 1977; González-Fontes et al. 2016) and noteworthy, the growth of roots of tomato and sunflower seedlings could be stopped within 3 h after transfer to boron-free media and an extended B deficiency (72 h) led to root tip necrosis (Kouchi and Kumazawa 1975). In our research, by counting the total lateral roots already formed along the primary root and determining their density, the greater lateral root formation capacity of the *med18-1* mutants became evident, which developed almost twice the number of lateral roots of WT plants at low BA concentrations. An increase in lateral root density in the WT could be recorded only in the WT reaching comparable density to *med18-1* seedlings at 4 mM BA, but diminishing the density in both genotypes upon 8 mM BA supplementation. These data show that the root branching response of

Arabidopsis seedlings to BA is dynamic and occurred in a phenotype and dose-dependent manner.

Our data are consistent with those of the classic report by Kouchi and Kumazawa (1975) in that B deficiency causes necrosis of root tips and its supplementation protects roots against necrosis. Striking similarities were also found in the manner by which BA diminishes cell death in primary root meristems of *med18-1* mutants of *Arabidopsis* to what is known in cell viability/proliferation in animals. First of all the protective effects were observed at concentrations lower than 1 mM, whereas higher concentrations exerted growth-repressing effects. Noteworthy, WT plants did not manifest cell death symptoms in the root meristems at the range of BA treatments applied (1–8 mM). In an experiment where borate was supplemented to HeLa cell cultures, treatments of 0.1 and 1.0 mM BA promoted mitosis, whereas higher doses decreased cell numbers (Park et al. 2005). Thus the range where BA promotes or affects cell proliferation is very narrow in both plants and animals.

Raya-Gonzalez et al. (2018) using Tunel assays demonstrated that the cell death in *med18-1* shares the typical signatures of an apoptotic behavior. The anti-apoptotic function of boric acid and borate has been coincidentally demonstrated in several recent reports in animals, where BA protects from cell death under different experimental conditions, including drug and heat stress and during cardiac function. Cyclophosphamide (CP) is an alkylating, anti-cancer agent that causes side effects on patients that damages healthy tissues. Boron treatments to rats exposed to CP protected from cellular damage due to anti-apoptotic and antioxidant activities (Ayhanci et al. 2020). In mouse cells cultured in vitro, heat stress affected cell viability, promoted apoptosis, and activated endoplasmic reticulum-stress markers. BA was protective against all these adverse effects (Xiong et al. 2021). Induction of ectopic apoptosis may cause embryo malformations in mammals, degeneration of neurons and diabetes, and BA effectively protected embryos from organ disruption (Routray and Ali 2019). Borax supplementation reduced myocardial fibrosis and apoptosis in rat-injured hearts, highlighting a protective effect of boron in the ischemic heart (Bouchareb et al. 2020). These effects were correlated with DNA protection since BA could reduce double-strand breaks in DNA and promoted wound healing (Tepedelen et al. 2016).

The cellular behavior imposed by BA was very different to that imposed by the oxyanion chromate (CrVI). Ruiz-Aguilar et al. (2020) hypothesized that exogenous CrVI could exacerbate the cell death in root meristems of *Arabidopsis med18* mutants due to its demonstrated genotoxic and mutagenic effects. Surprisingly, mild concentrations of the metal did not increase the apoptotic phenotype, but rather triggered the duplication in vivo of the meristem producing twin roots. These data indicate that the cellular targets and developmental consequences of CrVI and BA are not comparable. Intriguingly, our survey of the literature already published in plants and animals highlights the fact that CrVI strongly induces oxidative signaling, whereas BA at low doses acts to protect from oxidative damage the macromolecules, including DNA, proteins, and lipids.

The recovery of root growth of *med18-1* mutants by mild concentrations of BA was correlated with reduced cell death and lower expression of the ERF115 transcription factor. Heyman et al. (2013) discovered ERF115 as a rate-limiting factor for proliferation of the quiescent center, which signals initial cells to divide. When the meristem cells are damaged, *ERF115* is ectopically expressed in surrounding cells and it accounts for stem cell niche longevity. In our experiments, *ERF115*:*GFP* expression was not observed in root tips at all concentrations of BA applied, but in the *med18* mutants, strong expression was evident within pro-vasculature cells. The reduction of *ERF115*:*GFP* expression in *med18-1* root meristems can be explained by the anti-apoptotic effect of BA, which reduced tissue damage. *ERF115* is transcriptionally activated by jasmonate (JA), an oxylipin-derived phytohormone that mediates the wounding response in plants and triggers tissue regeneration after excision (Zhou et al. 2019; Lakehal et al. 2020). Furthermore, by analyzing the expression of *DR5*:*GFP* expression in WT and *med18-1* roots stained with propidium iodide, no damage was observed in the WT meristems and instead, a reduction of the red patch that marks the cell death zone could be observed in *med18-1* mutants. For this line, there are two independent zones of the WT root expressing the reporter gene, the first one is at the center of the root and the second encompasses the columella. In *med18* mutants, the fluorescence changes toward the root side due to the cellular damage within the pro-vasculature and 3 mM BA normalized the expression to WT levels, as the root width decreases. Moreover, the *DR5*:*GFP* expression at the root tip was gradually normalized in *med18-1* mutants, which indicates the restoration of normal auxin homeostasis. Thus, BA abolished cell death of pro-vasculature cells imposed by the loss of MED18 and restored normal auxin response within the root meristem. To the best of our knowledge, this is the first report demonstrating an anti-apoptotic activity of BA in plant cells, which could be possible by the use of a recently identified *Arabidopsis* mutant with genetically stable cell death in pro-vasculature cells. To what extend BA improves tissue repair, regeneration, or wound healing in plants or protects meristems from environmental stress is an interesting area to follow in order to improve boron application to field crops.

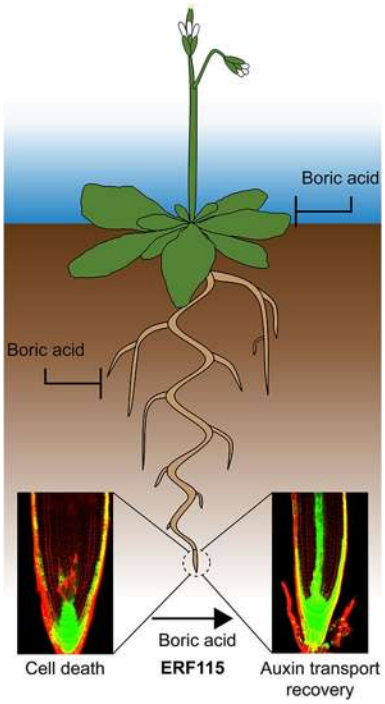

Fig. 7 Dual functions of boric acid include an overall growth-repressing effect at high concentrations and a protecting role from cell death in root meristems. Confocal images show the phenotypes of root tips of *med18-1* mutants expressing the auxin-inducible marker *DR5*:*GFP*. Note that BA decreases the root width of the root tip and normalizes the auxin pattern in pro-vasculature cells. The recovery of cell death in the mutants correlates with decreased ectopic expression of ERF115, a protein that specifically locates in neighbor cells adjacent to damaged areas

Conclusion

Figure 7 summarizes the results of the application of boric acid to *Arabidopsis* seedlings at earlier stages of development employing two genotypes, one being wild-type and a mutant deficient of the MEDIATOR18 protein, which manifest genetically stable cell death within the primary root meristem. Analysis of leaf formation, primary root growth, and lateral root formation in response to boric acid unveiled overall growth-repressing effects, but these depended upon the concentrations applied, the genotype, and developmental trait analyzed. Moreover, our study reveals for the first time the protective properties of mild BA concentrations against genetically fixed cell death in root meristems. This later aspect is very relevant toward protection of crops against environmental factors that trigger genotoxic stress such as UV light, salinity, and metals that are known to damage root cells.

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Author Contributions CETQ and JLB conceived and designed the experiments; CETQ, PIHV, and LFRH performed experiments; CETQ, LFRH, PIHV, and JLB analyzed the data; JLB contributed reagents/materials/analysis tools and wrote the manuscript. All authors read and approved the final draft.

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Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Declarations

Conflict of interest The authors have no conflict of interest or competing interests to declare.

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Mutation of *MEDIATOR16* promotes plant biomass accumulation and root growth by modulating auxin signaling

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A R T I C L E I N F O

Keywords:
MEDIATOR16
N-1-naphthylphthalamic acid (NPA)
Root development
Mitosis
Cell elongation

A B S T R A C T

The MEDIATOR complex influences the transcription of genes acting as a RNA pol II co-activator. The MED16 subunit has been related to low phosphate sensing in roots, but how it influences the overall plant growth and root development remains unknown. In this study, we compared the root growth of *Arabidopsis* wild-type (WT), and two alleles of *MED16* (*med16-2* and *med16-3)* mutants *in vitro*. The *MED16* loss-of-function seedlings showed longer primary roots with higher cell division capacity of meristematic cells, and an increased number of lateral roots than WT plants, which correlated with improved biomass accumulation. The auxin response reported by *DR5:GFP* fluorescence was comparable in WT and *med16-2* root tips, but strongly decreased in pericycle cells and lateral root primordia in the mutants. Dose-response analysis supplementing indole-3-acetic acid (IAA), or the auxin transport inhibitor *N*-1-naphthylphthalamic acid (NPA), indicated normal responses to auxin in the *med16-2* and *med16-3* mutants regarding primary root growth and lateral root formation, but strong resistance to NPA in primary roots, which could be correlated with cell division and elongation. Expression analysis of *pPIN1::PIN1::GFP*, *pPIN3::PIN3::GFP, pIAA14:GUS, pIAA28:GUS* and *35S:MED16-GFP* suggests that MED16 could mediate auxin signaling. Our data imply that an altered auxin response in the *med16* mutants is not necessarily deleterious for overall growth and developmental patterning and may instead directly regulate basic cellular programmes.

1. Introduction

Plants have an impressive ability to change their phenotype in response to environmental fluctuations through local and long-distance signalling between the root and shoot system, which helps to mitigate the negative effects of stressing factors and improve growth. The *Arabidopsis* root system is comprised of a single primary root and a network of lateral roots that originate from pericycle cells, both of which manifest a simple cellular organisation that facilitates their study [1]. The root system architecture, also defined as the spatial configuration of the primary and lateral roots, respectively, is dynamically modulated by environmental stimuli, such as water and nutrient availability, metals, temperature, soil pH and the presence of microorganisms [2]. This plasticity is controlled by a group of undifferentiated cells at the tip of the primary root, which is known as the stem cell niche (SCN). The position of the stem cells that surrounds two-to-four cells that rarely divide, the so-called quiescent center (QC), determines mitosis and organogenesis within the root tip [3].

The endogenous phytohormone levels and genetic elements reacting to changes in environmental conditions are involved in growth and root patterning. Auxin synthesized in the shoot system and the root tip affects overall plant development via two opposite streams [4,5]. Its transport from shoot to root is carried out by the efflux transporter PIN-FORMED (PIN) protein family and influx transporter AUXIN RESPONSE 1 (AUX1),

Abbreviations: APC/C, anaphase promoting complex/cyclosome; ARF, AUXIN RESPONSE FACTOR; AUX1, AUXIN RESPONSE1; CCS52A, CELL CYCLE SWITCH52A; DAG, days after germination; DEL1, DP-E2F-LIKE1/E2Fe; GFP, GREEN FLUORESCENT PROTEIN; IAA, indole-3-acetic acid; LRL, lateral root length; LRN, lateral root number; LRP, lateral root primordia; MED, mediator complex; MED16, MEDIATOR16; MS, Murashige and Skoog; NPA, naphthylphthalamic acid; PI, propidium iodide; PIN, PIN-FORMED; PRL, primary root length; POL II, DNA polymerase II; QC, quiescent center; SCN, stem cell niche; TF, transcription factor; WOX5, WUSCHEL-RELATED HOMEOBOX 5; WT, wild type.

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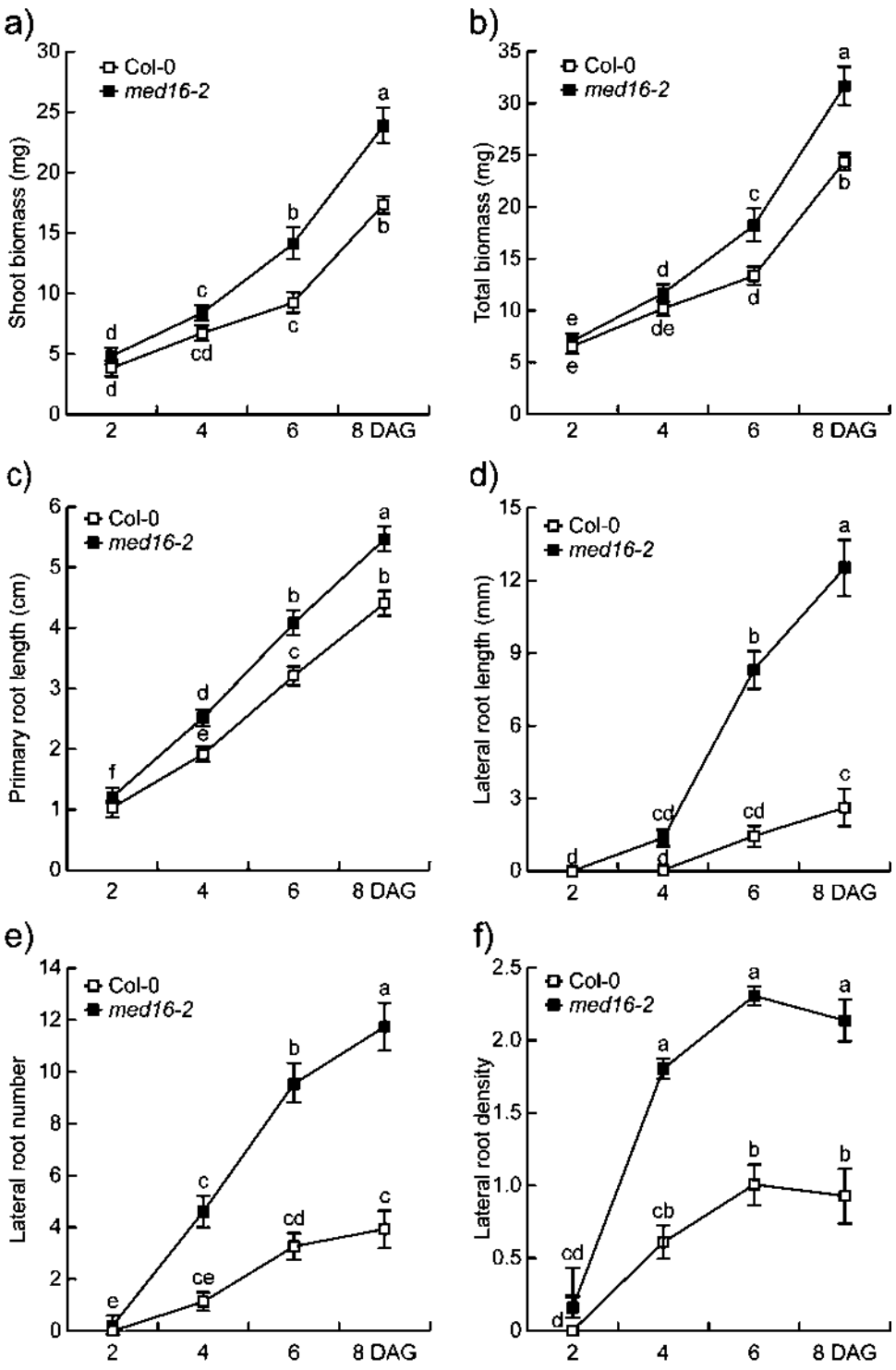

Fig. 1. The loss-of-function of MED16 enhances plant biomass production and root growth of *Arabidopsis*. Plant biomass and root architecture of WT (Col-0 ecotype) and *med16-2* seedlings, germinated and grown side by side on agar solidified 0.2x MS medium, were analysed at 2, 4, 6, and 8 days after germination (DAG). (a) Shoot biomass. (b) Total biomass (c) Primary root length. (d) Lateral root length. (e) Lateral root number. (f) Lateral root density. Data were analysed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p \leq 0.05$ from 15 seedlings analysed. The experiment was repeated three times with similar results.

forming a gradient with maximal accumulation in the QC [6,7], which positively influences the expression of WUSCHEL RELATED HOMEOBOX5 (WOX5) transcription factor to help in the establishment of the SCN [8]. Local auxin redistribution in meristems, elongation and differentiation zones acts through highly conserved AUXIN RESPONSE FACTORS (ARFs) influenced by the IAA family of auxin repressors (IAAs), which are degraded by the proteosome upon auxin binding to the receptors and enables root growth, initiation and growth of lateral roots and development of root hairs, which overall improve the potential for soil exploration and acquisition of resources [9,10].

In eukaryotes, most mRNA encoding genes are transcribed by RNA polymerase II (RNA pol II), whose enzymatic activity is driven by its interaction with proteins known as transcription factors (TFs), being these either activators or repressors of this process [11]. The MEDIATOR (MED) complex is a conserved multi-subunit protein assembly that is crucial for the positioning of RNA pol II to gene promoters [12]. The *Arabidopsis* MED complex has been identified and several of their subunits have been shown to mediate important morphogenetic and adaptive traits [13]. The MED16 subunit plays a role in flowering, freezing/osmotic stress tolerance, disease resistance, and iron/-phosphate homeostasis [14–22]. However, its role in the cellular and root developmental programmes that may impact overall plant biomass

production has been not assessed. In this study, through comparing the morphogenesis of *Arabidopsis* wild-type (WT) seedlings and two loss-of-function mutants of *MED16*, we uncovered a novel function of the encoded protein for mitosis and cell elongation processes through a process influenced by targets of NPA, an auxin transport inhibitor.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis thaliana seeds from wild-type (WT) Columbia-0 (Col-0), mutant plants (T-DNA insertion) of *med16-2* and *med16-3* [22], and transgenic lines *CycB1:uidA* [23], *DR5:GFP* [24], *pPIN1::PIN1::GFP* [25], *pPIN3::PIN3::GFP* [26], *pIAA14:GUS* [27], *pIAA28:GUS* [28] and *35S:MED16-GFP* [20] were used in this study. The *med16-2* plants were outcrossed with pollen of the transgenic lines, F1 plants were selected and allowed to self-pollinate to obtain the F2 and *med16* segregating plants were propagated until the fourth filial generation (F4) to obtain homozygous mutants carrying the different gene constructs [29]. Seeds were disinfected with ethanol (96 %) for 5 min and 20 % (v/v) commercial bleach for 5 min; subsequently, they were washed five times with sterilised distilled water. After cold treatment (4 °C) for 2 days, seeds were germinated and grown on plates with 0.2x MS medium, which contained 0.09 % (w/v) Murashige and Skoog Basal Salt Mixture (MS Salts; Sigma-Aldrich, St. Louis), 0.6 % (w/v) sucrose, and 1% (w/v) phytoagar (Phytotechnology Laboratories®). To test the auxin response, 0.2x

MS medium was supplemented with increasing concentrations of indole-3-acetic acid (IAA Sigma-Aldrich, St, Louis) or *N*-1-naphthylphthalamic acid (NPA; Sigma-Aldrich, St. Louis), where the control condition contained only the solvent. Plates were vertically placed in a plant growth chamber (Percival AR-95 L) with a photoperiod of 16 h of light/8 h of darkness, a light intensity of 300 μmol m^{-2} s^{-1}, and a temperature of 22 °C.

2.2. Measurements

The characterisation of root morphology was carried out for 15 individuals at 2, 4, 6, and 8 days after germination (DAG), or at a single time point. Analyses involving lateral root primordia were performed 4 DAG, and the experiments with IAA and NPA were performed 10 DAG. Primary root length (PRL) and lateral root length (LRL) were measured using a ruler, and lateral root number (LRN) was quantified using a stereoscopic microscope (Leica model EZ4D), and lateral root density was calculated by dividing LRN/PRL.

Lateral root primordia (LRP) were quantified and classified into seven stages until their emergence using a microscope (Leica) [30]. Root meristem length, root meristem width, number of meristematic cells expressing GUS, number of LRP cells expressing GUS, elongation zone length, number of cells in the elongation zone, length of cortex cells, root meristem relative fluorescence, and differentiation zone relative fluorescence were measured using the IMAGEJ software (https://imagej.nih.gov/ij/). Relative fluorescence was determined from green pixels in a defined area, and the control was normalised to 1 [31]. The data were statistically analysed using the Statistica 12 program (TIBCO Data Science-Statistica). Factorial ANOVA analysis with Tukey's post-hoc test was used to test the statistical differences. Different lowercase letters indicate means significantly different with a P value < 0.05.

2.3. Analysis of GUS activity and microscopy

A. thaliana seedlings carrying the reporter gene *uidA* were incubated with a *β*-glucuronidase (GUS) reaction buffer [1 mg/mL of 5-bromo4-chloro-3 indolyl-b-D-glucuronide, 50 mM NaH_2PO_4, 50 mM Na_2HPO_4, E.D.T.A. 10 mM, 0.1 % (v/v) Triton™ X-100, 2 mM $K_3Fe(CN)_6$, 2 mM $K_4Fe(CN)_6$; pH adjusted to 7 with KOH] overnight at 37 °C. The GUS

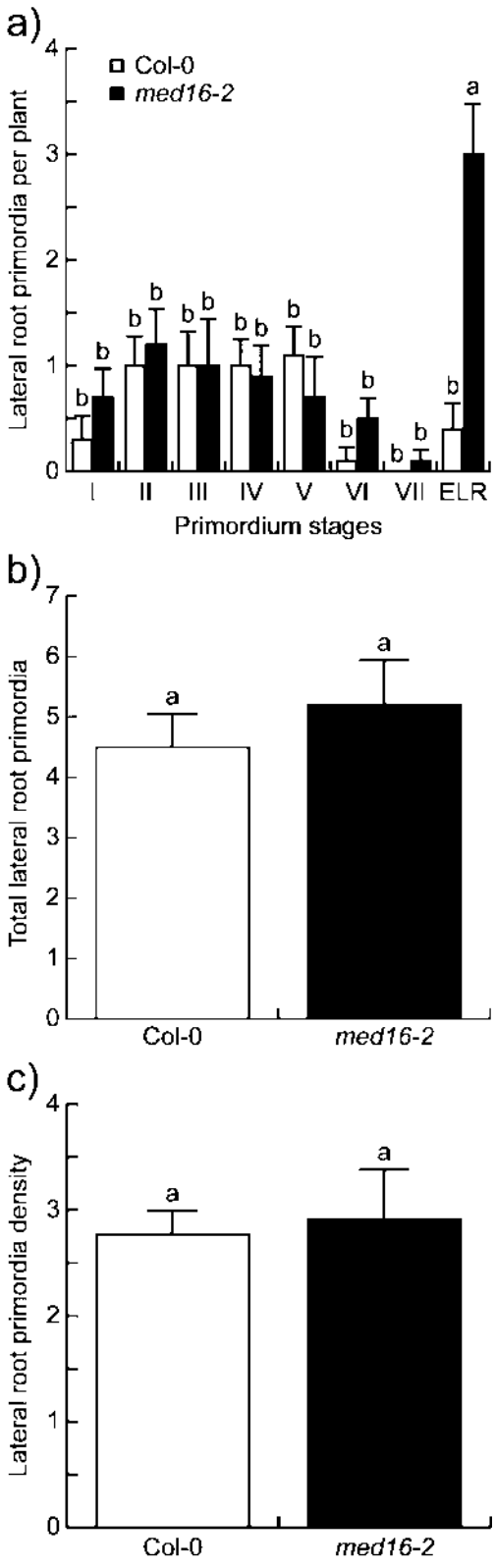

Fig. 2. Development of lateral root primordia in WT and *med16-2* mutants. Lateral root primordia (LRPs, I–VII) and emerged lateral roots were counted in a 10x objective in *Arabidopsis* WT (Col-0) and *med16-2* seedlings, which were germinated and grown for 4 DAGs on 0.2x MS medium. (a) Lateral root primordia per plant. (b) Total lateral root primordia. (c) Lateral root primordia density. Values were analysed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p \leq 0.05$ from 15 seedlings analysed. The experiment was repeated three times with similar results.

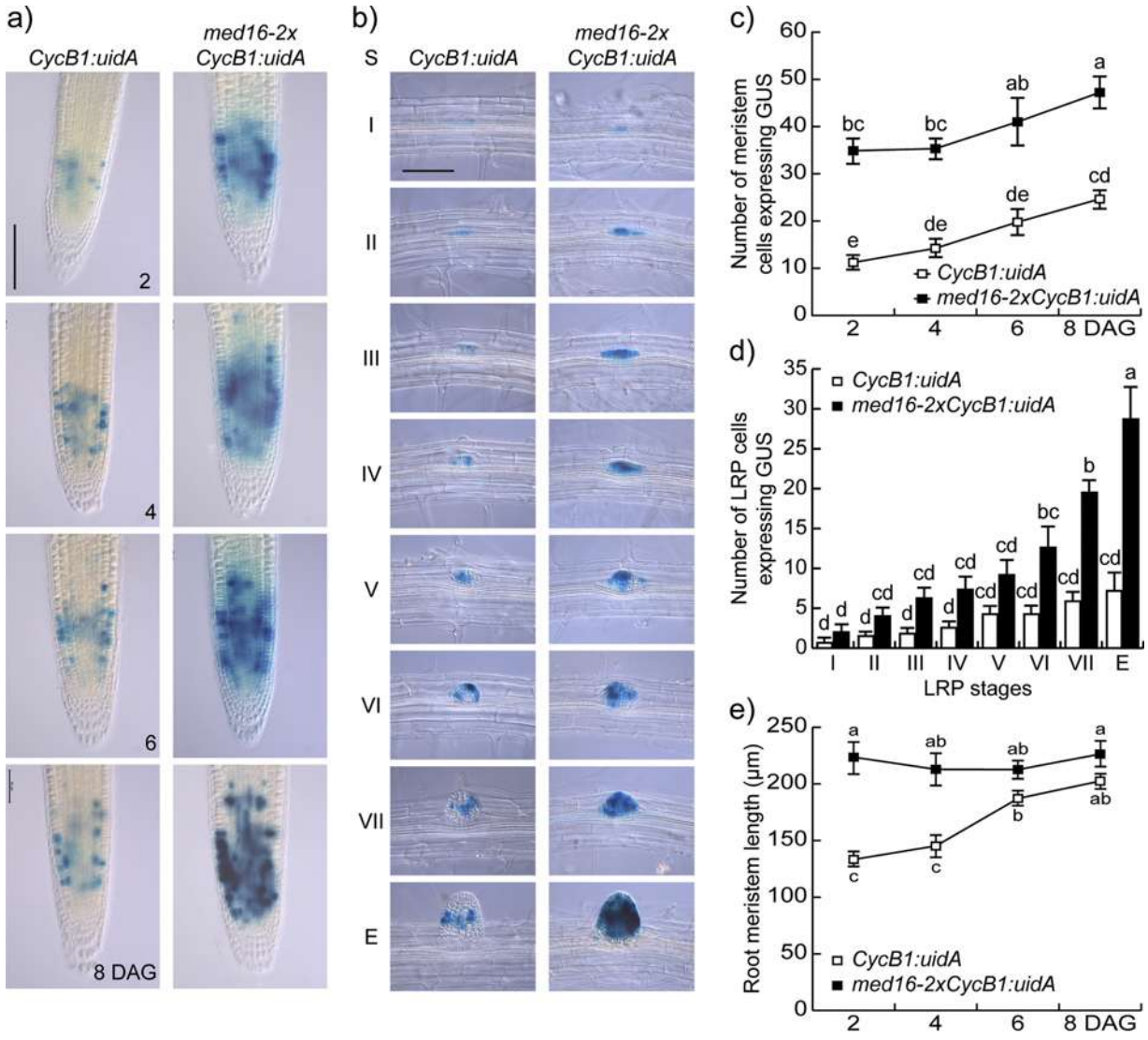

Fig. 3. *med16 Arabidopsis* mutants exhibit an enhanced cell mitotic activity in the primary root and lateral root primordia. *Arabidopsis thaliana* WT (Col-0) and *med16-2* seedlings harbouring the mitotic cell cycle reporter gene *CycB1:uidA* were germinated and grown on 0.2x MS medium for cell proliferation examination. (a) Representative Nomarski micrographs showing the cell mitotic cycle activity in the primary root meristem (at 2, 4, 6, and 8 DAG), (b) *CycB1:uidA* in lateral root primordial (I–VII stages), and emerged lateral roots. (c) Number of meristem cells expressing GUS in primary roots. (d) Number of LRP cells expressing GUS. (e) Length of the primary root meristem. The bar inside micrographs represents 100 μm. Data shown in (c-e) were analysed with the factorial ANOVA and Tukey's post hoc test. Different letters indicate statistical differences at $p < 0.05$ from 15 seedlings analysed. The experiment was repeated three times with similar results.

reaction buffer was removed and solution 1 [2% (v/v) of HCl; 20 % CH_3OH] was added for 60 min at 62 °C; subsequently, solution 1 was substituted with solution 2 [7% (w/v) of NaOH; 60 % (v/v) of CH_3CH_2OH] for 30 min at room temperature. During consecutive periods of 30 min, the seedlings were treated with 40 %, 20 %, and 10 % (v/v) of CH_3CH_2OH, and then placed in 50 % (v/v) glycerol inside glass cameras. Micrographs of the tissues were taken with a microscope (Leica DM5000 B) using differential interference contrast microscopy (Nomarski optics), in which the blue colour of GUS indicates the gene expression of interest in a tissue specific manner.

2.4. Detection of fluorescence by confocal microscopy

To visualise the GREEN FLUORESCENT PROTEIN (GFP) in plant tissues of seedlings carrying *GFP*, each individual was placed on a microscope slide with 90 μL of propidium iodide (PI) solution (0.5 mg/mL) and covered with a coverslip, and the samples were photographed using a confocal microscope (Olympus FV1200; Olympus Corp., Tokyo, Japan). Each sample was analysed separately for PI (an excitation peak at 568 nm, and an emission window between 585 nm and 610 nm) and GFP fluorescence (an excitation peak at 488 nm, and an emission window between 505 nm and 550 nm), and the two micrographs were merged to produce a final image, where the red colour of PI indicates the cell outline and the green colour of GFP indicates the protein of interest.

3. Results

3.1. MEDIATOR16 is a repressor of Arabidopsis biomass production and root growth

Previous reports indicated that loss of *MED16* affects the growth of *Arabidopsis* shoot organs, including hypocotyls, cotyledons, leaves, and petals [15,20,32]. To determine if MED16 regulates *Arabidopsis* biomass production and root development, WT (Col-0) seedlings and *med16-2* mutants were grown side by side in agar-solidified 0.2x MS medium. Then, fresh weight and root development were monitored at 2, 4, 6, and 8 DAG. Quantitative analysis demonstrated that starting at 6 DAG, shoot biomass and the total biomass increased in *med16-2* mutants (Fig. 1a, b).

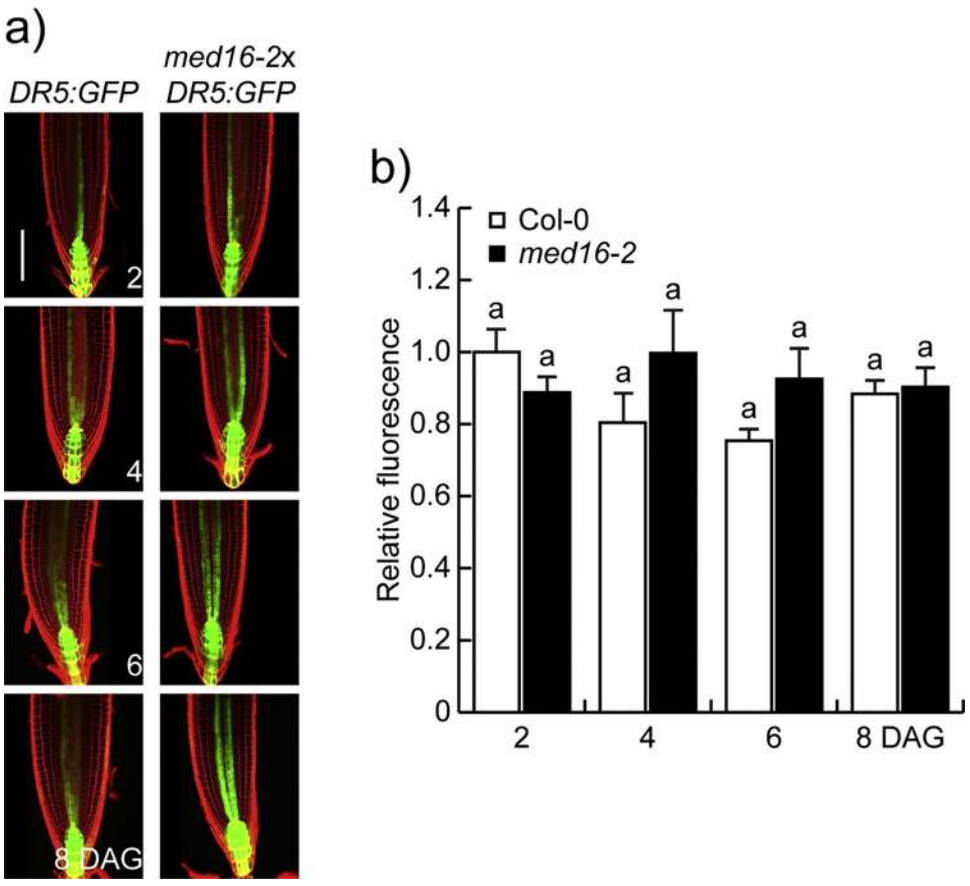

Fig. 4. *DR5:GFP* expression in the primary roots of WT and *med16* seedlings. *Arabidopsis thaliana* WT (Col-0) and *med16-2* seedlings harboring the auxin reporter *DR5:GFP* were germinated and grown on agar-solidified 0.2x MS medium. After 2, 4, 6, and 8 DAG the primary root of both plant lines was uploaded with propidium iodide and analyzed with a confocal microscope. (a) Representative confocal micrographs. (b) Fluorescence intensity of *DR5:GFP* reporter gene represented in relative fluorescence units. White scale bar inside micrographs represents 100 μm. Data shown in (b) were analyzed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p \leq 0.05$ from 15 seedlings analyzed. The experiment was repeated three times with similar results.

Additionally, *med16-2* mutants developed a primary root longer than the WT (Fig. 1c), and the length, number, and density of lateral roots increased approximately three-fold in these mutants from 4 DAG onwards (Fig. 1d–f). These observations indicate that MED16 subunit regulates root growth in *Arabidopsis*.

To gain insight into how MED16 influences the formation of lateral roots, we evaluated the lateral root primordia (LRP) stage distribution and quantified the number of LRP of 4 DAG in WT and *med16-2* seedlings. We found a homogeneous distribution of LRP stages in both lines, but the number of emerged lateral roots of *med16-2* increased six-fold when compared to the WT (Fig. 2a). The total LRPs and LRP density did not differ between WT and *med16-2* (Fig. 2b,c). Taken together these data suggest that mutation of *MED16* triggers the emergence of LRPs and may promote the initiation of new primordia since the number of LRP did not decrease in the mutants.

3.2. *MEDIATOR16 represses root cell division*

To further understand how MED16 regulates primary root growth and the emergence of lateral roots, we traced cell division over time assessing the expression of the reporter gene *CycB1:uidA* in the root meristem, LRPs and newly-emerged lateral roots of WT and *med16-2* seedlings. Interestingly, *med16-2* mutants presented an intense proliferative activity within the primary root meristem, I–VII stages of LRPs, and meristems of emerged lateral roots (Fig. 3a, b). In the kinetic analysis at 2, 4, 6, and 8 DAG and all stages of LRPs, we found that the number of cells expressing GUS in the primary root meristem and LRPs was increased roughly three-fold in *med16-2* than in the WT (Fig. 3c, d). We also found that the root meristem of *med16-2* was longer than that of WT for the first 4 DAG; however, from 6 DAG, its size was comparable (Fig. 3e). These data correlate the growth of the primary root and emergence of the lateral roots observed in *med16-2* with an enhanced mitotic activity.

3.3. *Mutation of MEDIATOR16 decreases auxin response in lateral root primordia*

The cell cycle is regulated by the auxin signalling pathway [33]. To investigate the possible correlation between the root phenotype of *med16* mutants and auxin signalling, we compared the expression of the auxin-responsive reporter gene *DR5:GFP* in root meristem and LRPs of WT and *med16-2* seedlings that were grown for 2, 4, 6, and 8 DAG on agar plates containing agar-solidified 0.2x MS medium. We did not find any significant difference in *DR5* expression within the root meristem of either line (Fig. 4). Conversely, *DR5* expression was down-regulated in the vasculature, I–VII stages of LRP, and newly-emerged lateral roots of *med16-2* (Fig. 5).

To further assess the root sensitivity to auxin, we examined the root architecture of WT, *med16-2* and *med16-3* mutants that were grown for 10 DAG in agar-solidified 0.2x MS medium supplemented with 0, 0.025, 0.05, and 0.1 μM IAA. The application of IAA reduced the length of the primary root in a dose-dependent manner in all three genotypes (Fig. 6a, b). 0.1 μM IAA enhanced both lateral root number and density to comparable levels in the WT and both mutant alleles (Fig. 6c, d).

3.4. *The loss of MEDIATOR16 confers primary root growth resistance to N-1-naphthylphthalamic acid*

NPA disrupts auxin transport and alter auxin levels in roots, affecting root growth, and lateral root development [34–38]. Given that *med16-2* manifests a weak expression of *DR5:GFP* in the vasculature, LRP, and newly-emerged lateral roots, we now determined whether the configuration of the root system could be modulated by NPA. WT seedlings, *med16-2* and *med16-3* mutants were grown side by side for 10 DAG on

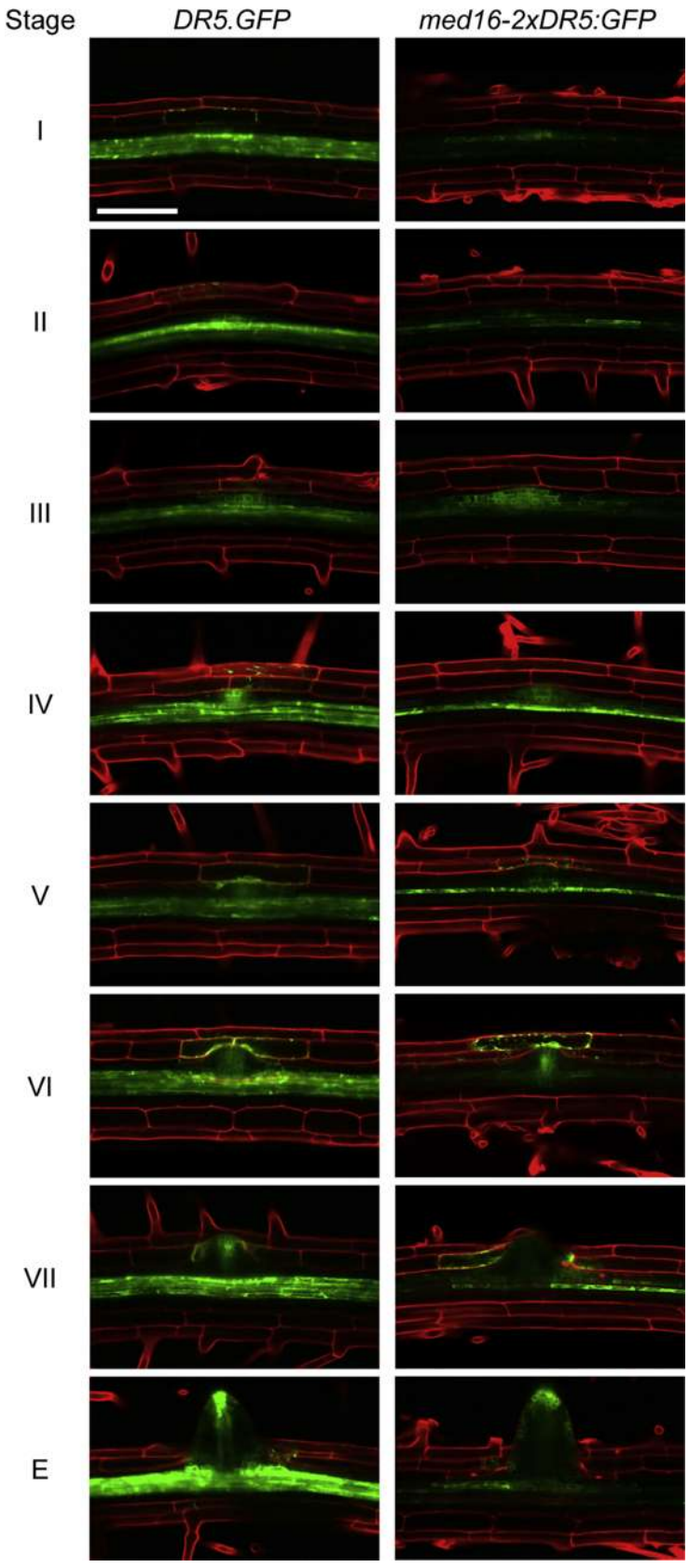

Fig. 5. *DR5:GFP* expression during lateral root formation in WT and *med16* seedlings. *Arabidopsis thaliana* WT (Col-0) and *med16-2* seedlings harboring the auxin reporter *DR5:GFP* were grown for 6 DAG on agar-solidified 0.2x MS medium under standard growth conditions. Both plant lines were uploaded with propidium iodide and analysed by confocal microscopy. Each micrograph shown is representative of the LRPs (I–VII stages) and emerged lateral roots. The white scale bar inside the micrograph represents 100 μm.

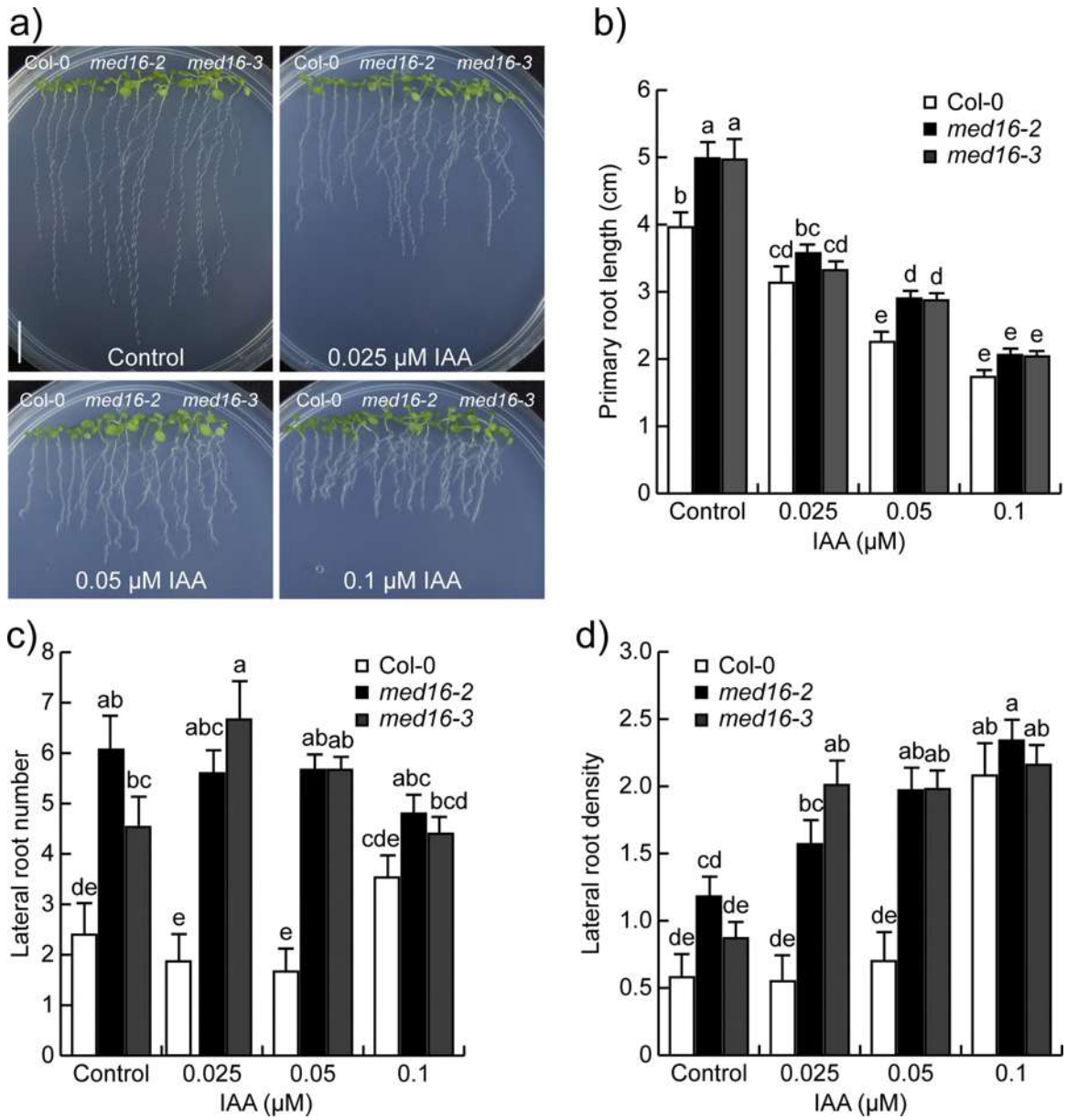

Fig. 6. Effects of índole-3-acetic acid (IAA) on root architecture of WT and *med16* seedlings. (a) Representative photographs of *Arabidopsis* WT (Col-0), *med16-2* and *med16-3* seedlings grown side by side for 10 DAG on agar-solidified 0.2x MS medium supplemented with increasing IAA concentrations. (b) Primary root length. (c) Lateral root number per plant. (d) Lateral root density per plant. White scale bar represents 1 cm. Data shown in (b-d) were analyzed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p < 0.05$ from 15 seedlings analyzed. The experiment was repeated three times with similar results.

agar plates supplemented with 0.5, 1, 2, and 4 µM NPA. NPA repressed the primary root growth of WT seedlings in a dose-dependent manner. Interestingly, the primary root of both mutant alleles of *MED16* resisted NPA-induced growth inhibition (Fig. 7a, c). However, NPA arrested the lateral root emergence of both plant lines (Fig. 7b, c), indicating that the auxin transport to developing LRP is critical for lateral root formation in these lines.

3.5. Auxin response in WT and med16 mutants subjected to NPA treatment

Primary root development depends of auxin distribution within the root tip, which mainly involves the root meristem and the elongation zones. We evaluated *DR5:GFP* expression in WT and *med16-2* seedlings grown for 7 DAG on medium supplemented with 4 µM NPA. Relative fluorescence intensity and representative images showed that *DR5:GFP* expression in the differentiation zone of WT seedlings was about two-fold higher than that of *med16-2* mutants after NPA treatment and it correlates with denser and longer root hairs being formed in the mutants (Fig. 8a, b). On the other hand, confocal imaging indicated that the WT seedlings developed wider meristems than the mutants upon NPA treatment (Fig. 8c-d) which may help explain their differential sensitivity in root growth.

3.6. Expression patterns of PIN1 and PIN3 in Arabidopsis WT and med16 mutants treated with NPA

It has been shown that NPA directly binds PIN proteins to inhibit auxin efflux transport [36,37]. To explore if MED16 mediates the sensitivity to NPA during the auxin transport inhibition through influencing the expression of auxin transporters, we analyzed *pPIN1::PIN1::GFP* and *pPIN3::PIN3::GFP* expression in the primary root of WT and

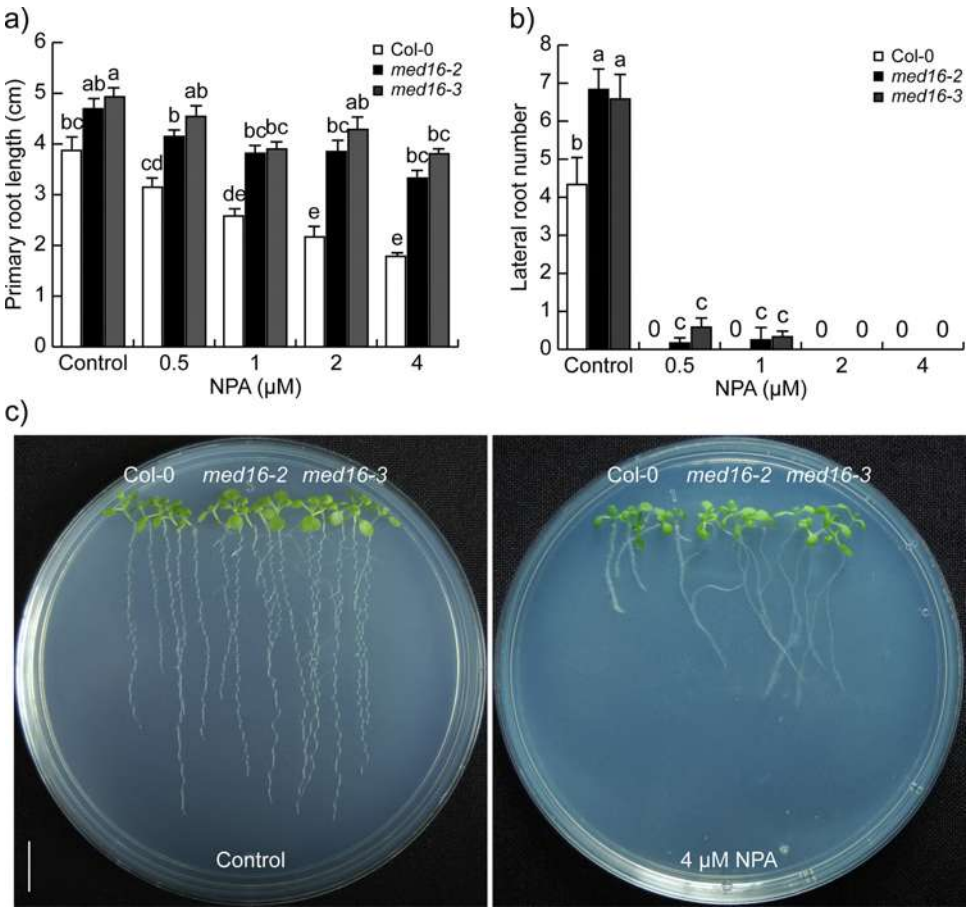

Fig. 7. Effects of *N*-1-naphthylphthalamic acid on primary root growth and lateral root formation in WT and *med16* seedlings. *Arabidopsis* WT (Col-0), *med16-2* and *med16-3* seedlings were germinated and grown side by side for 10 DAG on agar-solidified 0.2x MS medium supplemented with increasing NPA concentrations. (a) Primary root length. (b) Lateral root number. (c) Representative photographs of *in-vitro* experiments. White scale bar represents 1 cm. Data shown in (a, b) were analysed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p < 0.05$ from 15 seedlings analysed. The experiment was repeated three times with similar results.

med16-2 seedlings. Determination of relative fluorescence indicated that loss of *MED16* down-regulated *PIN1* expression in the root meristem (Fig. 9a, c), but not in the differentiation zone of either WT or *med16-2* mutants (Fig. 9b). NPA supplementation increased significantly *PIN1* expression in the root meristem of WT seedlings, but not in *med16-2*, where it remained comparable with or without NPA (Fig. 9a, c). On the other hand, NPA exposure increased *PIN3* expression in the differentiation zone and the root meristem of WT and *med16-2* seedlings (Fig. 9d–f). Thus, there seems to be no link between NPA sensitivity and *PIN1* or *PIN3* expression.

3.7. Expression patterns of IAA14 and IAA28 in Arabidopsis WT and med16 mutants

The reason why *med16* mutants grow longer primary roots and more lateral roots than the wild type in steady state may be explained by its role downstream of auxin response. To assess this point, we performed experiments to evaluate expression of master genes controlling the auxin response and lateral root formation, namely *IAA14* and *IAA28* [28, 39–41] through histochemical detection of *pIAA14:GUS* and *pIAA28:GUS* in *Arabidopsis* primary roots and particularly in vasculatures, at sites where lateral root primordia are initiated. It was found that in the *med16-2* background, GUS expression driven by the *IAA14* and *IAA28* promoters strongly decreased in primary roots (Fig. 10a, c) and root vasculatures (Fig. 10b, d). Since both IAA14 and IAA28 act as repressors of root branching via ARF7/19-LBD transcription factors, it may explain why *med16* mutants develop more branched root systems.

3.8. MED16 is located in nuclei of primary root tips and vasculature

According to Fornero and coworkers (2019) [42], *MED16* was strongly expressed in the cotyledon vasculature. To further define whether it could be detected in root growth zones and/or in vasculatures, the expression of *35S:MED16-GFP* construct in *Arabidopsis* primary roots was assessed via confocal microscopy. It could be appreciated that the green fluorescence is located in nuclei within the primary root meristem including the pro-vasculature and external cell layers (Fig. 11a). At the root differentiation region, it could be detected in the vasculature and developing lateral root primordium (Fig. 11b). These data show the location of the MED16 protein in root cells.

3.9. Cell expansion is a target of the differential growth response in the WT and med16 mutants affected by NPA

To understand in more detail if the growth repressing effects of NPA in primary roots could be due to arrested cell division, elongation or both, WT and *med16-2* seedlings harbouring the *CycB1:uidA* gene construct were grown for 8 DAG on agar plates supplemented with 0.5, 1, 2, and 4 μM NPA, and root development zones were examined. NPA did not change the number of cells expressing *CyCB1:uidA* (Fig. 12a, b), but induced the widening and shortening of the primary root meristem in WT seedlings, mainly at 4 μM concentration (Fig. 12c, d). It also decreased the number of cells and length of the elongation zone (Fig. 12e–g), and reduced the size of differentiated cells in primary roots of WT seedlings (Fig. 12h). In contrast, the cells in the meristem, elongation, and differentiation zones of the *med16-2* primary roots were less sensitive to NPA (Fig. 12). Our data clearly indicate that NPA inhibits cell elongation and that MED16 is involved in such developmental programme.

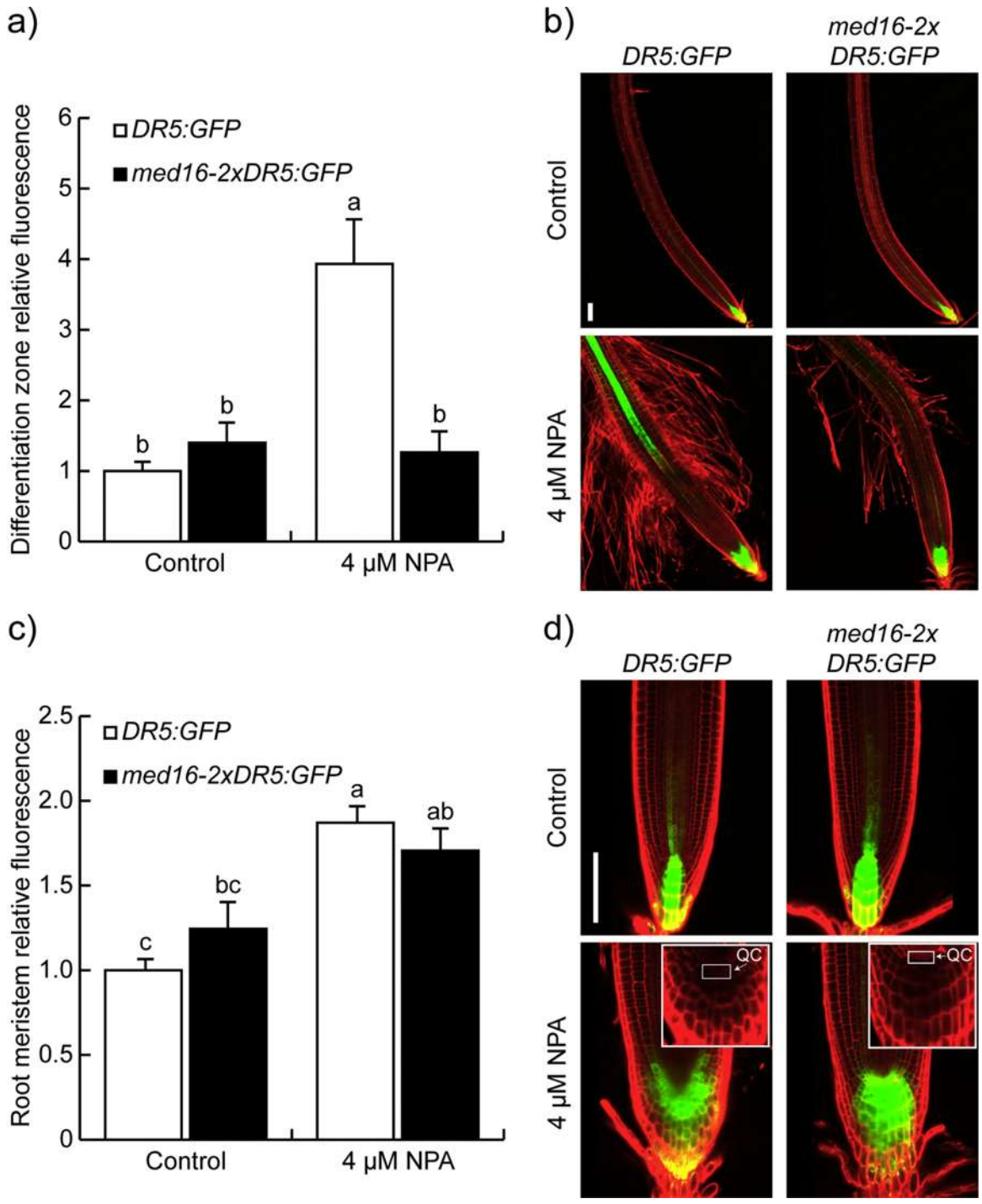

Fig. 8. Effect of *N*-1-naphthylphthalamic acid in *DR5:GFP* expression of WT and *med16-2* primary root tips. *Arabidopsis DR5:GFP* and *med16-2xDR5:GFP* seedlings were grown on agar-solidified 0.2x MS medium supplemented with 4 µM NPA. After 8 DAG, seedlings were stained with propidium iodide and were analysed by confocal microscopy. (a) Relative fluorescence of differentiation zone and (c) root meristem. (b, d) Representative micrographs of *DR5:GFP* expression in the primary root tip of WT and *med16-2* seedlings at different magnification. White scale bars represent 100 µm. Values were analysed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p < 0.05$ from 15 seedlings analysed. The experiment was repeated twice with similar results.

4. Discussion

The MED16 subunit of the mediator complex regulates the transcription of specific genes that allow plant survival under unfavourable growth conditions, such as cold and osmotic stress or pathogen infection [15–19]. However, MED16 may also coordinate shoot and root system architecture by modulating iron and phosphate homeostasis [20–22]. In this report, we unveiled a critical role of MED16 in basic cellular programmes in *Arabidopsis* roots. The phenotype shown in seedlings defective in two alleles, *med16-2* and *med16-3* may be due to their reported higher ploidy level and an increase in cell growth because MED16 interacts with DP-E2F-LIKE1/E2Fe (DEL1) to repress the expression of *CELL CYCLE SWITCH52 A1* (*CCS52A1*) and *CCS52A2*, which are activators of the anaphase promoting complex/cyclosome (APC/C) [32]. Furthermore, the primary root of *med16* seedlings grown under $\geq$1.0 mM phosphate showed increased cell division and

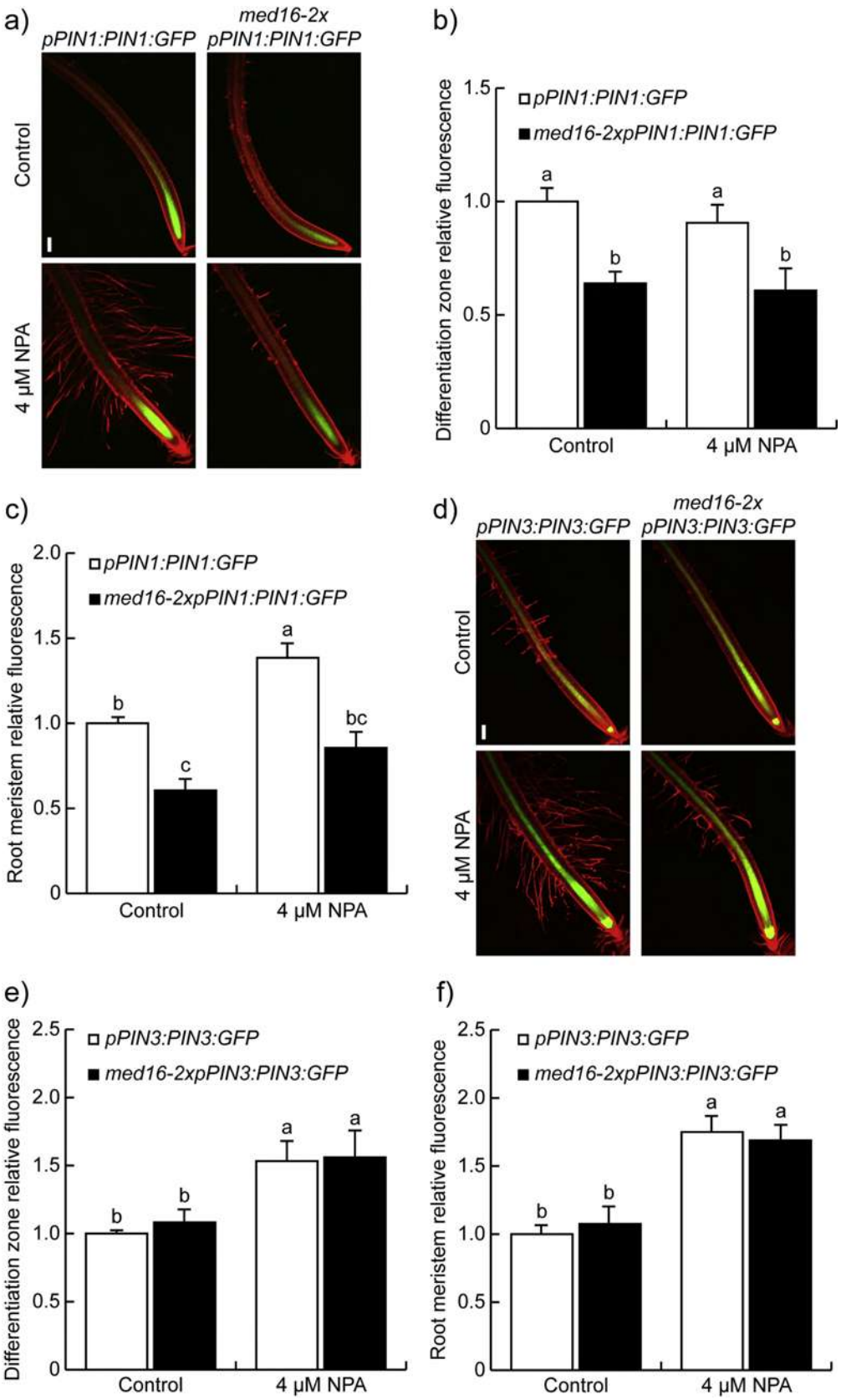

Fig. 9. Effect of *N*-1-naphthylphthalamic acid on auxin transporters *PIN1* and *PIN3* in the primary root of WT and *med16* seedlings. *Arabidopsis* WT (Col-0) and *med16-2* seedlings harboring the auxin transport reporter lines *pPIN1:PIN1:GFP* and *pPIN3:PIN3:GFP* were germinated on 0.2x MS medium supplemented with 4 µM NPA. 8 DAG seedlings were uploaded with propidium iodide and analyzed by confocal microscopy. (a) Representative micrographs of *pPIN1:PIN1:GFP* in the primary root of WT and *med16-2*. (b) Relative fluorescence of *pPIN1:PIN1:GFP* in the differentiation zone. (c) Relative fluorescence of *pPIN1:PIN1:GFP* in the root meristem. (d) Representative micrographs of *pPIN3:PIN3:GFP* in the primary root of WT and *med16-2* seedlings. (e) Relative fluorescence of *pPIN3:PIN3:GFP* in the differentiation zone. (f) Relative fluorescence of *pPIN3:PIN3:GFP* in the root meristem. The white scale bar inside (a) and (d) panels represents 100 µm. Data shown were analyzed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p \leq 0.05$ from 15 seedlings analyzed. The experiment was repeated twice with similar results.

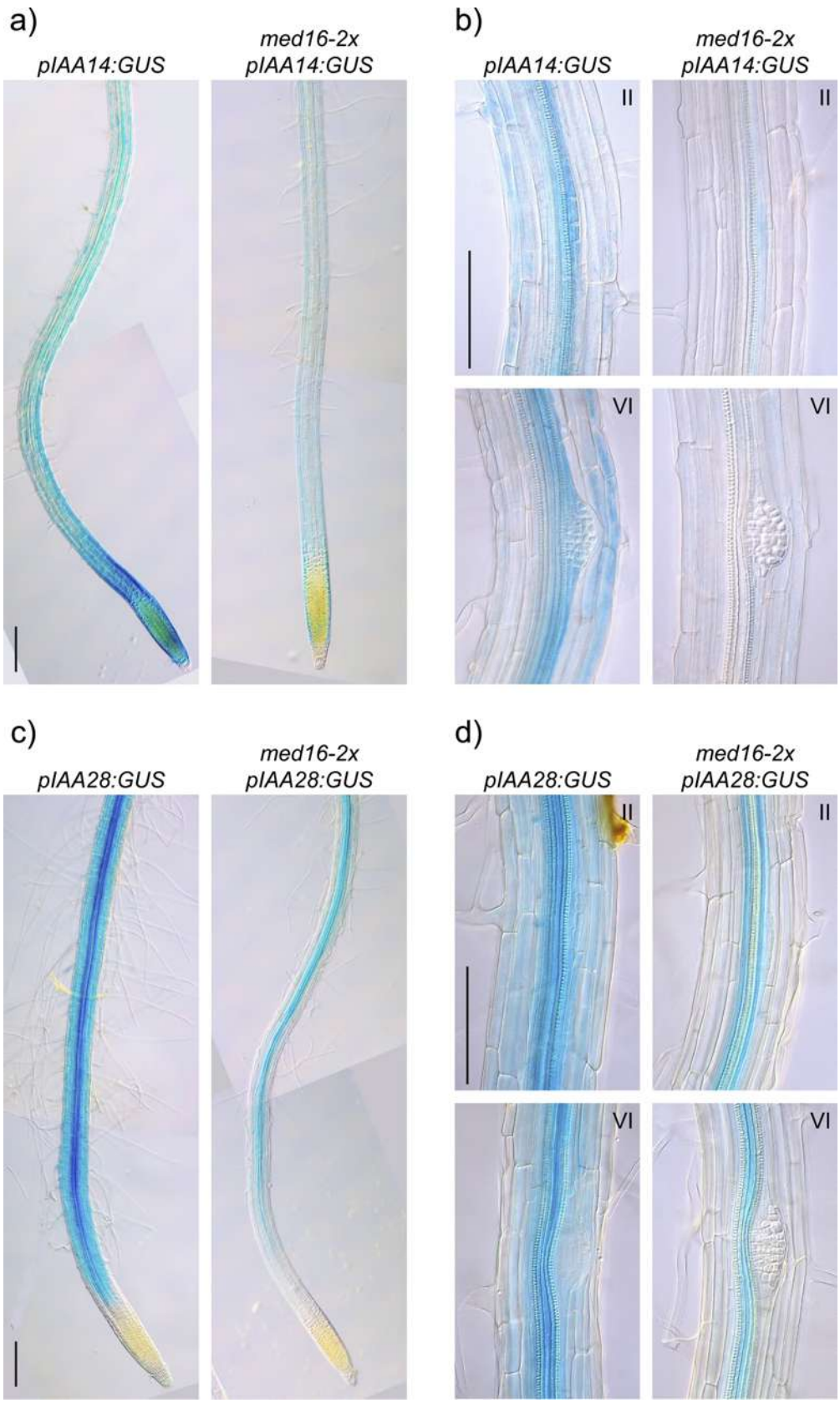

Fig. 10. Expression of *pIAA14:GUS* and *pIAA28:GUS* gene constructs in the primary root and vasculature of WT and *med16-2* seedlings. *Arabidopsis* WT (Col-0) and *med16-2* seedlings harboring *pIAA14:GUS* and *pIAA28:GUS* were grown for 8 DAG on agar-solidified 0.2x MS medium under standard growth conditions. Seedlings were processed for histochemical detection of β-glucuronidase activity, then clarified and analyzed via Nomarski optics. Representative micrographs of (a, b) *pIAA14:GUS* and (c, d) *pIAA28:GUS* expression in the primary root and LRP (II and VI stages) of WT and *med16-2* seedlings. The black scale bar inside (a) and (c) panels represents 200 μm, and the black scale bar inside (b) and (d) panels represents 100 μm. The experiment was repeated twice with similar results.

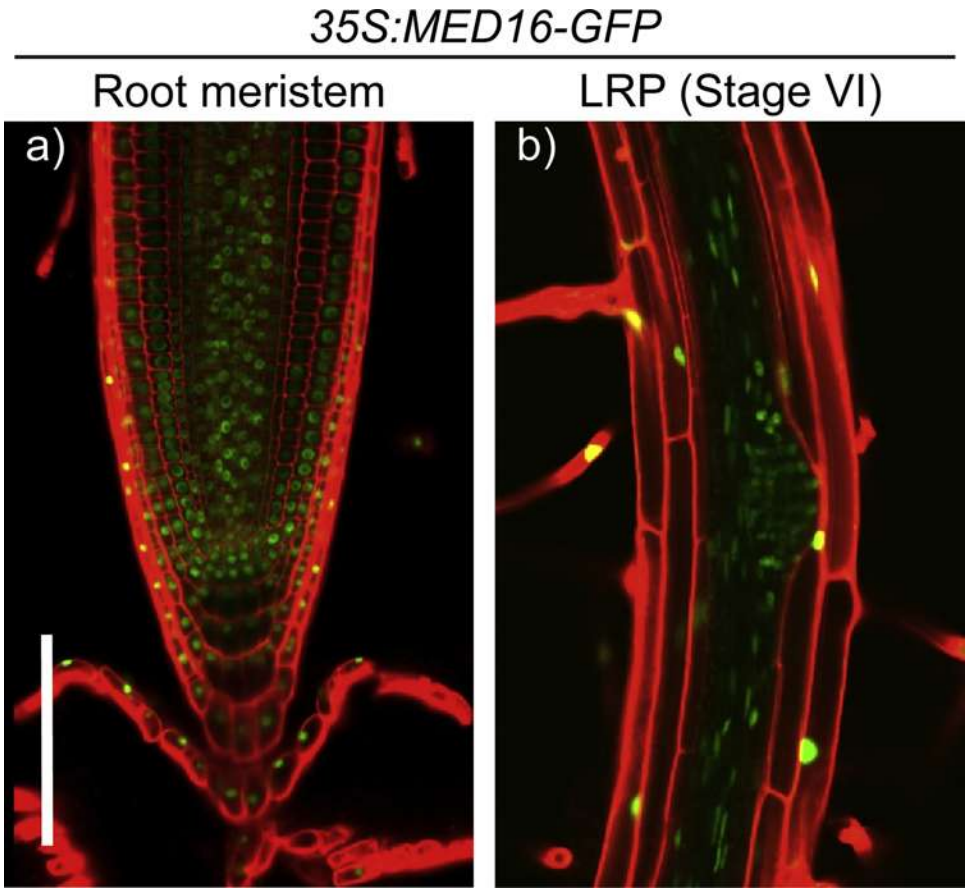

Fig. 11. Expression pattern of *MED16* in *Arabidopsis* primary roots. *Arabidopsis* transgenic *35S:MED16-GFP* seedlings were germinated and grown for 8 DAG on agar-solidified 0.2x MS medium under standard growth conditions and *MED16* expression was analyzed via confocal microscopy. Representative micrographs of the (a) root meristem, and (b) stage VI of LRP from roots stained with propidium iodide. The experiment was repeated two times with similar results. The white bar represents 100 μm.

elongation suggesting that MED16 may be involved in the *Arabidopsis* root growth regulation directly influencing mitosis [22].

In the current study, we show that *med16-2* and *med16-3* mutants grown in a 0.2x MS medium under standard growth conditions increased biomass production and developed longer primary roots and more lateral roots than WT seedlings. The root systems with strong apical dominance and more branches may provide better anchorage and improve soil exploration in the search for water and nutrients. In *Arabidopsis*, the lateral root development overall consists of four successive steps: LRP positioning in pericycle cells, LRP initiation, LRP development, and lateral root emergence [5]. Our analysis indicates that WT seedlings and *med16* mutants had a similar abundance of LRPs but *med16* mutants emerged more lateral roots than WT seedlings. Thus, although the loss of *MED16* increased the emergence of lateral roots in a dynamic manner, we cannot exclude an enhanced formation of primordia since the overall numbers did not decrease in the mutants. Additionally, expression analysis of the mitotic gene reporter *CycB1:uidA* showed that enhanced primary root growth and lateral root maturation of *med16* was related to an increased cell proliferation activity at all LRP stages and the primary root meristem. This confirmed that MED16 controls primary and lateral root growth by negatively modulating cell proliferation.

Cell cycle progression is regulated by auxin levels. In this regard, the maximum auxin concentration close to the root tip promotes cell proliferation, whereas low levels of auxins lead to a down-regulation of the mitotic cell cycle genes *CYCA2;3* and *CYCB1;1* [33,43]. To understand whether MED16 regulates cell proliferation by modulating auxin concentration within the root meristem and lateral root formation zone, we analysed the expression of *DR5:GFP* auxin response reporter. We found that *DR5:GFP* expression in the root tip of *med16* mutants was comparable to that in WT seedlings. In *Arabidopsis,* the root tip represents an important site for auxin biosynthesis during the first stages of plant development [4]. Given that the mutation of *MED16* did not affect *DR5:GFP* expression in the root tip, we conclude that auxin biosynthesis was not disturbed in *med16* mutants. The increment of the auxin reporter gene *DR5:GFP* signal in the lateral root formation zone has been related to the enhanced production of lateral roots [34]. Therefore, it was somewhat surprising that *DR5:GFP* signal expression was decreased in the lateral root formation zone of *med16* since these mutants developed more lateral roots than WT seedlings. One possible explanation is that MED16 acts on the auxin signaling pathway to modulate root branching. This is supported by the reduced expression of the auxin response factors IAA14 and IAA28, whose gain of function strongly impairs lateral root formation [28,39–41].

Auxins (e.g. IAA) or auxin precursors modify root architecture by decreasing primary root length and stimulating lateral root emergence and root hair formation [44]. Our results revealed that the primary root of *med16* mutants showed a similar response to IAA that the primary roots of WT seedlings. Thus, the loss of *MED16* did not alter root sensitivity to IAA. Strikingly, *med16* mutants showed also an increasing number of lateral roots upon IAA supplementation, which excludes the possibility that major changes in auxin biosynthesis or sensitivity may be occurring in *med16* mutants. In contrast, comparison of root architecture of WT seedlings and *med16* mutants exposed to NPA further indicated a different sensitivity of the WT and *med16* impairing primary root development. For instance, NPA suppressed the formation of lateral roots in both WT seedlings and *med16* mutants at comparable level, but the mutants were resistant to NPA-induced root growth arrest.

Mutation of several *PIN* genes, which are part of a family of proteins that transport auxin, affects both the meristem and elongation zone of the primary root [33]. Therefore, *MED16* may be involved in the control of the meristem and elongation zone developmental process through the regulation at some level of auxin distribution via PIN proteins. Our data revealed that NPA increases *DR5:GFP* signal in a spatially separated

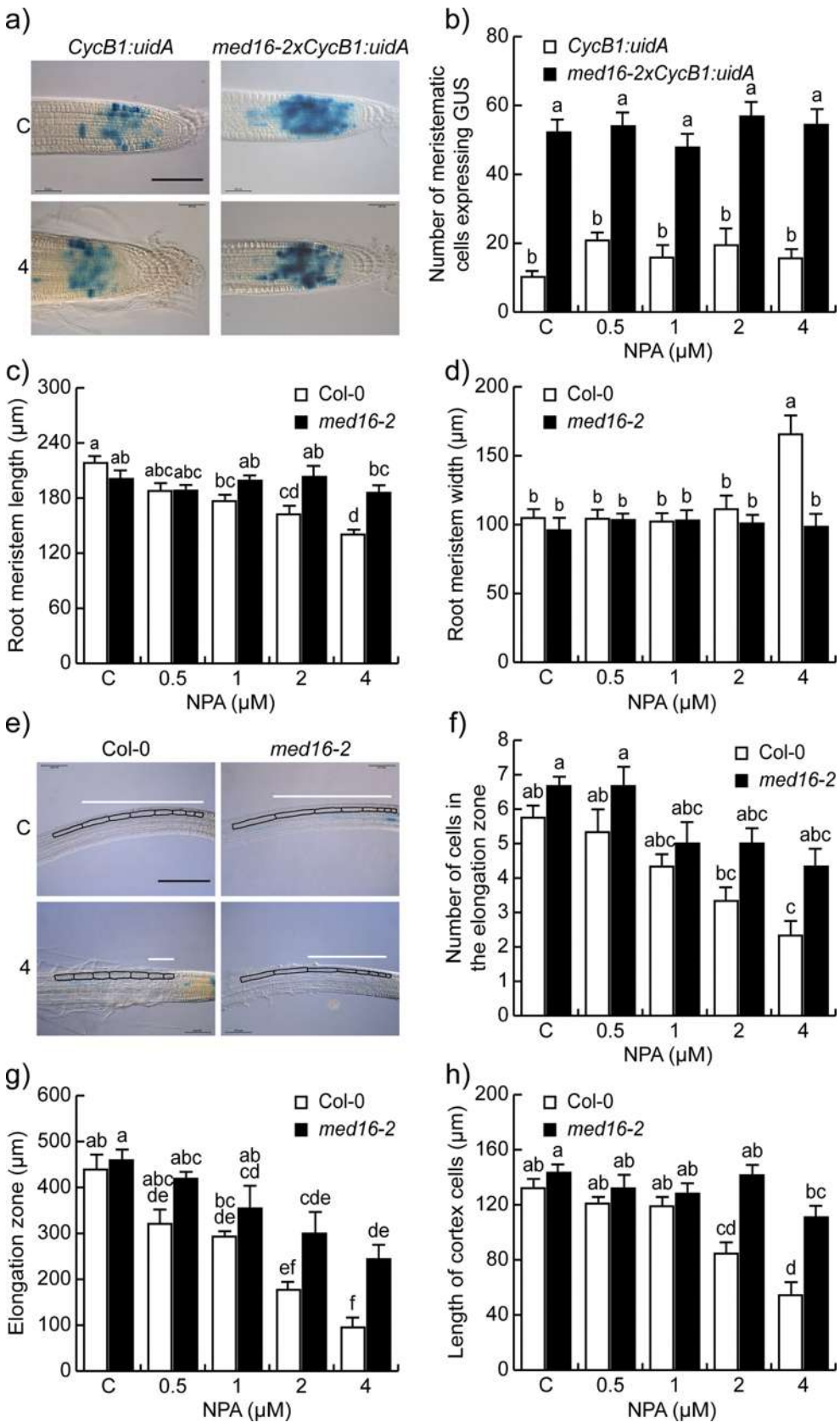

Fig. 12. Cellular parameters in WT and *med16* primary roots in response to *N*-1-naphthylphthalamic acid. *Arabidopsis thaliana CycB1:uidA* and *med16-2xCycB1:uidA* seedlings were grown for 8 DAG on agar-solidified 0.2x MS medium supplemented with increasing NPA concentrations. (a) Representative Nomarski images of the proliferative activity of meristematic cells of *CycB1:uidA* and *med16-2xCycB1:uidA* seedlings. (b) Number of root meristematic cells expressing GUS. (c) Root meristem length. (d) Meristem width. (e) Representative Nomarski images of the elongation zone of the primary root tip. (f) Number of cells in the elongation zone. (g) Elongation zone length. (h) Length of cortex cells. Black scale bars inside micrographs represents 100 μm. White bars in panel (e) indicate the length of the elongation zone. Values were analysed with the factorial ANOVA and Tukey's post hoc test. Letters indicate statistical differences at $p \leq 0.05$ from 15 seedlings analysed. The experiment was repeated twice with similar results.

manner forming two domains in the primary root of WT seedlings: one in the differentiation zone and the other on the root meristem, both of which were reduced in *med16* mutants. The fact that NPA increased *DR5:GFP* signal in WT root meristem and differentiation zone indicates that NPA prevented auxin transport and induced its accumulation in these developmental zones. Confocal imaging of the root of *Arabidopsis* WT seedlings grown in NPA treatment of 4 μM increased *PIN1* expression in vasculature cells of the root meristem and *PIN3* expression in vasculature cells of the root meristem and the differentiation zone. By contrast, NPA did not increase significantly *PIN1* expression in vasculature cells of *med16* mutant root meristem. Furthermore, the analysis of root tip revealed that the mutation of *med16* caused the repression of *PIN1* expression. According to two recent reports, NPA interacts directly with PIN dimers in cell membranes to inhibit auxin transport [36,37], and this interaction is dependent on *PIN* expression status [37]. Accordingly, *PIN* expression associates directly with NPA activity. Overall, our data excludes the possibility that the loss of *MED16* confers resistance to NPA-induced auxin transport inhibition in the differentiation zone as a consequence of *PIN1* or *PIN3* repression in the primary root. It might be rather function through ARF7/19 module [28,39–41], which influences lateral organ boundary (LBD) genes involved in lateral root development.

Auxin controls the elongation of cells and participates in the maintenance of the SCN. It has been reported that NPA increases auxin levels in the root tip, which leads to the loss of *WOX5* expression, allowing cell division of QC cells and promoting columella stem cell differentiation [8]. Our data indicated that columella stem cells of *med16* mutants were resistant to NPA-induced cell differentiation. On the other hand, auxin regulates several steps of cell elongation, for example: (I) It triggers the activation of H^+-ATPases to acidify the apoplast, resulting in an increase in turgor pressure and the activation of cell wall loosening enzymes [45,46], and (II) auxin regulates the actin cytoskeleton and the abundance of SNARE proteins to control vacuole morphology and cell size [47,48]. Hence, the reduction of auxin levels or inhibition of the auxin signalling pathway abolishes the cellular expansion of root cells [45]. Here we showed that the inhibition of auxin efflux transport induced by NPA predominantly reduced the elongation zone size and the final length of the cortex cells in the WT seedlings. Conversely, the loss *MED16* mutants provided resistance to NPA-induced reduction of elongation zone size and the length of cortex cells. Therefore, in this study we unveil a novel function of MED16 for cell elongation that underlies an improved root growth. Organogenesis depends on both cell division and elongation, how the MEDIATOR complex influences the transcription at the meristem and cell elongation regions, two developmental and spatially separated zones remains to be investigated.

Author contributions

PIHV, JLB and JRG planned and designed the research. PIHV, LFRE, SBO, CMLG and JLB performed the experiments and analyzed data. PIHV and JLB wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgments

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