Programa de Doctorado en Bioingeniería

TESIS DOCTORAL

Nuevas funciones génicas en la formación de raíces adventicias en Arabidopsis

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- **Sergio Ibáñez**, Helena Ruiz-Cano, María Á. Fernández, Ana Belén Sánchez-García, Joan Villanova, José Luis Micol, José Manuel Pérez-Pérez (2019). A network-guided genetic approach to identify novel regulators of adventitious root formation in *Arabidopsis thaliana*. *Frontiers in Plant Science* **10**: 461. doi: 10.3389/fpls.2019.00461.

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INFORMA:

Que D. Sergio Ibáñez López ha realizado bajo mi supervisión el trabajo titulado **“Nuevas funciones génicas en la formación de raíces adventicias en Arabidopsis”** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmo para los efectos oportunos, en Elche.

Director de la tesis

Prof. Dr. D. José Manuel Pérez Pérez.

La Dra. Dña. Piedad de Aza Moya, Coordinadora del Programa de Doctorado en Bioingeniería de la Universidad Miguel Hernández de Elche

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Que D. Sergio Ibáñez López ha realizado bajo la supervisión de nuestro Programa de Doctorado el trabajo titulado **"Nuevas funciones génicas en la formación de raíces adventicias en Arabidopsis"** conforme a los términos y condiciones definidos en su Plan de Investigación y de acuerdo al Código de Buenas Prácticas de la Universidad Miguel Hernández de Elche, cumpliendo los objetivos previstos de forma satisfactoria para su defensa pública como tesis doctoral.

Lo que firmo para los efectos oportunos, en Elche

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El trabajo recogido en esta memoria ha sido realizado en el marco de los siguientes proyectos de investigación:

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Lo que firmo para los efectos oportunos, en Elche

Investigador Principal

Prof. Dr. D. José Manuel Pérez Pérez

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6-BAP	6-benzilaminopurina
ADN	Ácido desoxirribonucleico
ADN-T	ADN de transferencia
AIA	Ácido indol-3-acético
ANF	Ácido 1-naftalenacético
AP2/ERF	APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING FACTORS
ARF	AUXIN RESPONSIVE FACTOR
ARN	Ácido ribonucleico
ARR	ARABIDOPSIS RESPONSE REGULATOR
AtGA20ox1	*ARABIDOPSIS THALIANA GIBBERELLIN 20-OXIDASE 1*
AtRH7/PRH75	*ARABIDOPSIS THALIANA RNA HELICASE 7/PLANT RNA HELICASE 75*
ATXR2	*ARABIDOPSIS TRITHORAX-RELATED 2*
Aux/IAA	AUXIN/INDOLE-3-ACETIC ACID
axr2-1	*auxin resistant 2-1*
BAR	Bio-Analytic Resource for Plant Biology
bZIP	FD BASIC-LEUCINE ZIPPER
CEP	cys-ENDOPEPTIDASE
Col-0	Columbia-0
CRISPR	CLUSTERED REGULARLY INTERSPACED SHORT PALINDROMIC REPEATS
CUC	*CUP-SHAPED COTYLEDON*
CYCB1;1	*CYCLIN B1;1*
CYCB1;2	*CYCLIN B1;2*
CYP83A1	*CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE 1*
dde	días después de la escisión
EXT	EXTENSIN
GA1	*GA REQUIRING 1*
GFP	GREEN FLUORESCENT PROTEIN
GLM	*Generalized Linear Model*
GRAS	GAI, RGA y SCR
GUS	GLUCURONIDASE BETA
hde	horas después de la escisión
HRGP	HYDROXYPROLINE-RICH GLYCOPROTEIN
IAA28	ACETIC ACID INDUCIBLE 28
IPT	ARABIDOPSIS THALIANA ISOPENTENYLTRANSFERASE

KRP2	KIP-RELATED PROTEIN 2
lars	*less adventitious roots*
LBD	*LATERAL ORGAN BOUNDARIES DOMAIN*
LEC	*LEAFY COTYLEDON*
LOG	*LONELY GUY*
LSD	*Least Significant Difference*
mars	*more adventitious roots*
MeJa	Metil jasmonato
Mpb	Megapares de bases
MSE	Metanosulfonato de etilo
NAC	NAM, ATAF1/2 y CUC2
NASC	*Nottingham Arabidopsis Stock Centre*
PLT	*PLETHORA*
PtRR13	*POPULUS TRICHOCARPA RESPONSE REGULATOR 13*
REF2	*REDUCED EPIDERMAL FLUORESCENCE 2*
SHR	*SHORT-ROOT*
SKP2B	*ARABIDOPSIS HOMOLOG OF HOMOLOG OF HUMAN SKP2 2*
slr-1	*solitary root-1*
sur2-1	*superroot 2-1*
TAIR	The Arabidopsis Information Resource
TDZ	Tidiazurón
WIND1	*WOUND INDUCED DIFFERENTIATION 1*
WOX	*WUSCHEL-RELATED HOMEOBOX*
WUS	*WUSCHEL*
XYP1	*XYLOGEN PROTEIN 1*
YUC	*YUCCA*

Abstract

Adventitious root formation is a key developmental process for plant survival. In many plant species, they arise after some stress events, usually abiotic stress such as floods or droughts and, especially mechanical stress or injury, to restore feeding and/or structural support functions. Furthermore, many commercially important plant species are propagated by cuttings, so research aimed at understanding the physiological and molecular mechanisms involved in the adventitious rooting process is of vital importance.

In this thesis we intend to study the genetic, morphological, and hormonal process of adventitious root regeneration using two different experimental systems in the model organism *Arabidopsis thaliana*: (1) whole leaf explants excised from the mother plant by manual dissection and (2) hypocotyl explants after excision of the main root system. Both experimental systems have been used to characterise the origin and kinetics of adventitious root development.

In the whole-leaf system, adventitious root formation occurs in a localized region at the base of the petiole in response to leaf excision and without the exogenous addition of hormones. Using marker lines, we have spatially and temporally characterised the main molecular events that occur during adventitious root development. The use of additional mutant lines has allowed us to characterize the action of certain hormonal pathways or the involvement of some key regulators in the process of adventitious organogenesis, such as IAA18.

The hypocotyl system has been used primarily to identify novel regulators of adventitious root formation by screening 112 homozygous T-DNA mutants with altered leaf phenotypes, which were selected based on their dynamic gene expression profiles in tissue regeneration experiments published elsewhere. This screen has produced forty-seven T-DNA

homozygous lines showing low rooting ability (*less adventitious roots*; *lars*) and eight lines with increased rooting ability relative to their wild-type genetic background (*more adventitious roots*; *mars*). A large percentage of the genes identified encode ribosomal proteins, although genes related to gibberellin biosynthesis and signalling, auxin homeostasis and xylem differentiation also stand out. Almost all mutants studied in the hypocotyl system showed similar rooting responses in whole-leaf explants, suggesting that their affected genes are involved in shared regulatory pathways.

Finally, and as an example of the complexity of the regulation of regenerative processes, a bibliographic review is included that collects the most current information on the mechanisms and key actors in plant regeneration processes, ranging from total organogenesis to tissue repair, including also somatic embryogenesis.

Resumen

La formación de raíces adventicias es un proceso del desarrollo esencial para la supervivencia de las plantas en la naturaleza. En muchas especies vegetales, las raíces adventicias surgen tras algunos eventos de estrés, generalmente de estrés abiótico, como inundaciones o sequías, y especialmente estrés mecánico o heridas, en un intento por restaurar las funciones de alimentación y/o soporte estructural. Además, un gran número de especies vegetales de interés comercial se propagan mediante esquejes, por lo que las investigaciones destinadas a entender los mecanismos fisiológicos y moleculares implicados en el proceso de enraizamiento adventicio son de vital importancia.

En esta Tesis se propone el estudio a nivel genético, morfológico y hormonal del proceso de regeneración de raíces adventicias empleando para ello dos sistemas experimentales diferentes en el organismo modelo *Arabidopsis thaliana*: (1) explantos de hoja completa escindidos de la planta madre mediante disección manual y (2) explantos de hipocótilos mediante la escisión de la raíz principal. Ambos sistemas experimentales han sido utilizados para caracterizar el origen y la cinética del desarrollo de las raíces adventicias.

En el sistema de hoja completa, el desarrollo de raíces adventicias se origina en una zona delimitada en la base del peciolo como respuesta al estímulo de escisión foliar y en ausencia de hormonas exógenas. A través del empleo de líneas marcadoras, ha sido posible la caracterización espacial y temporal de los eventos moleculares más relevantes que juegan un papel fundamental durante el proceso de formación de las raíces adventicias. El uso de líneas mutantes adicionales nos ha permitido caracterizar la acción de determinadas rutas hormonales o la participación de algunos reguladores clave en el proceso de organogénesis adventicia, como *IAA18*.

El sistema de hipocótilo ha sido principalmente utilizado para identificar

nuevos reguladores de la formación de raíces adventicias, mediante el análisis de 112 mutantes homocigotos de ADN-T con fenotipo foliar, que fueron seleccionados en función de los perfiles de expresión dinámica de sus genes en experimentos de regeneración tisular publicados por otros autores. En este cribado se han identificado cuarenta y siete líneas homocigotas de ADN-T que mostraban una baja capacidad de enraizamiento (*less adventitious roots*; *lars*) y ocho líneas con mayor capacidad de enraizamiento (*more adventitious roots*; *mars*) que su fondo genético silvestre. Un gran porcentaje de los genes identificados codifican proteínas ribosómicas, aunque también destacan genes relacionados con la biosíntesis y señalización de giberelinas, la homeostasis de auxinas y la diferenciación del xilema. Casi todos los mutantes estudiados en el sistema de hipocótilo mostraron respuestas de enraizamiento similares en explantos de hoja completa, lo que sugiere que sus genes afectados participan en vías reguladoras compartidas.

Finalmente, y como muestra de la complejidad de la regulación de los procesos regenerativos, se incluye una revisión bibliográfica que recoge la información más actual en cuanto a los mecanismos y jugadores clave en los eventos de regeneración vegetal, que comprenden desde la organogénesis total a la reparación de tejidos, pasando, también, por la embriogénesis somática.

1. INTRODUCCIÓN GENERAL

1.1 *Arabidopsis thaliana* como sistema modelo

El término "organismo modelo" se empezó a utilizar de forma general en la comunidad científica en la década de los 90 del siglo pasado, con el avance del Proyecto Genoma Humano [1]. Los organismos modelo empleados en el estudio de la biología vegetal son especies poco complejas y fáciles de analizar, que nos permiten obtener conocimiento y aplicarlo o extrapolarlo a otras especies más complejas y, por lo general, de mayor interés para el ser humano [1]. Para el estudio de la biología vegetal de dicotiledóneas, *Arabidopsis thaliana*, en adelante Arabidopsis, ha sido históricamente la especie vegetal más utilizada por los centros de investigación de todo el mundo [2]. Fruto de este uso tan amplio, la cantidad de bibliografía publicada en Pubmed asciende hasta los 38 459 resultados si buscamos artículos que contengan la palabra "Arabidopsis" en su título (julio de 2022). El ciclo vital de Arabidopsis es de seis semanas, un periodo muy corto si lo comparamos con otras especies vegetales [3], lo que permite obtener varias generaciones de forma rápida (**Figura 1**). Además, tiene un tamaño reducido que permite cultivarla fácilmente en interiores con iluminación artificial, así como establecerla en cultivo *in vitro* con densidades altas [3]. Por otra parte, el pequeño tamaño de su genoma (~132 Mpb), junto con la disponibilidad temprana (año 2000) de la secuencia completa y anotada del mismo, hizo que fuera la especie preferente para la investigación en Genética [2]. No obstante, los primeros mapas genéticos datan de la década de los 80 del siglo pasado [4]. Además, la capacidad de la planta para someterse a la autopolinización y tolerar un alto grado de homocigosidad, así como su fácil transformación con ADN exógeno, la hacen especialmente adecuada para los estudios genéticos [2].

El amplio uso de Arabidopsis como sistema modelo ha permitido la generación de una gran cantidad de herramientas genéticas, entre las que destacan las colecciones indexadas de mutantes; (1) puntuales de

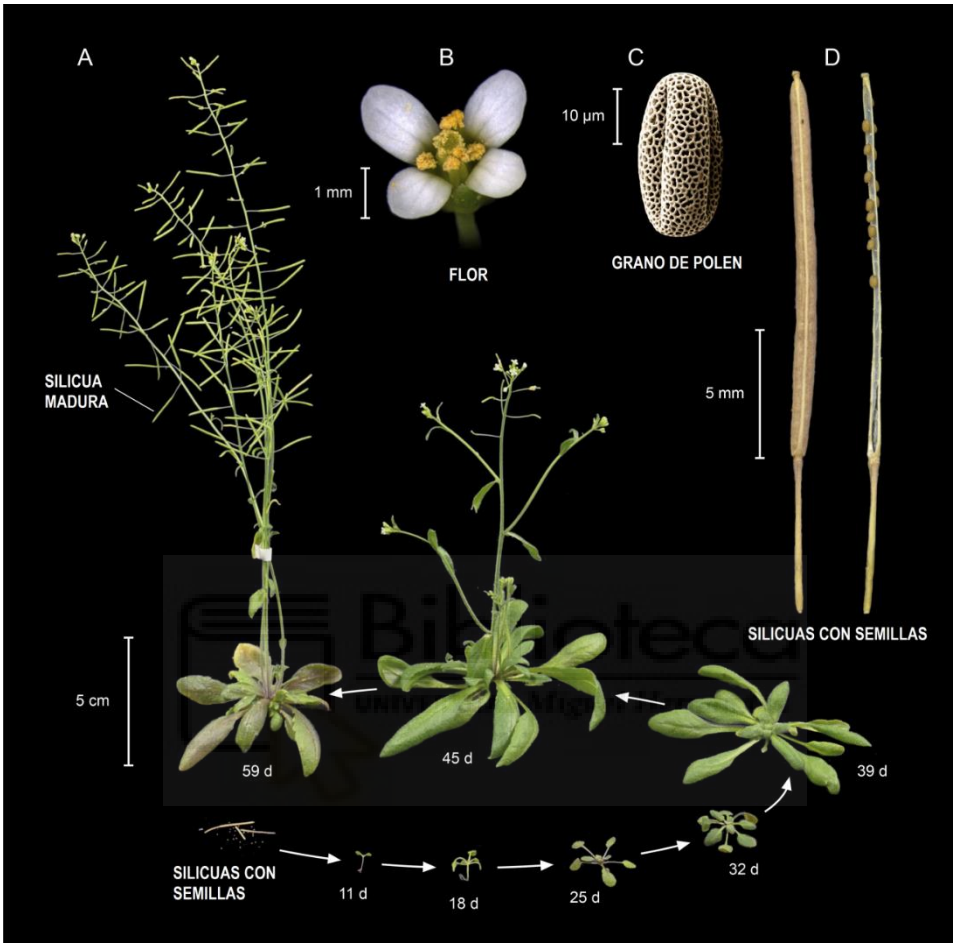

Figura 1.- Visión general del ciclo vital y la morfología de *Arabidopsis thaliana*. Imagen obtenida de [5], con ligeras modificaciones.

metanosulfonato de etilo (MSE), (2) insercionales de ADN de transferencia (ADN-T) y transposones [6] y, más recientemente, (3) puntuales mediante CRISPR/Cas9 [7]. Por otra parte, también se han desarrollado numerosas herramientas en línea con el objetivo de albergar, ordenar y facilitar datos de esta especie para toda la comunidad científica, como *The Arabidopsis Information Resource* (TAIR) [8].

Existen referencias bibliográficas [9] que confirman que muchos de los

resultados obtenidos en Arabidopsis se han mantenido válidos en plantas cultivables.

1.2 Estudio de la regeneración en Arabidopsis

Las plantas se distinguen por su capacidad para regenerar tipos celulares, tejidos y órganos específicos (formación de órganos *de novo*) e incluso organismos completos [10]. Los eventos de regeneración requieren la intervención de redes génicas que regulan procesos complejos de muerte celular programada, desdiferenciación y proliferación celular, además del establecimiento de nuevos patrones tisulares que permitan, en última instancia, la formación del nuevo órgano o la recuperación de los fragmentos perdidos [10].

Los estudios realizados en Arabidopsis proponen la existencia de dos mecanismos diferenciados que pueden iniciar eventos de regeneración tisular. Por una parte, se ha descrito la existencia y activación de células que permanecen en estado desdiferenciado y que se encontrarían presentes en los tejidos vegetales adultos [11]. En contraposición, también se han descrito eventos de regeneración en los que la desdiferenciación y reprogramación de células somáticas ya diferenciadas es crucial [12, 13]. En cuanto a la formación de órganos *de novo*, esta puede ocurrir a través de dos vías diferentes llamadas regeneración directa y regeneración indirecta [11]. Durante la regeneración indirecta, los explantos producen el nuevo órgano a través de una etapa intermedia llamada callo, mientras que en la regeneración directa los explantos pueden evadir la formación del callo y producir el órgano adventicio directamente [11].

La formación de órganos adventicios es un proceso de interés agronómico ya que permite la propagación vegetativa de especies de interés comercial. Así, existe una gran cantidad de estudios en Arabidopsis, encaminados a identificar y caracterizar reguladores de este proceso, con el objetivo de trasladar estos hallazgos a especies de interés comercial.

1.2.1 Herida y daño tisular

Las plantas están constantemente sujetas a daños mecánicos por factores bióticos como herbívoros, insectos y nematodos, así como factores abióticos como vientos fuertes o lluvias torrenciales. Las lesiones de los órganos aéreos incluyen la interrupción vascular debido, principalmente, a la rotura de tallos mientras que las lesiones en la raíz suelen producirse por nematodos que habitan en el suelo. En las últimas, las lesiones van desde el daño en células individuales hasta la pérdida del ápice radicular completo.

En Arabidopsis, la regeneración de tejidos vasculares se ha observado tanto en el tallo como en las hojas [14]. La regeneración vascular en los órganos aéreos de Arabidopsis requiere la actividad de los genes *PLETHORA 3, 5* y *7 (PLT3*/*5*/*7*) y *AINTEGUMENTA* [14]. En este modelo, PLT3, 5 y 7 se unen directamente al promotor del gen *CUP-SHAPED COTYLEDON 2* (*CUC2*), en respuesta a la lesión, aumentando sus niveles de expresión. PLT3/5/7 y CUC2, de forma conjunta, potencian la biosíntesis local de auxina, esencial para guiar los tejidos vasculares recién formados hacia los extremos fracturados [14].

En cuanto a los órganos subterráneos, los experimentos llevados a cabo en Arabidopsis muestran que la escisión del ápice radicular provoca la especificación de las identidades celulares perdidas y el restablecimiento de un nicho de células madre funcional que permite la regeneración completa del órgano, así como su crecimiento posterior [12]. Se ha demostrado que estas células madre se originan *de novo* a partir de múltiples tipos celulares cercanos a la herida, en un proceso que requiere la activación del factor de transcripción MONOPTEROS, normalmente asociado a la formación de la raíz principal durante la embriogénesis [15].

1.2.2 Embriogénesis somática

La embriogénesis somática se define como la formación de embriones a partir de diferentes tipos de células y explantos sin la fusión previa de gametos [16]. Los avances obtenidos en embriogénesis somática en

Arabidopsis han permitido la caracterización de algunos genes implicados en los mecanismos moleculares que la regulan. Se ha descrito la participación de algunos factores de transcripción clave, que se inducen durante la embriogénesis somática: *BABY BOOM*, *PLT1*, *PLT2*, *AGAMOUS-LIKE 15*, *FUSCA 3*, *LEAFY COTYLEDON 1* y *2*, *RWP-RK DOMAIN-CONTAINING 4*, *ABA INSENSITIVE 3* y *WUSCHEL* [17]. La expresión ectópica de algunos de estos genes puede reprogramar directamente las células somáticas e inducir la embriogénesis somática en ausencia de estímulos exógenos [18].

Varias referencias bibliográficas apoyan la hipótesis de que las auxinas desempeñan una función crítica en la reprogramación de las células somáticas durante la embriogénesis somática [19]. En muchos protocolos de inducción de embriogénesis somática, el tratamiento con auxinas exógenas da como resultado la reprogramación celular, mientras que, para permitir el avance del proceso regenerativo, es necesaria la posterior eliminación de las auxinas del medio de cultivo [20]. Además, durante la embriogénesis somática se ha observado la activación de la biosíntesis de auxinas mediada por los genes *YUCCA* (*YUC*), *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* y *TRYPTOPHAN AMINOTRANSFERASE-RELATED 2*, el transporte polar de esta, mediado por PIN-FORMED 1 (PIN1), así como la participación de reguladores de las familias AUXIN/INDOLE-3-ACETIC ACID (*Aux/IAA*) y *AUXIN RESPONSE FACTOR* (*ARF*) [21].

1.2.3 Formación de órganos adventicios

La formación de órganos adventicios engloba tanto la formación de tallos adventicios como la de raíces adventicias. Se ha descrito que la formación de un órgano u otro viene condicionada por los niveles endógenos de auxinas y citoquininas en la región regenerativa del explanto [11, 22].

La formación de tallos adventicios puede llevarse a cabo a través de procesos de regeneración directa e indirecta. Durante la regeneración indirecta de tallos adventicios, los explantos vegetales (raíces o hipocótilos)

se incuban en medios de cultivo suplementados con cantidades equimolares de auxina y citoquinina, produciendo un callo pluripotente, generalmente a partir de células del pericíclo adyacentes al xilema [23]. La naturaleza molecular de este callo se ha estudiado ampliamente para revelar los reguladores que controlan su formación y la adquisición de pluripotencia.

Una vez generado el callo, la regeneración de los tallos adventicios requiere la activación de los *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) de tipo B y la eliminación de las marcas de represión epigenética de los reguladores de las células madre iniciadoras [24, 25]. Durante el proceso de regeneración, se forman una suerte de brotes iniciales, llamados progenitores, antes de la formación y estabilización del meristemo apical del tallo que darán continuidad al crecimiento del nuevo órgano [26]. Aunque todas las células del callo son teóricamente pluripotentes, los progenitores de brotes no se inician a partir de todas las células, sino que surgen estocásticamente de unas pocas células del callo [26].

Se ha descrito que este proceso regenerativo está controlado por un mecanismo molecular de dos pasos que comprende *PLT3*, *PLT5* y *PLT7*. Estos genes controlan el posicionamiento de los órganos laterales durante el desarrollo normal de raíces y tallos [27, 28] y, durante el primer paso de dicho mecanismo regenerativo, activan los reguladores de las células madre de la raíz, haciendo que el callo adquiera la competencia necesaria para formar los progenitores de tallos adventicios. En el segundo paso del mecanismo propuesto, *PLT3* y *PLT7* activan la expresión de *CUC2* y otros reguladores para lograr la estabilización del meristemo y, finalmente, la regeneración del tallo adventicio [29]. Este mecanismo de dos pasos actúa en todos los explantos, independientemente de su origen tisular [29].

La formación de tallos adventicios también se puede lograr directamente sin la formación de un callo. Se ha descrito que la incubación de primordios de raíces laterales con concentraciones altas de citoquininas en el medio de cultivo puede inducir un evento de transdiferenciación en el

que el primordio de raíz lateral se reprograma para generar directamente un tallo adventicio [29, 30]. La regeneración directa de tallos adventicios también parece estar regulada por la tríada *PLT3*/*5*/*7*, ya que el mutante *plt3,5,7*, no experimenta la transdiferenciación, ni siquiera en presencia de abundante citoquinina exógena [29].

En contraste con los procesos de regeneración de tallo, en los que es necesario un aporte exógeno de hormonas, la formación de raíces adventicias es un proceso regenerativo que se da de manera natural en plantas (**Figura 2**) [31].

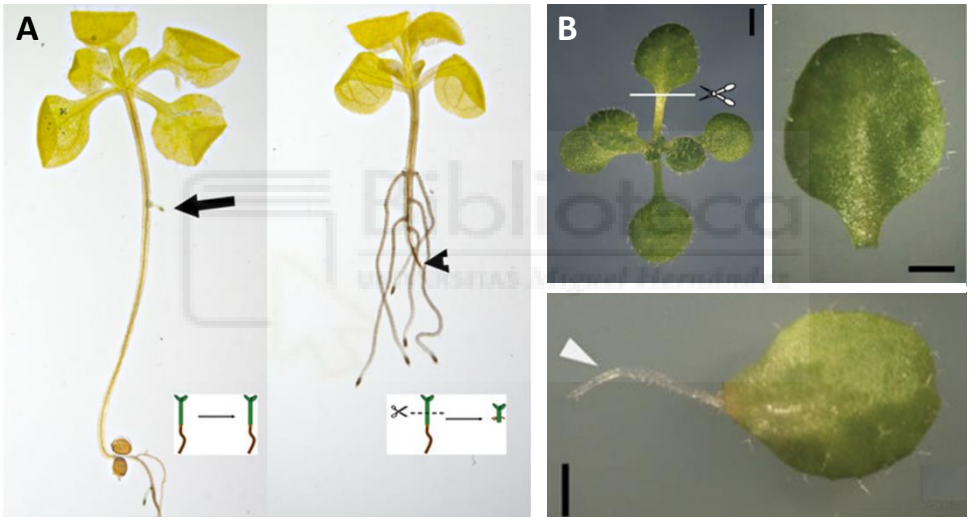

Figura 2.- Formación de raíces adventicias en distintos tejidos de Arabidopsis sin aporte exógeno de hormonas vegetales. Formación de raíces adventicias en (A) hipocótilos y (B) explantos foliares (Barra de escala: 1 mm). Imágenes obtenidas de [32, 33], con ligeras modificaciones.

Raíces adventicias son todas aquellas que se originan a partir de tejidos no radiculares como tallos u hojas [34]. Respecto a la regulación de la formación *de novo* de raíces, numerosos estudios han demostrado que las auxinas presentan un rol protagonista en este proceso [31, 35]. Una elevada concentración endógena de estas hormonas se vincula con un mayor éxito

del enraizamiento adventicio de los explantos [36, 37].

La formación de raíces adventicias puede darse a partir de distintos órganos de la planta [31]. En explantos foliares de Arabidopsis sin peciolo, la escisión del explanto induce la producción de ácido jasmónico que, indirectamente, aumenta la biosíntesis de auxinas en toda la hoja [38]. Posteriormente, las auxinas se transportan activamente a la región próxima a la herida, donde promueven la especificación de las células fundadoras de la raíz a través, presumiblemente, de la expresión de *WUSCHEL-RELATED HOMEOBOX 11* (*WOX11*) [39]. A su vez, *WOX11* y su homólogo *WOX12*, junto con otros factores de respuesta a auxinas, inducen la expresión de *WOX5* y *LATERAL ORGAN BOUNDARIES DOMAIN 16* (*LBD16*), que desencadenan finalmente el desarrollo de los primordios radiculares [40].

1.3 Raíces adventicias. Regulación hormonal y redes génicas reguladoras

La formación *de novo* de raíces a partir de esquejes es clave en el éxito de los sistemas de propagación vegetativa de las plantas. Se reconocen dos pasos principales en la formación de raíces adventicias. La primera se conoce como fase de inducción, donde las auxinas parecen jugar un papel crucial para la reprogramación de células competentes que lleven a cabo el desarrollo posterior de la raíz [41]. Seguidamente, la segunda etapa, llamada fase de formación, comprende la división celular, la diferenciación y formación del meristemo y su posterior elongación y emergencia [41, 42]. La activación de las células competentes se suele dar por las condiciones ambientales a las que se exponen los explantos. Los tratamientos con auxina exógena, las heridas, eventos de sequía, inundaciones e incluso la exposición a la luz activan una red compleja de regulación que inicia el proceso de formación de raíces *de novo* [43].

Los factores moleculares más importantes que determinan la señalización de auxinas durante la formación de raíces adventicias son los genes *Aux*/*IAA* y *ARF*. Se ha descrito que los genes *ARF6* y *ARF8* actúan como

reguladores positivos de la formación de raíces adventicias en hipocótilos de Arabidopsis, mientras que *ARF17* actuaría como un regulador negativo de este proceso [44]. Aguas abajo de estos, actuarían otros factores de transcripción de la familia GRAS como *SHORTROOT* (*SHR*), *SCARECROW*, y miembros de las familias *WOX*, *LBD* y *APETALA2/ETHYLEN RESPONSIVE FACTOR* (*AP2/ERF*), que intervendrían en el establecimiento de las células fundadoras de raíces, la formación del nuevo meristemo radicular, el mantenimiento del centro quiescente, así como la diferenciación en los distintos tipos celulares del meristemo [43, 45]. También se ha confirmado la participación de factores de transcripción de las familias ZINC-FINGER, WRKY, NAC y bZIP. Algunos ejemplos concretos de ellos podrían ser *LATERAL ROOT PRIMORDIA1* [46], WRKY75 [47], NAC1 [48] o bZIP53 [49], respectivamente. Estos genes estarían involucrados tanto en la formación del primordio radicular como en su emergencia [45].

La emergencia del primordio radicular a través de las células del córtex y la epidermis requiere la degradación y reconstrucción de la pared celular [50]. Se ha descrito que las proteínas EXTENSIN (EXT), usualmente vinculadas a la cicatrización de heridas, podrían limitar la emergencia del ápice radicular durante la formación *de novo* de raíces [48]. Por ejemplo, se ha demostrado que la generación de heridas induce la expresión de *EXT* durante el enraizamiento adventicio en esquejes de tallo de *Vitis vinifera* [51]. En explantos foliares sin peciolo de Arabidopsis, la herida induce la expresión de *NAC1*, que induce, a su vez, la expresión del gen *Cys-ENDOPEPTIDASE* (*CEP*). La elevada actividad de CEP promueve la degradación de EXT, facilitando, por tanto, la emergencia del primordio radicular a través del mecanismo molecular de cicatrización de heridas [48]. Se ha demostrado la participación de otros genes en la remodelación de la pared celular durante la emergencia del primordio. Por ejemplo, *HYDROXYPROLINE-RICH GLUCOPROTEIN* (*HRGP*), un gen de la familia de proteínas asociadas a la pared celular se activa específicamente durante la iniciación de la raíz adventicia en esquejes de tabaco [52]. Otro gen, *ROOT*

HAIR DEFECTIVE 3, interviene en la biosíntesis de la pared celular y la organización de los filamentos de actina, los cuales son esenciales para la expansión celular durante la formación *de novo* de raíces en Arabidopsis y *Populus* spp. [53, 54].

En cuanto a la acción que ejercen otras hormonas distintas de la auxina, la bibliografía otorga a las citoquininas un papel represor en la diferenciación de primordios radiculares y la formación de raíces adventicias [55, 56]. Históricamente, las citoquininas han tenido un papel antagonista al de las auxinas, presumiblemente a través de la inducción directa de algunos genes *AuX/IAA*, que inhibirían a su vez la expresión de los canales PIN, dificultando el mantenimiento de altos niveles de auxinas en una región determinada [57, 58]. En Arabidopsis se ha demostrado que las citoquininas inhiben la expresión de *PIN1* y *LIKE AUX1 3*, bloqueando parcialmente el flujo de auxinas. Las citoquininas también limitan la expresión de *WOX5* y *YUC6* en la región distal del ápice radicular [59]. Se ha descrito que mutantes de Arabidopsis en genes *ARR* de tipo B, componentes fundamentales en la vía de señalización de las citoquininas, se caracterizan por la insensibilidad a las citoquininas y por la formación ectópica de raíces adventicias en sus hipocótilos [60]. En esquejes de *Populus tremula*, *PtRR13* regula transcripcionalmente la señalización de citoquininas aguas abajo para reprimir el enraizamiento adventicio [56].

Durante la formación de raíces adventicias también se ha descrito la interacción entre las giberelinas y las auxinas. Tanto en álamo híbrido como en Arabidopsis, la sobreexpresión de los genes *AtGA20ox1*, *PttGID1.1* o *PttGID1.3*, involucrados en la biosíntesis y señalización de giberelinas, respectivamente, muestran defectos en la formación de raíces adventicias, debido, presumiblemente, a la interacción negativa de las giberelinas sobre el transporte polar de auxinas [61]. Un estudio reciente mostró que la sobreexpresión del gen de *HDT902* de *Populus trichocarpa* (*PtHDT902*), perteneciente a la familia *HISTONE DEACETYLASE 2*, provocaba la expresión ectópica de genes biosintéticos de giberelinas e inhibió la formación de

raíces adventicias [62]. Estos resultados ponen de manifiesto la estrecha relación existente entre la epigenética y la regulación hormonal, así como la interacción entre las vías de percepción y señalización de estas.

1.4 Objetivos de mi Tesis Doctoral

La formación de raíces adventicias es un proceso de gran importancia para la propagación vegetativa de numerosas especies vegetales de interés comercial. Las mejoras en el enraizamiento adventicio se verían limitadas sin una base en investigación que las sustente y ponga sobre la mesa nuevas herramientas y nuevos procedimientos para la generación de estas últimas. En esta línea, el presente trabajo pretende contribuir al conocimiento actual de los mecanismos moleculares implicados en la formación de raíces adventicias desde una perspectiva básica. La descripción detallada de este proceso organogénico y la identificación de nuevos reguladores constituye la base para los procedimientos futuros de mejora vegetal.

En este contexto, se enmarcan los trabajos científicos presentados en esta memoria y que constituyen la presente Tesis Doctoral, encaminada a:

- Establecer un sistema de análisis del enraizamiento adventicio en órganos aéreos de *Arabidopsis thaliana*, llevando a cabo un enfoque doble en el que se analicen tanto explantos de hoja completa como de hipocótilo.
- Caracterizar los cambios morfológicos y los factores hormonales que intervienen en la regulación del enraizamiento adventicio de los explantos de hoja completa e hipocótilo.
- Identificar nuevos reguladores o la participación de otros ya descritos en el proceso de organogénesis adventicia, empleando para ello un abordaje basado en la Genética inversa.
- Establecer un protocolo de selección de genes candidatos basado en datos de expresión génica, previo al cribado fenotípico, aumentando

así las probabilidades de obtener fenotipos interesantes durante el cribado.

- Determinar si dichos reguladores conservan su función durante la formación de raíces adventicias en explantos de hojas completas o hipocótilo.

2. RESUMEN GLOBAL DE MATERIALES Y MÉTODOS

2.1 Regulación del control hormonal, reprogramación celular y patrones tisulares durante la organogénesis radicular *de novo*

Para el estudio de los mecanismos moleculares implicados en la regeneración de raíces adventicias a partir de explantos foliares de Arabidopsis se ha llevado a cabo el análisis de varias líneas marcadoras, así como de estirpes mutantes y sobreexpresoras de algunos de los genes analizados con el objetivo de validar su implicación en el proceso regenerativo.

Hemos utilizado las líneas marcadoras *ProIPT3:GUS*, *ProIPT5:GUS* [63], *ProLOG4:GUS* [64], obtenidas del RIKEN, y *ProARR5:GUS* [65] para caracterizar la biosíntesis y señalización de citoquininas durante el enraizamiento adventicio. Las líneas *ProYUC8:GUS*, *ProYUC9:GUS* [66] y *ProDR5:GUS* [67] se utilizaron para analizar la biosíntesis y señalización de auxinas, mientras que *ProPIN3:PIN3:GFP* [68] y *ProPIN4:GUS* [69] se utilizaron como marcadores del transporte de éstas. Para rastrear los mecanismos moleculares durante la formación de raíces *de novo*, usamos: *ProWIND1:GU*S [70], *ProWOX11:GUS* [39], *ProWOX5:GUS* [71] y *ProSKP2Bs:GUS* [72]. Las líneas J0121 y J0661 [73] se utilizaron para localizar células similares a las células del periciclo en los explantos foliares. La participación de ciertos elementos de la señalización de auxinas, citoquininas y otros reguladores relacionados con el control del ciclo celular se confirmó mediante el análisis del fenotipo organogénico de las siguientes líneas mutantes: *ahp1 ahp2 ahp3* [74], *ahp2 ahp4 ahp5*, *arr1 arr10 arr12* [75], *aux1-22* [76], *axr2-1* [77], *slr-1* [78], *crane-2* [79], *sur2-1* [80] e *iaa28-1* [81], que se obtuvieron del NASC; y *pin1*, *pin2 pin3*, *pin2 pin3 pin7* [82], *fwr* [83], *cyclinb1;1* (*cycb1;1*) y *cycb1;2* [84], *CDKB1;1* DN161 y *Pro35S:KRP2* [85, 86], cedidas por varios investigadores.

2.1.1 Cultivo *in vitro* y tratamientos químicos y hormonales

Las semillas de las líneas expuestas anteriormente se esterilizaron y establecieron en cultivo *in vitro* tal y como se explica en el apartado de materiales y métodos del segundo artículo, en la página 96. Doce días después de la germinación, se cortó el primer par de hojas en el punto de unión del peciolo con el tallo. Los explantos foliares se transfirieron a medio de cultivo con la composición especificada en el apartado de materiales y métodos del segundo artículo en la página 96. Tras la escisión, los explantos de hoja completa se cultivaron en oscuridad a 22 °C, de forma rutinaria durante 10 días, o durante el número de días indicado en el experimento correspondiente. Para cada línea se sembraron 48 semillas por placa Petri y se realizaron tres réplicas técnicas (144 muestras por línea). La estirpe Columbia-0 (Col-0) se incluyó en todas las siembras como estirpe de referencia.

Para los tratamientos hormonales exógenos, se utilizaron stocks de ácido indol-3-acético (AIA), 6-bencilaminopurina (6-BAP) o tidiazurón (TDZ), previamente esterilizados por filtración, que fueron añadidos al medio de cultivo antes de verterlo en las placas de Petri. Las concentraciones de uso de estas hormonas se detallan en el apartado de materiales y métodos del segundo artículo, en la página 96. La inhibición química del transporte polar de auxinas se llevó a cabo mediante la aplicación local de un anillo de lanolina con ácido 1-naftalenacético (ANF) en la unión del peciolo con el limbo de los explantos foliares. El ANF se disolvió en lanolina estéril a una concentración final del 1% m/m. Para los ensayos relacionados con el bloqueo del ciclo celular, los explantos de hoja completa se incubaron en medio de cultivo suplementado con hidroxiurea. Las concentraciones utilizadas se especifican en el apartado de materiales y métodos del segundo artículo en la página 97.

2.1.2 Parámetros medidos y análisis estadístico

Para realizar los análisis fenotípicos de las líneas estudiadas se llevó a cabo la caracterización de varios parámetros descriptores. En primer lugar, el porcentaje de regeneración se estipuló como el número de explantos foliares que mostraron alguna o varias de las siguientes características: (1) engrosamiento de los peciolos, (2) proliferación de células vasculares y asociadas o (3) crecimiento de raíces en la región proximal del pecíolo. Estas características se contabilizaron a los 7 días después de la escisión (dde) o en el punto temporal indicado en el experimento correspondiente. El porcentaje de enraizamiento se describió como el número de explantos foliares que mostraban raíces a los 10 dde, clasificados en categorías concretas en función del número de raíces de cada explanto. Esto nos permitió generar perfiles de enraizamiento que podían ser comparados entre sí mediante las pruebas estadísticas correspondientes.

Los valores relativos al porcentaje de enraizamiento, porcentaje de regeneración y área de los tejidos vasculares o de callo, se analizaron estadísticamente mediante un modelo lineal general univariado (*Generalized Linear Model*; GLM) y un análisis de varianza ANOVA con una prueba posthoc de diferencia mínima significativa (*Least Significant Difference*; LSD). Para el análisis de la capacidad de enraizamiento se realizó la prueba del χ^2, con el objetivo de comprobar si había diferencias en la frecuencia de distribución de fenotipos radiculares entre líneas, analizadas dos a dos. Las herramientas informáticas utilizadas para realizar dichos análisis estadísticos se especifican en el apartado de materiales y métodos del segundo artículo en la página 97.

2.1.3 Tinciones y microscopía

Con el objetivo de caracterizar los procesos de muerte celular utilizamos una tinción con lactofenol y azul de tripán que nos permitió identificar fácilmente las células muertas en los explantos foliares tras una incubación con dicha solución en las condiciones indicadas en el apartado

de materiales y métodos del segundo artículo en la página 97. Para el análisis de las líneas marcadoras basadas en el gen de la β-glucuronidasa se utilizó la tinción GUS. Las condiciones de incubación, fijación y lavado previo a la visualización microscópica se pueden consultar en el apartado de materiales y métodos del segundo artículo en la página 97. Las imágenes de estas líneas marcadoras se tomaron en un microscopio de campo claro equipado con un sistema de microfotografía. El área de los tejidos en proliferación de las líneas ensayadas se dibujó manualmente a partir de imágenes microscópicas con una tableta gráfica y las áreas y los diámetros de sus elipses se midieron con el software ImageJ [87].

Las líneas marcadoras fluorescentes también sufrieron un proceso de fijación y decoloración tal y como se detalla en el apartado de materiales y métodos del segundo artículo en la página 97, previo a su análisis microscópico. La obtención de imágenes de estas líneas se llevó a cabo mediante microscopía confocal de fluorescencia. Los parámetros relativos a las longitudes de onda de excitación y emisión de las proteínas fluorescentes marcadoras se especifican en el apartado de materiales y métodos del segundo artículo en la página 97. Para excluir la autofluorescencia se ajustaron los valores de intensidad de excitación y de recolección de la señal fluorescente de tal forma que los explantos foliares de la estirpe silvestre Col-0 no emitieran señal lumínica, especialmente en los haces vasculares, al inicio de cada análisis tal y como se describe en [88].

2.2 Un enfoque genético guiado por redes de expresión para identificar nuevos reguladores de la formación de raíces adventicias en Arabidopsis

Para la identificación de nuevos mecanismos moleculares implicados en la regeneración de raíces adventicias a partir de explantos de hipocótilo se ha llevado a cabo el análisis de numerosas líneas mutantes de Arabidopsis, que fueron sometidas al proceso organogénico (la inducción del proceso organogénico se tratará con más detalle en el apartado 2.2.2).

2.2.1 Obtención de datos de expresión y selección de candidatos

Para la elección de las líneas de ADN-T se llevó a cabo una representación gráfica de los niveles de expresión de los genes interrumpidos durante diferentes procesos regenerativos recogidos en la bibliografía [12, 23, 89]. Los datos de expresión génica se obtuvieron del *Arabidopsis eFP Browser*, englobada en el *Bio-Analytic Resource for Plant Biology* (BAR) [90]. Se recogieron los datos de expresión génica de 339 genes indexados en la colección de mutantes de ADN-T *PhenoLeaf* [91] que estaban disponibles al comienzo de este proyecto (véase la tabla suplementaria S1 y la figura suplementaria S2 del tercer artículo, en la página 121).

En cada experimento relativizamos el valor de expresión de las diferentes condiciones ensayadas respecto a su fondo genético de referencia y reajustamos los valores mediante una transformación logarítmica en base 2. De esta forma, conseguimos tanto la visualización de valores atípicos como una visualización más homogénea y conveniente a nivel gráfico. Posteriormente, se calcularon las matrices con la distancia euclidiana entre genes (filas) para construir las diferentes agrupaciones (*clusters*) dentro del dendrograma. El conjunto de datos procesados de expresión génica, así como el dendrograma organizador, nos permitió generar un mapa de calor capaz de agrupar conjuntos de genes con perfiles de expresión similares durante los procesos organogénicos detallados en [12, 23, 89]. Para obtener dicha representación gráfica se utilizó el paquete *Pheatmap* de R [92].

2.2.2 Cultivo *in vitro* e inducción de la organogénesis adventicia en explantos de hipocotilo

El protocolo empleado para la inducción del proceso organogénico se detalla en el apartado de materiales y métodos del tercer artículo en las páginas 96 y 97. El número de raíces adventicias en cada hipocótilo se

registró diariamente durante 6 dde. Cada placa de Petri contenía plántulas de dos líneas diferentes y el fondo Col-0 (ocho plántulas por genotipo). El experimento se realizó por triplicado.

La estirpe silvestre Col-0 y las líneas homocigotas confirmadas de ADN-T y analizadas en estos experimentos se obtuvieron de la colección PhenoLeaf [91], facilitada por el Dr. José Luís Micol y pueden ser consultadas en la figura Suplementaria S2 del tercer artículo en la página 121.

2.2.3 Aislamiento de mutantes adicionales y análisis del enraizamiento adventicio en explantos de hoja completa

Las líneas insercionales empleadas para aislar mutantes adicionales de ADN-T de genes candidato se obtuvieron del NASC, el cual proporciona semillas y recursos de información para el Proyecto Internacional del Genoma de Arabidopsis y la comunidad de investigación en general, y se pueden consultar en el apartado de materiales y métodos del tercer artículo, en las páginas 96. Otras líneas adicionales utilizadas en el estudio y que fueron obtenidas de diferentes autores también se explicitan en el apartado de materiales y métodos del tercer artículo en la página 96. Las secuencias de los cebadores empleados en este trabajo pueden consultarse en la tabla 1 del tercer artículo en la página 97, así como el proceso de aislamiento de ADN genómico y el genotipado posterior. Para analizar la organogénesis de raíces *de novo* en hojas seguimos el protocolo descrito en [93], expuesto ya en el apartado 2.1.1 del presente trabajo.

2.2.4 Tratamientos químicos, tinciones y microscopía óptica de campo claro

Llevamos a cabo un estudio de la inhibición de la función ribosómica mediante el uso de antibióticos. En estos experimentos los explantos foliares se incubaron en un medio de cultivo suplementado con estreptomicina 30 mg/mL, que afecta específicamente a la subunidad pequeña de los

ribosomas cloroplásticos.

La tinción de β-glucuronidasa (GUS) se llevó a cabo siguiendo el protocolo ya descrito en el apartado 2.1.3 de la presente memoria. El área de los tejidos vasculares en proliferación se dibujó manualmente a partir de imágenes microscópicas utilizando una tableta gráfica y las áreas se midieron con el software ImageJ [87]. Las imágenes de rosetas, hipocótilos y hojas frescas se obtuvieron con un microscopio con zoom estereoscópico equipado con una cámara digital, mientras que las imágenes microscópicas se tomaron en un microscopio de campo claro equipado con un sistema de microfotografía, tal y como se describe en el apartado de material y métodos del tercer artículo en la página 107.

2.2.5 Análisis estadísticos

Los estadísticos descriptivos (promedio, desviación estándar, mediana, máximo y mínimo) se calcularon utilizando los programas StatGraphics Centurion XV (StatPoint Technologies, Estados Unidos) e IBM SPSS Statistics 21 [94]. El análisis de la bondad de ajuste entre la distribución de los datos y una distribución normal teórica se llevó a cabo mediante la prueba de Kolmogorov-Smirnov. Para comparar los datos de una variable dada realizamos la prueba de ANOVA o LSD de Fisher (*Least Significant Difference*). Las diferencias significativas fueron identificadas con un nivel de significación del 5% (valor de $p<0.05$), a menos que se indique lo contrario.

Los parámetros descritos en el modelo de explanto de hoja completa (capacidad de regeneración, capacidad de enraizamiento, etc.) se analizaron según lo expuesto en el apartado 2.1.2 del trabajo que se presenta.

3. DISCUSIÓN

3.1. Regulación de la formación de raíces *de novo* en explantos de hojas completas de Arabidopsis

Las plantas poseen capacidades regenerativas extraordinarias, entre ellas la capacidad de generar nuevos órganos a partir de tejidos postembrionarios, así como reconstituir órganos dañados tras sufrir una herida [95]. Curiosamente, la regeneración de meristemos radiculares conlleva la activación de vías de señalización embrionarias [15], mientras que la formación de órganos postembrionarios completos parece servirse de otras vías de señalización, como las implicadas en la formación de raíces laterales [96]. Muchas especies como el tomate (*Solanum lycopersicum* L.), la higuera (*Ficus carica* L.) o el chopo (*Populus* spp.) son capaces de generar nuevos órganos a partir de explantos (foliares, radiculares, etc.) sin necesidad de un suplemento exógeno de hormonas [43, 97]. La formación de raíces *de novo* requiere de la formación de los diferentes tejidos y tipos celulares del nuevo órgano. Todas las raíces tienen los mismos tejidos, aunque el número de capas y el número de células y tipos celulares en los mismos puede variar [98-100].

En esta Tesis se ha llevado cabo un análisis extensivo para investigar la regeneración radicular a partir de órganos aéreos de la planta. Las hojas completas escindidas de la planta madre son capaces de dar lugar a individuos completos en medios de cultivo sin aporte hormonal exógeno en una gran cantidad de especies vegetales [101]. Decidimos, por tanto, utilizar hojas completas de Arabidopsis en lugar de explantos foliares consistentes en limbos sin peciolos, ya que se ajusta más a lo observado en la naturaleza y además permite estudiar la formación de raíces adventicias en un solo conjunto de haces vasculares convirtiéndolo en un sistema más homogéneo. En este trabajo se han identificado cuatro estadios principales durante la formación de raíces adventicias: (1) la proliferación de algunos tejidos asociados al xilema, formando una masa de células sin estructura aparente

llamado callo endógeno; (2) la especificación de las células fundadoras de raíces dentro del callo endógeno; (3) la iniciación de los primordios radiculares y formación de patrones tisulares; y (4) la activación del meristemo radicular y la emergencia de la nueva raíz (véase la figura 8 del segundo artículo en la página 94). Además, se han identificado algunos de los factores reguladores, como *IAA28*, que intervienen en dichos estadios del desarrollo radicular adventicio.

3.1.1. Proliferación de algunas células vasculares y formación del callo endógeno

El engrosamiento y proliferación de algunas células vasculares en la zona próxima a la herida es el primer cambio morfológico que se puede apreciar durante la organogénesis *de novo*. A los 2 dde, las células adyacentes al xilema comenzaron a proliferar, formando capas estratificadas a partir de los 3 dde, que empujaron los conductos del xilema y desplazaron el colénquima. La proliferación de la vasculatura y la posterior formación de primordios provocaron que el peciolo proximal se engrosara formando lo que hemos llamado callo endógeno (véase la figura 1 y figura suplementaria 1 del segundo artículo en las páginas 83 y 100, respectivamente). El análisis de las líneas fluorescentes J0121 y J0661, marcadoras de las células del pericicloo, mostró una inducción de la expresión de la GREEN FLUORESCENT PROTEIN (GFP) en las células circundantes al xilema durante el engrosamiento del peciolo. Además, la línea J0661 también mostró expresión de la GFP en células del procámbium. Fueron las células marcadas en la línea J0661 las que mostraron altas tasas de proliferación, aunque ciertos grupos de células en división también mostraban fluorescencia en la línea J0121 (véase la figura 1 del segundo artículo en la página 83). Las células del pericicloo y otras estrechamente asociadas, en concreto las que expresan el marcador J0121, se han asociado a procesos regenerativos y morfogénicos como posible origen de células reprogramables [23, 102]. La competencia regenerativa asociada al

marcador J0121 en este proceso muestra paralelismos con la expresión de este marcador durante la formación de callos y raíces laterales. El seguimiento del linaje celular utilizando marcadores clonales y tecnologías de imagen *in vivo*, como la microscopía de haz de luz fluorescente, podrían ayudar a diseccionar la fuente exacta de células durante la regeneración de raíces *de novo* en hojas enteras. Podemos decir que la importancia de la proliferación celular en este proceso organogénico es obvia. Hemos demostrado que la inhibición química o genética de la proliferación vascular afecta negativamente a la organogénesis *de novo*. Además, el análisis de mutantes afectados en la progresión del ciclo celular, tanto en la transición G1/S como en la transición G2/M mostró fenotipos que diferían significativamente del observado en el fondo genético silvestre Col-0. El porcentaje de peciolos que muestran proliferación asociada a los haces vasculares se redujo significativamente en las líneas *Pro35S:KRP2* y *CDKB1;1DN161*. Además, la línea *Pro35S:KRP2* mostró un bloqueo total en la formación de raíces adventicias mientras que las líneas *cycb1;1* y *CDKB1;1DN161* mostraron una reducción significativa en el número de explantos foliares que regeneran raíces respecto al fondo silvestre Col-0, aunque mucho menos severa que *Pro35S:KRP2* (véanse las figuras 1 y 5 del segundo artículo en las páginas 83 y 90, respectivamente). Los ensayos con hidroxiurea produjeron un fenotipo intermedio entre los observados en las líneas *cycb1;1 y Pro35S:KRP2.* Además, también encontramos que las células vasculares en proliferación expresan *WOUND INDUCED DEDIFFERENTIATION 1* (*WIND1*) (véase la figura 5 del segundo artículo en la página 90), un regulador positivo de la formación de callos inducida por heridas [103], lo que refuerza, junto con el análisis de J0121, que estos tejidos en proliferación componen un callo y que este sería “endógeno” ya que no se surge en respuesta al aporte exógeno de hormonas vegetales. Se ha observado cierta analogía en este proceso respecto a otros procesos regenerativos, incluso en el reino animal, donde se ha descrito la proliferación inicial de células madre previa a la organogénesis con el

objetivo de generar una fuente suficiente de células que sean capaces de dar lugar al nuevo órgano o tejido. Según Lancaster y Knoblich [104], durante la regeneración tisular del sistema nervioso en mamíferos, las células madre neurales se expanden inicialmente mediante divisiones simétricas. Una vez se ha constituido un número suficiente de células, éstas llevan a cabo divisiones asimétricas para dar lugar a células madre autorenovadoras y otros tipos celulares más diferenciados como las neuronas.

3.1.2. Función de las auxinas y las citoquininas en la inducción del proceso organogénico

Durante la formación del callo endógeno también se producen cambios dinámicos en la señalización de auxinas y citoquininas a pesar de que éstas no procedan del medio de cultivo, sino de fuentes endógenas. Encontramos un incremento de la expresión de *ProIPT3:GUS* en los peciolos justo después de la escisión. Identificamos la expresión de *ProIPT5:GUS* en las células asociadas a los haces vasculares en la base del peciolo a los 2 dde, mientras que *ProLOG4:GUS*, que se expresó originalmente en los tejidos vasculares del explanto foliar a los 0 dde, aumentó significativamente su expresión en la base del peciolo 2 días después (véase la figura 2 del segundo artículo en la página 85). La señalización de las citoquininas, estimada según la expresión de GUS en la línea *ProARR5:GUS*, se restringió a un subconjunto de células asociadas a los haces vasculares cerca de la base del peciolo a los 2 dde y no mostró expresión el resto del proceso organogénico (véase la figura 2 del segundo artículo en la página 85). En contraste con las primeras nociones de que las citoquininas se producen solo en las raíces, recientemente se ha descrito que pueden ser sintetizadas en otras partes de la planta [105]. Nuestros resultados son consistentes con este hallazgo, dada la expresión de *ISOPENTENYL TRANSFERASE 3* (*IPT3*), *IPT5* y *LONELY GUY 4* (*LOG4*), que estarían mediando de forma local la biosíntesis de citoquininas en la base del peciolo para contribuir a la proliferación vascular durante la regeneración radicular. El estudio de mutantes afectados en la señalización

nos permitió confirmar el papel crucial de esta hormona en la formación de raíces adventicias a partir de explantos foliares (véase la figura 2 del segundo artículo en la página 85). Los fenotipos observados en estas líneas sugieren un papel regulador positivo de las citoquininas en la proliferación vascular que conduce a la formación del callo endógeno mientras que, en contraste, podrían actuar como un regulador negativo en cuanto a la capacidad de enraizamiento.

Las citoquininas son hormonas clave en la regulación del desarrollo vascular durante el crecimiento primario y secundario de la planta. Durante el crecimiento primario de las raíces de Arabidopsis, las citoquininas promueven la proliferación de células del procámbium y regulan el patrón vascular junto con las auxinas. Recientemente se ha descrito que las citoquininas activan directamente la expresión de dos genes *LBD*; *LBD3* y *LBD4* y que otros dos homólogos, *LBD1* y *LBD11*, se inducen únicamente tras un tratamiento prolongado con citoquininas. Estos resultados describen un mecanismo de dos etapas aguas abajo de la señalización de citoquininas: mientras que LBD3 y LBD4 regulan la activación del crecimiento secundario, LBD1, LBD3, LBD4 y LBD11 promueven conjuntamente un mayor crecimiento radial y el mantenimiento de las células madre del cámbium [106]. Por otra parte, es ampliamente conocido que los reguladores LBD16 y LBD29, inducidos por auxinas, intervienen en la formación de callos y raíces laterales, presumiblemente a través de la actuación del complejo ATXR2-ARF, el cual se une específicamente a sus promotores y activa su expresión mediante el depósito de marcas H3K36me3, lo que estimula la proliferación de células competentes del periciclo y, además, confiere características de primordio radicular en el callo [107]. Curiosamente, en nuestros experimentos pudimos observar que la adición de auxinas exógenas al medio de cultivo rescataba el porcentaje de regeneración reducido en las líneas mutantes de citoquininas, lo que sugiere una interacción muy estrecha y parcialmente redundante entre ambas hormonas durante la formación del callo endógeno. Aunque nuestros resultados son

preliminares, es tentador especular con la posible interacción entre ambas hormonas a través de estos módulos de señalización mediados por LBD.

En cuanto al papel regulador negativo que ejercen las citoquininas en la capacidad de enraizamiento de los explantos foliares, existe una gran cantidad de literatura científica alineada con ello. Una revisión reciente [108] expone resultados muy claros de otros autores en los que las citoquininas muestran un papel totalmente antagonista al de las auxinas en la formación de raíces adventicias: las citoquininas reprimen la diferenciación de primordios y la formación de raíces adventicias en hipocótilos de pepino y en esquejes de *Populus tremula* [55, 56]. Las citoquininas regulan negativamente la función de las auxinas mediante la inducción de la expresión de algunos genes *Aux/IAA* y la represión de la expresión de algunos transportadores PIN [57, 58]. En esquejes de *Populus tremula*, *PtRR13* regula transcripcionalmente la señalización de citoquininas para reprimir el enraizamiento adventicio [56]. Además, *PtRR13* promueve la expresión de *CONTINUOUS VASCULAR RING 1*, un regulador negativo de la proliferación vascular, y *PLEIOTROPIC DRUG RESISTANCE TRANSPORTER 9* (*PDR9*), que codifica un transportador de auxinas, lo que afecta aún más la formación de tejido vascular durante el enraizamiento adventicio. En este contexto, *PDR9* sería un ejemplo claro de componente clave para la interacción entre auxinas y citoquininas.

En cuanto a la biosíntesis y señalización de auxinas, encontramos expresión de *ProDR5:GUS* en células asociadas a los tejidos vasculares de la región proximal del peciolo 12 horas después de la escisión (hde), para aumentar rápidamente 1 dde, manteniéndose activa durante todo el proceso organogénico (véase la figura 3 del segundo artículo en la página 86). La eliminación de la fuente de auxinas mediante la escisión del limbo foliar o la adición de ANF en la transición limbo-peciolo produjo un descenso significativo en el porcentaje de regeneración y la capacidad de enraizamiento de los peciolos además de la pérdida de la expresión de *ProDR5:GUS* en la región proximal del peciolo (véase la figura 4 del segundo

artículo en la página 88). Confirmamos el papel indispensable que ejercen las auxinas en la formación de raíces adventicias a partir de explantos foliares mediante el análisis de mutantes afectados en la señalización de éstas. Nuestros resultados indican que los genes *IAA28* y *CRANE* podrían actuar como reguladores positivos de la formación de raíces adventicias (véase la figura 3 del segundo artículo en la página 86). Recientemente, se ha descrito que el mutante *crane-2* es incapaz de generar callos a partir de protoplastos. Esto es debido, presumiblemente, a su papel mediador de la respuesta a auxinas en estas células, junto a IAA3, ARF7 y ARF19, siendo responsables de la reactivación del ciclo celular [109]. Por su parte *IAA28* es necesario para la formación de callos mediante tratamiento exógeno hormonal. IAA28 induce la expresión de *GATA23* y confiere identidad de célula fundadora en los explantos sometidos al tratamiento hormonal, previo a la proliferación celular durante la formación de callos [101]. Según nuestros resultados, y basándonos en la literatura existente, podríamos decir que *IAA28* intervendría momentos antes de la proliferación vascular y la formación de callo endógeno mientras que *CRANE* se encontraría involucrado en fases posteriores, relacionadas con la especificación y proliferación de las células fundadoras de raíces, previa a la formación del meristemo radicular.

Respecto al origen de estas auxinas, nuestros resultados muestran que es necesario un sistema de transporte basípeto de larga distancia que concentre las auxinas generadas en el mesófilo del limbo foliar, presumiblemente a través de la acción de YUC8 y YUC9, en algunas células vasculares definidas en la base del peciolo (véase la figura suplementaria 7 del segundo artículo en la página 104). Se ha descrito que la expresión de *YUC9* responde al tratamiento con metil jasmonato (MeJA) de una manera dependiente de COI1 [66]. Dado que la generación de los explantos foliares implica una herida física y, por lo tanto, la producción de MeJA, este último podría activar la expresión de *YUC9* para aumentar rápidamente los niveles de auxinas. También encontramos que la expresión de *YUC8* se inducía en la

región vascular del peciolo asociada con la proliferación y, por lo tanto, es posible que YUC8 pueda tener un papel específico en el mantenimiento de los niveles de auxina durante la proliferación de la vasculatura o en pasos regenerativos posteriores. Curiosamente, en un artículo citado anteriormente [109], se demostró que la biosíntesis de auxinas es necesaria para llevar a cabo la desdiferenciación y adquisición de pluripotencia en protoplastos de células del mesófilo. Estos resultados indican que la acetilación alterada de histonas afecta negativa y predominantemente a la transcripción de genes de biosíntesis de auxinas. Adicionalmente, demostraron que se requiere la biosíntesis de auxinas para lograr la división celular inicial a través de la activación de los genes de la fase G2/M del ciclo celular [109]. Estableciendo cierta homología, es posible que la biosíntesis local de auxinas en la región organogénica, mediada por YUC8, sea necesaria también en etapas anteriores a la proliferación de las células vasculares o los pasos regenerativos posteriores que se han expuesto anteriormente.

Adicionalmente, hemos demostrado que la inhibición genética y química del transporte de auxinas afectó significativamente a la regeneración radicular. A pesar de la redundancia conocida entre los distintos transportadores de auxina [82, 110], detectamos fenotipos aberrantes en mutantes simples según su porcentaje de regeneración, lo que podría sugerir una compartimentación espacial de estos transportadores en las diferentes zonas del explanto foliar (véase la figura 4 del segundo artículo en la página 88). En línea con esta idea, encontramos que *PIN3* se expresaba en los haces vasculares del peciolo, mientras que *PIN4* se restringió más tarde a la región vascular en proliferación y callo endógeno. Dado que la expresión predominante de *ProDR5:GUS* se da en la región de los haces vasculares del peciolo proximal, es posible que sea necesario retener las auxinas en esta región. Nuestros datos sugieren un modelo en el que la localización subcelular de PIN3 cambia desde la membrana basal a la membrana apical en las células vasculares cercanas a la herida, para redirigir el flujo de auxina hacia atrás y así mantener altos

niveles de auxina en los haces vasculares del peciolo proximal. Curiosamente se ha descrito un cambio similar, dependiente de auxinas, en la polarización de PIN3, que contribuye a la inversión del flujo de auxinas durante la respuesta gravitrópica del tallo [111], donde se ha demostrado que el cambio basal a apical en la localización de PIN3 depende de su estado de fosforilación [112]. Por último, cabe destacar el fenotipo observado en el mutante *gnom*/*fewer roots*, el cual presenta defectos en la capacidad de internalización de los transportadores PIN [83]. Este mutante, en contraste con el fenotipo observado para los mutantes simples *pin*, también mostró un fenotipo aberrante respecto a la capacidad de enraizamiento, posiblemente debido a que varios transportadores de auxina se estarían viendo afectados simultáneamente (véase la figura 4 del segundo artículo en la página 88).

En conjunto, nuestros resultados desvelan una regulación fina entre los niveles de auxinas y citoquininas a lo largo de los distintos estadios del proceso regenerativo. De hecho, en nuestro modelo experimental, el potencial de regeneración de mutantes de señalización de citoquinina se restaura o compensa parcialmente mediante un aumento moderado de los niveles de auxinas. Estos resultados muestran la gran plasticidad de los procesos regenerativos y abren la puerta a diferentes abordajes hormonales para la regulación artificial del proceso organogénico en Arabidopsis y, posteriormente, en especies de interés comercial, en un contexto en el que las citoquininas nunca han tenido un papel especialmente relevante en comparación con las auxinas.

3.1.3. Especificación de células fundadoras de raíces y formación del meristemo radicular

Hemos detectado la expresión de *ProWOX11:GUS* tan solo 1 dde en algunas células asociadas al xilema en la base del peciolo. Además, esta expresión se potenció durante la formación del callo endógeno, aunque no simultáneamente en todas las células en proliferación. Posteriormente (5

dde), se observó la expresión de *ProWOX11:GUS* cerca de la zona central del callo endógeno pero excluida de los primordios radiculares (véase la figura 5 del segundo artículo en la página 90). Estos resultados sugieren que la expresión de *WUSCHEL-RELATED HOMEOBOX11* (*WOX11*) estaría asociada con la formación del callo endógeno y, quizás, con la especificación de células fundadoras de raíces, aunque en nuestro sistema no todas las células marcadas dieron como resultado la iniciación de primordios radiculares. La expresión de *WOX11* se ha asociado a la transición de células competentes a células fundadoras de raíces durante la formación de raíces adventicias en explantos foliares sin peciolo [39, 40]. En estos estudios se describió que WOX11 induce la expresión de *WOX5* durante la formación de raíces *de novo* en los explantos foliares sin peciolo; sin embargo, en nuestro trabajo, no encontramos expresión de *WOX5* en los callos endógenos durante la formación de raíces *de novo* a partir hojas enteras, lo que sugiere que, en nuestro sistema experimental, *WOX11* podría estar involucrado en un paso anterior en el proceso de reprogramación. Además, la expresión de *WOX11* podría vincularse a la proliferación de los haces vasculares en la región proximal del peciolo ya que el arresto del ciclo celular mediante abordajes químicos provoca una intensificación de su expresión y su persistencia en el tiempo (véase la figura 5 del segundo artículo en la página 90).

La expresión de *WOX5* en nuestro sistema experimental se produce tras la expresión de *ARABIDOPSIS HOMOLOG OF HOMOLOG OF HUMAN SKP2,* también llamado *SKP2B*, observándose, además, focalizada en pequeñas agrupaciones de células con una estructura compatible con un pro-meristemo, lo que indicaría que la expresión de este marcador no sería necesaria para el establecimiento de los meristemos más que para un correcto desarrollo y emergencia de estos. En contraste, el marcador SKP2B, ya vinculado previamente con la definición de células fundadoras de raíces laterales [72], ha mostrado una expresión temprana en nuestro modelo experimental, coincidente con la localización en la que posteriormente se establecerán los nuevos meristemos radiculares. Además, esta expresión se

mantiene en el meristemo radicular hasta la emergencia del primordio de raíces adventicias, en contraste con la expresión de *WOX5*.

En conjunto, nuestros resultados indican que la expresión de *WIND1* y *WOX11* se asociaría con la proliferación vascular que conduce a la formación del callo endógeno en la base del peciolo, mientras que *ProSKP2B:GUS* está restringido a algunas células fundadoras de raíces muy determinadas que se encontrarían dentro del callo y que adquieren rápidamente la expresión de *WOX5* asociada a la división celular del meristemo radicular. Algunos autores han relacionado la expresión de *SKP2B* con la especificación de células precursoras de tallos mediada por los genes *PLT3, PLT5* y *PLT7* dentro de callos inducido por hormonas [29], lo que indica que la adquisición de la pluripotencia podría ser un proceso estrictamente regulado en algunos subconjuntos de células y no una característica intrínseca de la totalidad del callo como se creía inicialmente [113]. Se desconoce cómo se especifican las células fundadoras de raíces en los callos endógenos formados durante el enraizamiento de la hoja completa, pero podría requerir los reguladores PLT1, PLT2 y SHR, ya que los explantos foliares del triple mutante *shr plt1 plt2* fueron incapaces de generar raíces adventicias (véase la figura 6 del segundo artículo en la página 86). Esto sugiere un presunto papel para dichos reguladores en la adquisición de la pluripotencialidad y capacidad formadora de raíces durante la formación de raíces adventicias en los callos endógenos de los explantos foliares.

3.2. Identificación de nuevos reguladores de la formación de raíces *de novo*

3.2.1. Caracterización del sistema de hipocótilo y obtención del mapa de calor

En este estudio se optimizó un protocolo para estudiar la formación de raíces adventicias inducida por heridas en hipocótilos de Arabidopsis, que es un procedimiento adecuado para el cribado de alto rendimiento de

mutantes. Además, se llevó a cabo un estudio comparativo en el que se evaluó la conservación funcional de ciertos reguladores del enraizamiento adventicio en hipocótilos en relación con la formación de raíces adventicias a partir de explantos foliares, siguiendo el sistema experimental expuesto en el apartado anterior de la presente memoria. De esta forma pudimos establecer paralelismos y diferencias entre ambos procesos organogénicos en función del órgano de partida.

El mapa de calor generado a partir de los datos de expresión génica de los 339 genes anotados en la colección *PhenoLeaf* nos permitió la agrupación y visualización sencilla de patrones de expresión compartidos (véase la figura 2 del tercer artículo en la página 110). Cabe esperar que un regulador positivo de la regeneración tisular inducida por hormonas aumentaría su expresión con el tratamiento hormonal. Por el contrario, los perfiles que mostraron un descenso de la expresión génica con el tratamiento hormonal podrían postularse como reguladores negativos de la regeneración tisular inducida por hormonas. Al usar estos criterios, seleccionamos 112 genes con perfiles de expresión dinámicos para llevar a cabo el cribado fenotípico de mutantes en el sistema experimental de explantos de hipocótilo.

La caracterización inicial de la organogénesis radicular adventicia en explantos de hipocótilo del fondo silvestre Col-0 nos permitió observar que la escisión completa de la raíz desencadenaba la especificación de nuevos focos sensibles a auxina (*ProDR5:GUS*) y el desarrollo y crecimiento de focos sensibles a auxina establecidos previamente dentro del hipocótilo, lo que llevó a un aumento significativo en el número de raíces adventicias unos días después de la escisión (véase la figura 1 del tercer artículo en la página 108). Las raíces adventicias derivadas del hipocótilo se originaron a partir de células del pericliclo anexas al xilema, en un proceso que se asemeja a la iniciación de las raíces laterales [31, 35]. En el modelo actual para la formación de raíces adventicias inducida por heridas en hipocótilos [32]; [114], la escisión de la raíz principal mejora el transporte polar de auxinas a través del hipocótilo, mientras que la acumulación de auxinas en los tejidos

próximos a la escisión impulsa la especificación localizada de las células fundadoras de raíces adventicias en el periciclo. En los hipocótilos intactos, el transporte polar de auxinas a través del hipocótilo y hacia los meristemos radiculares activos reduce la acumulación de auxina en las células del periciclo del hipocótilo, lo que, a su vez, limita la aparición de raíces adventicias. Al combinar datos de perfiles genéticos y un cribado fenotípico sistemático, hemos sido capaces de identificar una gran cantidad de mutantes foliares que presentaban un fenotipo pleiotrópico en la formación de raíces adventicias en hipocótilos después de la escisión de la raíz completa. En nuestro estudio, 47 (41.6 %) y 8 (7.1 %) de los mutantes de la colección *PhenoLeaf* analizados mostraron, respectivamente, significativamente menos y más raíces adventicias inducidas por herida en el hipocótilo que el fondo genético Col-0 (véanse la tabla suplementaria 2 y la figura suplementaria 2 del tercer artículo en la página 121).

En la mayoría de las especies, no obstante, la formación de raíces adventicias parte de tejidos no radiculares, en un proceso que requiere la desdiferenciación celular y, presumiblemente, la participación de vías reguladoras diferentes a las implicadas en la formación de raíces adventicias a partir de hipocótilos, que podrían asimilarse más a las raíces laterales en este aspecto [41]. Por tanto, analizamos la organogénesis radicular en explantos foliares de hoja completa en una selección de mutantes identificados en el cribado anterior. Todos los mutantes estudiados también mostraron respuestas organogénicas similares en explantos de hoja completa, lo que sugiere que los genes afectados en estos mutantes participarían en vías reguladoras compartidas, que serían requeridas para la formación de órganos *de novo* a partir de diferentes órganos (véase la figura 4 del tercer artículo en la página 112).

3.2.2. Cribado sistemático e identificación de nuevos reguladores negativos. Conservación funcional en el sistema de hoja completa

En nuestro cribado hemos identificado un número relativamente alto de mutantes en genes implicados en la traducción de proteínas y genes que codifican proteínas ribosómicas que mostraron una reducción significativa del número de raíces adventicias en el hipocótilo después de la escisión de la raíz principal (n=11; 23.4% de los 47 mutantes que hemos denominado *less adventitious roots* [*lars*]). Algunos ejemplos son el mutante *m274*, homocigoto para una inserción de ADN-T en el gen At4g16720, que codifica una proteína ribosómica de la familia L23/L15e [115], y el mutante *m285*, que presenta una inserción de ADN-T en el gen *PIGGYBACK 1* que codifica la subunidad ribosomal L10a [116]. La inserción en la línea *m405* afecta al gen At3g09720, que codifica la subunidad grande de una GTPasa requerida para la maduración de la subunidad ribosómica 60S y cuya pérdida de función causa la alteración del transporte, distribución y respuesta a las auxinas, afectando consecuentemente a múltiples procesos de desarrollo [117]. Nuestros resultados están en línea con un papel específico de los ribosomas como reguladores clave en el desarrollo de las raíces adventicias. Una posibilidad es que la función de los ribosomas influya en la capacidad de la célula para activar las divisiones celulares durante las primeras etapas de la formación de raíces adventicias o, alternativamente, que ciertos conjuntos de genes implicados en respuestas específicas de raíces adventicias puedan requerir una configuración ribosómica particular para producir la respuesta organogénica. Se han descrito algunos alelos mutantes de genes codificantes de proteínas ribosómicas que presentan una disminución significativa y particular en la traducción de algunos factores de respuesta a auxinas específicos [118], favoreciendo la última hipótesis, aunque su confirmación requerirá estudios adicionales. Dada la aparición de una gran cantidad de fenotipos pleiotrópicos que afectaban severamente a la morfología de las plántulas, los ensayos en el sistema experimental de hoja

completa fueron descartados.

Varias líneas de evidencia apoyan la hipótesis de que las giberelinas son críticas para el desarrollo de la raíz primaria mediante del control del tamaño del meristemo de la raíz [119, 120]. No obstante, resultados obtenidos en otras especies vegetales sugieren que las giberelinas tienen un efecto inhibitorio en el desarrollo de las raíces adventicias [61, 121]. El mutante *m240* contiene una inserción homocigota de ADN-T en la región codificante del gen At4g02780, llamado *GA REQUIRING 1* (*GA1*), y el análisis del enraizamiento en explantos de hipocótilo mostró un fenotipo *lars*. Los ensayos en el sistema experimental de hoja confirmaron dicho fenotipo, mostrando la conservación de la función de *GA1* en la formación de raíces adventicias en ambos órganos de partida. El análisis de líneas mutantes adicionales afectadas en la biosíntesis y señalización de las giberelinas nos permitió confirmar el papel de las giberelinas como reguladores positivos de la formación de raíces adventicias en el sistema experimental de hoja completa (véase la figura 5 del tercer artículo en la página 113). Nuestros resultados contrastan con los obtenidos en álamo híbrido, ya que se ha descrito que líneas transgénicas sobreproductoras de giberelinas o hipersensibles a la misma produjeron menos raíces adventicias que su fondo silvestre en esquejes de tallos, probablemente por la interacción negativa que se produce entre las giberelinas y el transporte polar de auxinas [61]. Por su parte, el mutante *lars m482* contiene una inserción homocigota de ADN-T en el octavo exón del gen At5g62190, que codifica la helicasa de ARN AtRH7/PRH75 DEAD-box, involucrada en el procesamiento del ARN ribosómico [122]. Mientras que la capacidad de enraizamiento de los explantos foliares de *m482* mostró diferencias sutiles, pero no significativas, respecto al fondo silvestre Col-0, las líneas adicionales Salk_062900 y Salk_016729 mostraron fenotipos totalmente opuestos (véase la figura 6 del tercer artículo en la página 114) lo que sugiere un efecto fenotipo dependiente de la posición de la inserción en el gen. Además, para confirmar si los defectos en el procesamiento del ARN ribosómico que producen una

conformación alterada del ribosoma podrían causar el fenotipo observado en explantos de hipocótilo de mutantes *m482*, incubamos explantos de hoja completa del genotipo silvestre Col-0 con estreptomicina, y encontramos una reducción muy intensa de la capacidad de enraizamiento debido a un retraso en la emergencia de raíces adventicias. En conjunto, nuestros resultados indican que las mutaciones de *AtRH7/PRH75* podrían afectar el ensamblaje adecuado de los ribosomas, aunque una caracterización más profunda de estas inserciones será necesaria para confirmar esta hipótesis.

Otras líneas *lars* (*m039*, *m143*, *m240*, *m274*, *m482*, *m608*, *m617* y *m626*) identificadas en el cribado inicial se analizaron en el sistema experimental de explantos foliares. Todas las líneas *lars* evaluadas mostraron una reducción en la capacidad de enraizamiento respecto al fondo genético silvestre en explantos de hoja completa (véase la figura 4 del tercer artículo en la página 112) lo que sugiere una gran conservación en los mecanismos de formación de raíces adventicias independientemente del órgano de origen.

3.2.3. Cribado sistemático e identificación de nuevos reguladores positivos. Conservación funcional en el sistema de hoja completa

Los ocho mutantes *more adventitious roots* (*mars*) que hemos identificado (*m065*, *m232*, *m258*, *m525*, *m530*, *m667*, *m678* y *m681*,) podrían definir reguladores negativos de la formación de raíces adventicias en explantos de hipocótilo. Los mutantes ensayados en el sistema experimental de hoja (*m232* y *m678*) mostraron la conservación de su fenotipo *mars* en este sistema experimental (véase la figura 4 del tercer artículo en la página 112).

En base a nuestros resultados, podríamos sugerir la clasificación de los reguladores de la formación de raíces adventicias en dos grandes categorías: (1) reguladores generales de la regeneración y (2) reguladores específicos de tejido, siendo los primeros, probablemente, los más relevantes para

estudios futuros. No son muchas las referencias bibliográficas que abordan el estudio de los componentes centrales del proceso regenerativo. Se ha descrito que la pérdida de la función del complejo represor Polycomb 2 provoca desdiferenciación celular, la formación de callos y la formación de embriones somáticos a partir de pelos radiculares completamente diferenciados [123]. Estudios previos [124] proponen la existencia de un núcleo de señalización ARF-LBD común que participaría en varios procesos de organogénesis: la formación de tallos a partir de tejido calloso, el desarrollo de nódulos y la formación de haustorios. Además, este autor sugiere que los reguladores del estado de la cromatina, así como otros reguladores epigenéticos, podrían constituir otra capa de reguladores comunes en diferentes procesos de reprogramación celular [124]. Nuestros resultados muestran una gran conservación en la función de los reguladores del enraizamiento adventicio, reforzando la hipótesis de que existen reguladores maestros que intervendrían de forma común en los diferentes procesos regenerativos y organogénicos de la planta.

El mutante *m232* presenta una inserción homocigota en el tercer exón del gen *XYLOGEN PROTEIN 1* (*XYP1*), que codifica un factor de diferenciación del xilema llamado xilógeno [125]. Además, los resultados obtenidos en el sistema de hoja completa permiten confirmar la participación de este gen en los procesos de enraizamiento adventicio independientemente del órgano de partida ya que, tanto el mutante *m232*, como las líneas adicionales Sail_896_G05 y Salk_147826 mostraron una capacidad de enraizamiento significativamente más alta que su fondo genético silvestre en explantos de hoja completa (véase la figura 7 del tercer artículo en la página 115). El xilógeno se secreta direccionalmente desde las células vasculares en diferenciación, se mueve por el apoplasto hacia las células mesófilas indiferenciadas adyacentes y las “conduce” hacia su misma ruta de diferenciación celular [125]. En muchas especies, el cámbium vascular se ha identificado como el tejido de origen de las raíces adventicias derivadas del tallo [31, 41]. Se ha propuesto que dentro del cámbium vascular reside

una población definida de células iniciadoras, no diferenciadas, que producen células madre del xilema hacia adentro y células madre del floema hacia afuera del mismo cámbium [126], actuando como un meristemo causante del crecimiento secundario de la planta. Es posible, por tanto, que la diferenciación reducida del xilema en los mutantes *xyp1* aumente el número de estas células del cámbium iniciadoras, lo que permite una especificación mediada por auxina de una gran población de células fundadoras de raíces adventicias y, por lo tanto, aumenta el número de raíces adventicias formadas en estos mutantes. Serán necesarios estudios adicionales que utilicen líneas marcadoras para la especificación de células fundadoras de raíces adventicias [93] para confirmar esta hipótesis.

Otro mutante con mayor capacidad de enraizamiento en hipocótilos fue *m678*, que es homocigoto para una inserción de ADN-T en el gen At4g13770, llamado *REDUCED EPIDERMAL FLUORESCENCE* 2 (*REF2*) y que codifica la enzima CYP83A1, responsable de la conversión de aldoximas a tiohidroximatos en la ruta de biosíntesis de los glucosinolatos independiente del triptófano [127, 128]. Curiosamente, el desarrollo anormalmente abundante de raíces adventicias en hipocótilos es un rasgo bien conocido del fenotipo superproductor de auxinas del mutante *superroot2-1* (*sur2-1*), con una pérdida de función en *CYP83B1*, que comparte un 63% de identidad de aminoácidos con CYP83A1 [129, 130]. Los estudios llevados a cabo en el sistema experimental de explantos foliares mostraron que tanto *m678* como el mutante *ref2-1* mostraron un incremento significativo de la capacidad de enraizamiento respecto a su fondo genético silvestre (véase la figura 7 del tercer artículo en la página 115). Los mutantes *ref2-1* y *sur2-1* muestran niveles reducidos de glucosinolatos y niveles aumentados de sus precursores en las hojas, lo que sugiere una interacción compensatoria entre CYP83A1 y CYP83B1 en algunos órganos [131]. La canalización de indol-3-acetaldoxima hacia la producción de AIA o glucosinolatos está estrictamente controlada y podría explicar los fenotipos de los mutantes *ref2-1* y *sur2-1*. Otros mutantes de biosíntesis de glucosinolatos también tienen niveles elevados de AIA y,

por lo tanto, respuestas mejoradas de auxina, lo que indica una interacción directa entre las vías biosintéticas de los glucosinolatos y las auxinas [132].

Los otros seis mutantes *mars* identificados en el cribado sistemático del sistema de hipocótilo no fueron evaluados en explantos de hoja completa. No obstante, algunos de estos genes podrían también ser de interés ya que la confirmación de su papel como reguladores negativos del enraizamiento adventicio supondría el establecimiento de nuevos candidatos para futuras aplicaciones en la mejora genética vegetal. Un ejemplo de los mutantes *mars* identificados en el sistema experimental de hipocótilo pero que no fue posteriormente ensayado en el sistema de hoja completa fue el mutante *m667*. *m667* presenta una inserción homocigota de ADN-T en At2g45310, llamado *UDP-D-GLUCURONATE 4-EPIMERASE 4*, uno de los seis genes que codifican las UDP-D-glucuronato 4-epimerasas involucradas en la biosíntesis de pectina [133, 134]. Según fuentes bibliográficas, el mutante *atpme3-1*, con niveles bajos de metilesterasas de pectina, también mostró un gran aumento (>30%) en el número de raíces adventicias que emergen del hipocótilo [135], confirmando así el papel fundamental de la mecánica de la pared celular en la formación raíces adventicias en explantos de hipocótilo. De acuerdo con estos resultados, el mutante *m667* podría contener niveles alterados de pectina en el hipocótilo, lo que interferiría con la localización de PIN1 y la respuesta de auxina durante la formación de raíces adventicias. Sería interesante establecer si la reducción de los niveles de pectina en las hojas afecta a la capacidad de regeneración o la capacidad de enraizamiento en explantos foliares.

En resumen, hemos utilizado un enfoque genético guiado por perfiles de expresión en una colección de mutantes de ADN-T bien caracterizada (*PhenoLeaf*) que nos permitió mostrar algunos genes como reguladores novedosos del desarrollo de raíces adventicias. Con la llegada de nuevas herramientas de la biología de sistemas [136] y los nuevos avances en robótica, los genes candidatos se seleccionarán en función de la expresión específica de la célula, la interacción proteína-proteína y proteína-ADN, y el

cribado de alto rendimiento de fenotipos de raíces adventicias se llevará a cabo de forma automatizada. La identificación de los reguladores que intervienen en la formación de raíces adventicias permitirá detallar los aspectos moleculares de la formación de estos órganos adventicios y constituirán un gran potencial para modular estos comportamientos de manera artificial en especies de interés comercial.

Adicionalmente, para un pequeño número de mutantes *mars* y *lars* en explantos de hipocótilo, hemos comprobado la conservación funcional de los genes implicados en la organogénesis radicular a partir, también, de explantos de hoja completa. Aunque estos resultados apuntan a que las redes génicas que intervienen en la organogénesis radicular adventicia lo hacen con independencia del órgano de partida, no podemos descartar la existencia de mecanismos particulares para cada órgano iniciador. En cierto modo, el establecimiento de varios sistemas de estudio para la formación de raíces adventicias (hipocótilo y hoja completa) nos permite tener más herramientas para diseccionar el proceso organogénico en cuanto a parámetros cuantificables (número de máximos de auxinas, número de raíces adventicias, capacidad de regeneración, perfiles de capacidad de enraizamiento, engrosamiento del área vascular, etc.) y además nos permitirá detectar diferencias dependientes del órgano de origen si los resultados en ambos modelos muestran comportamientos discordantes.

4. CONCLUSIONES Y PROYECCIÓN FUTURA

4.1 Conclusiones

- En este trabajo hemos caracterizado la formación de raíces adventicias en explantos foliares de *Arabidopsis thaliana* empleando un abordaje sistemático en 17 líneas mutantes, 1 línea sobreexpresora y 15 líneas marcadoras. Nuestros estudios nos han permitido describir de una manera más detallada la interacción funcional de las auxinas y citoquininas en la formación de raíces *de novo* así como en los procesos de desdiferenciación, proliferación y adquisición de nuevas identidades celulares.
- La proliferación del tejido vascular y formación de un callo endógeno se produce en respuesta a la herida y como consecuencia de la acción conjunta de auxinas (*INDOLE-3-ACETIC ACID INDUCIBLE 18*, *INDOLE-3-ACETIC ACID INDUCIBLE 28*) y citoquininas (*WOODEN LEG*, *ARABIDOPSIS RESPONSE REGULATOR 1*, *10* y *12*). Este callo endógeno expresa marcadores asociados a la desdiferenciación como *WOUND INDUCED DEDIFFERENTIATION 1*. La especificación posterior de las células fundadoras de raíces dentro del callo endógeno viene marcada por la expresión de los genes *WUSCHEL RELATED HOMEOBOX 11* y *ARABIDOPSIS HOMOLOG OF HOMOLOG OF HUMAN SKP2 B*.
- Hemos desarrollado un protocolo de cribado sistemático basado en redes de expresión génica que nos ha permitido identificar nuevos reguladores de la formación de raíces *de novo* a partir de una colección de mutantes de hoja estudiados previamente. Además, hemos confirmado la implicación de algunos de estos reguladores en dicho proceso mediante el uso de líneas mutantes adicionales.
- Hemos identificado 47 mutantes *lars* y 8 mutantes *mars* entre los 112 mutantes analizados de la colección *PhenoLeaf*. Estos mutantes

mostraron significativamente menos y más raíces adventicias en el hipocótilo, respectivamente, que su ancestro silvestre Columbia-0.

- Hemos encontrado reguladores comunes en la formación de raíces adventicias en hipocótilos y explantos foliares. Nuestros resultados parecen indicar la existencia de mecanismos comunes al proceso organogénico, independientemente de los órganos de origen, aunque también muestran la existencia de reguladores que actuarían de forma específica en cada uno de ellos.
- En este trabajo hemos confirmado que los genes *GA REQUIRING 1*, *REDUCED EPIDERMAL FLUORESCENCE 2* y *XYLOGEN PROTEIN 1*, relacionados con la biosíntesis y la señalización de giberelinas, la homeostasis de las auxinas y la diferenciación del xilema, respectivamente, participan en la formación de raíces adventicias.
- Los resultados obtenidos en este trabajo permiten ahondar más en los mecanismos moleculares de la regeneración radicular y contribuyen al entendimiento general del enraizamiento adventicio. Avances en esta línea de investigación permitirán la identificación de otros reguladores implicados en la regeneración de raíces y su posible uso eventual como marcadores moleculares asociados a las respuestas de enraizamiento en otras especies.

4.2 Proyección futura

En la presente Tesis Doctoral se han identificado nuevos reguladores de la formación de raíces adventicias relacionados con vías de señalización muy diversas. Con relación al estudio detallado de la formación de raíces adventicias en explantos foliares completos, tenemos previsto que este sistema experimental siga aportando información de nuevos reguladores mediante el análisis sistemático de líneas mutantes y sobreexpresoras. Por otra parte, la identificación de estos reguladores nos permitirá llevar a cabo análisis funcionales más detallados de los mismos. Será interesante explorar la interacción entre los reguladores CRANE, IAA28 y SKP2B y los reguladores

PLT1, PLT2 y SHR, así como la posible intervención de los genes *LBD* regulados por citoquininas. Por otra parte, es probable que se realicen experimentos encaminados a evaluar la conservación funcional de estos reguladores en tomate (*Solanum lycopersicum* L.), una especie con la que el laboratorio del Dr. José Manuel Pérez Pérez trabaja de forma habitual.

Sobre el cribado fenotípico de la colección *PhenoLeaf* que hemos llevado a cabo en el sistema de hipocótilo, seguiremos dos abordajes principales: (1) utilizar el sistema de hipocótilo para la identificación de más reguladores implicados en la organogénesis adventicia y (2) ahondar en la función de algunos de los reguladores encontrados. El análisis funcional del gen *XYP1* arrojará luz sobre los mecanismos implicados en la regulación de la diferenciación de las células pluripotentes del cámbium vascular y cómo la proliferación anormal de estas células puede favorecer la formación de órganos adventicios. En el contexto legislativo de la Unión Europea respecto a los organismos modificados genéticamente, la identificación de reguladores negativos del enraizamiento siempre es muy interesante dado que una mutagénesis sencilla puede aportar fenotipos útiles para la industria agroalimentaria de forma rápida. Por último, proponemos el uso de nuevos datos de expresión génica (secuenciación de ARN) durante eventos de regeneración que se han venido publicando en los últimos años para optimizar la búsqueda dirigida de fenotipos interesantes en colecciones de mutantes preexistentes.

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6. ANEXOS

- **Sergio Ibáñez**, Elena Carneros, Pilar S Testillano, José Manuel Pérez-Pérez (2020). Advances in plant regeneration: shake, rattle and roll. *Plants* **16**: 897. doi: 10.3390/plants9070897.

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- **Sergio Ibáñez**, Helena Ruiz-Cano, María Á. Fernández, Ana Belén Sánchez-García, Joan Villanova, José Luis Micol, José Manuel Pérez-Pérez (2019). A network-guided genetic approach to identify novel regulators of adventitious root formation in *Arabidopsis thaliana*. *Frontiers in Plant Science* **10**: 461. doi: 10.3389/fpls.2019.00461.

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Review

Advances in Plant Regeneration: Shake, Rattle and Roll

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Abstract: Some plant cells are able to rebuild new organs after tissue damage or in response to definite stress treatments and/or exogenous hormone applications. Whole plants can develop through de novo organogenesis or somatic embryogenesis. Recent findings have enlarged our understanding of the molecular and cellular mechanisms required for tissue reprogramming during plant regeneration. Genetic analyses also suggest the key role of epigenetic regulation during de novo plant organogenesis. A deeper understanding of plant regeneration might help us to enhance tissue culture optimization, with multiple applications in plant micropropagation and green biotechnology. In this review, we will provide additional insights into the physiological and molecular framework of plant regeneration, including both direct and indirect de novo organ formation and somatic embryogenesis, and we will discuss the key role of intrinsic and extrinsic constraints for cell reprogramming during plant regeneration.

Keywords: hormone-induced callus; wound-induced callus; somatic embryogenesis; stress-induced microspore embryogenesis; root tip regeneration

1. Introduction

Unlike what happens in animals, plants have a high regenerative capacity and, under natural conditions, they are able to form new organs and even complete individuals from a few cells present in adult tissues, either in response to injury or to the alteration of their environment [1]. Classical in vitro culture experiments of plant tissues indicated that the exogenous auxin and cytokinin (CK) balance control plant organogenesis, so that a high CK-to-auxin balance induces the production of shoots, an elevated auxin-to-CK balance induces the formation of roots, while intermediate levels of both hormones induces the formation of an amorphous cell mass dubbed callus [2]. Application of stress treatment or exogenous auxin can induce somatic embryogenesis, an intriguing process that exemplifies plant cell totipotency expression. Here, we provide an update on the key molecular and signaling events on three different regenerative processes in plants: (i) hormone-induced callus formation; (ii) tissue regeneration after micro-surgical excision of the root tip; and (iii) embryo induction in somatic cells from different cell types and explants without the fusion of gametes.

2. Transcription Factor Networks and Epigenetic Regulators during Hormone-Induced Callus Formation

Callus formation is experimentally induced from a variety of plant tissues by their incubation on an auxin-rich callus-inducing medium (CIM), and relies on the re-deployment of lateral root (LR) developmental programs from existing pericycle-like cells (Figure 1a), which are functionally

analogous to animal stem cells [3]. Hence, mutants defective in LR formation, such as *aberrant lateral root formation 4* (*alf4*) and *solitary root 1* (*slr1*, also known as *iaa14*) are also impaired in auxin-induced callus formation [3]. ALF4 is required for the formative divisions of xylem pole pericycle (XPP) cells during LR formation [4]. Through its binding to the RBX1 subunit of the SCF E3 ligases, ALF4 interferes with the interaction between E2 and RBX1 [5]. As a result, several SCF^{TIR1} substrates, such as AUXIN RESISTANT 3 (AXR3), are miss-regulated in the *alf4* mutants and this might explain the auxin-related phenotypes of *alf4* seedlings [5]. AXR3 physically interacts with MONOPTEROS, also known as AUXIN RESPONSE FACTOR 5 (ARF5), and the resulting AXR3-ARF5 complex functions as a transcriptional repressor at low auxin levels [6], which has also been shown to control plant stem cell maintenance and differentiation during embryogenesis [7].

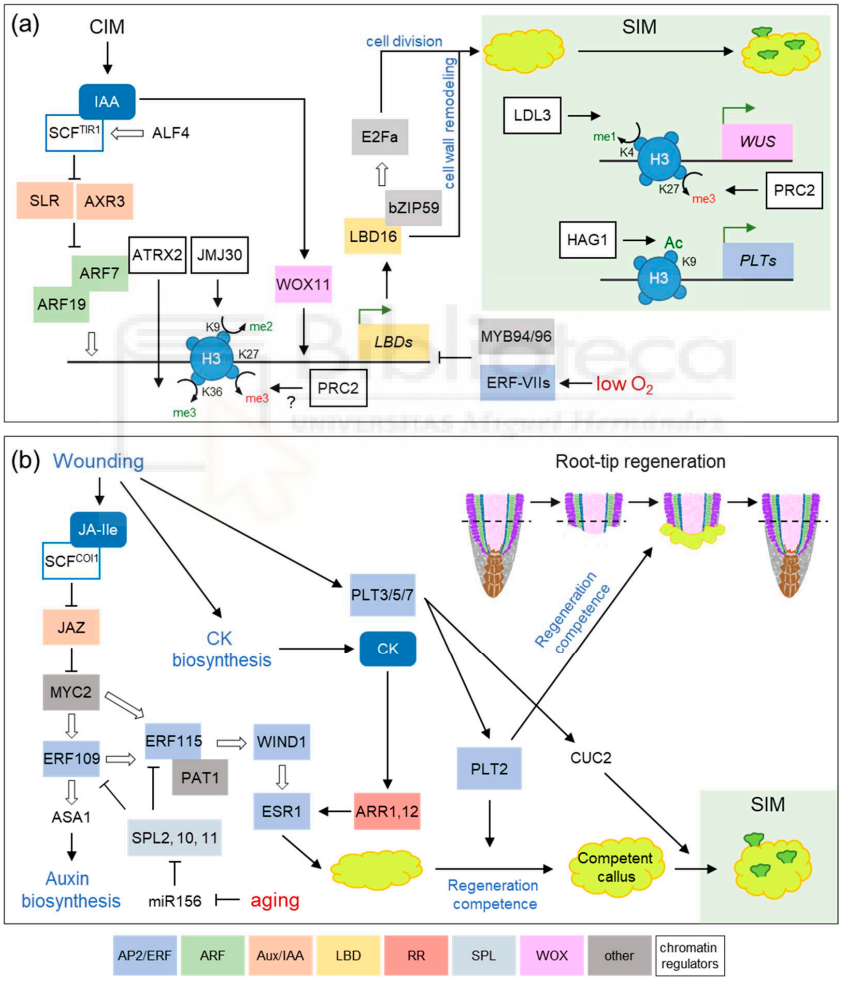

Figure 1. Transcriptional networks involved in cell reprogramming during regeneration. (**a**) Hormone-induced shoot organogenesis. (**b**) De novo root formation after root-tip excision. Positive (wounding, callus-inducing medium (CIM), auxin, etc.) and negative (low O_2, aging, etc.) signals are shown in blue and red, respectively. Transcriptional and epigenetic regulators (Table 1) are depicted inside boxes of different colors. Each color represents a given DNA binding domain (see main text for legends). White arrows indicate direct upregulation via promoter binding.

Table 1. Some key regulatory factors (transcription factors, epigenetic regulators and others involved in auxin homeostasis) involved in plant regeneration.

Genes	Abbreviations	Function in Plant Regeneration	Molecular Function
ABA INSENSITIVE 3	*ABI3*	Quantitatively regulates BBM-mediated somatic embryogenesis. Acts as a positive regulator	Dof-type transcription factor
ABERRANT LATERAL ROOT FORMATION 4	*ALF4*	Formative divisions of XPP cells during LR formation. Callus formation upon CIM induction	SCF^{TIR1} regulation
AGAMOUS LIKE 15	*AGL15*	Activates auxin biosynthesis, leading to totipotency acquisition and SE initiation	MADS domain transcription factor
ANTHRANILATE SYNTHASE α1	*ASA1*	Tryptophan biosynthesis	Oxo-acid-lyase enzyme
ARABIDOPSIS RESPONSE REGULATOR 1 and *12*	*ARR1 and 12*	Involved in CK-mediated ESR1 induction in order to promote shoot regeneration	Type-B Arabidopsis response regulator transcription factors
ARABIDOPSIS TRITHORAX-RELATED 2	*ATXR2*	Positively regulates *LBD16* and *LBD29* expression upon CIM induction	Histone lysine methyltransferase
AUXIN RESISTANT 3	*AXR3, IAA17*	Transcriptional repressor upon low auxin levels. Controls stem cell maintenance	Aux/IAA corepressor
AUXIN RESPONSE FACTOR 7 and *19*	*ARF7 and 19*	LR formation / Positively regulates LBD16 and *LBD29* expression upon CIM induction	Auxin-responsive transcription factor
BABY BOOM	*BBM, PLT4, AIL5*	Its ectopic expression can also directly reprogram somatic cells and induce SE in the absence of exogenous stimuli	AP2/ERF transcription factor
BASIC REGION/LEUCINE ZIPPER MOTIF 59	*bZIP59*	Interacts with LBD16 upon CIM induction	bZIP transcription factor
E2 PROMOTER BINDING FACTOR a	*E2Fa*	DNA replication	E2F transcription factor
ENHANCER OF SHOOT REGENERATION 1	*ESR1*	Induces the expression of key shoot regulators (*CUC1*, *RAP2.6L*, *ESR2*, *WUS*, and *STM*) to promote shoot regeneration	AP2/ERF transcription factor
ETHYLENE RESPONSE FACTOR 109	*ERF109*	Up-regulates *ERF115* expression. Up-regulates ASA1 expression, probably involved in the auxin biosynthetic pathway	AP2/ERF transcription factor
ETHYLENE RESPONSE FACTOR 115	*ERF115*	Acts as as a rate-limiting factor for quiescent center (QC) cell division after DNA damaging stress. Involved in *WIND1* up-regulation upon wound signaling	AP2/ERF transcription factor
FUSCA 3	*FUS3*	Involved in embryo development. Essential for successful SE	B3 domain-containing transcription factor

Table 1. *Cont.*

Genes	Abbreviations	Function in Plant Regeneration	Molecular Function
GENERAL CONTROL NONREPRESSED 5	*GCN5*, *HAG1*	Root stem cell niche maintenance. Callus pluripotency and shoot induction upon SIM	Histone acetyltransferase
JASMONATE-ZIM DOMAIN PROTEINS	*JAZ PROTEINS*	Represses de novo root formation in Arabidopsis leaf explants. Their destabilization allows the action of positive regulators	Jasmonate zinc-finger inflorescence meristem domain transcription factor
JUMONJI C DOMAIN-CONTAINING 30	*JMJ30*, *JMJD5*	Positively regulates *LBD16* and *LBD29* expression upon CIM induction	Histone lysine demethylase
LATERAL ORGAN BOUNDARIES DOMAIN 16, *17*, *18* and *29*	*LBD16*, *17*, *18 and 29*	Callus formation upon CIM induction	LOB-domain transcription factor
LEAFY COTYLEDON 1	*LEC1*	Its ectopic expression can also directly reprogram somatic cells and induce SE in the absence of exogenous stimuli	B3 domain-containing transcription factor
LEAFY COTYLEDON 2	*LEC2*	Its ectopic expression can also directly reprogram somatic cells and induce SE in the absence of exogenous stimuli	B3 domain-containing transcription factor
LYSINE-SPECIFIC DEMETHYLASE 1-LIKE 3	*LDL3*	Presumably removes H3K4me2 during callus formation. It may allow the genes for shoot initiation to be expressed after SIM treatment	Histone lysine demethylase
microRNA156	*miRNA156*	Reduces *SPL2*, *10* and *11* expression, promoting AR formation	microRNA molecule
MONOPTEROS	*MP*, *ARF5*	Hypophysis specification during embryogenesis	Auxin-responsive transcription factor
MYB94 and *96*	*MYB94 and 96*	Regulates *LBD29* expression upon CIM induction	MYB transcription factors
MYC2	*MYC2*	Acts upstream of *ERF109* as a positive regulator	bHLH transcription factor
PHYTOCHROME A SIGNAL TRANSDUCTION 1	*PAT1*	Acts as a partner of ERF115 and induces *WIND1* expression	GRAS transcription factor
PIN-FORMED 1	*PIN1*	Auxin transport	Auxin efflux facilitator
PLETHORA 3, *5* and *7*	*PLT3*, *5* and *7*	Induce the expression of genes involved in regeneration competence acquisition (*PLT2*) and differentiation factors (i.e., *CUC2*)	AP2/ERF transcription factor
POLYCOMB REPRESSIVE COMPLEX 2	*PRC2*	Di- and tri-methylation of Lys27 on histone H3. PRC2 activity blocks hormone-mediated SE	Histone lysine methyltransferase

Table 1. *Cont.*

Genes	Abbreviations	Function in Plant Regeneration	Molecular Function
RWP-RK DOMAIN-CONTAINING 4	*RKD4, GRD*	Induces early embryo-specific genes when overexpressed in seedlings. Its ectopic expression can also directly reprogram somatic cells and induce SE in the absence of exogenous stimuli	RWP-RK-type transcription factor
SOLITARY ROOT 1	*SLR1, IAA14*	Formative divisions of XPP cells during LR formation	Aux/IAA corepressor
SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 2, 10 and *11*	*SPL2, 10* and *11*	Their up-regulation is linked to a decrease in wound-induced ARs, presumably due to the repression of ABR1, ERF109, ERF115 and RAP2.6L, among others	SPL transcription factor
TAA-RELATED 2	*TAR2*	Auxin biosynthesis	Tryptophan aminotransferase enzyme
TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1	*TAA1*	Auxin biosynthesis	Tryptophan aminotransferase enzyme
WOUND INDUCED DEDIFFERENTIATION 1	*WIND1, RAP2.4*	Establishes and maintains dedifferentiated cell status	AP2/ERF transcription factor
WUSCHEL	*WUS*	Shoot induction upon SIM	Homeobox transcription factor
WUSCHEL RELATED HOMEOBOX 11 and *12*	*WOX11 and 12*	Positively regulates *LBD16* and *LBD29* expression upon CIM induction	Homeobox transcription factor
YUCCA 1 and 4	*YUC1 and 4*	Auxin biosynthesis	Flavin-containing monooxygenase enzymes

Several LATERAL ORGAN BOUNDARIES DOMAIN (LBD) transcription factors (LBD16, LBD17, LBD18 and LBD29) act downstream of the auxin-responsive transcription factors ARF7 and ARF19 to induce callus formation, to some extent through regulation of the E2 PROMOTER BINDING FACTOR a (E2Fa) transcription factor that promotes cell division [8]. Accordingly, the *arf7 arf19* double mutants display reduction of auxin-induced callus formation [9], while the ectopic expression of each of these LBD genes is sufficient to trigger callus formation in the absence of exogenously applied auxin [8]. In addition, auxin activates the expression of *WUSCHEL RELATED HOMEOBOX 11* (*WOX11*) and its homolog *WOX12*, which in turn have been shown to induce *LBD16* and *LBD29* expression during hormone-induced callus formation [10].

The chromatin context influences the accessibility of transcriptional regulators and thereby gene expression profiling during cell reprogramming and regeneration (Figure 1a) [11–13]. The ARABIDOPSIS TRITHORAX-RELATED 2 (ATXR2) is a histone lysine methyltransferase that stimulates the deposition of the active H3K36me3 mark at the *LBD16* and *LBD29* promoters through its direct interaction with ARF7 and ARF19 transcription factors [9]. Hence, ATRX2 contributes to the auxin-mediated epigenetic regulation of *LBD* expression during callus formation (Figure 1a) [9]. JUMONJI C DOMAIN-CONTAINING 30 (JMJ30), also known as JMJD5, is a member of the JmjC domain subgroup of histone demethylases that is involved in diverse developmental processes, including circadian regulation and temperature-dependent flowering control [14,15]. JMJ30 is recruited to the promoters of the *LBD16* and *LBD29* genes by ARF7 and ARF19, and removes the repressive H3K9me3 mark to ensure chromatin-dependent activation of *LBD* expression during hormone-induced callus formation [16]. Moreover, the ARF-JMJ30 complex further recruits ATXR2, and the multimeric protein complex ensures stable *LBD* activation during callus formation [16].

Di- and tri-methylation of Lys27 on histone H3 (H3K27me2/3), catalyzed by the Polycomb repressive complex 2 (PRC2), is a key repressive mark of many developmental processes in eukaryotes [17]. Earlier work suggested a central role of H3K27me3 mark during plant regeneration, particularly on genes on the auxin biosynthesis and root development pathways, where the H3K27me3 levels decreased during callus formation [18]. In leaf explants, the early activation of the auxin biosynthesis genes *YUCCA1* (*YUC1*) and *YUC4* during de novo root regeneration is accompanied by decreasing H3K27me3 levels at their promoters [19]. The high CK levels of the shoot-inducing medium (SIM) gradually reduced H3K27me3 levels at the *WUSCHEL* (*WUS*) locus in a cell cycle-dependent manner allowing its expression and an efficient shoot regeneration [20]. Callus-promoting LBDs are known H3K27me3 target genes [21] and it would be interesting to test whether removal of this repressive histone mark in the *LBD* promoters is required for auxin-induced callus formation.

LYSINE-SPECIFIC DEMETHYLASE 1, LSD1 (also known as KDM1A), is a conserved histone demethylase in metazoans that specifically removes H3K4me1/me2 or H3K9me1/me2 marks, and can function as a transcriptional repressor or activator [22]. The Arabidopsis genome contains four *LSD1* paralogs, *FLOWERING LOCUS D* (*FLD*), *LDL1*, *LDL2* and *LDL3*, which have been linked to seed dormancy, circadian clock and flowering time regulation [23–25]. In a recent report, *LDL3* was found upregulated on CIM and presumably removes H3K4me2 during callus formation, which then may allow the genes for shoot initiation to be expressed after SIM treatment [26]. In human cells, LSD1 participates in the maintenance of stem cell pluripotency through the control of the levels of H3K4 methylation at the regulatory regions of some Oct4-regulated developmental genes involved in the cellular balance between self-renewal and differentiation [27]. One possible scenario for LDL3 function is that stepwise histone modifications take place between the LDL3-mediated primed H3K4me2 demethylation in CIM treatment and the gene activation in the subsequent SIM treatment [26].

The Arabidopsis histone acetyltransferase HAG1, also known as GENERAL CONTROL NONREPRESSED 5 (GCN5), was previously reported to affect the stem cell niche maintenance in roots by regulating *PLETHORA1* (*PLT1*) and *PLT2* expression [28]. HAG1 plays a pivotal role in the establishment of pluripotency in callus and subsequent shoot regeneration [29]. In developing CIM-induced callus, HAG1 catalyzes histone acetylation at several root-meristem loci, including *PLT1*,

PLT2, *SCARECROW* (*SCR*) and *WOX5*, which drives their transcriptional activation allowing successful shoot regeneration after incubation on SIM [29]. In human gastrointestinal endocrine cells, LSD1-mediated H3K9me2 demethylation facilitate subsequent histone H3K9 acetylation by histone acetyltransferases, leading to gene activation [30]. Likewise, in Arabidopsis shoot regeneration, HAG1 might play roles in the LDL3-mediated gene priming, a hypothesis that now might be tested.

In a search for additional regulators of hormone-induced callus formation, the BASIC REGION/LEUCINE ZIPPER MOTIF 59 (bZIP59) transcription factor was identified on a screen for LBD17-partners, and its physical interactions with the other LBDs involved in auxin-induced callus formation were confirmed [31]. Interestingly, CIM or auxin treatment induced a post-translational accumulation of bZIP59 specifically in pericycle-like cells, and that enhanced its interaction with LBD16. Further results confirmed that bZIP59 and LBD16 act synergistically on a subset of LBD target genes that might directly contribute to callus formation [31]. Among the upregulated LBD targets identified so far [32], genes involved in cellular oxygen availability and activation of reactive oxygen species (ROS), cell wall remodeling and lipid metabolism deserve further investigation.

Additional regulation of callus-promoting LBD function by two MYB-domain transcription factors, have been recently described [33]. MYB94 and MYB96 regulate *LBD29* expression during callus formation through direct binding to its promoter, likely through the inhibition of pericycle-like cell competence in a novel, unknown regulatory pathway [33]. These MYB-domain transcription factors are involved in lipid metabolism in response to ABA or abiotic stress (i.e., drought and cold) by regulating the biosynthesis of very-long-chain fatty acids (VLCFAs). In plants, VLCFAs participate in the regulation of organ regeneration processes through its negative role in pericycle-like cell competence during auxin-induced callus formation [34]. Wild-type plants treated with a VLCFA biosynthesis inhibitor and mutants with altered VLCFA biosynthesis exhibited an over proliferation of cells in the leaf vasculature, a phenotype that was dependent on endogenous CK levels [35]. VLCFAs or their derivatives act non-cell autonomously to restrict pericycle-like cell competence and thereby prevent excess callus formation in response to external cues [34]. Interestingly, plasma membranes across juxtaposed cells display enrichment in sterols and sphingolipids with saturated VLCFAs that functionally define the plasmodesmata domain [36]. A direct link between VLCFA metabolism, plasmodesmata function and cell-to-cell trafficking has been recently established between sieve elements and phloem pole pericycle cells [37]. It is tempting to speculate that analogous cell-to-cell trafficking of an unknown non-cell autonomous signal (maybe acting on ALF4 regulation) between XPP and neighboring cells might restrict pericycle cell competence during regeneration.

3. Wound Signaling Regulates Tissue Regeneration through Conserved Gene Regulatory Networks

Our understanding of the molecular networks involved in wound-induced tissue regeneration has gained from recent results (Table 1) [38]. In *Arabidopsis thaliana*, the micro-surgical excision of the root tip leads to a quick re-specification of lost cell identities and to the re-establishment of a functional stem cell niche that allows complete organ regeneration (Figure 1b) [39]. By a combination of lineage tracing, single-cell RNA sequencing and marker analysis, it was shown that stem cells originate de novo from multiple tissues near the wound, on a process that required the activation of the MONOPTEROS transcription factor which is normally required for hypophysis specification during the formation of the embryonic root [40]. In addition, self-organizing auxin and CK interactions near the wound reset cell identities in this region and provide new positional cues to the dividing cells of the remaining meristem for the re-establishment of the developmental axes within the newly formed tissues [40].

The AP2/ERF transcription factor WOUND INDUCED DEDIFFERENTIATION 1 (WIND1), also known as RAP2.4, was identified as a central regulator for wound-induced cellular reprogramming in plants [41]. WIND1 is sufficient to establish and maintain dedifferentiated cell status without the exogenous addition of auxin and CKs. WIND1 is induced at the wound site where it promotes cell proliferation by the direct upregulation of ENHANCER OF SHOOT REGENERATION 1 (ESR1) [42].

Based on expression data and mutant analyses, CKs activate ESR1 expression through the B-type ARABIDOPSIS RESPONSE REGULATOR 1 (ARR1) and ARR12 [41,42]. Indeed, the *arr1 arr12* double mutants displayed reduced callus formation at wounded hypocotyls after shoot excision [43] but decreased rooting capacity from leaf explants [44], suggesting a complex regulation of CK signaling during tissue regeneration.

A recent study has contributed to clarify the intriguing results found for ARR1 and ARR12 in different regeneration models. ARR12 is a central enhancer of both callus formation and shoot regeneration whereas ARR1 inhibits regeneration through transcriptional activation of *AXR3* and that indirectly repress *WUS* expression [45]. Interestingly, MONOPTEROS binds the promoter of ESR1 and directly represses its transcription, providing a mechanistic model for auxin and CK crosstalk during regeneration [7]. ETHYLENE RESPONSE FACTOR 115 (ERF115), which was initially described as a rate-limiting factor for quiescent center (QC) cell division after DNA damaging stress [46], has been found to upregulate *WIND1* expression through its heterodimerization with PHYTOCHROME A SIGNAL TRANSDUCTION 1 (PAT1) [47]. These results are in agreement with a role of ERF115-PAT1 complex in driving the regeneration potential of root meristem cells in response to local cell death caused by wounding. However, the direct link between the wound signal and WIND1 expression have remained elusive until recently. Latest studies have shown that wounding produces changes in the H3K9/14 and H3K27 acetylation state of key reprogramming genes such as *WIND1*, *ERF113* or *LBD16* [48]. Moreover, it has been described that the histone variant HISTONE THREE RELATED 15 (H3.15), which lacks the PRC2-targeted K27 residue, is quickly induced after wounding. The absence of the H3K27me3 repressive mark in the H3.15 histones causes the de-repression of several key developmental genes, amongst which is *WOX11* [49]. The repressive mark H3K27me3 seems to be conserved in regenerative processes along the plant lineage. Indeed, the ectopic expression of the AP2/ERF-encoding gene *STEM CELL-INDUCING FACTOR 1* (*STEMIN1*) in *Physcomitrella patens* leaves causes the acquisition of stem cell properties in leaf cells through local reduction of H3K27me3 marks before cell division in a subset of STEMIN1 targets [50].

The stress hormone jasmonic acid (JA) plays well-established roles in wounding and defense responses. Downstream of the JA signal, the F-box protein CORONATINE INSENSITIVE1 (COI1) binds to JA and destabilizes the JA ZIM domain (JAZ) repressor proteins, allowing the positive regulators, such as the basic helix-loop-helix (bHLH)–domain containing MYC transcription factors, to induce their target genes [51]. JA promotes de novo root formation in Arabidopsis leaf explants [52]. After leaf excision, free JA and its active form JA-isoleucine (JA-Ile) are quickly upregulated within 10 to 30 min, and a time-course RNA-seq analysis identified the *ERF109* gene as a key factor for root regeneration [52]. Additionally, ERF109 was found to directly upregulate *ANTHRANILATE SYNTHASE α1* (*ASA1*), which encodes an enzyme involved in the tryptophan biosynthesis pathway [52]. Tryptophan is the precursor of auxin which, in turn, is upstream of the WOX11 activation required for hormone-induced callus formation (see above), as well as for de novo root regeneration [53]. The role for ERF109 in tissue regeneration, downstream of MYC2, was independently confirmed using the root-tip excision model [54]. Interestingly, the levels of *ERF109* induction after root tip excision depended on the position of the cut along the proximodistal axis of the root, which may correlate with the regeneration capacity of remaining tissues and was restricted to the root-ward region of the meristem [54]. Additionally, ERF109 was found to upregulate *ERF115* expression in cooperation with unknown auxin transcriptional regulators [54].

The molecular mechanism that restricts regenerative capacity during tissue culture along with plant age has been well-documented [55]. microRNA156 (miRNA156) repress the expression of several *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes, which causes progressive decline in shoot regeneration [56]. In addition, a role for miR156 during de novo root formation was proposed based on the reduced number of wound-induced ARs of plants transformed with *35S::MIM156*, which blocks the activity of miR156 and causes an increase in SPL expression [57,58]. In older leaves, SPL2, 10 and 11 directly bind to the promoters of a subset of wound-induced AP2/ERF transcription

factors, such as ABR1, ERF109, ERF115 and RAP2.6L, among others, and attenuate their induction, thereby dampening auxin accumulation at the wound (Figure 1b) [59].

Wound stress activates a set of AP2-ERF transcriptional regulators, including WIND1, WIND3, RAP2.6L, ERF114, ERF115, PLT3, PLT5 and PLT7, and they contribute to callus formation at wound sites [55]. It was described that *PLT3*, *PLT5* and *PLT7* regulate de novo shoot formation in root and hypocotyl Arabidopsis explants under CIM and subsequent SIM culture conditions [60]. *PLT3, PLT5* and *PLT7* are upregulated in mitotically active cells of callus tissue, regardless of the explant type, and their expression is progressively confined in clusters of cells forming the new shoot primordia upon their transfer to SIM [60]. Although the incubation of *plt3 plt5-2 plt7* explants in CIM can successfully achieve the formation of a callus mass, later culture of these *plt3 plt5-2 plt7* calluses in SIM did not produce any adventitious shoots, indicating their function is not essential during the reversion of the explant identity or during callus proliferation, but required for shoot initiation [60]. The authors demonstrated that PLT5-mediated induction of *PLT2* is required for calluses to develop shoot primordia, as this root stem cell regulator confers the regeneration competence required for shoot initiation from callus tissue. LR primordia exposed to high concentrations of CKs ectopically express *PLT3, PLT5* and *PLT7*, which induce subsequent *PLT2* expression and lead to direct de novo shoot regeneration [60]. In line with the proposed role for PLT2 regarding regeneration competence acquisition during indirect adventitious shoot formation, LR primordia of *plt3 plt5-2 plt7* mutants were not able to induce *PLT2* expression and no successful direct de novo shoot regeneration process was observed [60]. In this context, it was proposed that PLT2 is also responsible for the regeneration competence in ablated or completely RAM-excised roots, which undergo root meristem regeneration. The endogenous gradient of PLT2 of undamaged root tips determines the competence for root tip regeneration, and the transient overexpression of PLT2 confers regeneration potential to differentiating cells beyond the regeneration competence region, which usually comprises the last 210-250 μm of the root meristem [61]. In addition, the reduction of retinoblastoma-related (RBR) levels enhances the effect of PLT2 overexpression and leads to the re-entry of differentiated cells into organ formation programs [62]. Interestingly, the JA-triggered activation of root stem cells through the RBR-SCR network and stress response protein ERF115 leads to the restoration of root tip lost after resection [54]. As, such that the decline of PLT2 towards the shoot-ward end of meristem is causal for the drop in regeneration capability at this region [61].

A recent paper [63] shows that PLT3, PLT5 and PLT7 promote YUC4-mediated local auxin biosynthesis to induce procambium proliferation and vascular regeneration in damaged aerial organs, although, in this process, they seem to perform through *CUP-SHAPED COTYLEDON 2* (*CUC2*) induction, instead of *PLT2* [63]. As the adventitious roots arise from cambium tissue in the majority of plant species, it would be interesting to explore whether this regeneration module is conserved in other types of regeneration processes.

4. Somatic Embryogenesis: Stress, Auxin and Epigenetic Modifications as Key Players of Cell Totipotency Expression

The high regeneration competence of plants derives from the extreme developmental plasticity of plant cells that allows the formation of organs and bipolar embryos under specific conditions. Somatic embryogenesis (SE), the induction of embryos from different cell types and explants, without the fusion of gametes, is one of the best examples of plant cell totipotency [64,65]. SE induction can lead to the formation of embryos directly from a cell or group of cells of the explanted tissue (direct SE), or to the proliferation of masses of embryogenic cells that further produce embryos (indirect SE) [66,67] (Figure 2). Despite this process having been extensively studied as a plant regeneration model, an understanding of the regulatory mechanisms at the molecular and cellular levels is still elusive.

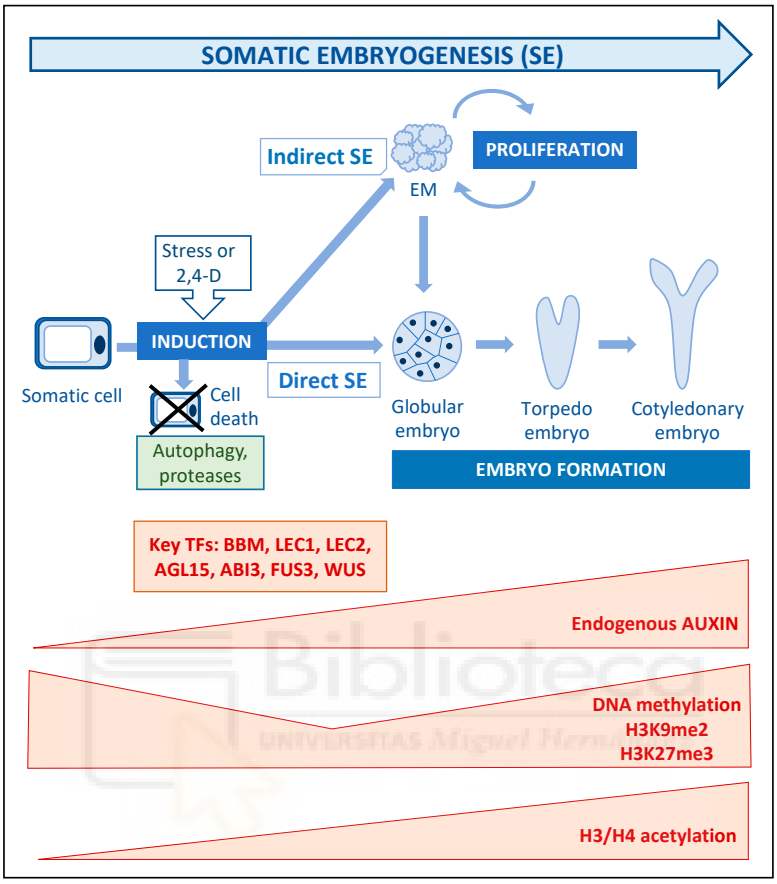

Figure 2. Schematic overview of somatic embryogenesis stages along the process. The presence and intensity of main regulatory factors (transcription factors, epigenetic modifications and auxin) are indicated in orange boxes (Table 1). Triggering factors and collateral cell death related processes are indicated in grey and green boxes, respectively. EM: embryogenic masses.

SE is considered a very powerful tool in plant biotechnology, as a feasible in vitro procedure for plant cloning and regeneration purposes [64]. Due to its great potential for large-scale clonal propagation and the cryopreservation of elite genotypes, as well as for production of genetically modified plants with improved traits, SE has been proven to be very useful for propagation of species with long reproductive cycles or low seed set in a large variety of crop and forest species [64,68–70]. In the case of microspore embryogenesis, the microspore (haploid cell, precursor of pollen grain) is reprogrammed towards an embryogenic pathway, by stress treatment [71]. The resulting haploid embryo, after spontaneous or chemically-induced diploidization, will produce doubled-haploid plants [72–74], which are widely used by seed and horticulture companies, since they provide unique source of new genetic variability, are homozygous at all genomic loci, and the allele fixation is accomplished very quickly, as compared to assortative mating schemes, like self-pollination [75,76]. Although SE is currently widely exploited, it is still highly, or even completely, inefficient in many species of economic interest. The low efficiency of embryo production in recalcitrant species presents serious limitations for widespread application of SE in the fields of agriculture and forestry. Together with its biotechnological application, SE represents a very interesting model to study cell reprogramming, totipotency acquisition and embryogenic

development, processes that involve the action of a complex signaling network which is not well understood yet.

The induction of SE is a multi-factorial developmental process that is usually initiated in response to exogenous stimuli produced by hormones, certain stress treatments (low or high temperature, osmotic shock, drought), or by a combination of both types of inductive conditions [65,66]. The stress treatment applied to switch the cell developmental program can also produce cell damage, and even partial or complete cell death. Recent reports have indicated that stress-induced cell death is a major factor that greatly reduces the yield of SE in various in vitro systems, particularly in microspore embryogenesis [77,78]. Markers of cellular death such as autophagy, the major catabolic process of eukaryotic cells, and cell death proteases (metacaspases, cathepsins and proteases with caspase 3-like activity) are activated during stress-induced microspore embryogenesis [71,79–82]. Pharmacological treatments with inhibitors of autophagy and proteolytic activities lead to the reduction of cell death, consequently increasing the embryogenesis initiation rate [79–81]. These novel findings are paving the way for new intervention pathways to increase cell viability in SE cultures.

The progress obtained on somatic embryogenesis in Arabidopsis has allowed the characterization of some genes involved in the molecular mechanisms underlying the complex regulatory networks that control SE. Exogenous auxins, either alone or in combination with other plant growth regulators, or stress, induce SE and the expression of different genes. Key transcription factors that have been found upregulated during the induction of SE in different species are members of the AINTEGUMENTA-LIKE (AIL) family, like BABY BOOM (BBM), PLT1 and PLT2, and others, such as AGAMOUS LIKE 15 (AGL15), FUSCA 3 (FUS3), LEAFY COTYLEDON 1 and 2 (LEC1, LEC2), RWP-RK DOMAIN-CONTAINING 4 (RKD4), ABA INSENSITIVE 3 (ABI3), and WUSCHEL (WUS) [66,83,84] (Table 1 and Figure 2). Some of these genes, such as *WUS*, *LEC1*, *LEC2* or *BBM*, have been reported to be responsible for the meristem/embryo identity during normal development, and their ectopic expression can also directly reprogram somatic cells and induce SE in the absence of exogenous stimuli [83].

The evidence supports the notion that auxins play a critical role in the reprogramming of somatic cells to SE [69,71]. In many SE protocols, treatment with exogenous auxin (usually 2,4-dichlorophenoxyacetic acid, 2,4-D) results in cell reprogramming, while SE initiation requires the subsequent elimination of auxin from culture media [65]. It has been proposed that the addition of 2,4-D to the culture medium induces an embryogenic response that is associated with the increase of the endogenous levels of indole-3-acetic acid (IAA) [84]. In various species, endogenous IAA levels have been shown to increase during SE initiation and embryo development [66,85,86]. In the microspore embryogenesis of *Brassica napus* and *Hordeum vulgare*, cell reprogramming is induced by stress without exogenous auxin in the culture media. However, endogenous auxin levels are highly increased in these species from the first embryogenic divisions in 2-3 cell proembryos [85,87] (Figure 2). Furthermore, SE is accompanied by the activation of endogenous auxin biosynthesis, polar transport and signaling pathways, as indicated by the up-regulation of auxin biosynthesis genes *YUC*, *TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1* (*TAA1*), and *TRYPTOPHAN AMINOTRANSFERASE-RELATED 2* (*TAR2*), polar transport gene *PIN-FORMED 1* (*PIN1*) and signaling genes *Aux/IAA* and *ARF* [85–89]. Interestingly, the use of inhibitors of auxin biosynthesis (kynurenine), polar auxin transport (N-1-naphthylphthalamic acid), and auxin antagonists (α-(pchlorophenoxy)-isobutyric acid), drastically impairs SE in monocot and eudicot species [85,87], indicating the key role played by this hormone in the process.

Together with hormones, epigenetic marks play an important role during SE induction and progression. Chromatin-modifying factors regulate conformational states of chromatin and its accessibility to transcriptional machinery. Epigenetic modifications, mainly DNA methylation and histone methylation and acetylation, are key factors contributing to the functional status of chromatin, that regulates gene expression, during cell proliferation and differentiation in both animals and plants [90]. In SE studies, the totipotency of cells was found to be associated with an open chromatin conformation [91]. Many studies have reported the ubiquitous epigenetic changes associated with SE

initiation. In particular, it has been found in a number of species that initial stages of cell reprogramming and embryogenesis initiation usually involve widespread DNA hypomethylation [91–94], histone H3K9 demethylation [95–97] and histones H3 and H4 acetylation [83,96] (Figure 2). In Arabidopsis, H3K27 methyltransferases of PRC2 have been associated with the prevention of pluripotency during cell differentiation, while PRC2 activity blocks hormone-mediated SE [98]. Compounds that inhibit enzymatic activities responsible of these epigenetic marks have been used in several in vitro embryogenesis systems, to manipulate ubiquitous epigenetic changes for promoting SE. Some epigenetic modulators that have been shown to promote SE induction are the inhibitors of DNA methyltransferases azacytidine and zeburaline [99,100], the inhibitor of histone methyltransferase, specific for H3K9, BIX-01294 [95], and the inhibitors of histone deacetylases trichostatin A or suberoylanilide hydroxamic acid [101,102]. Supplementing the culture medium with these small molecules induces widespread epigenetic changes that produce higher rates of initiation of SE. However, these epigenetic inhibitors also impaired embryo maturation. This effect can be explained by the fact that SE progression and embryo development are characterized by epigenetic features of cell differentiation, particularly by DNA hypermethylation and increasing H3K9me2 [94,95,100,103]. These findings reveal the crucial role of the epigenetic reprogramming in SE induction. Moreover, these studies are opening new possibilities to improve the efficiency of in vitro embryogenesis by the use of epigenetic modulators, which could extend the application of SE into propagation, breeding and conservation programs.

Investigation during recent years has suggested that cell reprogramming, totipotency acquisition and expression during SE is regulated by a complex interacting network, that includes crosstalk of epigenetic marks, transcription factors and auxin, a network that is repressed in somatic cells, but can be activated by exogenous stimuli, like stress or exogenous 2,4-D. In Arabidopsis, it has been proposed that the induction of SE leads to the removal of epigenetic repressor marks as DNA methylation, H3K9me2 or H3K27me3, and to increase histone acetylation, permitting the expression of specific transcription factors, such as LEC1, LEC2, BBM or AGL15, which would activate auxin biosynthesis and signaling, finally leading to totipotency acquisition and SE initiation [83,104]. This proposed SE regulatory network also involves the direct and indirect interactions between transcription factors and auxin homeostasis pathways and regulatory feedback loops [86]. However, much less is known on the regulatory mechanisms of SE in species other than Arabidopsis, and future studies will be necessary to determine the signaling pathways involved in crop and forest species, where SE is routinely developed, and to gain knowledge for the efficient manipulation and application of SE in recalcitrant species of agronomic and environmental interest.

5. Concluding Remarks

Although it was assumed that all plant cells are totipotent, recent studies suggest that only some of them remain in a pluripotent state throughout the plant life cycle, and it is from these cells that new organs develop in response to hormonal induction [105]. In addition, self-organizing auxin and CK interactions reset cell identities after wounding, and provide new positional cues for the re-establishment of the missing tissue through the re-deployment of embryonic development pathways [40]. During cell fate reprogramming in mammalian fibroblasts, the OSK (Oct4, Sox2 and Klf4) transcription factors act as pioneer factors to unwrap condensed chromatin and to induce pluripotent stem cell formation [106]. We propose that ARF7 might act as a pioneer transcription factor during hormone-induced callus formation, through their direct interaction with histone methylation modifiers [16]. The maintenance of the pluripotent state of animal stem cells requires hypoxic conditions, whereas higher oxygen tension promotes cell differentiation [107]. Transcriptional responses to hypoxia in Arabidopsis are mainly controlled by a group of five ERF-VII transcription factors, whose abundance is linked to oxygen levels [108,109]. A link between the establishment of hypoxic niches and plant stem function was recently established in the shoot apical meristem [110]. Additionally, in the LR primordia, the ERF-VII transcription factors bind to the promoters of the auxin-induced genes *LBD16* and *LBD18*, and repress their expression [111]. Hence, low oxygen levels within the new LR primordium

might directly interfere with auxin signaling, and could contribute to hindering the auxin-induced activation of neighboring pericycle cells, thus allowing a proper spacing between LRs [111]. As new hypoxia-responsive markers are now available [110], the contribution of low oxygen availability to hormone-induced callus formation could now be elucidated (Figure 1a).

Wounding also promotes tissue regeneration through an orchestrated network of AP2/ERF transcription factors that drive local auxin biosynthesis. Stress conditions can induce somatic cell reprogramming and totipotency expression in a number of cell types, through epigenetic regulators, a complex network of TFs and auxin homeostasis genes that promote embryo formation and plant regeneration (Table 1). Cell trafficking of transcription factors, the establishment of hypoxic niches and step-wise epigenetic reprogramming of regeneration-competent cells are emerging regulators of the tissue regeneration process, and further experiments using single-cell RNA sequencing, marker analysis and protein-protein and protein-DNA complex purification will enhance our understanding in this fascinating research field.

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Regulation of Hormonal Control, Cell Reprogramming, and Patterning during De Novo Root Organogenesis[1][OPEN]

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Body regeneration through formation of new organs is a major question in developmental biology. We investigated de novo root formation using whole leaves of Arabidopsis (*Arabidopsis thaliana*). Our results show that local cytokinin biosynthesis and auxin biosynthesis in the leaf blade followed by auxin long-distance transport to the petiole leads to proliferation of J0121-marked xylem-associated tissues and others through signaling of INDOLE-3-ACETIC ACID INDUCIBLE28 (IAA28), CRANE (IAA18), WOODEN LEG, and ARABIDOPSIS RESPONSE REGULATORS1 (ARR1), ARR10, and ARR12. Vasculature proliferation also involves the cell cycle regulator KIP-RELATED PROTEIN2 and ABERRANT LATERAL ROOT FORMATION4, resulting in a mass of cells with rooting competence that resembles callus formation. Endogenous callus formation precedes specification of postembryonic root founder cells, from which roots are initiated through the activity of SHORT-ROOT, PLETHORA1 (PLT1), and PLT2. Primordia initiation is blocked in *shr plt1 plt2* mutant. Stem cell regulators SCHIZORIZA, JACKDAW, BLUEJAY, and SCARECROW also participate in root initiation and are required to pattern the new organ, as mutants show disorganized and reduced number of layers and tissue initials resulting in reduced rooting. Our work provides an organ regeneration model through de novo root formation, stating key stages and the primary pathways involved.

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Plants have striking regeneration capacities, and can produce new organs from postembryonic tissues (Hartmann et al., 2010; Chen et al., 2014; Liu et al., 2014) as well as reconstitute damaged organs upon wounding (Xu et al., 2006; Heyman et al., 2013; Perianez-Rodriguez et al., 2014; Melnyk et al., 2015; Efroni et al., 2016). Intriguingly, root regeneration upon stem cell damage recruits embryonic pathways (Hayashi et al., 2006; Efroni et al., 2016), whereas in contrast, postembryonic formation of whole new organs, such as lateral roots, appears to use specific postembryonic pathways (Lavenus et al., 2013).

Cross talk between auxin and cytokinin signaling is required for many aspects of plant development and regeneration (El-Showk et al., 2013), although how their synergistic interaction is implemented at the molecular level has not been clarified (Skoog and Miller, 1957; Chandler and Werr, 2015). Exogenous in vitro supplementation of these two hormones results in continuous cell proliferation, to form a characteristic structure termed "callus". Callus emerges as a common regenerative mechanism for almost all plant organs through in vitro culture (Atta et al., 2009; Sugimoto et al., 2010). There is increasing evidence that callus formation requires hormone-mediated activation of a lateral and meristematic root development program in pericycle-like cells defined by expression of the J0121 marker

(Sugimoto et al., 2010). Accordingly, many regulators of lateral root development, such as AUXIN RESPONSE FACTOR7 (ARF7), ARF19, LATERAL ORGAN BOUNDARIES DOMAIN16 (LBD16), LBD17, LBD18, and LBD29, are required for hormone-induced callus formation (for review, see Ikeuchi et al., 2013).

Many species can regenerate new organs from explants (e.g. roots from leaves) without exogenous supplementation of hormones (Bellini et al., 2014). Making roots de novo requires generating the different tissues and cell types of the new organ. All roots have the same tissues, although the number of layers and cells types of these may vary (Kuroha et al., 2006; Lucas et al., 2011). Tissues are continuously formed by asymmetric division of initial cells, which are stem cells, followed by proliferative divisions of their daughter meristematic cells. Stem cell activity is maintained by a quiescent center (QC; van den Berg et al., 1997; Drisch and Stahl, 2015) and auxin activity (Della Rovere et al., 2013). Auxin accumulation in the QC area triggers a dose-dependent and slow response that activates PLETHORA (PLT) factors. PLT proteins form a gradient in the root meristem, which is required to position the QC, maintain stem cell activity, and trigger proliferation of meristematic cells (Aida et al., 2004; Mähönen et al., 2014). Position and activity of the QC also requires radial information delivered by the mobile factor SHORTROOT and its downstream target SCARECROW (Sabatini et al., 2003; Levesque et al., 2006; Moubayidin et al., 2016). In addition, WUSCHEL-RELATED HOMEOBOX5 (WOX5) is confined by auxin signaling into the QC and represses differentiation of the stem cell niche, primarily from the QC (Sarkar et al., 2007; Forzani et al., 2014; Pi et al., 2015; Zhang et al., 2015). Tissue formation in the primary root meristem also requires lineage-specific factors that function as cell fate determinants and as tissue endogenous signaling factors to incorporate positional information into patterning (Moreno-Risueno et al., 2015). However, little is known about how tissues are formed de novo.

Recently, a hormone-free method to study de novo root organogenesis in excised leaf blades has been described (Chen et al., 2014). *YUCCA*-mediated auxin biosynthesis was shown to be ubiquitously enhanced in the leaf mesophyll and indirectly contribute to auxin accumulation near the excision site to trigger localized auxin signaling in the vasculature (Liu et al., 2014; Chen et al., 2016). Formation of new roots involves formation of competent cells through auxin-induced expression of WOX11 transcription factor, which has been defined as a first step for cell fate transition during de novo organ regeneration (Liu et al., 2014). WOX11 and its homolog WOX12 can in addition promote callus formation and up-regulate the callus formation factors LBD16 and LDB29 (Fan et al., 2012; Liu et al., 2014), suggesting that de novo root formation might share similar regulatory mechanisms with callus formation. Subsequently in leaf blade rooting, WOX11 and WOX12 activate WOX5 and WOX7 factors, which are expressed in dividing cells forming root primordia, whereas WOX11/12 expression quickly decreases in dividing cells (Liu et al., 2014; Hu and Xu, 2016). Activation and maintenance of WOX5/7 expression also requires auxin signaling in an unknown pathway different from WOX11/12. Mutants in these WOX factors reduce the number of roots regenerated per leaf blades and affect rooting rate of leaf blades. As a considerably high percentage of leaf blades still root in these mutants, additional regulation must exist.

We have performed an extensive study to further understanding root regeneration from aerial organs. Whole leaves of many species can regenerate entire functional plants in hormone-free medium, and thus we used whole leaves with petioles of Arabidopsis (*Arabidopsis thaliana*) instead of excised leaf blades. We identified four developmental stages: 1) proliferation of some xylem-associated tissues, forming an endogenous callus; 2) specification of root founder cells within the callus; 3) root primordia initiation from founder cells and patterning; and 4) root meristem activation and emergence. We have also characterized a number of factors regulating these developmental stages. Some auxin and cytokinin signaling factors appear as critical for endogenous callus initiation and formation whereas some stem cell regulators control initiation and patterning of newly formed organs. These results define key stages and regulators required for leaf rooting establishing a developmental framework for de novo organ formation in plants.

RESULTS

Vasculature-Associated Cell Proliferation Is Required for De Novo Organ Regeneration in Arabidopsis

We found that excised whole leaves of Arabidopsis can root without hormone supplementation, similarly to leaf blades as previously described (Chen et al., 2014) and at similar percentages (Supplemental Fig. S1, A and B). Because some species can regenerate entire functional plants from whole leaves without the aid of external hormones, we performed our studies using whole leaves. As de novo formed roots emerged from the petiole base of whole leaves (Fig. 1A), petioles were microscopically observed (Fig. 1B). All petioles showed the same morphological changes during de novo organ regeneration. Although asynchrony was observed in the regeneration process, by 10 d after excision (dae) most leaves (85% to 100%) had regenerated at least one root. At 2 dae, cells adjacent to xylem started to proliferate, forming stratified layers from 3 dae onwards (Fig. 1, C to E) that pushed away xylem conducts and displaced the collenchyma. Vasculature proliferation and subsequent formation of primordia caused the proximal petiole to thicken (Supplemental Fig. S1C). First primordia were visible at 4 dae, and located at external layers of proliferating vasculature (Fig. 1F). At 5 dae, root primordia showed a layered pattern (Fig. 1G). Eventually, newly formed roots with well-organized

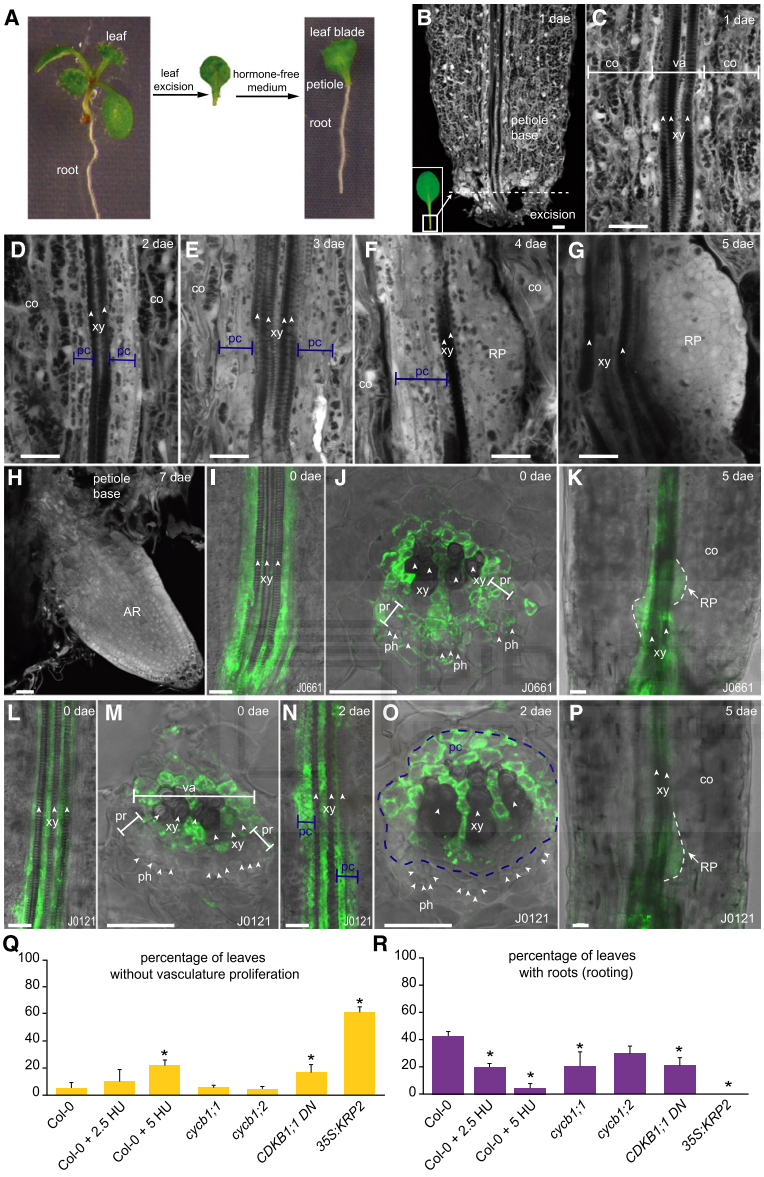

Figure 1. Developmental stages during rooting of Arabidopsis leaves. A, Whole leaf rooting procedure. B to H, Confocal microscopy pictures of petioles showing (C to E) proliferation of vasculature cells, (F and G) initiation and formation of primordia, and (H) de novo root emergence. I to K, J0661-GFP line in sections of petioles at (I and J) excision time or (K) 5 dae. L to P, J0121-GFP line in sections of petioles at (L and M) excision time, (N and O) 2 dae, and (P) 5 dae. J, M, and O, Transversal sections of petioles at the base. Scale bar: 25 μm. Q and R, Leaves of wild type and cell-cycle mutants, or upon treatment with HU, were assessed for (Q) vasculature proliferation and (R) rooting at 7 dae. Asterisks: statistically significant (P value < 0.05) by GLM and LSD posthoc test. AR, adventitious roots; co, collenchyma; pc, proliferating cells; ph, phloem; pr, procambium; RP, root primordium; va, vasculature; xy, xylem.

meristems emerged through petiole tissues from 7 dae onwards (Fig. 1H).

Pericycle-like cells (those expressing the J0121 reporter) have been associated to regenerative and morphogenic processes as the source of reprogrammable cells (Sugimoto et al., 2010; Chen et al., 2014). Sections of petioles at the time of excision revealed that the root-pericycle line J0661-GFP marks cells around xylem and procambium cells (Fig. 1, I to J), whereas the J0121-GFP line (Fig. 1, L and M) was restricted to a layer around xylem vessels, being excluded from procambium. Number of cells marked with J0661 and J0121increased quickly during first days of regeneration (Fig. 1, K, and N to P). We observed that all proliferating cells were marked with J0661-GFP whereas some proliferating cells in the J0121 line did not have the GFP (Fig. 1, K, and N to P), indicating that cell proliferation associated to the J0661 reporter. Although it cannot be ruled out

that J0661-GFP is activated in proliferating cells, it is possible that xylem and procambium proliferate as part of the reprogramming process. In addition, we observed that primordia at early stages of development were marked with J0121-GFP (Fig. 1P), establishing an association between de novo primordia formation and J0121 identity, similarly to other developmental or regenerative processes such as callus or lateral root formation (Dubrovsky el al., 2006; Sugimoto et al., 2010).

We next studied mutants defective in cell cycle progression, at the G1/S transition, such as the KIP-RELATED PROTEIN2 (KRP2) overexpressor, and at the G2/M transition, such as *cyclinB1;1* (*cycb1;1*) and *cycb1;2* mutants and a dominant negative form of the CDKB1;1 kinase (*CDKB1;1 DN161*). Percentage of petioles showing vasculature-associated proliferation was reduced in Pro_{35S}:*KRP2* and *CDKB1;1 DN161* lines (Fig. 1Q), whereas only size of proliferating mass of cells was reduced in the rest of the lines. In addition, Pro_{35S}:*KRP2* blocked de novo organ regeneration, whereas *cycb1;1* and *CDKB1;1 DN161* showed a significant reduction in the number of petioles regenerating roots (Fig. 1R). We also chemically inactivated the G1/S transition by incubating leaves with either 2.5 or 5 mM hydroxyurea (HU). We observed a significant decrease in rate of petioles showing vascular-associated proliferation and subsequent new root formation by the HU treatment (Fig. 1, Q and R; Supplemental Fig. S2, A and B). HU treatment did not associate with increased cell death around the vasculature near the leaf excision site upon Trypan Blue staining (Supplemental Fig. S2C). Taken together, these results indicate that cell division activation of vasculature cells is the first and required stage for de novo organogenesis during rooting of leaves.

Cytokinin Biosynthesis and Response during De Novo Root Regeneration

As we had found an association between de novo root regeneration and J0661 and J0121 identities, and callus originates from J0121-marked cells after hormonal induction (Sugimoto et al., 2010), we hypothesized that vasculature proliferation required for leaf rooting could be a type of callus. Cytokinin and auxin signaling are required for callus formation and regeneration, and thus we tested if these two hormones were involved in the developmental pathway leading to de novo organ regeneration from leaves.

First, we investigated cytokinin biosynthesis and signaling (Zürcher and Müller, 2016). We found enriched expression of Pro_{IPT3}:*GUS* in petioles right after excision (Fig. 2A). Pro_{IPT5}:*GUS* was highly expressed in vascular-associated cells in the petiole base at 2 dae (Fig. 2B), whereas Pro_{LOG4}:*GUS*, which was originally expressed in leaf vasculature at 0 dae, increased expression at the petiole base over time (Fig. 2C, arrowhead). Cytokinin signaling, as reported by Pro_{ARR5}:*GUS* (D'Agostino et al., 2000), was restricted to a subset of vascular-associated cells near the petiole base at 2 dae, which associated with proliferation of vasculature, to decrease at later time points (Fig. 2D) and it did not show expression during de novo primordia formation. Consistent with ARABIDOPSIS RESPONSE REGULATORS5 (*ARR5*) reporting primary cytokinin response during petiole vasculature proliferation (D'Agostino et al., 2000), incubation of leaf explants with synthetic cytokinin 6-benzylaminopurine (6-BAP) increased Pro_{ARR5}:*GUS* expression in the petiole vasculature and basal region (Fig. 2E).

Next, we studied the ability of several cytokinin signaling mutant combinations in *ARABIDOPSIS HIS KINASE4* (*AHK4*) and *ARABIDOPSIS HIS PHOSPHOTRANSFER PROTEIN1* (*AHP1*) to *AHP5* and *ARR1*, *ARR10*, and *ARR12* genes in regulating both vasculature proliferation and de novo root regeneration. We quantified petioles regenerating as petioles showing vasculature proliferation/thickening, root primordia formation, or visible roots. Leaf petioles of *wooden leg* (*wol*, a dominant negative mutant in AHK4 receptor) and *ahp1 ahp2 ahp3* and *arr1 arr10 arr12* loss-of-function mutants displayed lower regeneration percentage at 7 dae (Fig. 2F). Interestingly, *wol*, *ahp1 ahp2 ahp3*, and *arr1 arr10 arr12* mutants were also defective in hormone-induced callus formation from different tissue explants, such as leaves, cotyledons, and roots (Supplemental Fig. S3), indicating that specific cytokinin signaling is required for both callus formation and vasculature proliferation in leaf petioles. Despite cytokinin signaling being required for vasculature proliferation in petioles during rooting, for those leaf petioles of cytokinin signaling mutants that regenerated, we detected a higher number of roots (which we categorized by frequencies in numbers of roots and designated as "rooting capacity"; Fig. 2F). Higher auxin-to-cytokinin ratios have been shown to induce specification and growth of new root primordia (Müller and Sheen, 2008). Thus, we wondered if we could alter new primordia initiation by altering hormone ratios. Cytokinin treatment increased vascular proliferation in a concentration- and time-dependent manner (Supplemental Fig. S2, D and E). We observed that regeneration deficiencies of most cytokinin signaling mutants could be compensated by low levels of exogenous auxin (Fig. 2G) that also increased vasculature proliferation at the expense of reducing rooting capacity in the *ahp1 ahp2 ahp3* mutant (Fig. 2G). Taken together, these results indicate a dual role for cytokinin first as a positive activator of vasculature cell division, and second as a negative regulator of root primordia initiation.

Specific Auxin Signaling Factors Regulate De Novo Root Regeneration

Next, we investigated auxin signaling during rooting of leaves using the DR5 reporter line (Ulmasov et al., 1997). Pro_{DR5}:*GUS* was expressed in vascular-associated cells at the proximal region of the petiole, as early as 12 h after excision (hae), to increase quickly to 1 dae,

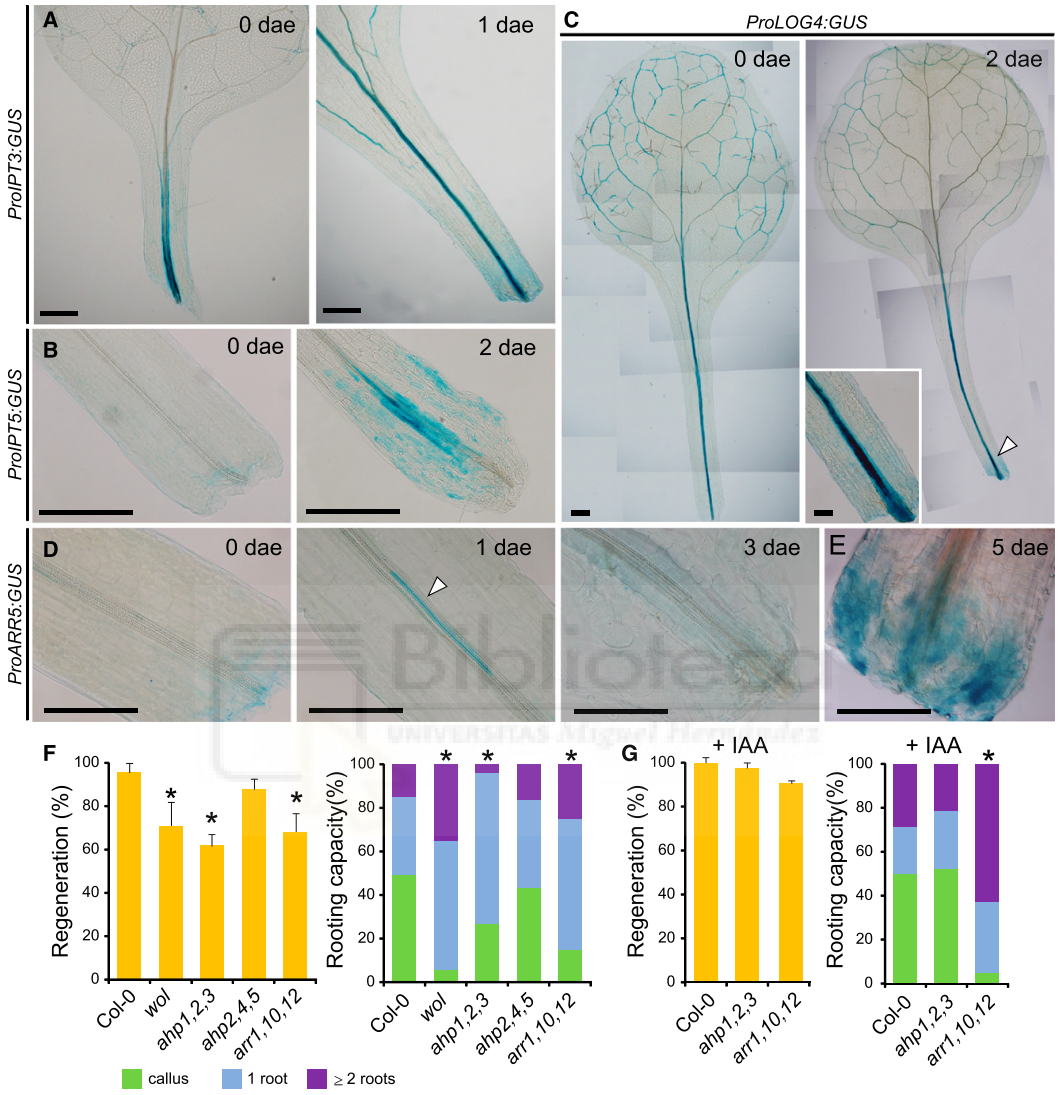

Figure 2. Cytokinin acts locally during de novo root regeneration. A and C, Expression of (A) Pro_{IPT3}:*GUS*, (B) Pro_{IPT5}:*GUS*, (C) Pro_{LOG4}:*GUS*, and (D and E) Pro_{ARR5}:*GUS* in petioles at indicated dae. E, Leaves were incubated with 5 μM 6-BAP. F, Mutants impaired in cytokinin signaling show defective regeneration (which accounts for petioles with vasculature proliferation, primordia, and roots; yellow barplots) and rooting capacity (multicolored barplots show frequencies of vasculature proliferation and of roots or primordia, indistinctly) at 7 dae. G, Cytokinin signaling mutant leaf explants incubated with 1 μM indole-3-acetic acid and measured for root regeneration responses as in (F). Scale bars: (A to C) 0.5 mm; (D and E) 0.25 mm. Asterisks: P value < 0.05 by GLM followed by LSD posthoc or χ^2 test.

remaining high during proliferative stages and decreasing over time coincident with deceleration of vasculature growth (Fig. 3, A and B). We observed high localized expression of Pro_{DR5}:*GUS* in clusters of cells at the time of primordia initiation and formation. Consistent with a regulatory role of auxin in rooting, local INDOLE-3-ACETIC ACID INDUCIBLE (IAA) application significantly increased root formation, along with expansion of Pro_{DR5}:*GUS* expression domain (Fig. 3, A and C). In addition, the auxin-overproducing

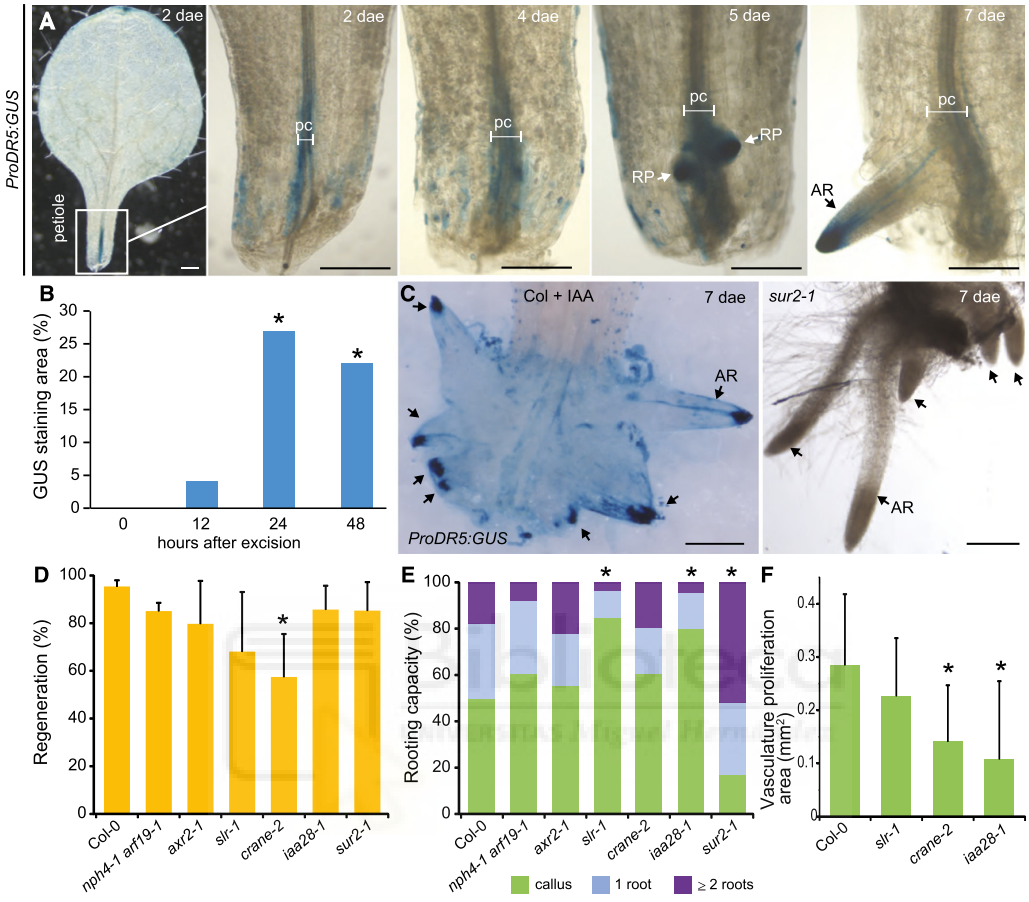

Figure 3. Distinctive auxin signaling pathways regulate de novo root regeneration. A and B, Auxin response reported by (A) Pro_{DR5}:*GUS* expression in Arabidopsis leaf petioles and (B) quantification of GUS-stained area. (C) Pro_{DR5}:*GUS* expression and de novo-formed adventitious roots in leaves supplemented with 1 μM indole-3-acetic acid (left) and in the *sur2-1* auxin overproduction mutant (right). D, Regeneration percentage and (E) rooting capacity at 7 d after excision of auxin signaling mutants (*nph4-1 arf19-1*, *axr2-1*, *slr-1*, *crane-2*, and *iaa28-1*) and auxin overproducer *sur2-1*. F, Vasculature proliferation area on Arabidopsis leaves cultivated for 14 d in hormone-free medium. Scale bars: 200 μm. Asterisks: P value < 0.05 by GLM followed by LSD posthoc or χ^2 test. AR, adventitious roots.

mutant *superroot2* (*sur2*; Barlier et al., 2000) also showed increased number of de novo formed roots at 7 dae, similarly to auxin-treated petioles (Fig. 3C).

Auxin signaling is regulated by IAA cofactors acting in combination with AUXIN RESPONSE FACTOR (ARF) transcriptional partners (Li et al., 2016). We reasoned that IAA factors regulating postembryonic (lateral) root formation (Goh et al., 2012) could be involved in de novo organ formation. We assessed the mutants *auxin resistant2-1* (*axr2-1*), *solitary-root-1* (*slr-1*), *crane-2*, *iaa28-1*, and the double mutant *nonphototrophic hypocotyl4-1 arf19-1* (see "Materials and Methods"); which are, respectively, gain-of-function mutants for the factors IAA7, IAA14, IAA18, IAA28, and a double loss-of-function mutant for ARF7 and ARF19. *crane-2* reduced de novo root regeneration, with approximately 40% of petioles not showing any sign of vasculature proliferation or de novo root formation (Fig. 3D). In addition, *slr-1* and *iaa28-1* showed reduced rooting capacity, although all petioles showed some vasculature proliferation (Fig. 3E). When we quantified vasculature proliferation area of petioles regenerating, we observed a reduction for *iaa28-1* and *crane-2* but not for *slr-1* (Fig. 3F). These results indicate that the auxin signaling module mediated by IAA18 is required for de novo root regeneration at stages of vascular proliferation, that of

IAA28 for vascular proliferation and root initiation, whereas IAA14 appears to be required only for de novo root initiation.

We also investigated if these mutants were affected in hormone-induced callus formation. We found that only *crane-2* showed reduction in all explants assayed after hormonal incubation, whereas *axr2-1* intriguingly showed an increase for callus formed from root explants (Supplemental Fig. S4, A to C). These results indicate that vasculature proliferation during rooting and hormone-induced callus use the auxin signaling pathway mediated by IAA18. Our previous results also showed that cytokinin signaling required for vasculature proliferation during de novo organogenesis was also required for hormone-induced callus formation, suggesting that vasculature proliferation is a type of callus. We investigated ABERRANT LATERAL ROOT FORMATION4 (ALF4) during leaf rooting, as *aberrant lateral root formation4* (*alf4*), in this case, *alf4-1* mutants, have been linked to callus formation (Sugimoto et al., 2010) and vascular connection during graft establishment (Melnyk et al., 2015). We observed that during de novo root formation, vasculature proliferation is reduced by 2.5-fold in *alf4-1* mutants, which is accompanied by 15-fold decrease in de novo formed root and primordia (Supplemental Fig. S5). Based on these results, we designated the vasculature proliferation developmental stage as the endogenous callus formation.

Auxin Signaling Factors Are Required for De Novo Organ Regeneration in Leaf Blades

In contrast to endogenous callus formation observed in petioles of whole leaves during rooting, limited vasculature proliferation was observed during rooting of leaf blades (Liu et al., 2014). We wondered to what extent auxin and cytokinin signaling factors regulating proliferation at the petiole base would be involved in rooting of leaf blades. When we assessed rooting capacity in the leaf blades of these mutants, we observed that *crane-2* and *iaa28-1* displayed a reduction in rooting capacity whereas *slr-1* presented moderate although nonsignificant reductions (Supplemental Fig. S6A). *wol* and *arr1 arr10 arr12* mutants were similarly affected as *slr-1*, whereas no change was detected for *ahp1 ahp2 ahp3* and *ahp2 ahp4 ahp5* mutants (Supplemental Fig. S6B). As IAA18 and IAA28 are required for endogenous callus formation during whole leaf rooting and are shared with leaf blade rooting, it is possible that de novo root regeneration in leaf blades could also involve an endogenous callus developmental program.

Local Auxin Accumulation at the Petiole Base Is Dependent on Polar Auxin Transport

As localized auxin signaling was required for whole leaf rooting, we wondered about the source of auxin. *YUCCA*-mediated auxin biosynthesis was shown to be ubiquitously enhanced in the mesophyll of leaf blades shortly after wounding (Chen et al., 2016). During rooting of whole leaves, we detected Pro_{YUC9}:*GUS*-enriched expression in leaf mesophyll cells at 12 hae, which progressively decreased at later time points (Supplemental Fig. S7A). Pro_{YUC8}:*GUS* expression was induced in proliferating vascular-associated cells at the petioles' bases 2 dae (Supplemental Fig. S7B). These results indicate two possible sources of auxin during first stages of regeneration, one from the leaf blade, and the other from the proliferating vasculature itself. To determine if there was differential contribution of these two auxin sources, we removed the leaf blade and found a significant decrease in regeneration and rooting capacity, which in most cases stopped at the endogenous callus stage (Fig. 4A). Local auxin response (Pro_{DR5}:*GUS* expression) in petioles without leaf blade was low or undetectable (Fig. 4B). We also locally inhibited polar auxin transport through application of N-1-naphthylphthalamic acid (NPA) at the blade-petiole junction, resulting in almost complete block of auxin response and subsequent regenerative response (Fig. 4, C and D).

We next characterized PIN-FORMED (PIN) expression. Pro_{PIN4}:*GUS* was ubiquitously expressed in the leaf vasculature at the time of excision; however, at 1 dae it was only expressed at the base of the petiole and in endogenous callus at 3 dae (Fig. 4E). Pro_{PIN3}:*PIN3*:*GFP* expression in the petiole at the time of excision was polarized toward the base in epidermal cell membranes and both laterally and basally localized in some vascular-associated cell membranes (Fig. 4F, arrowheads). From 12 hae to 1 dae, we found enriched expression of Pro_{PIN3}:*PIN3*:*GFP* in a subset of vascular-associated cells at the petiole base region (Fig. 4G). Interestingly, PIN3-GFP protein in cells proximal to the excision was oriented toward the apex (upper-left direction in Fig. 4G, arrowheads) whereas its orientation changed to the base in cells at the distal position from the excision.

We also investigated rooting in mutants of genes affected in auxin influx (*AUX1*) or auxin efflux (*PIN1*, *PIN2*, *PIN3*, and *PIN7*), which have been described to have low auxin transport rates (Petrásek et al., 2006). Consistent with our previous observations, the regenerative potential of leaves of *pin1*, *pin2 pin3*, *pin2 pin3 pin7*, and *aux1* mutants was reduced (Fig. 4H). GNOM loss-of-function mutants have altered polar auxin transport by interfering with PIN internalization (Kleine-Vehn et al., 2009). When we studied the GNOM mutant *fewer* (*fwr*; Okumura et al., 2013), we observed significant differences in regeneration and rooting capacity (Fig. 4H). *fwr* is a weak *gnom* allele but it is possible that several auxin transporters are simultaneously affected, which could explain why there is also a reduction in rooting capacity whereas no reduction was observed for single auxin transporter mutants.

Postembryonic Root Founder Cells Establish on Endogenous Callus Prior Primordia Formation

WOUND INDUCED DEDIFFERENTIATION1 (WIND1) is rapidly induced at the wound site to

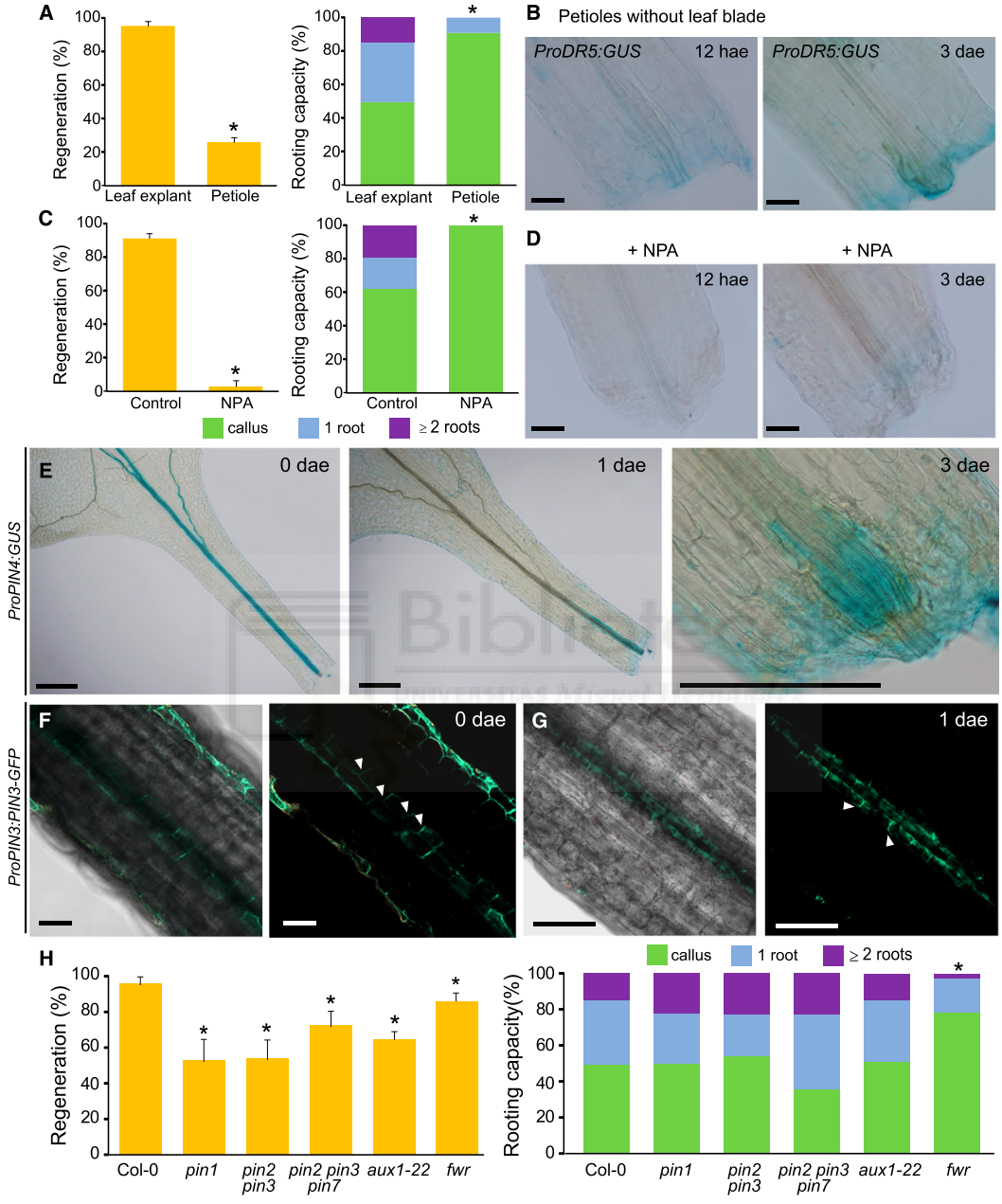

Figure 4. Polar auxin transport from the leaf blade to the petiole is required for rooting. A and B, Distal blade excision reduces (A) regeneration rate and rooting capacity at 7 dae and (B) Pro_{DR5}:*GUS* expression at 12 hae and 3 dae. C and D, Local treatment at the blade-petiole junction with 1% NPA reduces (C) regeneration rate and rooting capacity at 7 dae, and (D) Pro_{DR5}:*GUS* expression. E to G, Expression of (E) Pro_{PIN4}:*GUS* and (F and G) Pro_{PIN3}:*PIN3*:*GFP*. Arrowheads point to membrane-localized PIN-GFP. Scale bars: (B, D, F, and G) 0.2 and (E) 0.5 mm. H, Regeneration and rooting capacity in auxin transport mutants at 7 dae. Asterisks: *P* value < 0.05 by GLM followed by LSD posthoc or χ^2 test.

promote callus formation through the ARR-dependent signaling pathway (Iwase et al., 2011). From 1 to 4 dae, we found Pro_{WIND1}:*GUS* expression in vascular cells at the petiole near the excision site, whereas expression was downregulated in new root primordia and no expression was detected at the time of root emergence by 6 dae (Fig. 5A). Postembryonic development involves specification of organ founder cells (Chandler, 2011). Thus, we hypothesized that root founder cells (RFCs) could be specified within the endogenous callus to de novo form a root. It has been proposed that RFCs during de novo root formation in leaf blades could be marked by Pro_{WOX11}:*GUS* expression (Liu et al., 2014; Hu and Xu, 2016). We observed discrete Pro_{WOX11}:*GUS* signals early detected, at 1 dae, in a few xylem-associated cells at the petiole base (Fig. 5B). From 1 dae onwards, during callus formation, high Pro_{WOX11}:*GUS* expression was observed in many cells within this domain, but not simultaneously in all proliferating cells. Later on, Pro_{WOX11}:*GUS* expression was observed near the central zone of the endogenous callus but excluded from the dome-shape root primordia. WOX11 expression thus appears to associate with endogenous callus formation, although not all marked cells resulted in primordia initiation. We wondered whether cell division of vascular-associated cells was downstream of the WOX11 signal. Pro_{WOX11}:*GUS* expression was increased in vasculature of petioles of excised leaves incubated with the G1/S cell cycle inhibitor hydroxyurea, even when little vasculature proliferation was observed (Fig. 5, E and F).

Pro_{SKPB2s}:*GUS* expression has been shown to mark RFCs and their progeny during early stages of lateral root formation (Manzano et al., 2012). We detected Pro_{SKPB2s}:*GUS* expression at 3 dae restricted to few cells within the endogenous callus (Fig. 5C). From 4 to 5 dae, we observed marker expression in developing root primordia whereas its expression disappeared from functional primordia during the emerging process, remaining in some cells of the endogenous callus (Fig. 5C). Interestingly, RFCs did not appear to be specified simultaneously, supporting our initial observations about asynchrony in the regeneration process. To follow primordia formation, we used Pro_{WOX5}:*GUS*. WOX5 is expressed early during lateral root primordia formation (Tian et al., 2014; Goh et al., 2016), and also after root primordia initiation during de novo organogenesis (Liu et al., 2014; Hu and Xu, 2016). Expression of Pro_{WOX5}:*GUS* was first detected at 4 dae in clusters of cells within the endogenous callus (Fig. 5D), similarly to Pro_{SKPB2s}:*GUS* after root primordia initiation. At 6 dae, Pro_{WOX5}:*GUS* expression was enriched at root meristem tip, coinciding with meristem activation and growth prior emergence. Interestingly, we did not detect Pro_{WOX5}:*GUS* in the endogenous callus (Fig. 5D), although *WOX5* expression has been associated to hormone-induced callus (Sugimoto et al., 2010), suggesting specific regulation.

Cell division is required for primordia initiation and formation, and petioles of leaves treated with the cell cycle inhibitor HU, which started to form an endogenous callus, remained almost blocked at this stage (Fig. 5G). We wondered if general regulators of cell cycle progression would be involved in de novo primordia formation. Our results indicate that rooting capacity is compromised in *cycb1;1*, *cycb1;2*, and *CDKB1;1 DN161* mutants. Accordingly, overexpression of the cell cycle inhibitor KRP2 blocked progression to root initiation and formation, along with reduction in callus formation by 60% previously shown in Figure 1Q. Taken together, our results indicate that *WIND1* and *WOX11* expression associates with vasculature proliferation leading to endogenous callus formation at the petiole base, whereas Pro_{SKPB2s}:*GUS* is restricted to a few RFCs within the callus that quickly acquire *WOX5* expression associated to cell division, in turn mediated by CYCB1;1, CYCB1;2, CDKB1;1, and KRP2, to initiate de novo root primordia regeneration (Fig. 5G).

SHORT-ROOT, PLETHORA1, and PLETHORA2 Are Required for De Novo Initiation of Roots

We investigated whether factors specifying stem cell fate or their activity, such as PLETHORA (PLT), JACKDAW (JKD), BLUEJAY (BLJ), SCARECROW (SCR), SHORT-ROOT (SHR), SCHIZORIZA (SCZ), and WOX5 were required during de novo root regeneration. In addition to single loss-of-function mutants, double *plt1-4 plt2-2* and triple *blj-1 jkd-4 scr-4*, we generated and tested the double mutant *jkd-4 scz-1*. We found significant differences in percentage of leaves rooting and in rooting capacity for *shr-2* and *plt1-4 plt2-2* (Fig. 6, A and B). In addition, *scr-4*, *blj-1 jkd-4 scr-4*, *scz-1*, and *jkd-4 scz-1* were impaired in rooting capacity. These phenotypes suggested impairment in de novo root initiation or primordia formation. As in *shr-2* and *plt1-4 plt2-2*, many leaves failed to regenerate any root. We generated *shr-2 plt1-4 plt2-2*. Notably, leaves of *shr-2 plt1-4 plt2-2* were not capable of rooting (Fig. 6, A and C).

Petioles of *shr-2 plt1-4 plt2-2* were observed through confocal microscopy at 6, 10, and 20 dae. We did not observe any primordia at 6 dae in *shr-2 plt1-4 plt2-2*, whereas most control leaves had one or more primordia (Fig. 6D). Inspection at later days indicated that most leaves (97%, $n = 90$) of *shr-2 plt1-4 plt2-2* did not form any primordia up to 20 dae. The few primordia found (3%, $n = 90$) remained blocked or developed aberrant shapes with presence of mature xylem indicating premature differentiation. These primordia did not emerge through petiole tissues. We observed endogenous callus formation, which maintained growth over time up to 20 dae in all observed petioles of *shr-2 plt1-4 plt2-2*. These results indicate that the combined activity of SHR and PLT1 and PLT2 is required to de novo initiate root primordia. In addition, these factors maintain proliferative activity in the forming primordia; when these factors are removed, primordia differentiate.

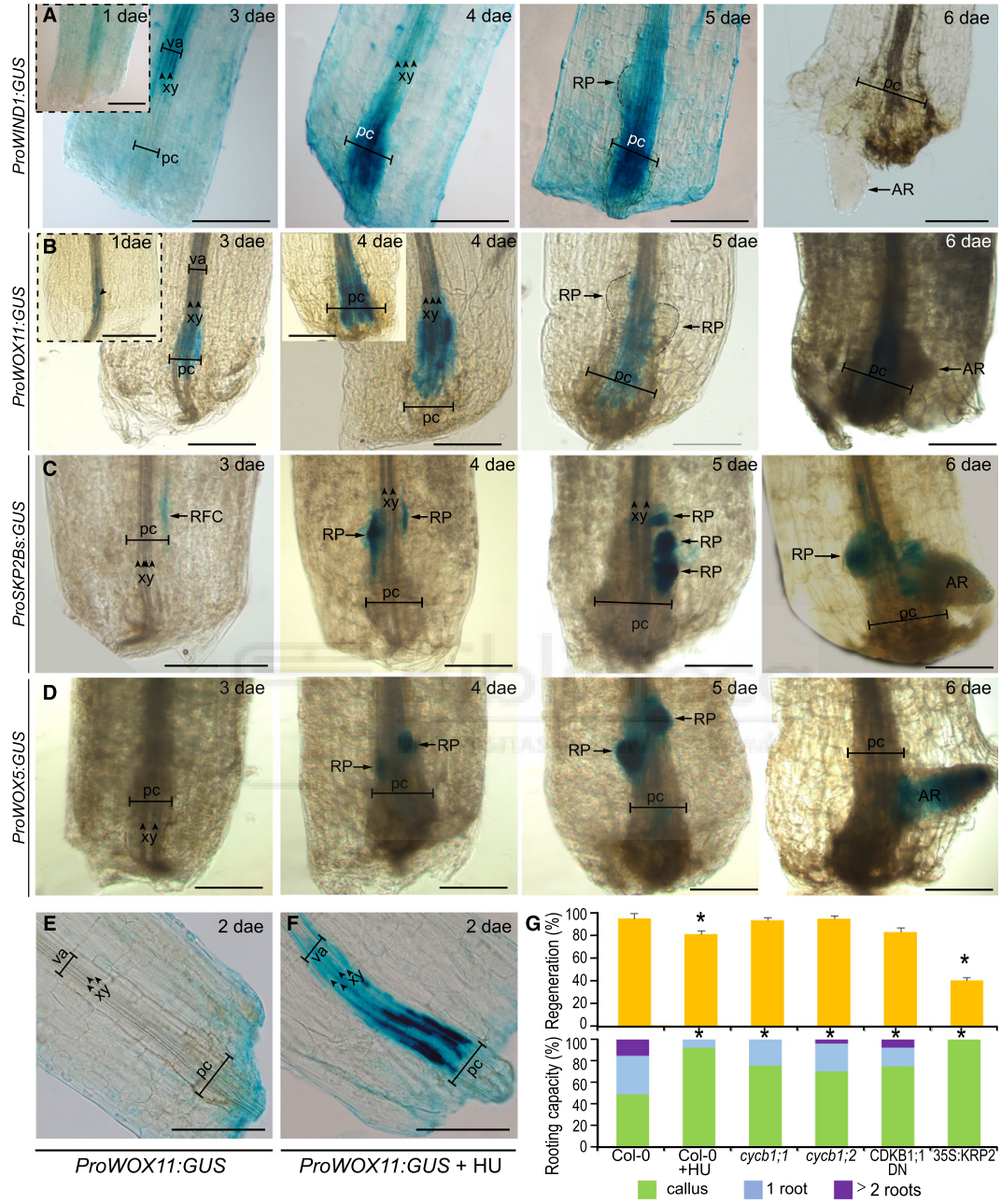

Figure 5. Root founder cells establish on endogenous callus prior de novo primordium formation. A to F, Expression of (A) Pro_{WIND1}:*GUS*, (B, E, and F) Pro_{WOX11}:*GUS*, (C) Pro_{SKP2Bs}:*GUS*, and (D) Pro_{WOX5}:*GUS* in leaf petioles at indicated dae. E, Control conditions or (F) upon 5 mM HU treatment. GUS staining time in (E) was set for no expression. Scale bars: 200 μm. G, Regeneration percentage and rooting capacity at 7 dae of mutants impaired in cell cycle progression and HU-treated Col-0 leaves. Asterisks: P value < 0.05 by GLM followed by LSD posthoc or χ^2 test. DN, de novo-formed root; pc, proliferating cells; RFC, root founder cell; RP, root primordium; va, vasculature; xy, xylem.

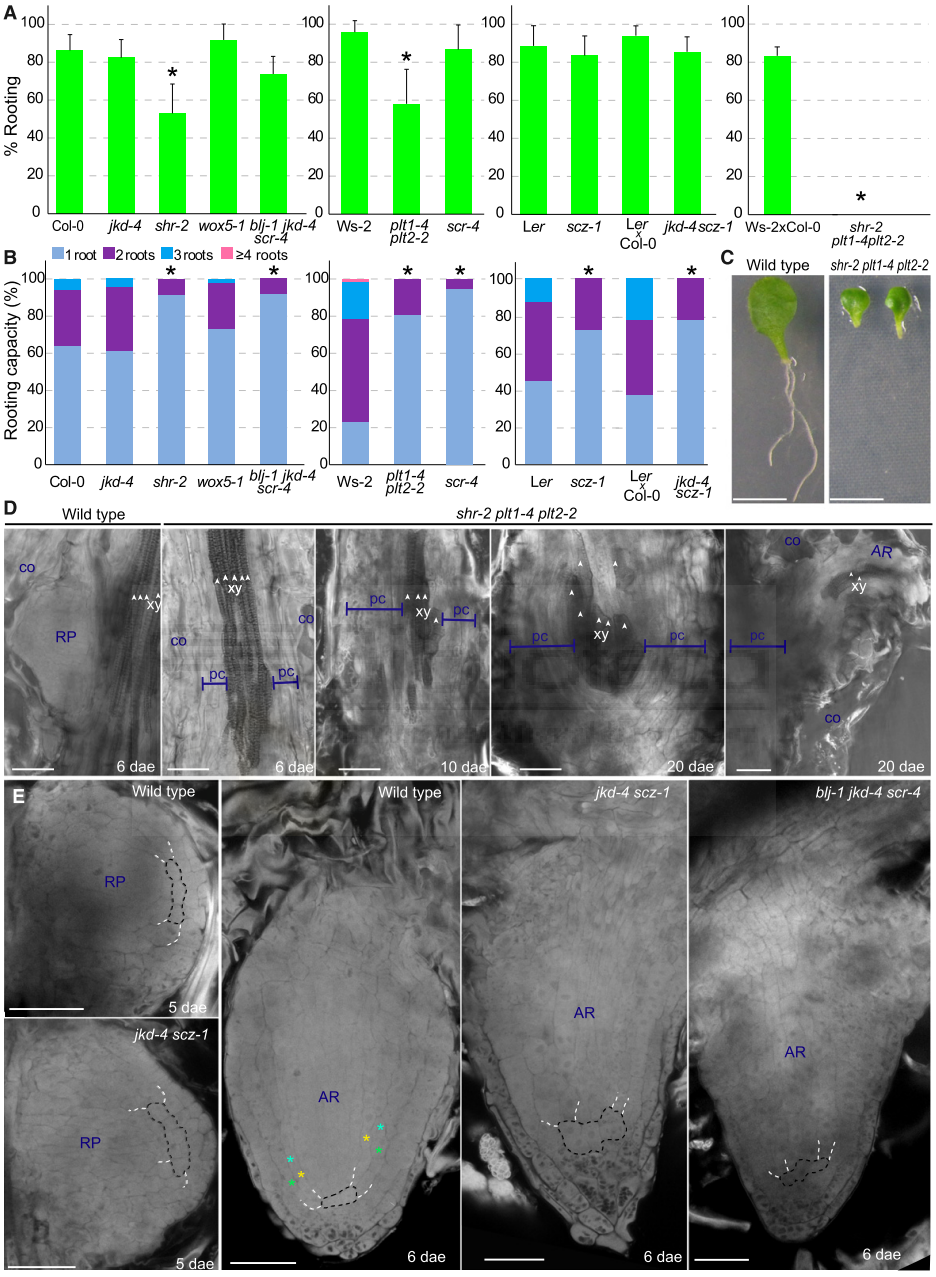

Figure 6. SHORT-ROOT, PLETHORA1, PLETHORA2, JACKDAW, BLUEJAY, SCARECROW, and SCHIZORIZA are required for de novo root primordia initiation and formation. A and B, Rooting percentage and rooting capacity, respectively, at 10 dae for loss-of-function mutants in stem cell regulators. Asterisks: *P* value < 0.05 by GLM followed by LSD posthoc or χ^2 test. C, Triple *shr-2 plt1-4 plt2-2* mutant does not regenerate roots at 10 dae. D, Confocal images of control and *shr-2 plt1-4 plt2-2* during root regeneration. Malformed primordia (3%, *n* = 90) in *shr-2 plt1-4 plt2-2* do not emerge through petiole tissues. E, Confocal images of de novo root primordia of wild type, *jkd4-4 scz-1*, and *blj-1 jkd-4 scr-4* at 5 and 6 dae. Black dashed line corresponds to cells in the

JACKDAW, BLUEJAY, SCARECROW, and SCHIZORIZA Are Required for De Novo Formation of Root Primordia

We observed formation of root primordia in petioles of *jkd-4 scz-1* and *blj-1 jkd-4 scr-4* through confocal microscopy. We observed abnormal formative divisions in *jkd-4 scz-1* primordia at 5 dae, which did not organize properly in layers or rows as compared to control roots (Fig. 6E). At 6 dae, we observed reduced and disorganized number of cell rows in *jkd-4 scz-1* and *blj-1 jkd-4 scr-4* primordia. Although endodermis, cortex, and middle cortex could be identified in control roots at this developmental stage based on position, corresponding rows in *jkd-4 scz-1* and *blj-1 jkd-4 scr-4* could not be identified (Fig. 6E). These results suggest that cell lineages or positional identity could not be correctly established in these mutants during de novo root formation.

JACKDAW, BLUEJAY, SCARECROW, SHORT-ROOT, and SCHIZORIZA Regulate Patterning of De Novo Formed Roots and of Lateral Roots

Primordia formation occurred incorrectly in *blj-1 jkd-4 scr-4* and *jkd-4 scz-1*, and therefore patterning of these de novo formed roots could be compromised after emergence. Longitudinal- and cross sections of de novo root meristems were examined through confocal microscopy after emergence at 10 dae (encompassing roots at 1 d to 3 d postemergence). The de novo wild-type roots showed ground tissue with middle cortex formation, a layer located between endodermis and cortex and associated to postembryonic development (Paquette and Benfey, 2005), whereas de novo roots of *shr-2*, *scr-4*, *blj-1 jkd-4 scr-4*, and *jkd-4 scz-1* had a single layer of ground tissue that we denoted the "mutant layer" (Fig. 7A; Fig. S8, A and B). In addition, the number of cell rows making the ground tissue, which indicates the number of tissues initializing, was reduced in de novo emerged roots of *blj-1 jkd-4 scr-4* and *jkd-4 scz-1* as compared to wild-type roots (Fig. 7E; Supplemental Fig. S8C). As a result, the stele region in these mutants was not delimited by a closed ring of ground tissue as in wild-type roots, as shown in cross sections (Fig. 7B). In addition, *shr-2*, *scr-4*, *blj-1 jkd-4 scr-4*, and *jkd4 scz-1* mutants also had a smaller stele region (Fig. 7, B and C; Supplemental Fig. 8, D and E).

Lateral roots are organs formed postembryonically the same as de novo-formed roots. We decided to investigate if stem cell regulators regulating de novo root formation could also regulate lateral root formation. We quantified lateral root capacity. In this assay, the root tip is cut to induce growth of lateral roots as described previously (van Norman et al., 2014). We confirmed, under the microscope, there were no unemerged primordia. We observed reduced lateral root capacity in *shr-2 plt1-4 plt2-2*, *blj-1 jkd-4 scr-4*, and *jkd-4 scz-1* mutant combinations indicating defects in founder cell specification or lateral root initiation (Fig. 7D), similarly to defects observed in these mutants during de novo root formation. We also observed patterning defects in emerged lateral roots of these mutants, which showed a single mutant layer of ground tissue and reduced number of cell rows as compared to wild-type lateral roots (Fig. 7E). In addition, we observed signs of premature differentiation in *shr-2 plt1-4 plt2-2*, with presence of mature xylem in the meristem similarly to de novo formed roots.

DISCUSSION

Plants have the remarkable ability to regenerate a new entire individual, specific organs, or their tissues from explants or even a few cells (Birnbaum and Sánchez Alvarado, 2008; Della Rovere et al., 2016). Plant regeneration through de novo organogenesis can be achieved through hormonal induction, directly or indirectly (Ikeuchi et al., 2013), but also in hormone free medium. The molecular pathways involved (Ikeuchi et al., 2016; Kareem et al., 2016) and the relationship between hormonal-induced and endogenous programs are not well understood. Moreover, callus formation, which is a prerequisite for hormonal-induced regeneration, does not appear to occur during endogenous organogenesis, although both processes share regulation (Sugimoto et al., 2010; Liu et al., 2014; Perianez-Rodriguez et al., 2014; Ramirez-Parra et al., 2017). Using a simple method to study de novo root organogenesis without hormone supplementation (Chen et al., 2014; Liu et al., 2014) but applied to whole leaves (Fig. 8A), we found that formation of an endogenous callus is a required step for de novo root organogenesis, and thus we established a direct connection between both regenerative processes. Our research has also identified the distinctive and required developmental stages leading to novo root organogenesis (Fig. 8B): 1) vasculature proliferation and endogenous callus formation, 2) root founder cell specification, 3) root primordia initiation and patterning, and 4) root meristem activation and emergence. Furthermore, we have genetically dissected the primary developmental pathways involved in its regulation and identified some of the key regulators involved (Fig. 8C).

Figure 6. (*Continued.*)
position of quiescent center; white dashed line cells correspond to the position of the ground tissue in contact to quiescent center. Green asterisk, cortex; turquoise asterisk, middle cortex; yellow asterisk, endodermis. Scale bars: (C) 5 mm and (D to I) 50 μm. AR, adventitious root; co, collenchyma; pc, proliferative cells; RP, root primordium; xy, xylem.

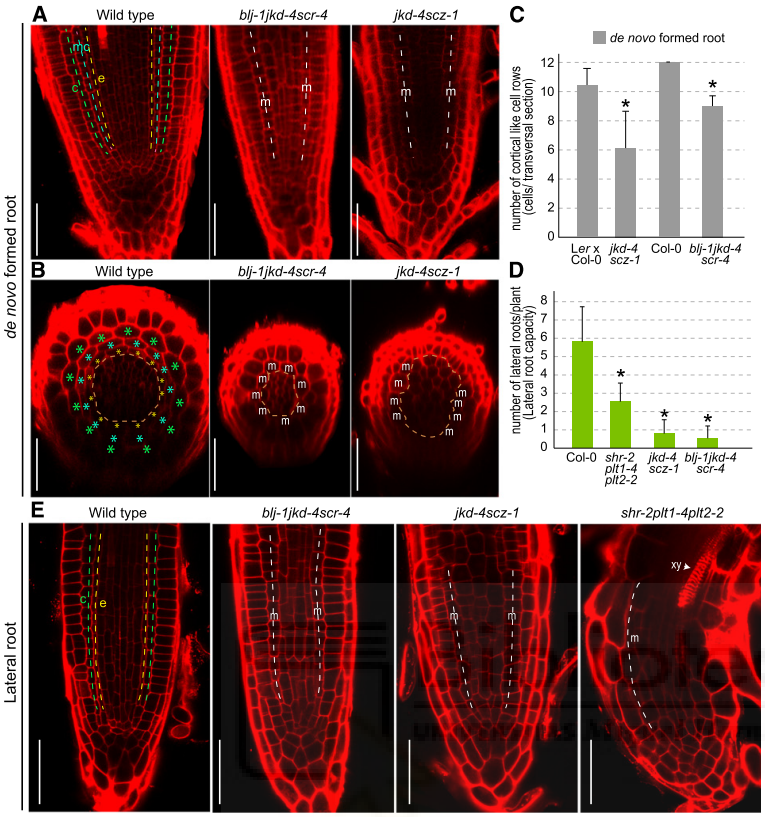

Figure 7. JACKDAW, BLUEJAY, SCARECROW, SHORT-ROOT, and SCHIZORIZA regulate patterning of postembryonic roots. A and B, Confocal (A) longitudinal and (B) transversal sections of de novo-formed roots at 10 dae (1 to 3 d post emergence). Transversal sections were taken at the end of the lateral root cap. Dashed lines and asterisks: green, cortex; turquoise, middle cortex; yellow, endodermis; brown, stele; white, mutant undivided ground tissue. C, Number of cortical-like cell or undivided mutant cell rows in control accessions, *jkd-4 scz-1* and *blj-1 jkd-4 scr-4*. D, Lateral root capacity in Col-0, *shr-2 plt1-4 plt2-2*, *jkd-4 scz-1*, and *blj-1 jkd-4 scr-4* and at 4 d after seed imbibition. E, Confocal longitudinal sections of emerged lateral roots of Col-0, *shr-2 plt1-4 plt2-2*, *jkd-4 scz-1*, and *blj-1 jkd-4 scr-4* at 7 d after seed imbibition. Scale bars: 50 μm. Asterisks: *P* value < 0.05 by GLM and LSD posthoc test. m, mutant; xy, xylem.

Vasculature Proliferation and Endogenous Callus Formation

Vasculature division is the first morphological change we detect, and by chemically or genetically inhibiting vasculature proliferation, we affected de novo root organogenesis. We also observed that root primordia originated from vasculature. Pericycle-like cells, particularly those expressing the J0121 marker, have been associated to regenerative and morphogenic processes as the source of reprogrammable cells (Sugimoto et al., 2010; Chen et al., 2014). When we assessed the J0661 and J0121 pericycle markers, we observed that vasculature proliferation associated to J0661 identity, whereas some proliferating cells were devoid of J0121 expression. Intriguingly, J0661 marks cells around xylem and procambium in petioles whereas J0121 only marks cells in contact to xylem. Proliferation competence, therefore, appears to involve different cell types. Closer examination showed that all primordia expressed a J0121 marker, which indicates that regeneration competence associates with J0121 identity and shows parallelisms with callus and lateral root formation. Cell lineage tracing using clonal markers and live imaging could dissect the exact source of reprogrammable cells during de novo root regeneration from whole leaves.

We also found that proliferating vascular cells express WIND1, a positive regulator of wound-induced callus formation (Iwase et al., 2011), suggesting together with J0121 analysis that these proliferating tissues could be a type of callus. Callus formation requires confluence of auxin and cytokinin responses in the same set of cells (Gordon et al., 2007). In agreement with this idea, our results show specific expression of auxin and cytokinin signaling reporters Pro_{DR5}:*GUS* and Pro_{ARR5}:*GUS*, respectively, in the vasculature. These results also indicate that specific regulation was required to induce auxin and cytokinin signaling at the petiole base. In contrast to early notions that cytokinins are produced only in roots, it is now recognized that they are synthesized throughout the plant (Zürcher and Müller, 2016). Our results are consistent with ISOPENTENYLTRANSFERASE3 (IPT3), IPT5, and LONELY GUY4 locally mediating cytokinin biosynthesis at the petiole base to contribute to vascular proliferation during root regeneration. Thus, cytokinin signaling mutants displayed reduced regeneration

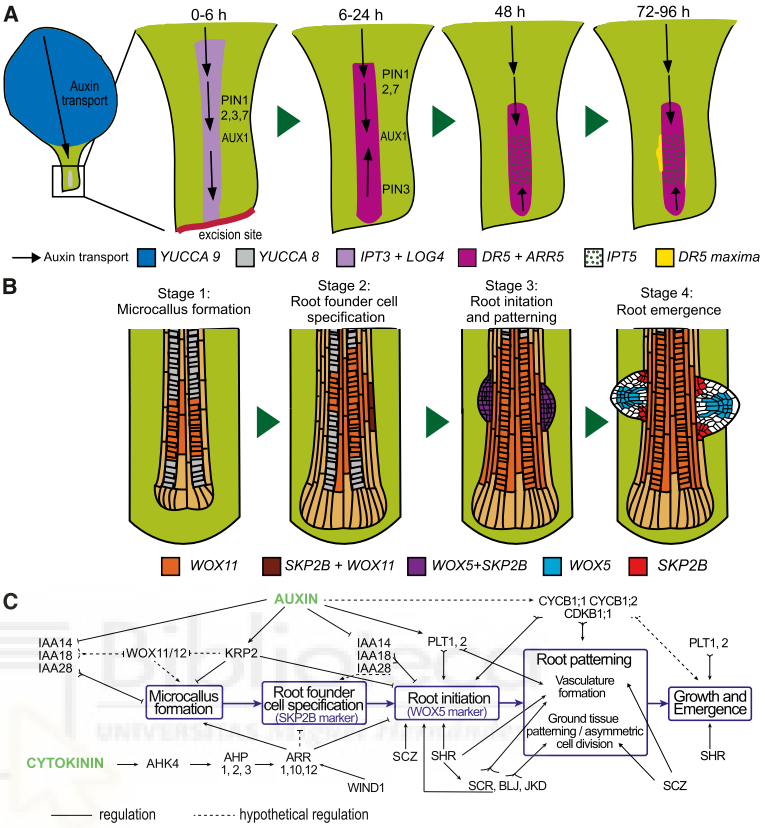

Figure 8. Model of de novo root regeneration in Arabidopsis leaves. A, Auxin is transported to the petiole base and acts in combination with locally produced cytokinin to induce endogenous callus formation: LOG4, IPT3, and IPT5 for cytokinin biosynthesis; ARR5 for cytokinin response; YUCCA8/9 for auxin synthesis; PIN1/2/3/7 and AUX1 for auxin transport; and DR5 for auxin response. B, Stages of de novo formation of roots based on morphological changes and expression of markers. C, Regulation of distinctive stages of de novo organogenesis based on mutant phenotypes. Regulation of functions that could not be directly assigned are indicated as hypothetical.

potential as well as defective hormone-induced callus formation. Interestingly, the triple cytokinin signaling mutant *ahp2 ahp4 ahp5* is affected in hormone-induced callus formation but not in vasculature proliferation during rooting, indicating the existence of specific genetic differences between hormone-induced callus formation and de novo root formation.

Pro$_{DR5}$:GUS expression in the proximal petiole vasculature could be indicative of auxin accumulation. A study in leaf blades has shown that YUCCA1 (YUC1) and YUC4 appear to mediate synthesis of auxin in mesophyll cells (Chen et al., 2016). If this occurred in our system, auxin would also need to be transported to cells near the wound to induce de novo root organogenesis. We observed that *YUC9* expression was induced in the leaf blade mesophyll but not in the petiole (Fig. 8A), suggesting a predominant function of the leaf blade mesophyll as source of auxin for regeneration. We confirmed this by removing leaf blades, which resulted in inhibition of regeneration. *YUC9* expression has been shown to respond to methyl-jasmonate (MeJA) treatment in a COI1-dependent manner (Hentrich et al., 2013). As excision of whole leaves or leaf blades involves wounding and therefore MeJA production, MeJA might activate *YUC9* expression to rapid increase auxin levels in leaf blades, likely in combination with YUC1 and YUC4 activity. We also found that *YUC8* was specifically upregulated in the vascular region of the petiole associated with proliferation, and thus, it is possible that YUC8 might have a specific role in maintaining auxin levels during vasculature proliferation or at later regenerative steps.

Our results indicate that a long distance basipetal transport system concentrates auxin generated in the leaf blade mesophyll toward defined vascular cells at the petiole base. We showed that genetic and localized chemical inhibition of auxin transport significantly affected regeneration. Despite known redundancy among auxin transporters (Blilou et al., 2005; Péret et al., 2012), we detected phenotypes in single mutants, suggesting spatial compartmentalization. Supporting this idea, PIN-FORMED3 (PIN3) was expressed in the petiole vasculature whereas PIN4 was later restricted to the proliferating vascular region. In contrast, more delocalized auxin transport is involved in rooting of leaf blades (Liu et al., 2014; Chen et al., 2016). As we

observed predominant expression of Pro_{DR5}:*GUS* in the proximal petiole vasculature, it is possible that auxin would need to be retained in this area. Our data suggest a model in which subcellular PIN3 localization shifts from basal to apical membranes in vascular cells near the wound to redirect auxin flow backward and thus maintaining high auxin levels in the proximal petiole vasculature. Interestingly, an auxin-dependent switch in PIN3 polarization contributing to auxin-flow reversal is involved in the shoot gravitropic response (Rakusová et al., 2016), where basal-to-apical shift in PIN localization has been described to depend on phosphorylation (Dai et al., 2012). It is thus tempting to speculate that auxin-dependent phosphorylation of PIN3 would be involved in maintaining high auxin levels in the petiole base vasculature during root regeneration.

The *alf4* mutant (DiDonato et al., 2004) has been linked to hormone-induced callus formation (Sugimoto et al., 2010), but not to wound-induced callus formation during graft establishment (Melnyk et al., 2015). We observed reduced vasculature proliferation along with great reduction in de novo root formation from whole leaves in *alf4-1* mutants. As vasculature proliferation during de novo root formation associates to J0121- and WIND1-marked cells, requires auxin and cytokinin signaling and involves ALF4, we propose it is a type of callus, and therefore we refer to it as "endogenous callus" to differentiate it from callus obtained by exogenous hormone supplementation. In our model, time-dependent auxin accumulation in a subset of vascular cells activates proliferation, whereas cytokinins might regulate the expression of genes that are directly involved in callus formation (such as *WIND1* or *ALF4*) or that are downstream targets of the auxin signal involved in callus formation (LATERAL ORGAN BOUNDARIES DOMAIN factors; Schaller et al., 2015). Particularly, we found that IAA18 and IAA28 are both involved in endogenous callus formation, although only mutations in IAA18 affect whole regeneration response, whereas ARABIDOPSIS HIS PHOSPHOTRANSFER PROTEIN1 (AHP1) to AHP3 and ARABIDOPSIS RESPONSE REGULATOR1 (ARR1), ARR10, and ARR12 were involved in vasculature proliferation and regeneration response.

Specification of Root Founder Cells

Hormone-induced callus is organized in layers showing root tissue identities that resemble a root meristem and therefore a new organ could be theoretically initiated through a differentiation process (Sugimoto et al., 2010). WUSCHEL-RELATED HOMEOBOX11 (WOX11) expression has been associated to first cell-fate transition from regeneration-competent cells to root founder cells during leaf blade rooting (Liu et al., 2014; Hu and Xu, 2016). WOX11 activates WOX5 during root formation in leaf blades; however, we did not find WOX5 expression in endogenous callus during de novo regeneration from whole leaves, suggesting that WOX11 could be involved in an earlier step in the reprograming process. On the other hand, specific expression associated to root founder cell specification (SKP2B) revealed the establishment of a cell lineage capable of forming a new root. These results indicate that additional reprogramming processes are required.

PLT1 and PLT2 have been shown to be required to establish pluripotency during de novo shoot regeneration (Kareem et al., 2015). Our results show that during de novo root formation, PLT1 and PLT2, in combination with SHR, could also be involved in specification of root founder cells, which are pluripotent. In addition, persistent expression of PLT1, PLT2, and SHR appears to be necessary during subsequent formative stages to maintain primordia growth, as the very primordia found in *shr-2 plt1-4 plt2-2* quickly differentiate. In contrast, in the de novo shoot regeneration system, transient induction of PLT2 has been shown to be sufficient to specify shoot progenitors, whereas subsequent expression of other regulators is required to accomplish de novo shoot formation from these progenitors (Kareem et al., 2015).

Root Primordia Initiation and Patterning

Multiple INDOLE-3-ACETIC ACID INDUCIBLE (IAA)-ARF modules cooperatively regulate lateral root formation (Goh et al., 2012). We observed that factors regulating auxin signaling, such as SOLITARY ROOT (IAA14), could be also involved in de novo root initiation; we detected decreased root capacity for *slr-1*, which could be indicative of fewer primordia initiation. In addition, the IAA28 module, which is upstream of lateral root founder cell specification (De Rybel et al., 2010), also regulates de novo root founder cell specification or initiation, although further experiments could dissect more precisely at which stage IAA28 is involved. Our results show that factors primarily involved in formation of lateral roots are also affected in rooting of leaves, suggesting the existence of partially overlapping auxin signaling modules during postembryonic root development. Conversely, cytokinin mutants (*ahp1 ahp2 ahp3* and *arr1 arr10 arr12*) showed increased rooting capacity and thus, a repressor role in de novo root initiation can be assigned for these factors, likely in an analog manner as their role during lateral root initiation (Lavenus et al., 2013; Chang et al., 2015). In agreement with this model, we restored regeneration potential of cytokinin signaling mutants by a moderate increase in auxin levels.

PLT1 and PLT2 expression is considered to be a slow readout of auxin response and prolonged auxin treatment results in PLT1 and PLT2 activation and the de novo specification of WOX5-marked stem cells (Mähönen et al., 2014). We found severe impairment in de novo primordia initiation in *shr-2 plt1-4 plt2-2*, and WOX5 expression during de novo primordia formation

requires auxin input through an unknown pathway (Hu and Xu, 2016). Therefore, it is possible that PLT1 and PLT2 are required for specification of WOX5-marked cells downstream of auxin during de novo organ initiation, which could occur in combination with activity of WOX5 transcription factor itself and/or SHR, in turn acting in an auxin-independent pathway. Further experiments will be required to dissect the molecular pathway including PLTs, SHR, and WOX5.

We have also identified specific factors involved in formation of de novo root primordia. We have found that the stem cell regulators BLJ, JKD, SCR, SHR, and SCZ regulate ground tissue patterning and vasculature formation prior emergence at the step of dome-shape primordia. Subsequently, more developed primordia are not properly organized in cell layers or rows and by the time of emergence, these defects persist and aggravate. Our results indicate that ground tissue patterning appears to be regulated in newly formed roots at two levels: First, we observed impairment in asymmetric divisions specifying cortex, middle cortex, and endodermis in mutants of SHR and SCR, although a few asymmetric divisions were still observed. In agreement, *shr* mutants have been shown to form endodermis in anchor roots (Lucas et al., 2011), which are a type of adventitious root. Furthermore, we observed that ground tissue asymmetric divisions were absent in mutant combinations of *scr-4* with *bjl-1* and *jkd-4* and in double mutants *jkd-4 scz-1*. Interestingly, when we studied if these mutant combinations had defects in lateral roots, which are also organs formed postembryonically, we also observed absence of ground tissue asymmetric divisions suggesting a conserved developmental program for endodermis and cortex specification. Second, we observed that SCR, JKD, BLJ, and SCZ could function as ground tissue lineage determinants during de novo root organogenesis. The combined action of BLJ, JKD, and SCR is required to maintain the ground tissue lineage postembryonically, and lacking these three factors results in missing ground tissue initializations (hence fewer ground tissue cell rows are observed in cross sections: Moreno-Risueno et al., 2015). We observed that de novo formed roots in *blj-1 jkd-4 scr-4* and *jkd-4 scz-1* mutants were missing ground tissue cell rows shortly after emergence, which indicates incorrect specification of ground tissue initials during primordia formation or later on in the course of development. Thus, it is possible that SCZ, SHR, PLT1, and PLT2 function as lineage or cell fate determinants during postembryonic development, and particularly so during de novo organogenesis.

MATERIALS AND METHODS

Growth Conditions

Seeds were surfaced-sterilized in 10% (m/v) NaClO and rinsed with sterile water before being transferred to 120 × 120 × 10 mm petri dishes containing 65 mL of one-half-Murashige & Skoog (MS) medium with 1% Suc and 10 g/L Plant Agar (Duchefa). After 2 d of stratification at 4°C in darkness, plates were transferred to an MLR-352-PE growth chamber (Panasonic) at 22°C, 16/8 photoperiod or continuous light (50 μmol·m^{-2}·s^{-1}). Twelve d after germination, the first pair of leaves was excised across the junction of the petiole with the stem and transferred to Gamborg B5 medium with 2.5% Suc, 10 g/L Difco Agar (Becton Dickinson), or 3 g/L Gelrite (Sigma-Aldrich) and Gamborg B5 vitamin mixture (Duchefa). Leaves after excision were grown in darkness at 22°C, routinely for 10 d, or for number of days indicated in corresponding experiment.

Hormonal and Inhibition Treatments

For exogenous hormone treatment, filter-sterilized indole-3-acetic acid, 6-BAP, or thidiazuron (TDZ) stock solutions were added to warm growth medium before pouring into plates to provide a final concentration of 1 μM indole-3-acetic acid, 5 μM 6-BAP, or 4 μM TDZ, respectively. NPA was applied locally by preparing a lanoline solution containing 1% w/w NPA. For cell cycle arrest, leaf explants were incubated with growth medium supplemented with 0.5, 2.5, or 5 mM hydroxyurea (Sigma-Aldrich).

Plant Material

Columbia-0 (Col-0), Landsberg *erecta* (L*er*), and Wassilewskija-2 (Ws-2) accessions were used as a genetic background as corresponding. The reporter lines Pro_{IPT3}:*GUS*, Pro_{IPT5}:*GUS* (Miyawaki et al., 2004), Pro_{LOG4}:*GUS* s (Kuroha et al., 2009) obtained from RIKEN, and Pro_{ARR5}:*GUS* (D'Agostino et al., 2000) were used for tracing cytokinin biosynthesis and signaling during rooting. Pro_{YUC8}:*GUS*, Pro_{YUC9}:*GUS* (Hentrich et al., 2013) and Pro_{DR5}:*GUS* (Ulmasov et al., 1997) were used to investigate auxin biosynthesis and signaling; Pro_{PIN3}:*PIN3*:*GFP* (Xu et al., 2006) and Pro_{PIN4}:*GUS* (Friml et al., 2004) were used for auxin transport. To trace the molecular mechanisms during de novo root formation we used: Pro_{WIND1}:*GUS* (Iwase et al., 2011), Pro_{WOX11}:*GUS* (Liu et al., 2014), Pro_{WOX5}:*GUS* (Sarkar et al., 2007), and Pro_{SKP2Bs}:*GUS* (Manzano et al., 2012), which corresponds to a promoter deletion containing 0.5 Kb upstream from the ATG. The J0121 and J0661 lines (Laplaze et al., 2005) were used to locate pericycle-like cells during rooting. The following mutant lines were used: *wol-1* (Scheres et al., 1995), *ahp1 ahp2 ahp3* (Hutchison et al., 2006), *ahp2 ahp4 ahp5*, *arr1 arr10 arr12* (Mason et al., 2005), *aux1-22* (Bennett et al., 1996), *axr2-1* (Timpte et al., 1994), *slr-1* (Fukaki et al., 2005), *crane-2* (Uehara et al., 2008), *sur2-1* (Delarue et al., 1998), and *iaa28-1* (Rogg et al., 2001), which were obtained from NASC; and *pin1*, *pin2 pin3*, *pin2 pin3 pin7* (Blilou et al., 2005), *fwr* (Okumura et al., 2013), *cyclinb1;1* (*cycb1;1*) and *cycb1;2* (Nowack et al., 2012), and *CDKB1;1 DN161* and Pro_{35S}:*KRP2* (Boudolf et al., 2004; Verkest et al., 2005). We also used the following stem cell niche mutants: *blj-1 jkd-4 scr-4* (Moreno-Risueno et al., 2015), *scr-4* (Fukaki et al., 1996), *shr-2* Pro_{SHR}:*SHR*:*GR* (Levesque et al., 2006), *plt1-4 plt2-2* (Aida et al., 2004), *jkd-4* (Welch et al., 2007), *scz-1* (Mylona et al., 2002), and *wox5-1* (Sarkar et al., 2007), and *jkd-4 scz-1* and *shr-2 plt1-4 plt2-2* Pro_{SHR}:*SHR*:*GR* (generated in this study).

Genotyping

The triple mutant *shr-2 plt1-4 plt2-2* Pro_{SHR}:*SHR*:*GR* was generated by crossing *shr-2* Pro_{SHR}:*SHR*:*GR* and *plt1-4 plt2-2*. F2 seedlings were grown and preselected on one-half-MS medium with 10 μM dexamethasone followed by DNA extraction and genotyping by PCR to finally obtain homozygous lines. The following oligonucleotides were used for genotyping *shr-2* (while discriminating Pro_{SHR}:*SHR*:*GR*): F 5′-CCAATACCATCCCGCCAC-3′ and R 5′-TGAACCGGTCATGCGGTTG-3′; for *plt1-4*: F 5′-AGACCGGCCACGCCAAGAC-3′ and R 5′CTAGTATCACGACATTATTTGC-3′; and for *plt-2-2*: F 5′-ACCTACAGTCGTCACTTGTGC-3′ and R 5′-ACTCTTGTCTCGTCATGTTTTTC-3′. T-DNA insertions of *PLT1* and *PLT2* were amplified using LB 5′-CATTTTATAATAACGCTGCGGACATCTAC-3′ and respective reverse primer. Double mutant *jkd-4 scz-1* was generated by crossing *jkd-4* and *scz-1*. DNA extraction from F2 seedlings was used for genotyping by PCR. The following oligonucleotides were used for genotyping *jkd-4*: F 5′-GGATGAAAGCAATGCAAAACA-3′ and R 5′-AATGTCGGGATGATGAACTCC-3′. The T-DNA insertion in the *jkd-4* line was genotyped using respective forward primer and RB 5′-TCAAACAGGATTTTCGCCTGCT-3′. For *scz-1* genotyping, a *SCZ* fragment was amplified using F 5′-CGAAGGTCAAGGCAAAGCTG-3′ and R 5′-GAGCAACAGGCTTGACATGG-3′primers, followed by digestion with *Nla*III restriction enzyme. Upon digestion, an *SCZ* fragment from *scz-1* renders a band of 900 bp, while the wild-type fragment is cut by the enzyme, resulting in two fragments of 530 bp and 360 bp.

Cell Death, GUS Staining, and Microscopy Analysis

In leaf tissues, dying cells were visualized by Lactophenol-Trypan Blue staining as described in Pavet et al. (2005). For GUS staining, leaf explants were incubated at 37°C for variable times (4 h to 24 h) in multiwell plates in the presence of the GUS staining solution as described in Pérez-Pérez et al. (2010) or Manzano et al. (2012). To bleach chlorophyll, two different methods were used indistinctly, with similar results. For the first method, samples were dehydrated after GUS staining using increasing ethanol concentrations (15, 50, 70, 96, and 100%) for 15 min in each one, and kept overnight in 100% ethanol; then samples were rehydrated following the same ethanol concentration series to 15% ethanol and mounted in 15% glycerol. For the second method, samples were fixed in 96% ethanol for 48 h and washed with 0.1 M P buffer (pH 6.8) before being transferred to a clearing solution (80 g chloral hydrate and 30 mL distilled water). Leaf explants were mounted on slides using a mixture of 80 g chloral hydrate, 20 mL distilled water, and 10 mL glycerol. After GUS staining, pictures were taken in a DM 2000 with a DCF300 camera (Leica), or in a bright-field AX70 microscope (Olympus) equipped with a PM-C35DX microphotography system (Olympus). The area of proliferating tissues (callus or vasculature) was manually drawn from microscopic images using a Bamboo tablet (Wacon) and areas or diameters of their best-fitting ellipses were measured with the software ImageJ (v1.50; National Institutes of Health).

Microscopy Analysis

Leaf petioles (for studying developmental stages during rooting) and mature embryos were stained using an Aniline Blue staining method as described in Bougourd et al. (2000). Processed samples were observed with a TCS SP8 laser scanning confocal microscope (Leica) with the settings described in Bougourd et al. (2000). A J0121 fluorescent reporter line was processed before imaging as indicated in Kurihara et al. (2015). Cross sections (10 μm of thickness) of J0121 petioles were obtained using a Vibratome 1000 Plus (Ted Pella) when indicated. Other fluorescent reporter lines were incubated in a methanol/acetone solution for 20 min at −20°C, and immediately transferred to a 0.1-M P buffer (pH 6.8). Imaging was performed by confocal microscopy using a Digital Eclipse C1 (Nikon) equipped with EZ-C1 control software (Nikon Instruments) or a TCS SP8 laser scanning microscope (Leica). GFP was excited with an Argon laser at 488 nm and emission was collected at 505 nm to 530 nm, whereas YFP was excited at 514 nm and emission collected at 535 nm to 560 nm. To exclude autofluorescence contamination, sample emission was collected at 605 nm to 675 nm before excitation with an He-Ne laser at 543 nm, and used as a background reference. Root samples for radial and longitudinal analyses in patterning studies were fluorescently stained with 10 mM propidium iodide (Sigma-Aldrich), and imaged using standard settings on a TCS SP8 confocal microscope (Leica).

Quantification and Statistical Analysis

Regeneration percentage was scored as the number of leaf explants that showed swelling of petioles, proliferation of vascular-associated cells, and/or outgrowth of roots at the proximal region of the petiole, at 7 dae or as indicated in corresponding experiments. Rooting percentage was scored as the number of leaf explants showing roots at 10 dae or as indicated. Proliferation of vascular-associated cells and number of de novo formed roots were scored on individual leaf samples and used to estimate rooting capacity categories at indicated days in corresponding experiments. Ten-dae de novo-formed roots were used for establishing differences in tissue patterning using number of cortical cells. The number of cortical cells was quantified using confocal cross sections taken at the site of lateral root cap ending. To determine lateral root capacity, root meristems of 4 d postimbibition seedlings were removed and the number of lateral roots quantified 3 d later. Roots were observed at the microscope to confirm there were not unemerged primordia. Data values referred to % rooting, % regeneration, number of cortical cells, lateral root capacity, and vasculature or callus area were statistically analyzed by a univariate general linear model (GLM) and ANOVA with a least significant difference (LSD) posthoc test, using SPSS Statistics 21 software (IBM). For rooting capacity, χ^2 test was performed to assay if there were differences in distribution frequency between lines, analyzed two-by-two, using the software Centurion XVI.I (STATGRAPHICS). Significant differences were collected with 5% level of significance (P value < 0.05).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Rooting of whole leaves and leaf blades of Arabidopsis wild-type accessions.

Supplemental Figure S2. Vascular proliferation and callus formation during rooting of whole leaves.

Supplemental Figure S3. AHK4, AHP1, AHP2, AHP3, AHP4, and AHP5, and ARR1, ARR10, and ARR12 cytokinin signaling factors regulate hormone-induced callus formation.

Supplemental Figure S4. IAA7/AXR2 and IAA18 auxin signaling factors regulate hormone-induced callus formation.

Supplemental Figure S5. ALF4 is required for vasculature proliferation and de novo root formation.

Supplemental Figure S6. IAA7/AXR2, CRANE/IAA18, and IAA28 signaling factors regulate de novo root formation in leaf blades.

Supplemental Figure S7. The *YUC8* and *YUC9* auxin biosynthesis genes are induced in excised leaves.

Supplemental Figure S8. SCARECROW and SHORT-ROOT regulate ground tissue patterning of de novo formed roots.

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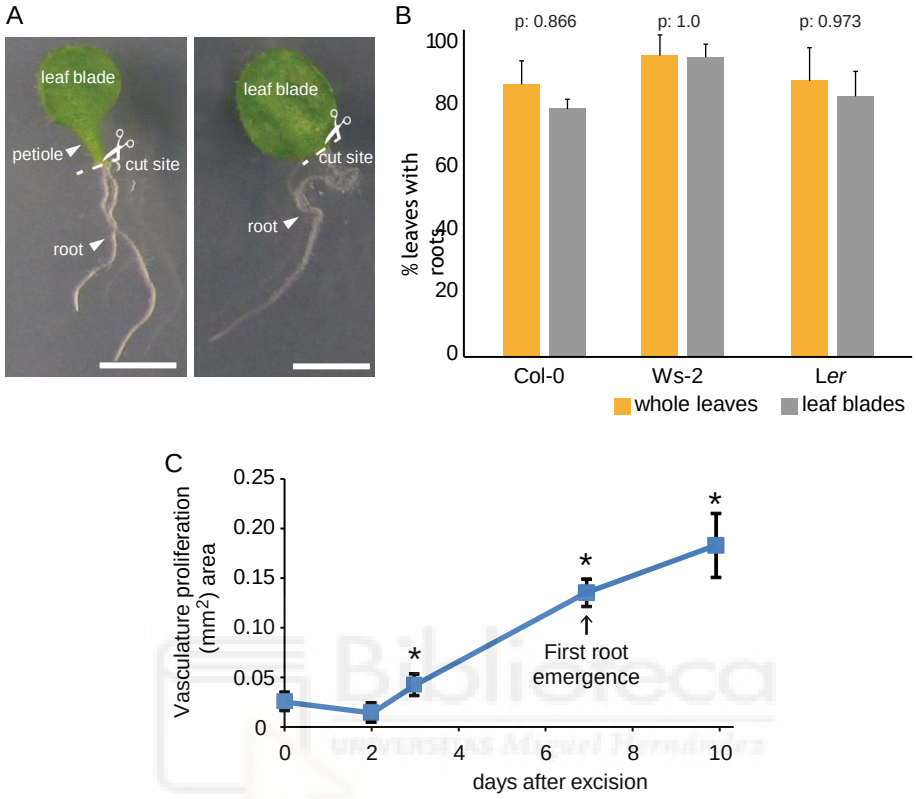

Figure S1. Rooting of whole leaves and leaf blades of Arabidopsis wild-type accessions. (A) Arabidopsis leaves were excised from mother plant at the site indicated and cultivated for 12 days in B5 hormone-free medium. (B) Percentage of whole leaves and leaf blades with roots of Columbia-0 (Col-0), Wassilewskija-2 (Ws-2), and Landsberg *erecta* (L*er*) accessions at 12 days after excision. p: p-value by General Linear Model (GLM) and DMS Post-Hoc test. (C) Increase in vasculature proliferation area during rooting. Area of the central cylinder of petioles undergoing regeneration was quantified up to 10 days after excision. Time when first root emerged is indicated. Asterisks: statistically significant (p-value<0.05) as compared with time 0 by General Linear Model (GLM) and DMS Post-Hoc test.

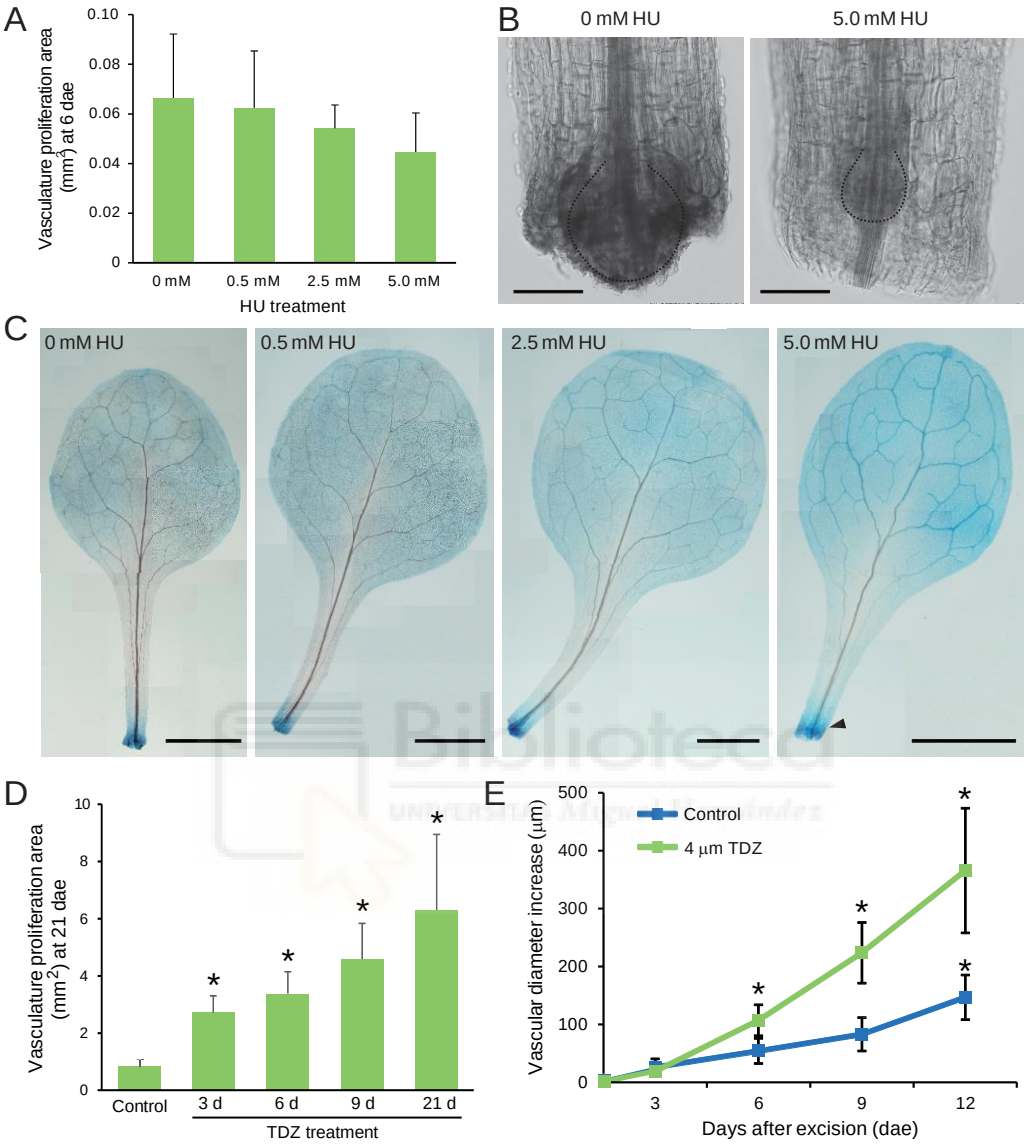

Figure S2. Vascular proliferation and callus formation during rooting of whole leaves. (A) Vasculature proliferation area was quantified on Arabidopsis leaves cultivated for 6 days in hydroxyurea (HU)-supplemented medium. (B) Callus formation on the proximal petiole at 10 days after excision (dae); dashed line corresponds to vasculature proliferation area. (C) Trypan blue staining of mock or HU-treated whole leaves at 6 dae. Black arrowhead marks staining at the leaf excision site. (D) Vasculature proliferation area at 21 dae was quantified on control leaves or cultured on TDZ-supplemented medium for 3, 6, 9 and 21 days. (E) Vascular diameter increase in petioles undergoing regeneration. Scale bars:

(B) 0.2 and (C) 2 mm. Asterisks: statistically significant (p-value<0.05) as compared with (D) control and

(E) time 0 by General Linear Model (GLM) and DMS Post-Hoc test.

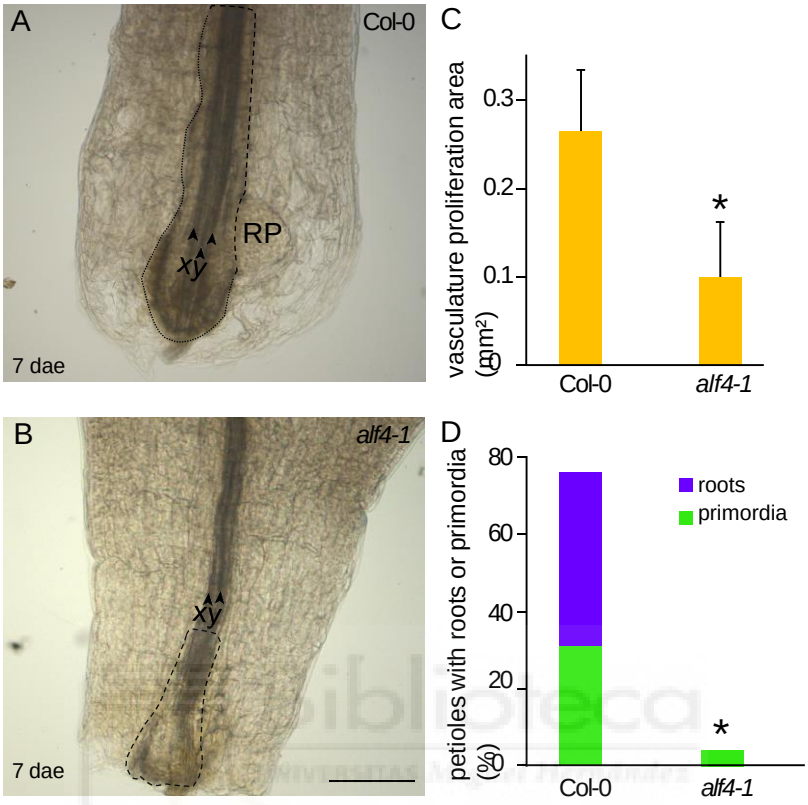

Figure S5. ALF4 is required for vasculature proliferation and *de novo* root formation. (A-B) Longitudinal sections of petioles of (A) Col-0 and (B) *alf4-1* near the excision site at 7 days after excision (dae). xy, xylem; dashed line marks vasculature proliferation area. Scale bar: 200 µm. (C) Vasculature proliferation area (mm^2) in Col-0 and *alf4-1* at 7 dae (n=50). (D) Percentage of petiole leaves with roots or primordia Col-0 and *alf4-1* at 7 dae (n=70). Asterisks: statistically significant (p-value<0.05) as compared to controls according to General Linear Model (GLM) followed by DMS Post-Hoc or Chi-square test.

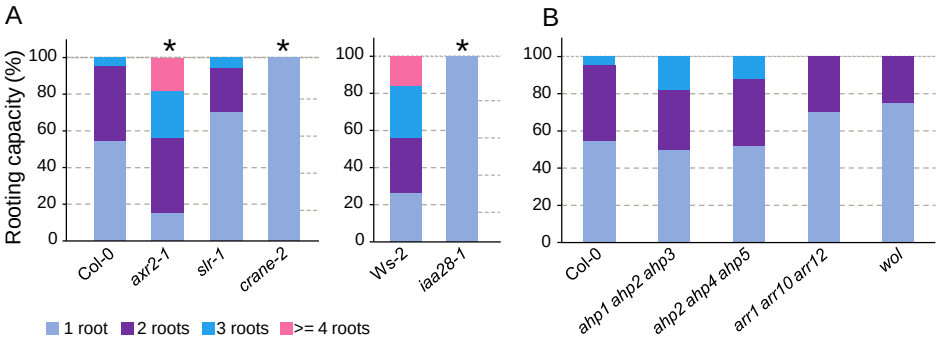

Figure S6. IAA7/AXR2, CRANE/IAA18 and IAA28 signaling factors regulate *de novo* root formation in leaf blades. Rooting capacity at 10 days after excision of (A) auxin signaling mutants *axr2-1*, *slr-1*, *crane-2*, and *iaa28-1* and (B) cytokinin *ahp1 ahp2 ahp3*, *ahp2 ahp4 ahp5*, *arr1 arr10 arr12* and *wol-1* as compared to their respective control backgrounds. Asterisks: statistically significant (p-value<0.05) as compared to controls according to Chi-square test.

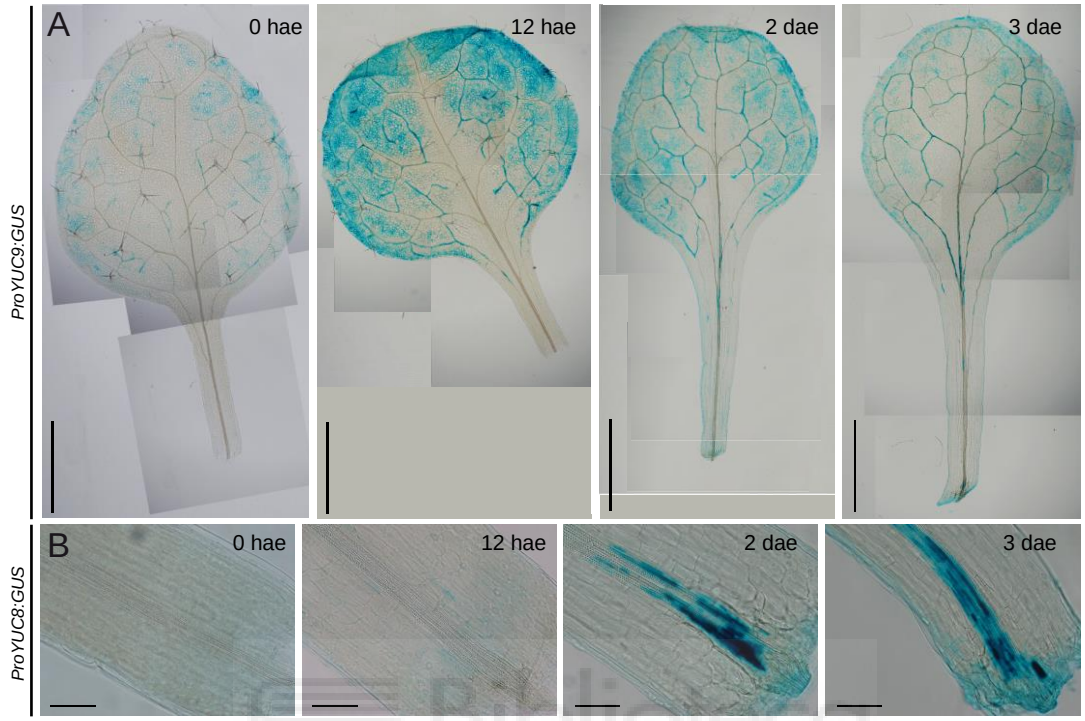

Figure S7. The *YUC8* and *YUC9* auxin biosynthesis genes are induced in excised leaves. (A) *Pro$_{YUC9}$:GUS* expression in leaf explants from 0 to 3 days after excision (dae). (B) *Pro$_{YUC8}$:GUS* expression in the petioles of excised leaves from 0 to 3 dae. Scale bars: (A) 2 mm and (B) 0.1 mm.

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A Network-Guided Genetic Approach to Identify Novel Regulators of Adventitious Root Formation in *Arabidopsis thaliana*

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Adventitious roots (ARs) are formed *de novo* during post-embryonic development from non-root tissues, in processes that are highly dependent on environmental inputs. Whole root excision from young seedlings has been previously used as a model to study adventitious root formation in *Arabidopsis thaliana* hypocotyls. To identify novel regulators of adventitious root formation, we analyzed adventitious rooting in the hypocotyl after whole root excision in 112 T-DNA homozygous leaf mutants, which were selected based on the dynamic expression profiles of their annotated genes during hormone-induced and wound-induced tissue regeneration. Forty-seven T-DNA homozygous lines that displayed low rooting capacity as regards their wild-type background were dubbed as the *less adventitious roots (lars)* mutants. We identified eight lines with higher rooting capacity than their wild-type background that we named as the *more adventitious roots (mars)* mutants. A relatively large number of mutants in ribosomal protein-encoding genes displayed a significant reduction in adventitious root number in the hypocotyl after whole root excision. In addition, gene products related to gibberellin (GA) biosynthesis and signaling, auxin homeostasis, and xylem differentiation were confirmed to participate in adventitious root formation. Nearly all the studied mutants tested displayed similar rooting responses from excised whole leaves, which suggest that their affected genes participate in shared regulatory pathways required for *de novo* organ formation in different organs.

Keywords: adventitious rooting, callus formation, gibberellin, ribosome, auxin homeostasis, xylem differentiation

INTRODUCTION

Adventitious roots (ARs) are formed *de novo* from non-root tissues (i.e., stems or leaves) after a stress episode, such as drought, flooding or physical damage (Steffens and Rasmussen, 2016). AR formation is a complex process influenced by a large set of exogenous and endogenous factors (Druege et al., 2018). In *Arabidopsis thaliana*, induction of ARs in the hypocotyl has been successfully achieved either by growing seedlings in the dark and transferring them to light conditions (Sorin et al., 2005) or upon whole root excision (Sukumar et al., 2013). In the hypocotyl,

ARs originate from a cell layer reminiscent to the root pericycle and the newly initiated ARs share histological and developmental characteristics with lateral roots (Bellini et al., 2014; Verstraeten et al., 2014). A local increase in auxin-induced marker expression was observed shortly after whole root excision in a defined region of the hypocotyl with the highest expression localized to xylem pole pericycle cells. This expression pattern was dependent on ATP BINDING CASSETTE SUBFAMILY B 19 (ABCB19)-mediated polar auxin transport from the shoot (Sukumar et al., 2013). In addition, mutations of *PIN-FORMED1* (*PIN1*) produced fewer ARs on de-rooted hypocotyls, while the PIN6 auxin transporter behave as a negative regulator of AR formation in this organ (Simon et al., 2016). In most species, however, ARs originate from non-root tissues, such as the vascular cambium, in a process that requires cell dedifferentiation and presumably different regulatory pathways as the hypocotyl-derived ARs (Bellini et al., 2014; Verstraeten et al., 2014).

Recent work has uncovered some of the molecular mechanisms that regulate the development of ARs from the hypocotyl (Gutierrez et al., 2009, 2012; Pacurar et al., 2014) and from excised whole leaves (Chen et al., 2014; Bustillo-Avendaño et al., 2018). Downstream of canonical auxin signaling pathway (Salehin et al., 2015), AUXIN RESPONSE FACTOR 6 (ARF6) and ARF8 are positive regulators of light-induced adventitious rooting in the hypocotyl, while ARF17 is a negative regulator of this process (Gutierrez et al., 2009). The ARF6/8/17 transcription factor network regulates the expression of three *GRETCHEN HAGEN 3* (*GH3*) genes, encoding acyl acid amido synthases, that lead to a net increase in jasmonic acid (JA) conjugation, which has been proposed to negatively regulate AR formation downstream of auxin (Gutierrez et al., 2012). Additional auxin signal transduction components involved in AR formation have been identified from a suppressor screen of the auxin overproducing *superroot2* (*sur2*) mutants, such as COP9 SIGNALOSOME SUBUNIT 4 (Pacurar et al., 2014, 2017), AUXIN RESPONSE 1 (AXR1), SHORT HYPOCOTYL 2 (SHY2), and RUB-CONJUGATING ENZIME 1 (RCE1), among others (Pacurar et al., 2014).

Based on the hypothesis that there are similar regulatory mechanisms in the formation of ARs and callus (Liu et al., 2014), the search for differentially induced genes in leaf explants and callus led to identification of *WOX11*, encoding a homeodomain transcription factor of the WUSCHEL HOMEOBOX (WOX) family (van der Graaff et al., 2009). In Arabidopsis leaf explants, *WOX11* directly responds to a wound-induced auxin maximum in and surrounding the procambium and acts redundantly with its homolog *WOX12* to upregulate *LATERAL ORGAN BOUNDARIES DOMAIN16* (*LBD16*) and *LBD29*, resulting in the first step of stem cell fate transition from procambial cells to root founder cells (Liu et al., 2014). Leaf explants displaying a constitutive overexpression of *WOX11* produced more ARs, while *wox11 wox12* explants or a dominant repressor mutant of *WOX11* produced fewer roots than the wild type (Liu et al., 2014). In turn, WOX11 and WOX12 activate WOX5 and WOX7 in dividing cells of the newly formed root primordia, while the subsequent WOX11and WOX12 expression quickly decreases in these cells (Liu et al., 2014; Hu and Xu, 2016). AR formation in leaf explants is also dependent on the endogenous basipetal transport system that concentrates the auxin generated in leaf blade mesophyll toward vascular cells near the cutting site (Liu et al., 2014; Chen et al., 2016). We recently proposed that an auxin-dependent switch in PIN3 polarization contributing to auxin-flow reversal is involved in maintaining high auxin levels in the vasculature near the cutting site during root regeneration (Bustillo-Avendaño et al., 2018). Factors primarily involved in lateral root formation, such as *CRANE* (also named as *IAA28*) and *SOLITARY ROOT* (also named as *IAA14*) are involved in rooting of leaves, suggesting the existence of partially overlapping auxin signaling modules during post-embryonic development (Bustillo-Avendaño et al., 2018).

Despite the remarkable advances in molecular-level understanding of the process of AR formation in Arabidopsis, not much is known about the downstream effectors of this complex response. Adventitious rooting requires activation of cell proliferation in root competent cells followed by founder cell specification in a subset of these cells, that they will be later committed to become a root (Bustillo-Avendaño et al., 2018). Based on the hypothesis that there are similar regulatory mechanisms in AR formation and other regenerative processes, such as callus formation (Lup et al., 2016), we selected a number of differentially expressed genes whose inactivation was previously known to affect leaf development (Wilson-Sánchez et al., 2014) to screen for mutants affected in wound-induced AR formation. Our results highlight novel regulation of ribosome function, gibberellin (GA) and auxin homeostasis that appears to be both complex and context specific.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis thaliana wild-type accession Columbia-0 (Col-0) and confirmed T-DNA homozygous lines were obtained from the PhenoLeaf collection[1] (Wilson-Sánchez et al., 2014). The following lines were used to isolate additional T-DNA homozygous mutants of the studied genes: N492755 (GK-967B07), N667578 (Salk_147826), N668393 (Salk_062900), N678155 (Salk_016729), and N840465 (Sail_896_G05) (**Table 1**). The *pDR5::GUS* (Ulmasov et al., 1997) line was used to investigate auxin response. Homozygous mutants of *DELLA* pentuple mutant (*dellaP*; Park et al., 2013), *gai-1* (Alabadí et al., 2008), *ga1-7* (Sun et al., 1992), *ga5-1* (Xu et al., 1995), and *ref2-1* (Hemm et al., 2003) were also used. Seedlings with T-DNA homozygous insertions in the studied genes were identified by sulfadiazine selection (N462401 and N492755) and PCR verification with T-DNA specific primers (the LBb1.3 primer for the Salk lines, the LB3 primer for the Sail lines, and the o8474 primer for the GABI-Kat lines) and a pair of gene-specific primers (**Table 1**). Genomic DNA isolation and genotyping of the T-DNA insertions were performed as described elsewhere (Pérez-Pérez et al., 2004).

Seeds were surfaced-sterilized in 2% (w/v) NaClO and rinsed with sterile water before being transferred to 120 × 120 × 10 mm

[1]http://genetics.edu.umh.es/query-phenoleaf-db/

TABLE 1 | Oligonucleotides used in this study.

Gene	NASC ID (PhenoLeaf ID)	Stock/primer	Oligonucleotide sequences (5′→3′)	
At4g02780	N656319 (*m240*)	Salk_027931	TTGCCTACCAATTTTGAATGC	AATCCAAAACAAATGCATTGC
	N492755	GK_967B07	GCGGTTCCATACATTGTTC	CTTGTAAGCTTTAGCTCTTTC
At4g13770	N667207 (*m678*)	Salk_123405	TAGGAAGCAGAACAATGGTGG	GGCCTAAACTCATCAGGGTTC
At5g62190	N662659 (*m482*)	Salk_060686	TTTTCGTAAGACAAACCGCAG	CTTGTAATAAGGCAGCCATGG
	N668393	Salk_062900	TTGGGTTTTGCTTATTATGCG	AGAAGCAAGCGAAAAGGTCTC
	N678155	Salk_016729	TCGGTATTGTGAATCTCCTGC	ATATCAGGAATCAACCGAGCC
At5g64080	N655791 (*m232*)	Salk_103127	CATTTTGTTTCCTTTCACTTTC	TGTTGCTCCAAGTACTGCTCC
	N667578	Salk_147826	ATTTTTGTTTGGAAACCCCTG	TGGAGCAGTACTTGGAGCAAC
	N840465	Sail_896_G05	CTGTAGATGAATCGTGGAGGC	CGAACAGTCTACAGACGGAGC
T-DNA		LBb1.3	ATTTTGCCGATTTCGGAAC	
		LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	
		o8474	ATAATAACGCTGCGGACATCTACATTTT	

Petri dishes containing 65 mL of one-half-Murashige and Skoog medium with 2% sucrose and 3 g L^{-1} Gelrite (Duchefa Biochemie, Netherlands). After 2 days of stratification at 4°C in darkness, plates were wrapped in aluminum foil and were transferred to an MLR-352-PE growth chamber (Panasonic, Japan) at 22 ± 1°C during 4 days in a nearly vertical position to induce hypocotyl elongation. Plates were unwrapped and grew during another 3 days with continuous light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The formation of ARs was then induced by removing the entire root system 1–2 mm above the hypocotyl-root junction with a sharp scalpel (**Figure 1A**). After whole root excision, seedlings were transferred to new Petri dishes containing growth media with 3% sucrose. The number of ARs in each hypocotyl was daily scored up to 6 days after excision (dae). Each Petri dish contained seedlings of two different lines and the Col-0 background (Eight seedlings per genotype). The experiment was performed in triplicate.

For assaying *de novo* root organogenesis in leaves, we followed the protocol described in Bustillo-Avendaño et al. (2018). Briefly, surface-sterilized seeds were sown in Petri dishes, and transferred to the growth chamber in horizontal position after 2 days of stratification at 4°C in darkness. 12 days after sowing (das), the first pair of leaves was excised across the junction of the petiole with the stem and the leaf explants, and they were transferred to new Petri dishes containing growth media with 3% sucrose. The number of ARs was scored up to 10 dae or for the number of days indicated in the corresponding experiment.

Antibiotic Treatments

For antibiotic inhibition of ribosome function, leaf explants were incubated on growth medium supplemented with 30 μg ml^{-1} streptomycin that targets the small subunit of the chloroplast ribosomes.

GUS Staining, Microscopic Observation, and Microphotography

For β-glucuronidase (GUS) staining, *pDR5::GUS* seedlings were incubated at 37°C for a minimum of 12 h in multi-well culture plates in the presence of the GUS staining solution as described in Pérez-Pérez et al. (2010). Leaf and hypocotyl samples were fixed in 96% ethanol for 48 h and washed with 0.1 M phosphate buffer (pH 6.8) before being transferred to clearing solution (80 g chloral hydrate and 30 mL distilled water) for chlorophyll removal. The area of proliferating vasculature was manually drawn from microscopic images using a Bamboo tablet (Wacom) and areas were measured with the software ImageJ (v1.50; National Institutes of Health). Bleached samples were mounted on slides using a mixture of 80 g chloral hydrate, 20 mL distilled water and 10 mL glycerol. Leaf pictures were obtained in a bright field Olympus AX70 microscope equipped with an Olympus PM-C35DX microphotography system (Olympus, Japan). Rosette, hypocotyl and leaf images were obtained with a SMZ-168-TL Stereo Zoom Microscope equipped with a Motic5+ digital camera (Motic, China).

Heat Map Representation

We searched available gene expression data regarding several plant tissue regeneration experiments (Che et al., 2006; Sena et al., 2009; Sugimoto et al., 2010) available at the Arabidopsis eFP Browser within the Bio-Analytic Resource for Plant Biology (BAR) website[2] (Winter et al., 2007). Gene expression data was retrieved for the 339 expressed genes with confirmed homozygous T-DNA insertions in the studied mutants of the PhenoLeaf collection (Wilson-Sánchez et al., 2014) that were available at the start of this project. In each experiment, we calibrated the expression value of the different conditions to its reference background and log2 transformed the output for outlier detection and convenient graphical representation. The standardized dataset obtained in this way (**Supplementary Table S1**) was processed using the pheatmap package of R version 3.3.2[3]. Euclidean distance matrixes between genes (rows) were calculated to build the dendrogram.

[2]http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi

[3]http://www.r-project.org/

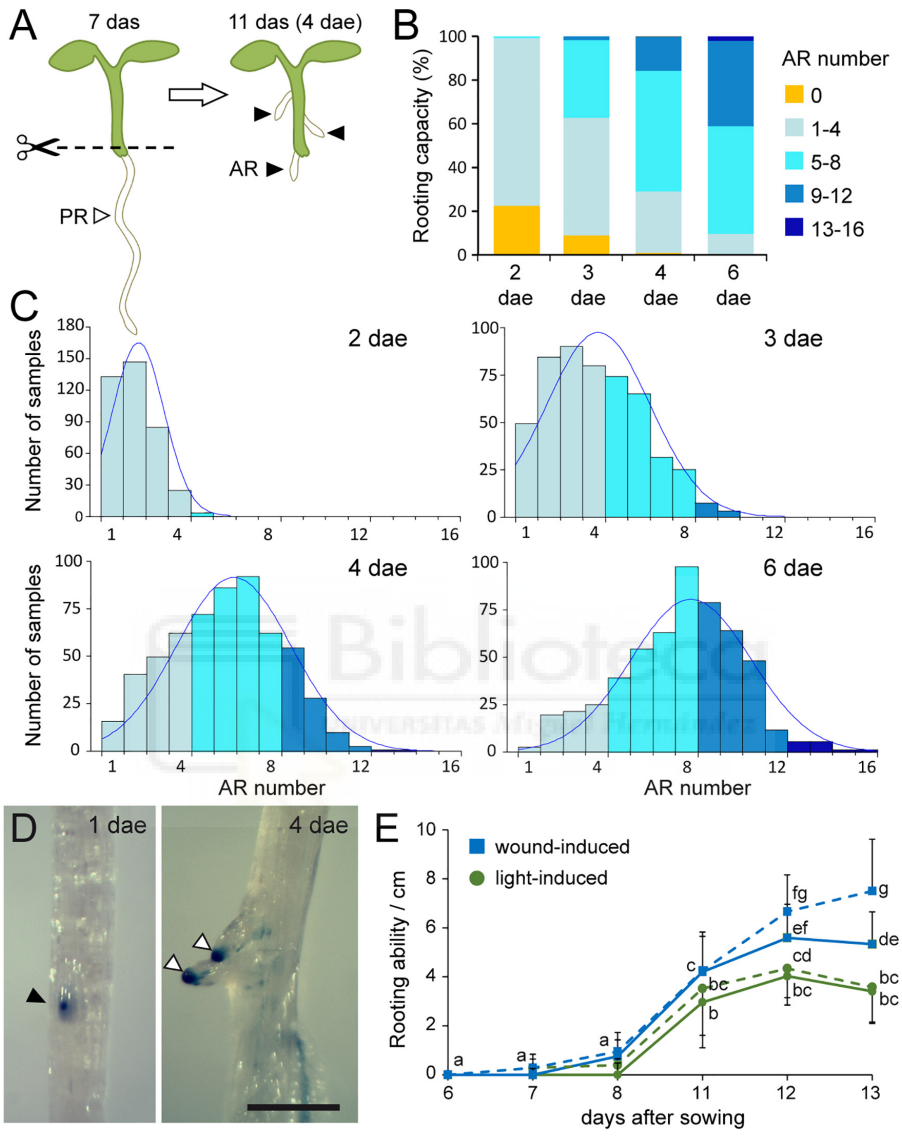

FIGURE 1 | Whole root excision promotes adventitious root formation in Arabidopsis hypocotyls. **(A)** Schematic representation of the induction system used. Dotted lines indicate the cut site. AR, adventitious root; dae, days after excision; das, days after sowing; PR, primary root. **(B)** Multicolored barplots show frequencies of ARs from 2 to 6 dae. **(C)** Histograms for AR number in a large sample of Col-0 seedlings with overlay of theoretical normal distribution. **(D)** Representative images of GUS expression foci (black arrowhead) in the hypocotyl of *pDR5::GUS* lines. White arrowheads mark the emerged AR primordia. Scale bar: 1 mm. **(E)** Rooting ability of the hypocotyl estimated by the presence of AR primordia (continuous lines) or GUS expression foci (dotted lines) of *pDR5::GUS* lines. Different letters indicate significant differences (LSD; p-value < 0.01) at each data point.

For a visual representation of the mutant phenotypes found, we built a data matrix containing normalized values for some of the estimated parameters (average, standard deviation, maximum, and minimum values) and a dendrogram was built with this dataset using Manhattan distances between mutant lines (rows).

Statistical Analysis

Descriptive statistics (average, standard deviation, median, maximum, and minimum) were calculated by using the StatGraphics Centurion XV software (StatPoint Technologies, United States) and SPSS 21.0.0 (SPSS Inc., United States) programs. One-sample Kolmogorov–Smirnov tests were performed to analyze the goodness-of-fit between the distribution of the data and a theoretical normal distribution. To compare the data for a given variable, we performed multiple testing analyses with ANOVA *F*-test or Fisher's LSD (Least Significant Differences) methods. For rooting capacity in excised leaves, χ^2 test was performed to assay if there were differences in distribution frequency between lines, analyzed two-by-two. Significant differences were collected with 5% level of significance (p-value < 0.05), unless otherwise indicated.

RESULTS

Adventitious Root Formation in Hypocotyls After Whole Root Excision

Whole root excision from young seedlings was previously used as a model to study AR formation in *A. thaliana* hypocotyls (Correa et al., 2012; Sukumar et al., 2013). We removed the entire root system 1–2 mm above the hypocotyl-root junction of 7 days-old seedlings and the number of ARs was visually scored between 1 and 6 (dae; **Figure 1A**). We characterized AR formation in the Col-0 accession, which has been used as a background reference for the Salk Unimutant and GABI-Kat collections of sequence-indexed T-DNA lines (Li et al., 2007; O'Malley and Ecker, 2010). As early as 2 dae, 76.7% of root-excised hypocotyls developed between one and four ARs, whereas no sign of AR formation was found for the remaining ones (**Figure 1B**). Interestingly, the number of ARs per hypocotyl increased significantly over time. At 6 dae, all root-excised hypocotyls developed at least one AR while 49.3% of the Col-0 hypocotyls included between five to eight ARs and some hypocotyls (2.0%) developed >12 ARs (**Figure 1B**). Distribution of AR number in the studied Col-0 population best fitted a normal function at 4 and 6 dae (**Figure 1C**).

Root excision-induced ARs in the hypocotyl emerged from the pericycle and were dependent on local auxin transport; previous results suggested that the internal auxin distribution was modified by the root excision, which, in turn, and drove enhanced AR initiation in the hypocotyls (Sukumar et al., 2013). We wondered whether root excision either induced the specification of new AR primordia within the hypocotyl or acted as a trigger to initiate the development of already-present AR founder cells within the hypocotyl. Indeed, lateral root founder cells are early specified in the oscillation zone of the primary root and later activated in the elongation zone upon additional shoot-derived signals (Laskowski and Ten Tusscher, 2017; Du and Scheres, 2018). To estimate the internal rooting ability of the hypocotyl and its eventual modification by the whole root excision, we quantified the number of foci (i.e., discrete regions) expressing the *pDR5::GUS* marker (**Figure 1D**), used previously as a direct read-out for endogenous auxin response maxima (Ulmasov et al., 1997). We found that the number of *pDR5::GUS* foci increased in intact hypocotyls between 8 and 12 das to a maximum of 4.3 ± 1.5 foci cm^{-1} (**Figure 1E**) and 95.2% of them developed as functional ARs in the absence of whole root excision. On the other hand, the number of *pDR5::GUS* foci in root-excised hypocotyls increased significantly from 11 das onward to a maximum of 7.5 ± 2.1 foci cm^{-1} at 13 das, but only 71.1% of them emerged as functional ARs (**Figure 1D**).

Candidate Regulators Selected From Gene Expression Data

To identify novel regulators of *de novo* root formation, we studied the annotated collection of T-DNA lines described previously (Wilson-Sánchez et al., 2014). 413 confirmed T-DNA homozygous lines that exhibit a leaf phenotype with full penetrance and constant expressivity were selected. To reduce the size of the screening population and to improve the frequency of the desired phenotypes, we prioritized candidate genes by using a network-guided genetic approach (Bassel et al., 2012; Ransbotyn et al., 2015). To this end, we gathered expression data for 339 of these genes from several Affymetrix microarray data sets related to hormone-induced and wound-induced tissue regeneration experiments (Che et al., 2006; Sena et al., 2009; Sugimoto et al., 2010; **Supplementary Table S1**), which were used to rank genes according to their expression profiles (**Figure 2**). Interestingly, while hormone-induced tissue regeneration followed an indirect morphogenesis pathway through callus formation a tissue with root primordium-like cell identity (Sugimoto et al., 2010), root tip regeneration proceeded through canonical WOX5, SCARECROW, and PLETHORA pathways required for root patterning and stem cell function (Sena et al., 2009; Lup et al., 2016). We reasoned that a positive regulator of hormone-induced tissue regeneration would increase its expression by the hormone treatment. In addition, such positive regulator will be expressed to a lesser extent during root tip regeneration as the reprogramming of this tissue proceeded by re-specification of lost cell identities in the absence of additional cell proliferation (Sena et al., 2009; Sena and Birnbaum, 2010). A contrasting expression profile was postulated for a negative regulator of hormone-induced tissue regeneration. By using these criteria, we selected 112 genes with dynamic expression profiles for further investigation (**Figure 2**).

Search for Mutants Affected in Wound-Induced AR Formation in Hypocotyls

We analyzed adventitious rooting in the hypocotyl after whole root excision of confirmed T-DNA homozygous lines in 112 selected genes (see section "Materials and Methods"). Mutant analysis was carried out in 11 consecutive sowings (S1 to S11) with Col-0 as a background reference. AR number in Col-0 ranged between 4.6 ± 1.5 (S6; $n = 125$) and 7.0 ± 2.2 (S7; $n = 77$) at 4 dae (**Supplementary Figure S1**). Normalized data for each mutant as regards Col-0 in the same sowing is shown in **Supplementary Table S2**. From the 112 mutants

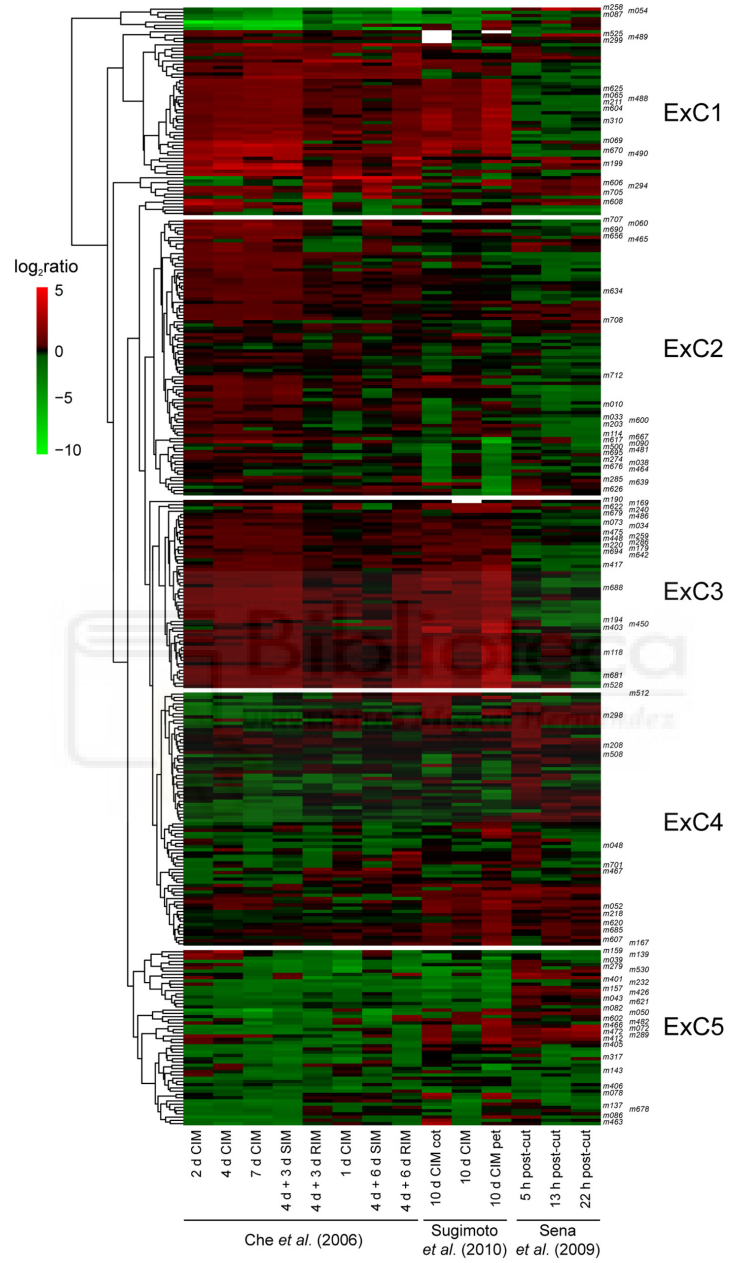

FIGURE 2 | Gene expression clustering of 339 genes in the confirmed homozygous PhenoLeaf collection. Each row corresponds to a single gene, and the color scale corresponds to the log2 ratio of fold gene expression from green (downregulated genes) to red (upregulated genes) as regards the expression level of the mock treatment in each experiment, as indicated; white color specifies no expression data available. Euclidean distance matrix between genes was calculated to build the dendrogram. Five expression clusters, ExC1 to ExC5, were defined based on this dendrogram. CIM, callus induction medium; cot, cotyledon explants; pet, petal explants; RIM, root induction medium; SIM, shoot-induction medium. The studied mutants are indicated in the right corner.

studied, 55 T-DNA homozygous lines (49.1%) showed a statistically significant difference (p-value < 0.05) in AR number as regards their Col-0 background in a minimum of two data points (**Supplementary Figure S2** and **Supplementary Table S2**). Twenty-seven and 12 lines grouped together on the same phenotypic clusters, PhC5 and PhC6, respectively, and were dubbed as the *less adventitious roots* (*lars*) mutants (**Supplementary Figure S2** and **Supplementary Table S2**). Eight remaining *lars* mutants were included in PhC4 and PhC3. Most *lars* mutants (e.g., *m039*, *m143*, *m240*, *m274, m482,* and *m602*) displayed low rooting capacity in the hypocotyl after whole root excision along the experiment (**Figures 3A,B**), indicating general defects in AR development. Other *lars* mutants either did not show clear defects in AR initiation but were delayed in subsequent AR emergence (e.g., *m078*) or were specifically affected at earlier time points (e.g., *m285*; **Figures 3A,B**).

We identified eight lines with enhanced rooting capacity as regards their Col-0 background (**Figures 3A,B**), five of them within the PhC1 cluster (**Supplementary Figure S2** and **Supplementary Table S2**), that we named as the *more adventitious roots* (*mars*) mutants. Most of these *mars* mutants displayed a significant increase in AR number in all studied time points (e.g., *m065*, *m232*, *m667*, *m678*, and *m681*). Interestingly, the *m525* line only displayed a significant increase in AR number at later time points (**Figure 3A**).

De novo Root Formation in Leaf Explants of Selected Mutants

Adventitious roots might develop from different cell types depending on the tissue of origin (Bellini et al., 2014). Using the experimental set up described previously (Bustillo-Avendaño et al., 2018; **Figures 4A,B**), we analyzed the competence for *de novo* root formation in the petiole base of excised whole leaves of eight *lars* and two *mars* mutants (**Figure 4C**). On the one hand, some of the *lars* mutants studied (*m039*, *m143*, *m240,* and *m608*), showed a high percentage of leaf explants with no sign of vascular proliferation at the excision site which ultimately led to low AR responses (**Figure 4C**). On the other hand, the *lars* mutants *m274*, *m617,* and *m626* were able to activate vascular proliferation in most leaf explants although ARs were rarely initiated, indicating specific defects in the ectopic specification of root founder cells and/or root primordia initiation in these mutants (**Figure 4C**). *m232* and *m678* lines were selected as *mars* for their increased AR formation in the hypocotyl (**Supplementary Figure S2**) and effectively displayed increased percentages of leaf explants with more ARs at 10 dae than those of the Col-0 background (**Figure 4C**). These results confirmed that the mutants identified previously in the wound-induced hypocotyl AR formation screen also displayed *de novo* root organogenesis phenotypes in whole leaves, indicating the putative participation of the damaged genes in shared developmental programs required for AR formation in both hypocotyls and proximal petioles of excised leaves. Further analyses will be required to confirm if these gene functions are conserved in AR formation from other organs.

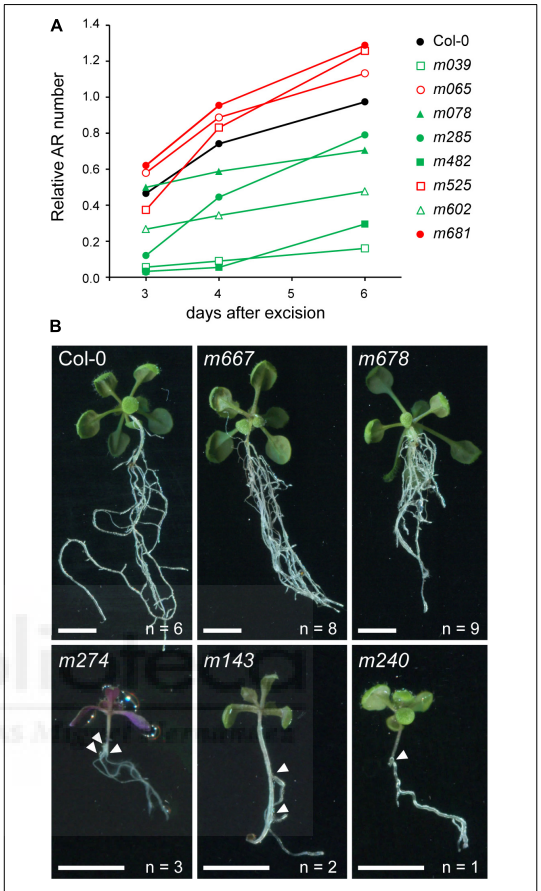

FIGURE 3 | Some AR formation mutants in the hypocotyl after whole root excision. **(A)** Temporal analysis of AR formation in a representative sample of the studied genotypes displaying highly significant differences (green, with lower AR values; red, with higher AR values; LSD, p-value < 0.05 at both 3 and 4 dae. See **Supplementary Table S2**) as regards their Col-0 background (shown in black). **(B)** Representative images of rooted hypocotyls of some mutants with altered AR formation. Arrowheads indicate the site of AR initiation. Numbers beneath images indicate ARs in each plant. Scale bars: 5 mm.

Analysis of *lars* Mutants Reveals a Positive Role for Gibberellins and Ribosome Function in AR Formation

We previously estimated that the annotated T-DNA was responsible for the observed phenotype in ∼47% of the lines in the PhenoLeaf collection and that their average number of T-DNA insertions was 2.1 (Wilson-Sánchez et al., 2014). To confirm that the observed AR phenotype of studied lines was caused by the homozygosity at the annotated T-DNA insertion and not because of other, non-annotated, T-DNA insertions, we

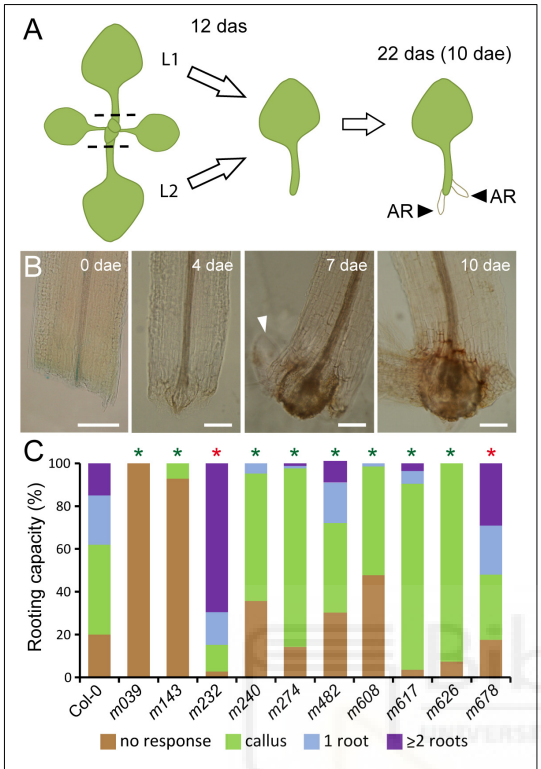

FIGURE 4 | *De novo* root formation in selected mutants. **(A)** Schematic representation of *de novo* root formation in Arabidopsis whole leaf explants. Dashed lines indicate the cut site. AR: adventitious root; L1 and L2: first node and second node vegetative leaves, respectively. **(B)** Morphological changes at the petiole base during *de novo* root formation in the Col-0 background. Arrowhead indicates the emergence of a root primordium. Scale bars: 200 μm. **(C)** Rooting capacity in leaf explants of selected mutants at 10 dae. Asterisks indicate significant differences (LSD; p-value < 0.01) with the Col-0 background; those with lower AR values are shown in green and those with higher AR values are in red.

selected additional mutant alleles of the putative causal genes of two of the studied *lars* mutants, *m240,* and *m482* (**Table 1**).

The *m240* mutant contained a homozygous T-DNA insertion at the 11th exon of the At4g02780 gene (**Figure 5A**), also named *GA REQUIRING 1*, which encodes the ENT-COPALYL DIPHOSPHATE SYNTHETASE 1 involved in a key step of GA biosynthesis (Michaels and Amasino, 1999). We analyzed rooting capacity in the petiole base of whole leaves of *ga1-7* mutants (**Figure 5A**) with a severe loss of GA1 function (Sun et al., 1992). *m240* and *ga1-7* leaf explants showed a strong reduction in their rooting capacity as regards their wild-type backgrounds, with most of the mutant explants showed lack or a severe delay of vascular proliferation (**Figures 5B,C**). In addition, we isolated additional T-DNA homozygous mutants within the *GA1* locus from the GK_967B07 line (**Figure 5A**) that also exhibited impaired rooting capacity in leaf explants (**Figures 5B,C**), supporting the correlation between defective AR formation and GA1 inactivation. To confirm the requirement for GA biosynthesis in AR formation, we studied the *ga5-1* mutant, which contains a loss-of-function in the GA 20-oxidase required for the later steps of GA biosynthesis (Xu et al., 1995). Consistently, *ga5-1* leaf explants showed a mild delay in AR formation (**Figures 5B,C**).

We wondered whether the effect of GAs in AR formation was dependent on canonical GA signaling pathway acting through DELLA repressors (Sun and Gubler, 2004). We analyzed AR formation in whole leaf explants of *gai-1*, bearing a deletion of the DELLA domain in GAI protein that renders this repressor constitutive and insensitive to GAs (Peng et al., 1997), and of the multiple mutant of all five *DELLA* genes (*dellaP*) that display constitutive GA responses (Park et al., 2013). Similar to GA-deficient mutants, *gai-1* leaf explants were partially defective on vascular proliferation and were consequently delayed in AR formation at 10 dae (**Figures 5B,C**). On the other hand, the *dellaP* mutants, with constitutive GA responses, also shown reduced regeneration percentage while the average number of ARs was not significantly different from those of the wild-type background (**Figures 5B,C**). Altogether, our results confirmed the requirement of GAs and tight regulation of their signaling through DELLA repressors to promote AR formation.

The *m482* mutant contained a homozygous T-DNA insertion at the 8th exon of the At5g62190 gene (**Figure 6A**), encoding the AtRH7/PRH75 DEAD-box RNA helicase involved in pre-rRNA processing which is active in regions undergoing cell division (Huang et al., 2016). We studied rooting capacity of *m482* (also named as *atrh7-2*) along with two additional T-DNA insertional lines of the *AtRH7/PRH75* locus (**Figure 6A**). All T-DNA homozygous mutants studied displayed a characteristic narrow leaf phenotype (**Supplementary Figure S3**) but only the Salk_062900 homozygotes displayed a significant lack of response during *de novo* root formation in the petiole base of whole leaves as compared with their Col-0 background (**Figures 6B,C**). Surprisingly, the Salk_016729 homozygotes displayed increased regeneration with a higher average of AR than in the wild-type background (**Figures 6B,C**). To confirm whether the defects in rRNA processing producing altered ribosome conformation might cause the observed AR phenotype of *AtRH7/PRH75* loss-of-function mutants, we incubated leaf explants on streptomycin that targets the small subunit of the chloroplast ribosomes and found a striking reduction of rooting capacity due to a delay in AR emergence (**Figures 6B,C**). Taken together, our results indicated that *AtRH7/PRH75* mutations might affect proper ribosome assembly, which is indeed required for AR development, an observation that requires further investigation.

Organ-Dependent Auxin Homeostasis Influences AR Formation

The *m678* mutant was identified as a *mars* mutant in our wound-induced hypocotyl AR formation screen. *m678* carried a homozygous T-DNA insertion in *REDUCED EPIDERMAL FLUORESCENCE 2* (*REF2*), encoding the

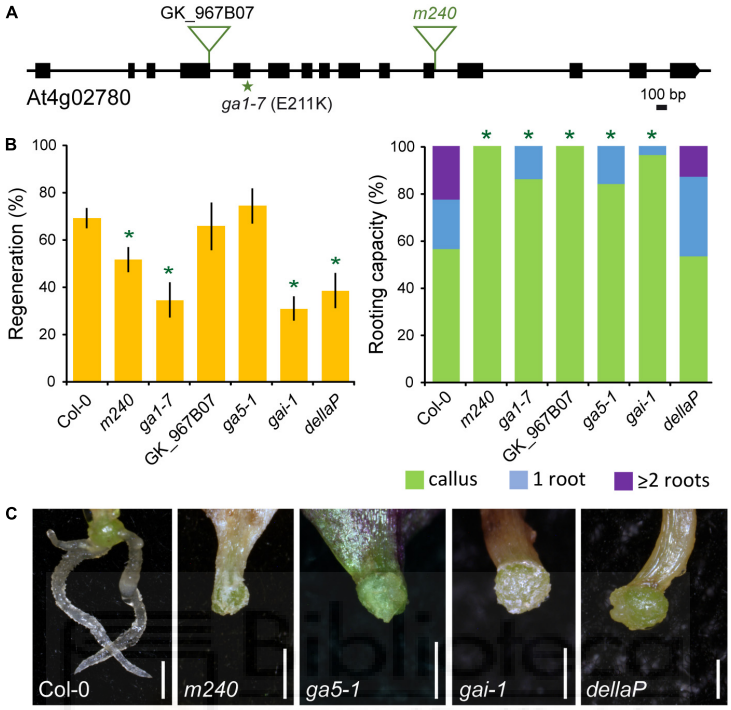

FIGURE 5 | Functional analysis of *GA1* during AR formation. **(A)** Gene structure of At4g02780. Exons are represented by boxes and introns are depicted as lines. The studied mutant lines and annotated T-DNA insertion (triangles) lines are indicated. **(B)** Regeneration and rooting capacity in leaf explants of selected lines at 10 dae. Asterisks indicate significant differences (LSD; p-value < 0.01) with the Col-0 background; those with lower AR values are in green. **(C)** Representative images of whole leaf explants of the studied lines at 14 dae. Scale bars: 2 mm.

CYP83A1 enzyme involved in the initial conversion of aldoximes to thiohydroximates in tryptophan-independent glucosinolate biosynthesis pathway (Bak and Feyereisen, 2001; Nintemann et al., 2018). One additional loss-of-function allele of the *REF2* gene was tested for *de novo* root formation in the petiole base of whole leaves (**Figure 7A**), which also produced vegetative rosettes with small curled down leaves (**Supplementary Figure S3**). Similar to *m678* mutants, *ref2-1* homozygotes activated vascular proliferation in most leaf explants and a significantly higher number of ARs were produced from these tissues (**Figures 7B,C**).

The Xylem Differentiating Factor XYP1 Is a Negative Regulator of AR Formation

The *mars* mutant *m232* was homozygous for a T-DNA insertion in the 3rd exon of the *XYLOGEN PROTEIN 1* (*XYP1*) locus (**Figure 7A**). XYP1 has been postulated as the xylogen factor for xylem differentiation (Motose et al., 2004). We identified homozygous mutants from two additional T-DNA insertional lines of the *XYP1* gene (**Figure 7A**). T-DNA homozygous mutants of the Sail_896_G05 line also developed more ARs than the Col-0 background (**Figure 7B**). Homozygous mutants in the Salk_147826 line showed a significant increase in rooting capacity of leaf explants at 10 dae as compared with those of Col-0 (**Figures 7B,D**). We wondered whether the increase number of ARs in leaf explants of *m232* and Salk_147826C homozygotes was caused by enhanced vasculature proliferation. The area of vascular proliferation on leaf explants at 7 and 10 dae was similar in these two mutants and the Col-0 background (**Figures 7E,F**), suggesting that the loss of XYP1 function enhanced post-embryonic root founder cell specification which ultimately lead to an increase in AR number.

DISCUSSION

We optimized a protocol to study wound-induced AR formation in *A. thaliana* hypocotyls, which is suitable for high-throughput mutant screens. Our results indicate that whole root-excision both triggered specification of new auxin-responsive (*pDR5::GUS*) foci and growth of already-specified auxin-responsive foci within the hypocotyl, leading to a significant increase in the number of ARs a few days after

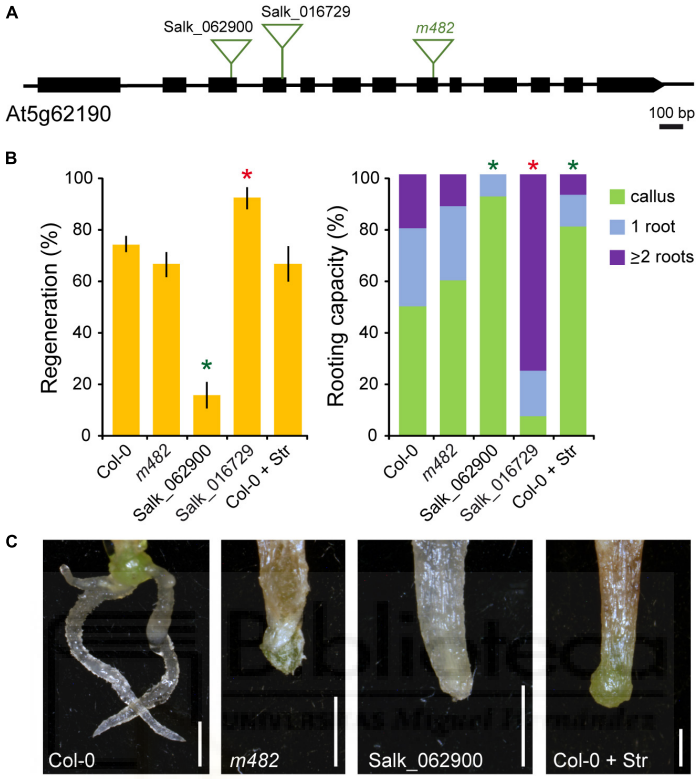

FIGURE 6 | Functional analysis of *AtRH7/PRH75* during AR formation. **(A)** Gene structure of At5g62190. Exons are represented by boxes and introns are depicted as lines. The studied mutant lines and annotated T-DNA insertion (triangles) lines are indicated. **(B)** Regeneration and rooting capacity in leaf explants of selected lines and treatments at 10 dae. Asterisks indicate significant differences (LSD; p-value < 0.01) with the Col-0 background; those with lower AR values are in green and those with higher AR values in red. **(C)** Representative images of whole leaf explants of the studied lines and treatments at 14 dae. Str: 30 μg ml^{-1} streptomycin. Scale bars: 2 mm.

the excision. We found substantial variation in the rate of AR formation in the wild-type background among experiments, indicating an environmentally mediated regulation of this developmental response. Hypocotyl-derived ARs originated from xylem-pole pericycle cells in a process resembling lateral root initiation (Bellini et al., 2014; Verstraeten et al., 2014). In the current model for wound-induced AR formation in hypocotyls (Sukumar et al., 2013), root excision enhances polar auxin transport through the hypocotyl while auxin accumulation at the excision site drives localized specification of AR founder cells within the pericycle. In intact hypocotyls, polar auxin transport through the hypocotyl and toward active primary (and lateral) root meristems reduced auxin accumulation in hypocotyl pericycle cells, which, in turn, limits following AR emergence.

By combining gene profiling data and a systematic phenotypic screen, we identified a large number of leaf mutants with a pleiotropic phenotype on AR formation in hypocotyls after whole root excision. In our study, 47 (41.6%) and 8 (7.1%) of studied PhenoLeaf mutants displayed, respectively, significantly less and more wound-induced ARs in the hypocotyl than the Col-0 background. In most species, however, AR formation aroused from non-root tissues, such as the vascular cambium, in a process that requires cell dedifferentiation and presumably different regulatory pathways as hypocotyl-derived ARs (Druege et al., 2018). Hence, we assayed *de novo* root organogenesis in excised whole leaves (Bustillo-Avendaño et al., 2018) of selected AR mutants. Nearly all the studied mutants displayed similar AR responses in excised whole leaves too, which suggest that the genes affected in these mutants participated in shared regulatory pathways required for *de novo* organ formation from different organs.

We have identified in our screen a relatively large number of mutants in protein translation- and ribosomal protein-encoding genes that displayed a significant reduction in AR number in the hypocotyl after whole root excision (n = 11; 23.4% of the *lars* mutants studied). Some of them lacked specific ribosomal

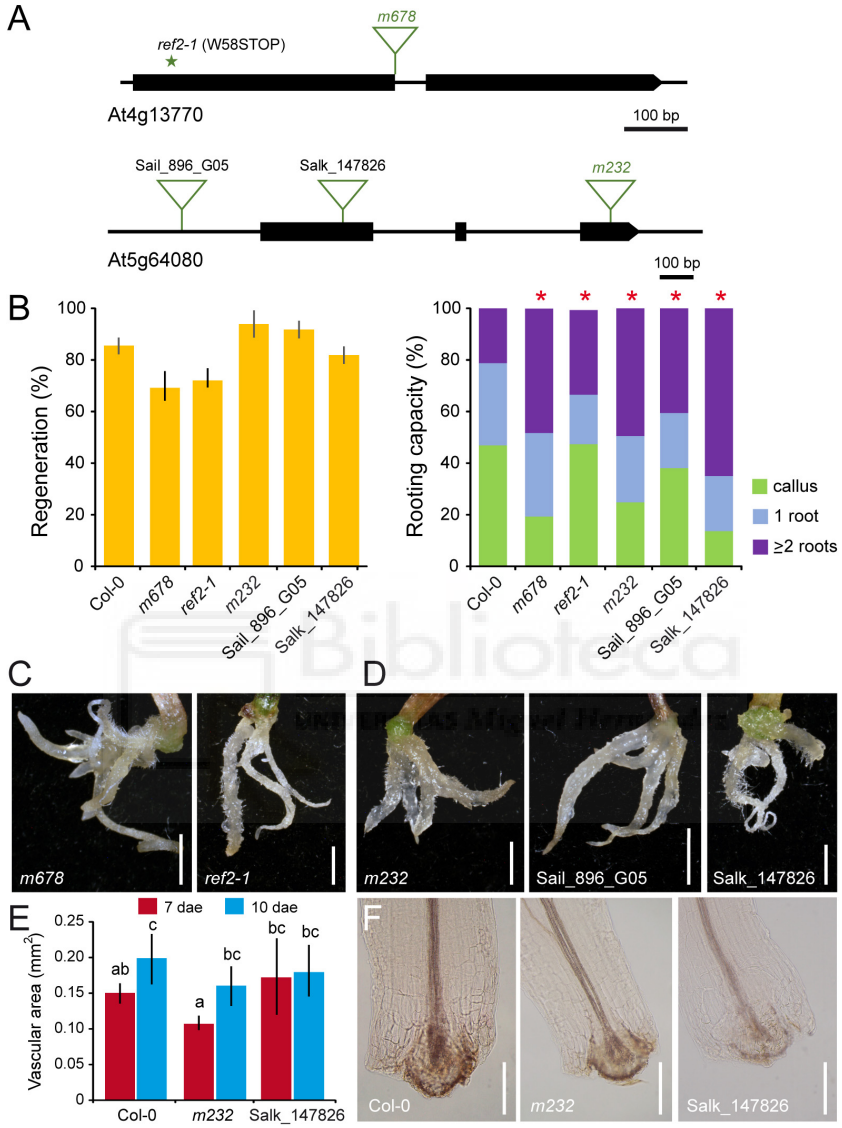

FIGURE 7 | Functional analysis of *REF2* and *XYP1* during AR formation. **(A)** Gene structure of At4g13770 and At5g64080. Exons are represented by boxes and introns are depicted as lines. The studied annotated T-DNA insertion (triangles) lines are indicated. **(B)** Regeneration and rooting capacity in leaf explants of selected lines at 10 dae. Asterisks indicate significant differences (LSD; p-value < 0.01) with the Col-0 background; those with higher AR values are in red. **(C,D)** Representative images of whole leaf explants of the studied lines at 14 dae. Scale bars: 2 mm. **(E)** Area of the vascular region in the proximal petiole at 7 and 10 dae of selected *xyp1* mutants. Different letters indicate significant differences (LSD; p-value < 0.05). **(F)** Vascular proliferation on the proximal petiole at 7 dae of selected *xyp1* mutants. Scale bars: 200 μm.

protein functions. An example of these were the *m274* mutant, which was homozygous for a T-DNA insertion in the At4g16720 gene encoding a ribosomal protein of the L23/L15e family (Carroll et al., 2008), and the *m285* mutant, which carried a T-DNA insertion in the *PIGGYBACK1* (*PGY1*) gene encoding the L10a ribosomal subunit (Pinon et al., 2008). Despite the

known genetic redundancy between ribosomal protein-encoding genes (Carroll et al., 2008), some of their mutants exhibited rare developmental phenotypes (e.g., pointed leaves and auxin-related phenotypes) that suggest non-equivalent functions of ribosome paralogs for the translational regulation of specific target mRNAs (Horiguchi et al., 2012). Other *lars* mutants related to ribosome function were *m405, m482*, and *m602*. *m482,* and *m602* carried homozygous insertions in two genes, respectively, encoding the DEAD-box RNA helicases AtRH57 (Hsu et al., 2014) and AtRH7/PRH75 (Huang et al., 2016). Both genes were required for pre-rRNA processing (Liu and Imai, 2018). Interestingly, the *root initiation defective1-1* (*rid1-1*) mutant identified as a temperature sensitive allele of another DEAH-box RNA helicase-encoding gene showed reduced hormone-induced AR formation from hypocotyl explants (Konishi and Sugiyama, 2003; Ohtani et al., 2013). *m405* affects At3g09720, which encodes the large subunit of a GTPase required for maturation of the 60S ribosomal subunit and whose loss-of-function caused the alteration of auxin distribution, auxin response, and auxin transport, and consequently affecting multiple auxin-regulated developmental processes (Zhao et al., 2015). Our results are in agreement with a specific role for ribosomes as regulators of key patterning events in AR development. One possibility is that ribosome function influences the cell's ability to undergo cell division during the early stages of AR formation (e.g., vasculature proliferation) or, alternatively, that certain set of genes involved in specific AR responses might require a particular ribosome conformation and therefore will be selectively regulated. Although the fact that mutant alleles of specific ribosomal protein-encoded genes caused a decrease in translational expression of particular auxin response factors (Rosado et al., 2012) favors the later hypothesis, its confirmation require further research.

Several lines of evidence support the hypothesis that active GAs are critical for primary root development through the control of root meristem size (Achard et al., 2009; Úbeda-Tomás et al., 2009). However, reports from various species suggest that GAs have an inhibitory effect on AR development (Niu et al., 2013; Mauriat et al., 2014). In hybrid aspen, transgenic plants with enhanced GA biosynthesis or signaling had significantly fewer ARs in stem cuttings, likely by the negative crosstalk of GAs with polar auxin transport (Mauriat et al., 2014). Analysis of GA-constitutive mutant *procera* (*pro*), a loss-of-function in a DELLA-like protein, also indicates that reduced levels or sensitivity to GA are associated with enhanced hormone-induced *in vitro* organogenesis in tomato (Lombardi-Crestana et al., 2012). However, we found that loss-of-function alleles of *GA1* and *GA5*, which are involved in key steps of GA biosynthesis (Xu et al., 1995; Michaels and Amasino, 1999), cause a significant decrease in AR numbers in both hypocotyl explants and excised leaves. In addition, we demonstrated that GA-related AR phenotypes were dependent on the growth-repressing DELLA function. Retarded growth of AR primordia in GA-deficient mutants was consistent with a positive role for GAs on both cell production and cell elongation in the root meristem (Achard et al., 2009; Úbeda-Tomás et al., 2009). In line with our results, intriguing results were found for GA function during AR formation in tobacco cuttings (Niu et al., 2013), which were interpreted as a consequence of GAs negatively regulating the early initiation step of AR formation but stimulating AR elongation. In all these examples, the relationship between GA biosynthesis and GA signaling appears to be both complex and context specific, which deserves further investigation.

The eight *mars* mutants that we identified might define negative regulators of AR formation. Among them, the *m667* mutants were homozygous for a T-DNA insertion in At2g45310 (*GAE4*), one of the six genes encoding UDP-D-glucuronate 4-epimerases involved in pectin biosynthesis (Molhoj et al., 2004; Usadel et al., 2004). Confirming the role for cell wall mechanics in AR initiation, the *atpme3-1* mutant, with low pectin methylesterase levels, also displayed a large increase (>30%) in the number of ARs emerging from the hypocotyl (Guenin et al., 2011). Indeed, fine-tuned crosstalk between microtubules (MTs), cell walls and auxin transport has been shown to be required for AR induction (Abu-Abied et al., 2015). In addition, MT perturbations caused a lack of PIN1 polarization and a loss of auxin maxima localization in the hypocotyl, which in turn lead to the formation of amorphous cell clusters and defective AR formation (Abu-Abied et al., 2015). In line with these results, the *m667* mutant might contain altered pectin levels in the wounded hypocotyl that interferes with PIN1 localization and auxin response during AR formation.

We identified two T-DNA insertions within the *XYP1* gene that caused a *mars* phenotype. *XYP1* is one of the genes encoding xylogen, an extracellular arabinogalactan protein that mediates local intercellular communication involved in xylem cell differentiation of *Zinnia elegans* cell cultures (Motose et al., 2004). According to previous studies, xylogen is secreted directionally from differentiating vascular cells, moves in the apoplast to the adjacent undifferentiated mesophyll cells and draws them into the pathway of vascular differentiation (Motose et al., 2004). In many species, the vascular cambium has been identified as the originating tissue for stem-derived ARs (Bellini et al., 2014; Druege et al., 2018). A definite population of indeterminate cambial initials that produce xylem mother cells inward and phloem mother cells outward from the cambium has been proposed to reside within the vascular cambium (Nieminen et al., 2015). It is therefore possible that reduced xylem differentiation in *xyp1* mutants will enlarge the number of these cambial initials allowing an auxin-mediated specification of a large population of AR founder cells and hence increasing the number of ARs formed in these mutants. Further studies using marker lines for AR founder cell specification (Bustillo-Avendaño et al., 2018) will help to confirm this hypothesis.

Another mutant with higher rooting capacity in wound-induced hypocotyls was *m678*, which is homozygous for a T-DNA insertion in *REF2*, encoding the CYP83A1 enzyme that catalyzes the conversion of aldoximes to thiohydroximates in the tryptophan-independent glucosinolate biosynthesis pathway (Bak and Feyereisen, 2001; Nintemann et al., 2018). Interestingly, the development of ARs from the hypocotyl is a well−known feature of the high−auxin phenotype of *superroot2-1* (*sur2-1*) mutant with a loss of function in CYP83B1, sharing 63% amino acid identity with CYP83A1 (Delarue et al., 1998;

Barlier et al., 2000). The *ref2-1* and *sur2-1* mutants displayed reduced glucosinolate levels and increased levels of its precursors in leaves, suggesting a compensatory interplay between CYP83A1 and CYP83B1 in some organs (Hemm et al., 2003). The contrasting results found for *ref2* and *sur2-1* mutants in wound-induced AR formation in hypocotyls and whole leaves might be due to unequal genetic redundancy between *REF2* and *SUR2* (Briggs et al., 2006). Indeed, indole-3-acetaldoxime channeling into production of either indole-3-acetic acid (IAA) or glucosinolates is tightly controlled and could explain the high-auxin phenotypes of *ref2-1* and *sur2-1* mutants. Other glucosinolate biosynthesis mutants also have increased levels of IAA and therefore enhanced auxin responses, which indicates a direct interaction between the biosynthetic pathways of glucosinolates and auxin (Malka and Cheng, 2017).

We used a network-guided genetic approach on a well-characterized T-DNA mutant collection (PhenoLeaf) that allowed us to identify novel functions in AR development for genes involved in foreseen housekeeping functions. With the advent of new systems biology tools (Waese et al., 2017), candidate genes will be selected based on cell-specific expression, protein-protein and protein-DNA interaction, and high-throughput screening for AR phenotypes in multiple T-DNA insertional lines of each gene will be conducted.

AUTHOR CONTRIBUTIONS

JMP-P was responsible for conceptualization and supervision. SI, JV, and JMP-P were responsible for methodology. SI, HR-C, MÁF, ABS-G, and JV were involved in the investigation. SI and JMP-P performed the formal analysis and wrote the original draft. SI, JLM, and JMP-P were involved in the review and editing of the manuscript. JMP-P provided the funding acquisition. JLM provided the PhenoLeaf mutants studied here.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.00461/full#supplementary-material

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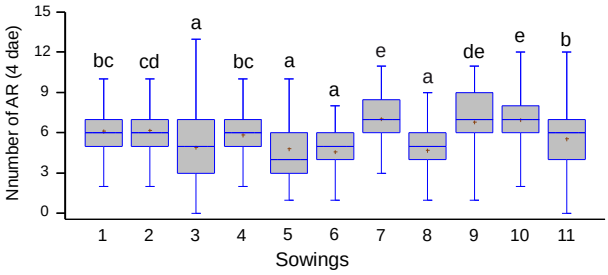

Figure S1.- Box-plots of adventitious root number in Col-0 hypocotyls according to the different sowings. Letters indicate statistically significant differences (LSD; p-value<0.05) between sowings. dae: days after excision.

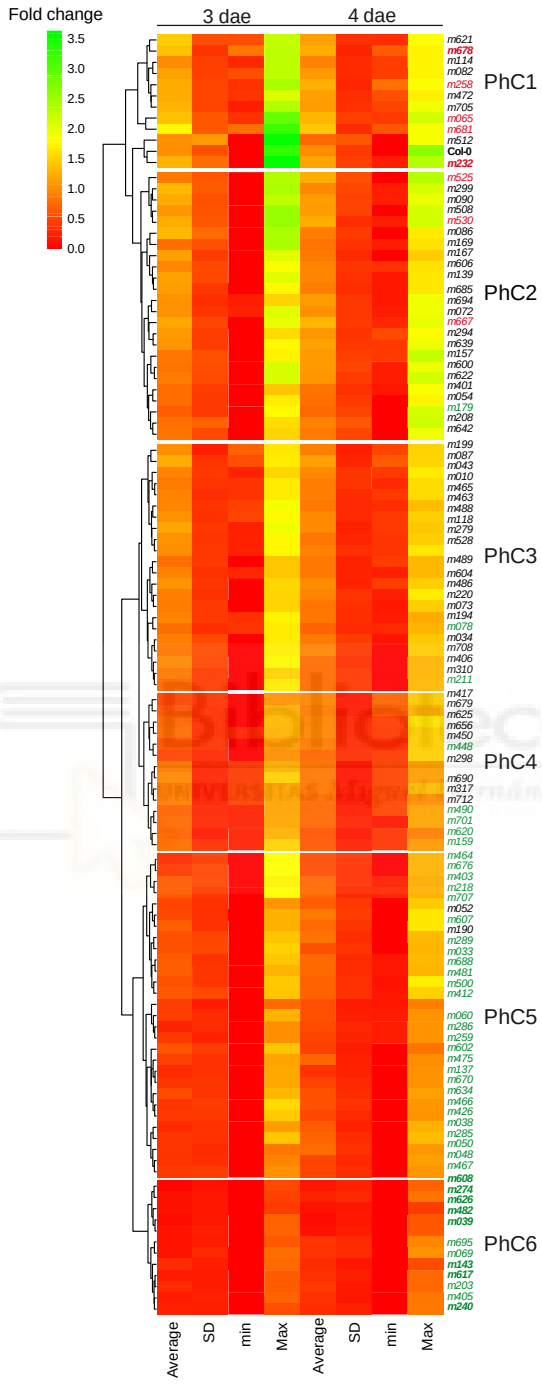

Figure S2. Heat map representation of relative rooting capacity in the studied lines. Each row corresponds to a single mutant, and the color scale corresponds to the fold change of rooting capacity values relative to the Col-0 background. Manhattan distance matrix between lines as regards their AR formation relative values was calculated to build the dendrogram. Six phenotypic clusters, PhC1 to PhC6, were defined based on this dendrogram. Mutant lines with significant changes over Col-0 are shown in green (lower AR values) or red (higher AR values), and mutant lines selected for further studies are indicated in bold; dae: days after excision.

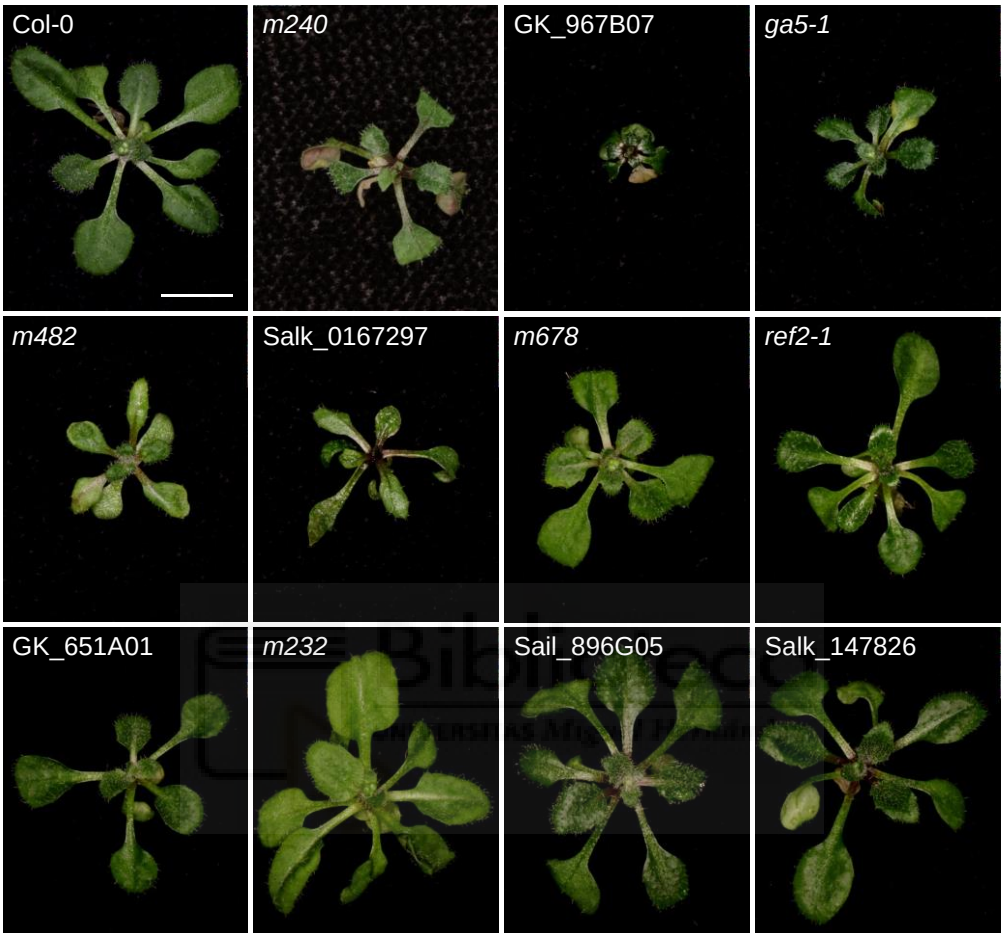

Figure S3. Rosettes of the *lars* and *mars* mutants studied in this work. Pictures were taken 21 d after sowing in Petri dishes. Scale bar: 5 mm.

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