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Desarrollo de herramientas para la mejora genética del enraizamiento de esquejes en clavel cultivado (*Dianthus caryophyllus* L.)

Joan Villanova Calatayud

Elche 2017

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JOSÉ MANUEL PÉREZ PÉREZ, Profesor Titular de Universidad en el área de conocimiento de Genética de la Universidad Miguel Hernández de Elche,

HAGO CONSTAR

que el presente trabajo ha sido realizado bajo mi dirección y recoge fielmente la labor realizada por el Ingeniero Joan Villanova Calatayud para optar al grado de Doctor;

que este documento se ha elaborado siguiendo la normativa de la Universidad Miguel Hernández de Elche para la presentación de Tesis con un conjunto de publicaciones, siendo éstas las siguientes:

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Eugenio Vilanova Gisbert, Catedrático de Toxicología y Director del Instituto de Bioingeniería,

HACE CONSTAR:

Que da su conformidad a la presentación de la Tesis Doctoral por compendio de publicaciones de Don **Joan Villanova Calatayud**, titulada **"Desarrollo de herramientas para la mejora genética del enraizamiento de esquejes en clavel cultivado (*Dianthus caryophyllus* L.)"**, que se ha desarrollado dentro del Programa de Doctorado en Bioingeniería de este Instituto, bajo la dirección del profesor Dr. José Manuel Pérez Pérez.

Lo que firmo en Elche, a instancias del interesado y a los efectos oportunos, a uno de febrero de dos mil diecisiete.

Eugenio Vilanova Gisbert

Catedrático de Toxicología

Director del Instituto de Bioingeniería

Universidad Miguel Hernández

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A mi familia

A Helena

A mis amigos

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Capítulo 1

Introducción general

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1. INTRODUCCIÓN GENERAL

1.1. El clavel cultivado como sistema modelo

1.1.1. Características de la especie a estudio

Una de las primeras descripciones de la flor del clavel la realizó el filósofo griego Teofrasto, cuando en el año 300 a.C. nombró a esta flor *Dianthus*, que significa *dios* (Dios) y *anthos* (Flor). Posteriormente, la "Flor divina", fue descrita por Linneo como *Dianthus caryophyllus* (Nau 2011; Tabla 1).

Tabla 1.- Clasificación botánica del clavel (Sánchez 1998)

Reino	Plantae (Plantas)
Subreino	Tracheobionta (Plantas vasculares)
Superdivisión	Spermatophyta (Plantas con semillas)
División	Magnoliophyta (Plantas con flores)
Clase	Magnoliopsida (Dicotiledóneas)
Subclase	Caryophyllidae
Orden	Caryophyllales
Familia	Caryophyllaceae
Género	*Dianthus*
Especie	*Dianthus caryophyllus* L.

Dianthus es un género monofilético, constituido por diferentes linajes bien definidos morfológica y geográficamente (Valente *et al*. 2010). Una gran mayoría de las especies de este género pertenecen al linaje euroasiático, que se caracteriza por haber experimentado una diversificación muy rápida en los últimos dos millones de años, sobre todo en la cuenca mediterránea. Esta diversificación parece estar relacionada con la estacionalidad de las temperaturas y la falta de lluvias en verano que caracterizan a esta región biogeográfica, produciéndose una fuerte adaptación local en los caracteres florales como respuesta a sus insectos polinizadores (Valente *et al*. 2010). Adicionalmente, algunos eventos de poliploidización (que son relativamente frecuentes en algunas especies de *Dianthus*) podrían haber contribuido al aislamiento reproductivo de las nuevas variedades (Balao *et al*. 2011; 2010).

La especie *Dianthus caryophyllus* L. es originaria del sur de Europa, principalmente de la zona mediterránea. Los claveles silvestres son plantas herbáceas anuales o perennes, con tallos glabros y erectos de hasta 80 cm de altura. Cada nudo

presenta un par de hojas con su base en forma de vaina, las cuales son opuestas, estrechas y alargadas. Sus inflorescencias son simples, están constituidas habitualmente por una sola flor situada en el extremo terminal del tallo. Las flores de clavel silvestre tienen un aroma característico del clavo de olor, lo que le ha valido su nombre común, *clavus* en latín. Las flores constan de 5 pétalos libres, flanqueados por cinco sépalos soldados que forman un cáliz largo y estrecho, cuya base contiene dos o tres pares de brácteas. En su interior las flores tienen diez estambres y sus dos carpelos forman un único pistilo súpero (Masalles *et al*. 1988; Figura 1A).

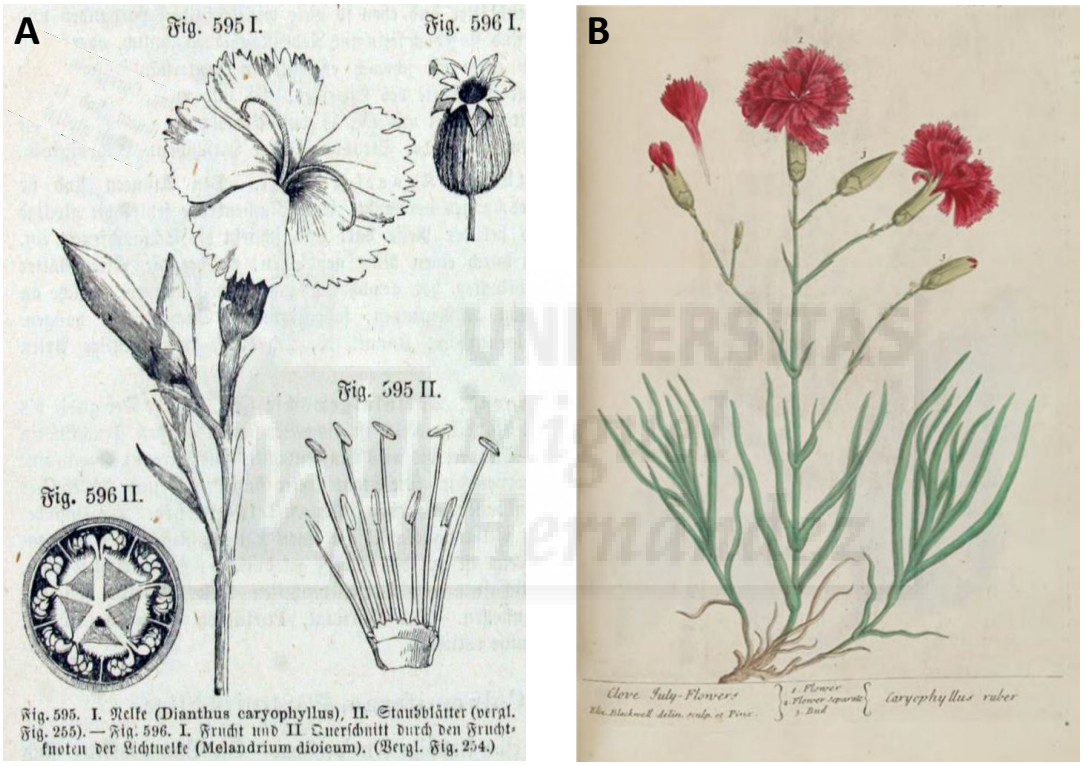

Figura 1. Morfología de la flor en el clavel silvestre (A) y el clavel cultivado (B). La ilustración en A se ha tomado de la fuente www.biolib.de**.** La ilustración en B se ha tomado del catálogo ilustrado de las plantas cultivadas de Blackwell (1737).

El clavel silvestre se ha utilizado como base para la creación de la mayoría de los claveles comerciales actuales, siendo una gran parte de ellos híbridos interespecíficos obtenidos para incorporar características deseables de otras especies de clavel (Sheela 2008). Tras un proceso intensivo de selección se han obtenido claveles con flores de mayor tamaño y gran variabilidad en colores, con un elevado número de pétalos y con tallos más largos y robustos que sus antecesores silvestres (Figura 1B). Además, los claveles cultivados florecen durante todo el año (Sheela

2008). Es de destacar que no se conocen variedades de clavel con flores de color azulado, ya que carecen de las enzimas en la ruta biosintética de las antocianinas, productoras del pigmento azul delfinidina (Nishihara y Nakatsuka, 2011).

1.1.2. Producción industrial de plantas de clavel

A nivel mundial, el clavel ocupa el quinto lugar en importancia en el campo de la flor cortada precedido, en este orden, por la rosa, el crisantemo, el tulipán y el lirio (Sheela 2008). En España, sin embargo, el clavel es el producto de mayor relevancia dentro del sector de la flor cortada, abarcando el 43% de la producción y el 30% de la superficie de cultivo (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2015). Para mantener una elevada productividad en el sector ornamental en general y en el del clavel en particular, se hace necesaria una reducción en los costes de producción y una mejora en la calidad del producto y en los procesos de producción. Por ello es importante tecnificar el proceso productivo y disponer de las variedades de clavel lo más definidas posible, dando relevancia a la realización de estudios que conlleven mejoras en la productividad de las empresas del sector (Ochoa *et al.* 2008; Peris 2008).

La empresa Barberet & Blanc, S.A. (http://www.barberet.es), proveedora de todas las muestras empleadas en la realización de este trabajo, se localiza en Puerto Lumbreras (Murcia). Esta empresa es una de las compañías líderes en la selección, propagación y comercialización de esquejes y plántulas de clavel. Exhibe una cuota en el mercado mundial del 26% y una superficie en invernaderos de más de 160.000 m^2. Desde 1965 ha lanzado más de 400 nuevas variedades protegidas, todas ellas con certificado de obtención vegetal. Los productos comercializados por Barberet & Blanc, S.A. se distribuyen en más de 60 países repartidos por los cinco continentes. Se estima que de cada cuatro claveles producidos a nivel mundial, uno de ellos tiene su origen en Barberet & Blanc, S.A.

En la Figura 2 se indica el procedimiento de propagación que se sigue habitualmente en la empresa Barberet & Blanc, S.A. A partir de los híbridos seleccionados por los mejoradores se establecen dos bancos de material vegetal, uno en el laboratorio, denominado banco *in vitro*, y otro en el invernadero, denominado banco *in vivo*. Los esquejes procedentes de las plantas del banco *in vivo* son plantados

en la reserva nuclear. De cada una de las plantas de la reserva nuclear se toman esquejes que se plantarán en la reserva de propagación (varios miles de plantas de cada cultivar). De la reserva de propagación se derivan las plantas madre, a partir de las cuales se obtendrán los esquejes comerciales.

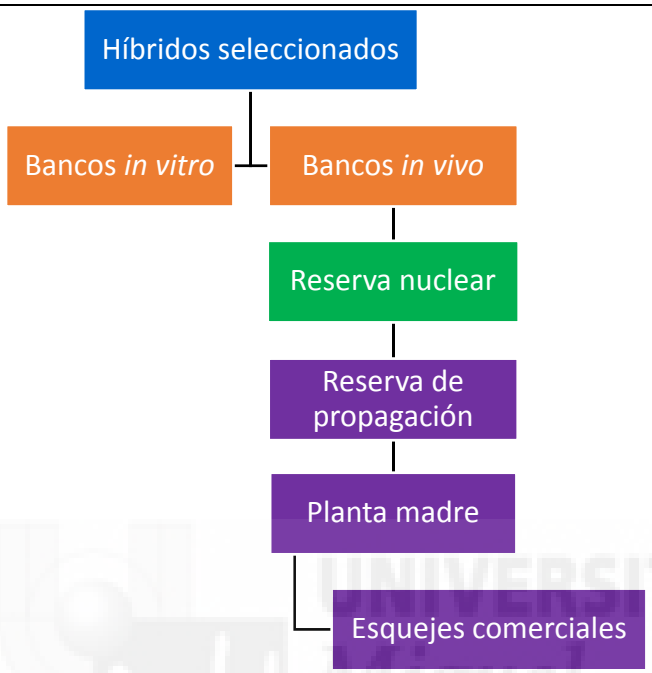

Figura 2. **Esquema de propagación de las variedades de clavel seleccionadas**. En cada una de las etapas presentadas en el diagrama se realizan ensayos moleculares para confirmar que las muestras están libres de virus. Adaptado, con ligeras modificaciones, de Agulló-Antón (2011).

Con este método de producción clonal se mantiene la uniformidad genética en los esquejes y se previene la aparición de enfermedades, al realizarse análisis continuados de la calidad sanitaria de los mismos. Las plantas madre se renuevan anualmente, siendo su ciclo de cultivo de unos siete meses y medio. De cada planta madre se escinden de forma manual entre 25 y 30 esquejes cada dos semanas, que se almacenan en cámaras refrigeradas. Los esquejes habitualmente se distribuyen a otros productores secundarios, que se encargan del cultivo y la distribución de la flor cortada del clavel.

Dado que la producción de flores de clavel se realiza, normalmente, en un lugar distinto al de la producción de los esquejes, es necesario que el enraizamiento de estos últimos se produzca de forma correcta y que las raíces estén en un estado óptimo de desarrollo. Para ello, se pueden transportar los esquejes sin enraizar, o bien se pueden enraizar los esquejes en sus lugares de producción (Figura 3). En cualquiera de los dos

casos, el enraizamiento de los esquejes es una etapa fundamental en la cadena de producción.

Reserva de propagación | Invernadero de plantas madre

Esquejes para su almacenamiento | Esquejes enraizados

Figura 3. Etapas en la producción de esquejes de clavel en la sede de la empresa Barberet & Blanc, S.A. en Puerto Lumbreras (Murcia).

1.1.3. Herramientas moleculares en clavel

Los mapas genéticos proporcionan información detallada sobre la ubicación cromosómica de los genes en relación con otros genes de posición conocida, y se calculan utilizando las frecuencias de recombinación entre ellos: una recombinación del 1% equivale a 1 unidad de mapa, o 1 centiMorgan (cM; Pierce 2010). En los estudios de Yagi *et al*. (2013; 2012; 2006), se determinaron los primeros mapas genéticos del clavel (Figura 4), que se han utilizado en el análisis de genes de caracteres cuantitativos o QTL (*Quantitative Trait Loci*) para la resistencia a la podredumbre bacteriana causada por *Burkholderia caryophylli* o para el contenido en antocianinas de sus flores (Yagi *et al*. 2013). Los análisis que se llevaron a cabo en estos

dos estudios permitieron asignar 635 loci a 15 grupos de ligamiento sobre un total de 970 cM.

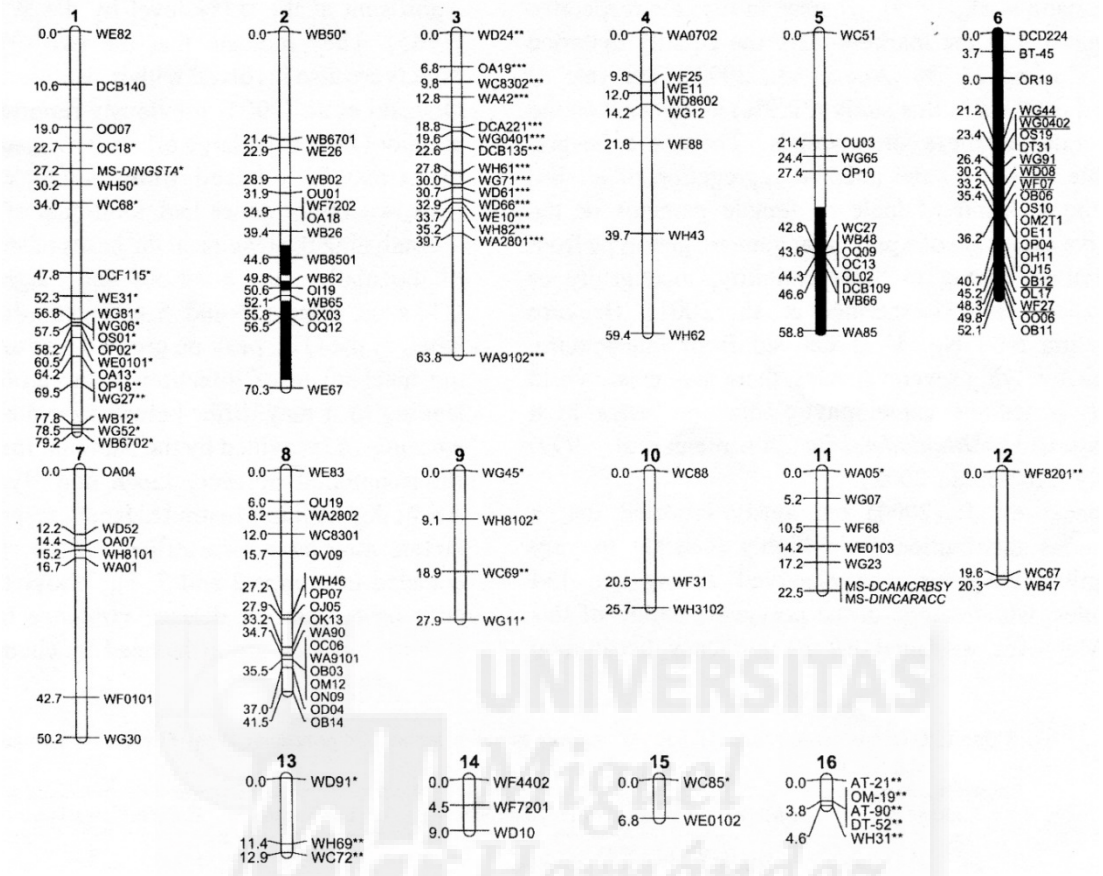

Figura 4. Uno de los primeros mapas genéticos del clavel. Imagen tomada de Yagi *et al*. (2006).

El clavel silvestre, *Dianthus caryophyllus* L., es una especie diploide (2*n* = 30). Aunque se conocen variedades de clavel cultivado tetraploides y hexaploides, la mayoría de las variedades comerciales de clavel son diploides. El clavel cultivado presenta un genoma nuclear haploide de unas 670 Mb (Agulló-Antón *et al*. 2013). En 2012 se inició la caracterización molecular del transcriptoma de clavel mediante la secuenciación de una librería de ADN complementario procedente de distintos tejidos en la variedad de referencia de clavel cultivado "Francesco" (Tanase *et al*. 2012). Estos estudios permitieron la obtención de polimorfismos en la longitud de secuencias simples (SSLP; *Simple Sequence Length Polymorphisms*) y la identificación de transcritos en genes asociados a distintos procesos, tales como la biosíntesis de carotenoides y antocianinas, la biosíntesis y degradación de clorofilas o la biosíntesis y señalización del etileno (Tanase *et al*. 2012). Utilizando técnicas de secuenciación de

última generación, el grupo de Yagi *et al*. (2014) ha obtenido recientemente unas 569 Mb de secuencia del genoma nuclear en la variedad de referencia de clavel cultivado "Francesco". Mediante la búsqueda de homólogos en las bases de datos, se han identificado 43.266 genes que codifican proteínas. Además, 238 de los 248 genes eucarióticos esenciales (*core eukaryotic genes*) se han identificado durante la secuenciación del genoma del clavel, lo que, junto a la estimación del tamaño del genoma por el método de los *k*-meros, implica que se conoce alrededor del 91% del total de la secuencia de su genoma nuclear. Los resultados de este trabajo se han depositado en una base de datos de acceso público (http://carnation.kazusa.or.jp/index.html). Esta información permitirá llevar a cabo estudios moleculares en esta especie. Un ejemplo destacado es la identificación de 217 genes de resistencia que contienen un dominio de unión rico en leucinas del tipo NBS-LRR (*Nucleotide-Binding Site Leucine-Rich Repea*t) y se ha determinado su posición en el genoma del clavel (Yagi *et al*. 2014), lo que permitirá el diseño de estrategias de mejora basadas en la selección asistida por marcadores para la introducción de los genes de resistencia entre distintas variedades.

1.2. Formación de raíces adventicias

El sistema radicular es determinante para el crecimiento vegetal por sus funciones básicas en la absorción de agua y nutrientes, como órgano de soporte y almacenamiento, y para la modulación de algunas respuestas frente a diferentes tipos de estrés (Rogers y Benfey, 2015). En plantas dicotiledóneas, como el clavel, el sistema radicular está formado por una raíz primaria o principal, de origen embrionario, a partir de la cual se desarrollan las raíces laterales. Por su parte, las raíces adventicias se desarrollan también de forma postembrionaria como las laterales, pero a partir de tejidos no radiculares, como el hipocótilo, los tallos o las hojas y en respuesta a distintos tipos de estrés o tras una herida (Steffens y Rasmussen, 2016; Bellini *et al*. 2014; Figura 5). En muchas especies, las células precursoras de las raíces adventicias no están preformadas y derivan de la desdiferenciación de una población muy definida de células vasculares, como las células del pericliclo en el hipocótilo (Sukumar *et al*. 2013) o las del cambium vascular en el tallo (Agulló-Antón *et al.* 2014; Verstraeten *et al*. 2014; Ahkami *et al*. 2013).

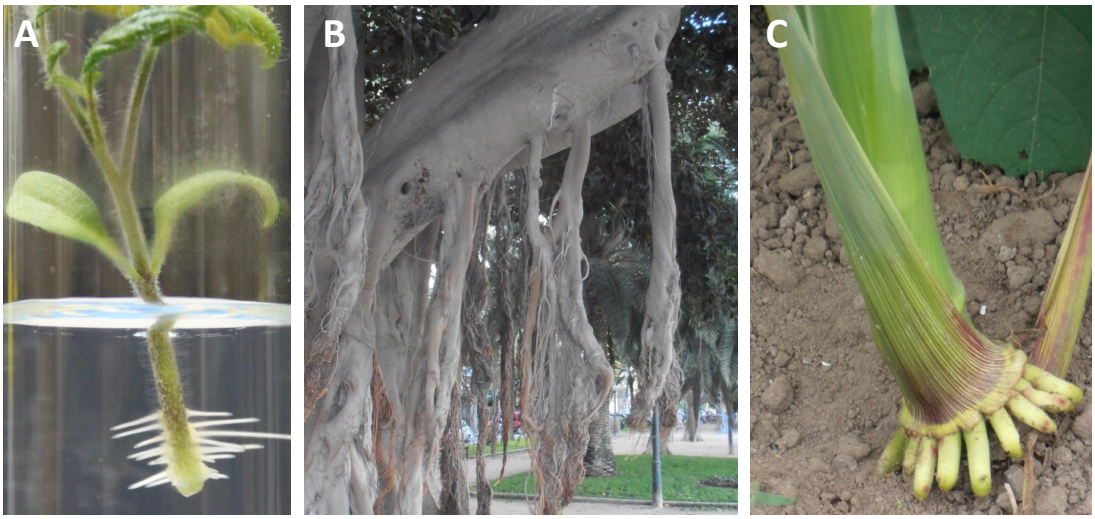

Figura 5. Algunos ejemplos de raíces adventicias. Raíces adventicias en el hipocótilo de tomate tras una herida (A). Raíces aéreas en tallos de ficus (B) y de maíz (C).

La formación de raíces adventicias es un proceso complejo regulado por factores tanto endógenos como ambientales, en el que las auxinas desempeñan una función clave (Pacurar *et al*. 2014). En *Arabidopsis thaliana*, las mutaciones de pérdida de función en el gen *ABCG19*, que codifica un transportador de auxinas, causan una reducción en el número de raíces adventicias, mientras que su expresión constitutiva las aumenta (Sukumar *et al.* 2013). Se ha observado que la expresión de *ABCG19* aumenta en el hipocótilo en respuesta a herida, lo que contribuye al incremento en el transporte de las auxinas desde las hojas (Sukumar *et al.* 2013). Las mutaciones en el ortólogo de *GNOM1* de arroz, *crown rootless4* (*crl4*), inhiben la formación de raíces en esta especie, tanto las adventicias (raíces en corona, *crown roots*) como las laterales (Liu *et al*. 2009). *GNOM1* codifica un factor que se requiere para la correcta localización polar del transportador de auxinas PIN-FORMED1 (PIN1). En consecuencia, el transporte polar desde las hojas y la posterior acumulación de auxinas en la base del tallo son esenciales en la formación de raíces adventicias en distintas especies. Otro de los genes que se requiere para la formación de las raíces adventicias en arroz es *CROWN ROOTLESS5* (Kitomi *et al.* 2011), que codifica un factor de transcripción de la familia APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) cuyo ortólogo en Arabidopsis se requiere en la regulación de la proliferación celular durante la formación de los órganos.

Además de las auxinas, las citoquininas son otros de los reguladores clave de la formación de raíces adventicias. Los mutantes deficientes en la biosíntesis de citoquininas o en su percepción muestran una mayor producción de raíces adventicias, mientras que el aumento de los niveles endógenos de citoquininas produce el efecto contrario (Rasmussen *et al.* 2012).

1.2.1. Formación de raíces adventicias a partir de esquejes

La formación de raíces adventicias a partir de tallos preformados escindidos de la planta madre (también denominados esquejes) se ha dividido tradicionalmente en tres etapas: (1) inducción, (2) iniciación, y (3) expresión (de Klerk *et al*. 1999). Durante la fase de inducción, algunas células en la base del esqueje revierten su identidad y se reprograman como células iniciadoras de los nuevos meristemos. A continuación, durante la fase de iniciación, se activa su proliferación celular y se forman los primordios radiculares. En la fase de expresión (también llamada fase de elongación), el crecimiento de los primordios radiculares es aparente, se establece su conexión vascular con el tallo y las raíces adventicias emergen y son funcionales (Cano *et al*. 2014; de Klerk *et al*. 1999).

Para el correcto enraizamiento de esquejes se requiere una acumulación localizada de las auxinas endógenas en la base del tallo del esqueje, que se produce como consecuencia de su transporte polar desde su lugar de síntesis (las hojas) hasta la zona de la herida (Ahkami *et al*. 2013; Acosta *et al*., 2009). En consecuencia, la aplicación exógena de auxinas induce la formación de raíces adventicias a partir de esquejes en incontables especies vegetales, tanto herbáceas como leñosas (Pacurar *et al.* 2014). Por otro lado, el tratamiento de los esquejes con citoquinina exógena suprime la formación de raíces adventicias (Bellini *et al*. 2014).

1.2.1.1. En especies forestales

En el álamo (*P. trichocarpa*) las primeras divisiones mitóticas, que conducen a la formación de primordios radiculares en la base del esqueje, se originan en el cambium y en los tejidos vasculares cercanos a la región del corte (Rigal *et al*. 2012). En un análisis transcriptómico realizado en esquejes de álamo durante la formación de raíces adventicias se identificó un factor de transcripción cuya actividad depende de las citoquininas, PtARR13, como un regulador negativo de la formación de raíces

adventicias en esta especie (Ramírez-Carvajal *et al*. 2009). La expresión de una forma constitutivamente activa de PtARR13 reprime la formación de raíces adventicias en esquejes de álamo, por lo que se ha propuesto que las citoquininas que se sintetizan en la raíz y se transportan a través del tallo inhiben la formación de raíces adventicias en plantas intactas mediante su regulación positiva de PtRR13. Por su parte, la eliminación de la fuente endógena de citoquininas tras la escisión del sistema radicular en los esquejes produce la inactivación de PtRR13 y permite el desarrollo de las raíces adventicias en la región de la herida (Ramírez-Carvajal *et al.* 2009). En otro trabajo se determinó que el factor de transcripción AINTEGUMENTA LIKE1 (PtAIL1), de la familia AP2/ERF, se expresa de manera específica en las primeras etapas de la formación de raíces adventicias y actúa como un regulador positivo de esta respuesta (Rigal *et al*. 2012). Las líneas de álamo transgénico que sobreexpresan *PtAIL1* exhibieron un mayor número de raíces adventicias, mientras que la reducción de los niveles endógenos de PtAIL1 utilizando ARN interferente produjeron plantas con una menor capacidad de enraizamiento adventicio (Rigal *et al*. 2012). En este modelo de regulación hormonal (Figura 6), la auxina promueve la activación de las células iniciadoras durante la inducción y la proliferación celular (Legué *et al*. 2014).

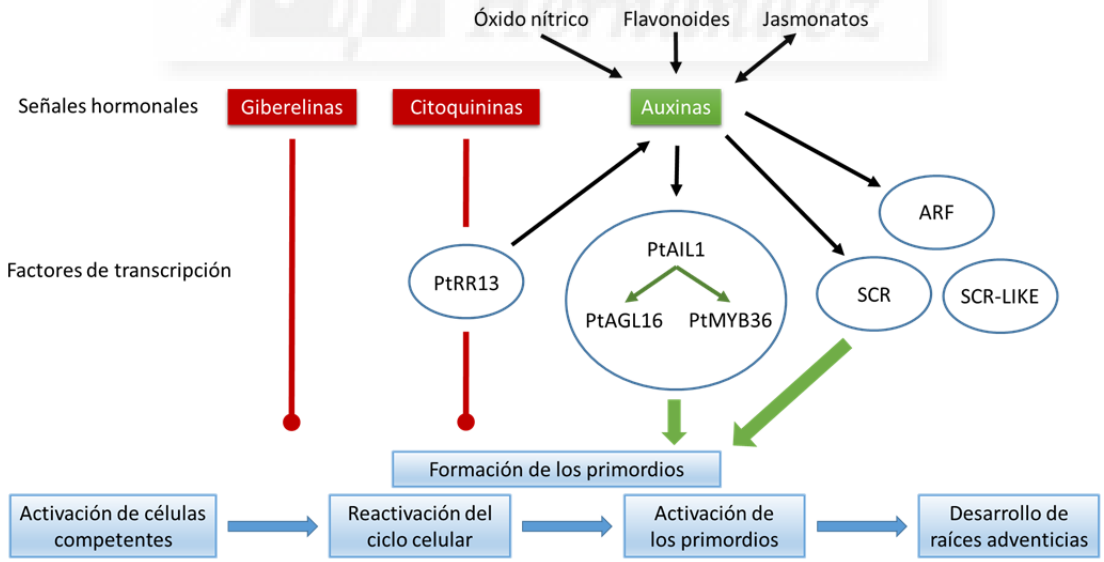

Figura 6. Posible regulación hormonal de la formación de raíces adventicias a partir de esquejes en especies leñosas. La imagen se ha tomado de Legué *et al*. (2014), con modificaciones. Las líneas rojas implican un efecto negativo de regulación, mientras que las líneas verdes indican una regulación positiva. Se muestran con líneas negras aquellos casos en los que no se conocen detalles sobre la regulación. Algunos de los factores de transcripción mostrados (PtAGL16, PtMYB36, SCR, SCR-LIKE y ARF) no se han descrito en el texto.

Recientemente, se han identificado en el álamo híbrido (*Populus deltoides* × *Populus euramericana*) dos ortólogos del factor de transcripción WUSCHEL-related HOMEOBOX11 (WOX11) de *Arabidopsis thaliana*, PeWOX11a y PeWOX11b, cuya sobreexpresión no solamente aumentó el número de raíces adventicias en esquejes, sino que también indujo la formación de raíces ectópicas en las partes aéreas del esqueje, como las hojas (Zhao *et al.* 2015). Por su parte, *OsWOX11*, el homólogo de *AtWOX11* en arroz, se expresa en las regiones en proliferación activa de los meristemos laterales del tallo y activa la aparición y el crecimiento de las raíces en corona (Zhao *et al*. 2009). Estos resultados indican que WOX11 puede actuar de forma redundante con otros miembros de la familia WOX en la regulación de la formación de raíces adventicias en distintas especies vegetales.

En especies forestales, la disminución de la capacidad de formar raíces adventicias a partir de esquejes es uno de los efectos más dramáticos de la maduración y ha sido objeto de numerosas investigaciones (Díaz-Sala 2014). *Eucalyptus grandis* se ha utilizado como sistema modelo para estudiar las diferencias en la expresión génica durante la formación de raíces adventicias en esquejes con diferente grado de maduración, dado que en esta especie se observa una disminución gradual de la capacidad de enraizamiento en tejidos fisiológicamente maduros (Abu-Abied *et al*. 2012). En el análisis transcriptómico comparativo que se llevó a cabo en este trabajo se identificaron unos 600 genes cuya expresión difirió significativamente entre esquejes juveniles y maduros. El análisis de los procesos biológicos en los que participaban estos genes reveló que los esquejes juveniles presentaban un alto potencial redox, que podría estar implicado en la transición rápida que se observa en los primordios radiculares entre la fase de iniciación (proliferación celular) y la fase de expresión (diferenciación y crecimiento de los primordios) (Abu-Abied *et al*. 2012). Otro de los procesos que se ha estudiado y que podría contribuir a las diferencias observadas en el enraizamiento entre esquejes juveniles y adultos es la regulación diferencial de la expresión de genes implicados en la formación del citoesqueleto (Abu-Abied *et al*. 2014).

Por otro lado, algunas especies de eucalipto que se utilizan para la producción de celulosa en la industria del papel, como *Eucalyptus globulus*, requieren de la adición

de auxina exógena para el enraizamiento de esquejes, mientras que otras son capaces de enraizar en ausencia de auxina endógena (de Almeida *et al*. 2015). Los resultados de este trabajo indicaron que los ortólogos en eucalipto de *TOPLESS* y *IAA12/BODENLOS*, relacionados con la regulación de la respuesta a auxina, y el gen *EgARR1*, relacionado con la respuesta a citoquininas, podrían actuar como reguladores negativos de la formación de raíces adventicias, posiblemente contribuyendo a la menor capacidad de enraizamiento observada en *E. globulus*.

1.2.1.2. En plantas ornamentales

Las especies del género *Petunia* pertenecen a la familia de las Solanáceas, son nativas de América del Sur, y tienen una gran importancia en la horticultura ornamental mundial. La extraordinaria diversidad fenotípica en las petunias actuales es el resultado de un proceso continuado de mejora genética que data de principios del siglo XIX, cuando se obtuvieron los primeros híbridos comerciales de *Petunia* × *hybrida* (Stehmann *et al*. 2009). La sencillez de su cultivo y propagación, el conocimiento de su secuencia genómica y la facilidad de su transformación genética hacen de *Petunia* × *hybrida* un sistema modelo atractivo para el análisis comparativo de la función génica (Bombarely *et al*. 2016). El grupo del Prof. Uwe Druege ha caracterizado la formación de raíces adventicias a partir de esquejes de *Petunia* × *hybrida*, lo que le ha llevado a proponer un modelo integrado de la regulación hormonal que tiene lugar durante este proceso (Druege *et al.* 2016; Figura 7). En un primer momento, la separación de los esquejes de la planta madre induce un aumento transitorio en la concentración del ácido jasmónico en la región de la herida, seguido de la expresión de genes regulados por esta hormona, algunos de los cuáles están implicados en el metabolismo de carbohidratos (Ahkami *et al*. 2009). En un trabajo reciente, se ha confirmado la función reguladora del ácido jasmónico en este proceso, dado que las líneas de *Petunia* × *hybrida* con niveles reducidos de ácido jasmónico muestran un retraso significativo en la formación de las raíces adventicias a partir de esquejes (Lischweski *et al*. 2015). Se ha determinado también que el mantenimiento de los esquejes en oscuridad durante varias semanas, de manera previa a su enraizamiento, incrementa la formación de raíces adventicias debido, en parte, a un aumento en la disponibilidad de carbohidratos en la base del tallo capaces de

sustentar el crecimiento de los nuevos primordios radiculares durante el enraizamiento adventicio (Klopotek *et al*. 2016; 2010).

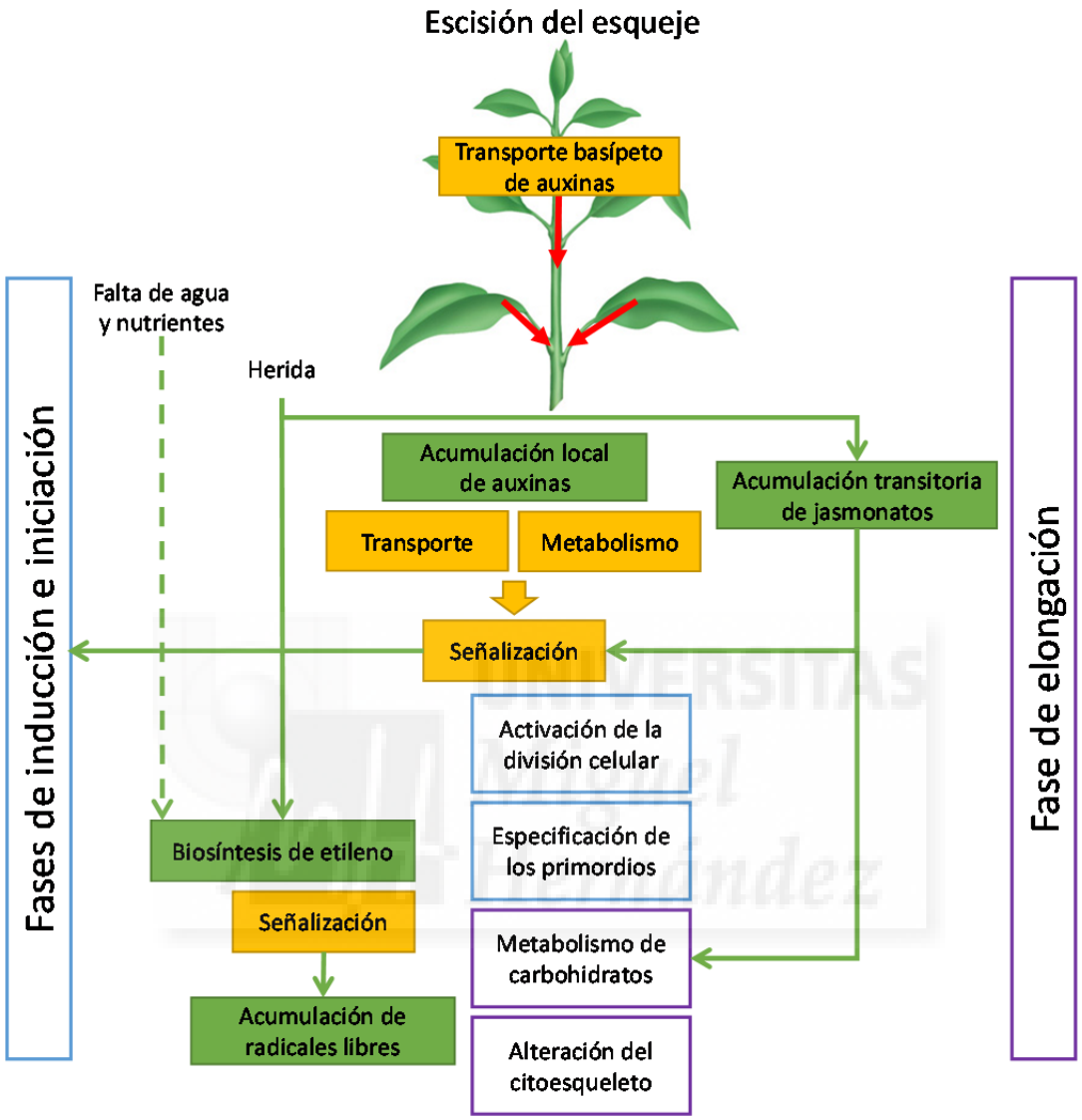

Figura 7. Un modelo hipotético de la regulación fisiológica durante la formación de raíces adventicias en esquejes de petunia. Se muestran con rectángulos en verde las distintas hormonas y segundos mensajeros implicados en el proceso y, en amarillo, su regulación. Los distintos procesos que tienen lugar durante la formación de raíces adventicias se han recuadrado en función de si ocurren, mayoritariamente, en las fases de inducción e iniciación (azul) o en la de elongación (morado). La imagen se ha tomado de Druege *et al*. (2016) y Ahkami *et al*. (2013), con modificaciones. La descripción de las diferentes fases de la formación de raíces en petunia se pueden encontrar en Ahkami *et al.* (2009).

Está ampliamente aceptado que las auxinas, principalmente el ácido indol-3-acético (AIA), actúan como inductores efectivos de la formación de raíces adventicias, mientras que niveles elevados de auxina tienen un papel inhibidor en etapas

posteriores (Ludwig-Müller, 2009; de Klerk *et al*. 1999). En los esquejes de petunia se ha estimado que los niveles absolutos de AIA en el momento de la escisión son mayores en las hojas que en la base del esqueje, con valores del 40% y del 11%, respectivamente (Ahkami *et al.* 2013). Además, los niveles de AIA aumentaron de forma rápida en la base del esqueje entre las 2 y las 24 h tras su escisión de la planta madre, y se redujeron considerablemente en tiempos posteriores, lo que se correlaciona bastante bien con los niveles de expresión del gen *GRETCHEN HAGEN3* (*GH3*) de respuesta a auxina. Estos resultados sugieren que la acumulación de AIA en la base del tallo, causada por el transporte polar de auxinas desde las hojas, induce la formación de raíces adventicias. Para comprobar esta hipótesis, los autores suprimieron el transporte polar mediante la adición exógena del ácido 1-naftiltalámico (*1-N-Naphthylphthalamic acid*) en las hojas y observaron una inhibición muy significativa de los niveles de auxina intracelulares en la base del esqueje y una reducción en la formación de raíces adventicias (Ahkami *et al.* 2013).

Los cultivares comerciales de clavel se propagan a partir de tallos terminales, de unos 4 a 6 pares de hojas, que se recolectan de forma periódica por personal especializado (Garrido *et al.* 1998; 1996). La formación de raíces adventicias en la base del esqueje es un proceso muy sensible y altamente dependiente del metabolismo celular y que está regulado por interacciones complejas entre los niveles endógenos de sacarosa y de diferentes fitohormonas (Agulló-Antón *et al*. 2011; Garrido *et al*. 2002). Una vez enraizados, los esquejes se transfieren a las cámaras de aclimatación antes de ser trasplantados a los campos de producción. Por lo tanto, la capacidad de enraizamiento de esquejes de las distintas variedades comerciales de clavel utilizadas es un aspecto importante a tener en cuenta en el escalado industrial del clavel. En algunas variedades, la producción de plántulas jóvenes se ve dificultada por la formación deficiente de raíces adventicias en la base del esqueje, lo que conduce a pérdidas de producción importantes (Agulló-Antón *et al*. 2011; Oliveros-Valenzuela *et al.* 2008; Garrido *et al*. 2002). El problema se ha superado, al menos en parte, optimizando el almacenamiento de los esquejes recién recolectados a temperaturas bajas (Garrido *et al.* 2003; Guerrero *et al*. 1999; Garrido *et al*. 1998; 1996).

1.3. Objetivos

A pesar de la enorme importancia que el enraizamiento adventicio desempeña en los métodos de propagación vegetativa en las distintas variedades comerciales de clavel, aún se desconoce la base genética que subyace a las interacciones hormonales que contribuyen a la formación de raíces adventicias en esta especie. En este contexto, se enmarcan los trabajos científicos presentados en esta memoria y que constituyen mi Trabajo de Tesis Doctoral, encaminados a:

- Caracterizar la variabilidad morfológica existente en la formación de raíces adventicias a partir de esquejes en una amplia colección de híbridos comerciales de clavel cultivado.
- Determinar los factores ambientales y fisiológicos que contribuyen a las diferencias observadas en el enraizamiento de esquejes en dos cultivares de clavel que difieren significativamente en su capacidad de enraizamiento adventicio durante su escalado industrial.
- Iniciar los estudios para la identificación de los eventos moleculares que regulan la señalización hormonal durante la formación de raíces adventicias en dos cultivares de clavel que difieren significativamente en su capacidad de enraizamiento adventicio.

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Capítulo 2

Resumen y conclusiones

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2. RESUMEN Y CONCLUSIONES

2.1. Resumen global

El clavel es una de las especies más importantes en el mercado mundial de la flor cortada. Las variedades comerciales de clavel se propagan vegetativamente mediante esquejes, tras su enraizamiento adventicio y aclimatación. Los esquejes de calidad comercial se caracterizan por la presencia de 30 a 40 raíces bien desarrolladas en su base, de entre 1 y 9 cm de longitud, y por un crecimiento sano y homogéneo tras un mes en la estación de enraizamiento. Los esquejes enraizados de calidad insuficiente se eliminan manualmente antes de su comercialización. En algunas variedades de clavel, la deficiente formación de raíces adventicias en la base del esqueje limita su escalado industrial debido a un aumento significativo en los costes de producción. Con este trabajo, hemos iniciado un enfoque genético-genómico (*Genetical Genomics*) para determinar la base molecular de las diferencias existentes en el enraizamiento adventicio en las distintas variedades comerciales de clavel.

A partir de las pérdidas durante el enraizamiento de esquejes en las 132 variedades comerciales de clavel que se propagaron vegetativamente en la estación de enraizamiento de Barberet & Blanc S.A. entre 2011 y 2013, se seleccionaron ocho variedades con valores extremos y opuestos en su porcentaje de pérdidas y otras dos representativas del comportamiento medio de este rasgo en toda la población de variedades. Para maximizar la contribución de los factores endógenos responsables de las diferencias en la formación de raíces adventicias en las variedades seleccionadas, se llevó a cabo la caracterización fenotípica del enraizamiento de esquejes sin la adición exógena de auxinas. El sistema radicular completo desarrollado en la base del esqueje se estudió a intervalos de tiempo regulares en la estación de enraizamiento de la empresa. Las diez variedades estudiadas mostraron diferencias significativas en varios parámetros cuantitativos de su enraizamiento adventicio, como por ejemplo el área total del sistema radicular. La tasa de crecimiento radicular fue uno de los mejores indicadores de la capacidad de enraizamiento de las variedades estudiadas. Hemos encontrado una correlación negativa y estadísticamente significativa entre el diámetro medio de la raíz y la capacidad de enraizamiento de los esquejes: las variedades con menor capacidad de enraizamiento adventicio mostraron raíces más gruesas que las

variedades con una elevada capacidad de enraizamiento. Para poder determinar de manera sencilla y objetiva la capacidad de enraizamiento adventicio de los esquejes en las nuevas variedades de clavel durante el proceso de mejora genética que se lleva a cabo en la estación de enraizamiento, se definió una escala cualitativa basada en siete etapas no solapantes de enraizamiento. Hemos encontrado una correlación negativa y estadísticamente significativa entre la capacidad de enraizamiento de los esquejes y su contenido en agua, lo que sugiere que una señal de estrés por sequía podría inducir la formación de raíces adventicias en esta especie. Sin embargo, se requieren experimentos adicionales para establecer el vínculo funcional entre el estrés hídrico, el contenido de nutrientes del esqueje y la formación de raíces adventicias en clavel. Con los resultados que hemos obtenido en la estación de enraizamiento hemos clasificado las diez variedades estudiadas en cuatro grupos distintos en función de su respuesta.

A continuación, para seis de las variedades de clavel previamente analizadas, se llevó a cabo un análisis temporal del enraizamiento adventicio de los esquejes mediante su cultivo *in vitro* en un entorno controlado. Mediante la utilización de un programa de análisis específico, se obtuvieron datos cuantitativos de la arquitectura del sistema radicular adventicio en la base del esqueje desde los 13 hasta los 29 días tras el trasplante. Aunque este programa se había diseñado para el estudio de la raíz primaria y las laterales, hemos confirmado su utilidad en el análisis cuantitativo del sistema radicular adventicio propio de los esquejes de clavel. Mientras que los esquejes de la variedad enana "*2101-02 MFR*" mostraron una elevada capacidad de enraizamiento, los de las variedades convencionales "*2003 R 8*" y "*2441-7 R*" mostraron un enraizamiento adventicio deficiente. El mal comportamiento en el enraizamiento adventicio de la variedad "*2003 R 8*" se corrigió, al menos en parte, mediante la aplicación exógena de auxina.

A partir de nuestros resultados en la caracterización detallada del enraizamiento adventicio de esquejes en las distintas variedades de clavel, tanto en condiciones *in vivo* como *in vitro*, se derivan una serie de principios generales que podrían explicar las diferencias observadas en la capacidad de enraizamiento entre variedades, de tal forma que las variedades con un enraizamiento adventicio deficiente se caracterizan por presentar uno o varios de los siguientes atributos: (1) un

retraso significativo en la iniciación de las raíces adventicias en la base del esqueje, (2) un número reducido de primordios radiculares en la base del esqueje, (3) una tasa de crecimiento reducida de las raíces adventicias primarias, y (4) una tasa de crecimiento reducida de las raíces adventicias secundarias.

En la producción industrial de plántulas de clavel, los esquejes recién recolectados suelen almacenarse a temperaturas bajas durante varias semanas antes de su comercialización. La duración del período de almacenamiento modifica la capacidad de enraizamiento de los esquejes en las diferentes variedades de clavel, lo que sugiere modificaciones en los niveles endógenos de auxinas o en la sensibilidad a las auxinas durante el almacenamiento en frío. Se observa también una disminución de la capacidad de enraizamiento adventicio durante la maduración, de tal forma que los esquejes recolectados de plantas más viejas enraízan peor que aquellos recolectados de plantas jóvenes. Aunque se ha propuesto que una mayor degradación de las auxinas endógenas en los esquejes recolectados de plantas maduras podría explicar su menor capacidad de enraizamiento adventicio, resultados adicionales sugieren que otras señales podrían contribuir a la pérdida de competencia en la formación de raíces adventicias durante la maduración.

Para determinar los factores adicionales que contribuyen a la formación de raíces adventicias en los esquejes de clavel durante su producción comercial, se evaluó una serie de parámetros morfométricos y fisiológicos en esquejes recolectados a intervalos regulares de plantas madre maduras (> 1 año de edad), que crecieron en condiciones de invernadero. Los esquejes fueron recolectados de manera periódica por técnicos especializados según sus características morfológicas. Para este estudio hemos seleccionado las variedades "*2101-02 MFR*" y "*2003 R 8*", que mostraron diferencias significativas en su capacidad de enraizamiento adventicio. La baja capacidad de enraizamiento adventicio en la variedad "*2003 R 8*" podría explicarse por el retraso en la aparición de raíces adventicias y su menor tasa de crecimiento, en comparación con las de la variedad "*2101-02 MFR*". Nuestro diseño experimental se planificó para determinar si (a) la edad de la planta madre y la temporada de recolección de esquejes, o (b) el almacenamiento a bajas temperaturas de los esquejes, afectaban a la capacidad de enraizamiento adventicio en cada una de las

variedades a estudio. Para minimizar el efecto del ambiente durante el enraizamiento adventicio, los esquejes se plantaron en las mismas condiciones de invernadero, tanto los recién recolectados como los que se habían almacenado previamente a bajas temperaturas.

Para las dos variedades estudiadas se encontró que la edad cronológica de las plantas madre afectaba negativamente a la producción y a la calidad de los esquejes, una observación que encaja perfectamente con la metodología productiva de Barberet & Blanc S.A., en la que se descartan las plantas madre de más de 16 meses de edad. El porcentaje de esquejes con raíces visibles a los 30 días después de la siembra fue significativamente mayor en la variedad "*2101-02 MFR*" que en "*2003 R 8*". La edad de la planta madre influyó negativamente en el porcentaje de enraizamiento de esquejes de la variedad "*2003 R 8*". Se encontró también una variación de dos veces en el área total de raíces adventicias y en la longitud del sistema radicular entre las variedades "*2101-02 MFR*" y "*2003 R 8*".

El almacenamiento en frío de los esquejes tras su recolección es un procedimiento habitual para preservar los esquejes de clavel antes de su enraizamiento. Varios estudios indican que el efecto del almacenamiento en frío sobre la capacidad de enraizamiento de los esquejes depende de la variedad de clavel utilizada. En nuestro experimento se encontró un efecto positivo del almacenamiento en frío sobre el porcentaje de esquejes enraizados y sobre el tamaño del sistema radicular solo en la variedad "*2101-02 MFR*". Se encontraron menores niveles de citoquininas en la base del esqueje tras su recolección en ambas variedades, que aumentaron de forma significativa tras el almacenamiento en frío, pero principalmente en el cultivar "*2003 R 8*". Dado que las citoquininas son reguladores negativos del crecimiento del sistema radicular adventicio en muchas especies, estos resultados podrían explicar por qué el almacenamiento en frío de los esquejes mejoró el enraizamiento adventicio en la variedad "*2101-02 MFR*" pero no en "*2003 R 8*", dado que esta última presentaba mayores niveles de citoquina endógena. Para ambas variedades hemos encontrado niveles más elevados de ácido 3-indol acético (AIA) en esquejes recién recolectados, que se redujeron considerablemente tras el almacenamiento en frío. La reducción de los niveles de auxina en la base del esqueje

que se observan durante el almacenamiento en frío puede ser debida a una mayor degradación de la auxina, a un transporte reducido de la auxina desde las hojas o a una combinación de ambos factores. Estos resultados indican que las diferencias en la relación existente entre las auxinas y las citoquininas en la base del esqueje contribuyen de manera significativa a explicar las diferencias en el enraizamiento de los esquejes almacenados en frío de los dos cultivares en estudio.

Hemos encontrado niveles elevados de AIA en esquejes recién recolectados y en los recolectados más tempranamente. Los esquejes recolectados de plantas cronológicamente más viejas en la variedad "*2003 R 8*" mostraron una tasa de iniciación de raíces adventicias más baja, y contenían una menor relación de auxinas y citoquininas en la base del esqueje tras su recolección. La regulación de la homeostasis de las auxinas en la base del esqueje a través de la regulación de su señalización, su transporte polar o su metabolismo, podría contribuir directamente a las diferencias en el enraizamiento entre los esquejes juveniles y los maduros, así como entre los esquejes recién recolectados y los esquejes almacenados en frío.

Finalmente, nos preguntamos si una combinación lineal de algunos de los parámetros hormonales cuantificados en la base de los esquejes antes del enraizamiento podía explicar las diferencias observadas en la capacidad de enraizamiento entre las variedades estudiadas. Se encontró una correlación estadísticamente significativa entre el área total del sistema radicular al finalizar el experimento y los niveles iniciales de citoquinina y de ácido abscísico (ABA). De manera consistente con una correlación fuerte y altamente significativa entre el área de enraizamiento y el ABA, se encontraron niveles elevados de ABA en la variedad "*2101-02 MFR*" después del almacenamiento en frío de los esquejes.

Nuestros resultados son coherentes con la hipótesis de que la iniciación de las raíces adventicias en los esquejes de clavel tras su recolección depende de los niveles relativos entre auxinas y citoquininas endógenas en la base del esqueje en el momento de su trasplante, mientras que el crecimiento posterior del sistema radicular adventicio está fuertemente influenciado por el estado fisiológico del esqueje en el momento de su recolección y durante el proceso de enraizamiento. Este parámetro podría estimarse indirectamente a partir de los niveles endógenos de ABA.

A continuación se llevó a cabo un abordaje basado en la secuenciación de última generación para estudiar los perfiles de expresión génica en la base del esqueje en dos variedades de clavel que mostraron diferencias significativas en su capacidad de enraizamiento adventicio. Para ello, hemos caracterizado la expresión génica y los cambios fisiológicos que acontecen en la base del esqueje durante las primeras etapas del enraizamiento adventicio en las variedades "*2101-02 MFR*" y "*2003 R 8*". Se secuenciaron varias bibliotecas de ADN complementario preparadas a partir de muestras de la región basal de los esquejes, en condiciones control y tratados con auxinas, a diferentes estadios temporales durante el enraizamiento (0, 6, 24 y 54 horas tras el trasplante). Mediante la utilización de diferentes modelos y contrastes estadísticos se estimó que las diferencias más significativas en los niveles de expresión génica venían determinadas en primera instancia por la variedad, luego por el tiempo tras el trasplante y, por último, por el tratamiento con auxinas.

Hemos identificado 1.286 genes que se expresaban de manera diferencial en las muestras procedentes de esquejes tratados con auxinas. La mayoría de estos cambios en la expresión génica tuvieron lugar en los estadios iniciales, entre 0 y 6 h tras el trasplante. En la variedad "*2003 R 8*" se encontraron 7.341 genes que se expresaban de manera diferencial durante la formación de raíces adventicias, tanto en condiciones control como en las muestras de esquejes tratados con auxinas. 12.525 genes presentaron una expresión diferencial durante la formación de raíces adventicias en condiciones control en la variedad "*2101-02 MFR*". Estos resultados indican que existe una modificación sustancial del perfil global de expresión génica en la base de los esquejes durante la formación de raíces adventicias en clavel. Se encontró además que la expresión de 9.645 genes se alteró específicamente durante la formación de raíces adventicias en la variedad "*2101-02 MFR*" después del tratamiento con auxinas, con una superposición significativa entre los dos conjuntos de datos. Sin embargo, para un pequeño conjunto de genes se encontraron cambios significativos en sus perfiles de expresión únicamente tras el tratamiento con auxinas.

Para determinar las redes génicas que están reguladas de manera específica durante la formación de raíces adventicias en cada una de las variedades, se llevó a cabo un análisis de ontologías génicas (*Gene Ontology*). Algunos genes que codifican

proteínas relacionadas con la división celular presentaron mayores niveles de expresión en los estadios iniciales, independientemente de la variedad y el tratamiento. Curiosamente, los genes que codifican ciclinas mitóticas mostraron un máximo de expresión entre las 24 y las 54 h después del trasplante, lo que coincide con los cambios celulares observados en la base de los esquejes durante la formación de raíces adventicias.

En un estudio previo se observó que el aumento de las divisiones celulares en el cambium vascular de la región basal del esqueje se produjo entre las 12 y las 24 h después del trasplante en la variedad de referencia "*Master*". En la variedad "*2101-02 MFR*" se observó una mayor frecuencia de grupos de células en división en la región del cambium vascular a tiempos iniciales (> 6 h), que se desarrollaron más tarde como primordios radiculares. En las muestras tratadas con auxina, los grupos de células se hicieron evidentes en el momento del trasplante, lo que es indicativo de la activación temprana de la división celular en esta variedad y en respuesta a la auxina. En ningún caso la adición exógena de auxinas incrementó las tasas de división celular para los estadios iniciales. En consecuencia, las diferencias en la capacidad de enraizamiento entre los cultivares "*2101-02 MFR*" y "*2003 R 8*" se deben a un retraso en la activación temprana de las divisiones celulares en este último. Estos resultados sugieren que la auxina actúa promoviendo las divisiones celulares en una subpoblación de células del cambium en lugar de aumentar el número de divisiones en aquellas células que ya se están dividiendo. Dado que ambas variedades son capaces de iniciar la formación de raíces adventicias en respuesta a la adición exógena de auxinas, cabe pensar que la respuesta a las auxinas en la variedad "*2003 R 8*" es funcional y que las diferencias de enraizamiento observadas entre las dos variedades se deben a las diferencias en los niveles endógenos de auxina.

El balance hormonal de auxinas y citoquininas desempeña una función determinante en la formación de raíces adventicias en los esquejes de clavel. La herida producida por la escisión del esqueje estimula la biosíntesis de etileno, que influye positivamente en la formación de raíces adventicias en algunas especies. Hemos encontrado niveles elevados de AIA en el momento del trasplante únicamente en la variedad "*2101-02 MFR*". En esta variedad, los niveles de citoquininas en la base del

esqueje fueron muy bajos. Los niveles de citoquininas en la base del esqueje en la variedad "*2003 R 8*" aumentaron tras el trasplante. Por tanto, la relación entre las auxinas y las citoquininas endógenas en los distintos estadios fue mucho mayor en la variedad "*2101-02 MFR*" que en la variedad "*2003 R 8*".

Los resultados que hemos obtenido nos permitirán identificar los genes involucrados en la formación de raíces adventicias en esquejes de clavel, lo que contribuirá a la comprensión básica de los eventos moleculares que conducen a esta respuesta compleja de desarrollo en plantas. Además, las herramientas moleculares que hemos desarrollado en este trabajo nos permitirán la implementación de nuevos procedimientos de mejora genética basados en la selección asistida por marcadores y que resultarán de utilidad a los mejoradores de la empresa.

2.2. Conclusiones

- Hemos caracterizado el sistema radicular en la base del esqueje en diez variedades de clavel comercial que mostraron valores extremos en su porcentaje de pérdidas durante el enraizamiento en condiciones de invernadero. En seis de estas variedades se llevó a cabo un análisis temporal de la arquitectura radicular mediante su cultivo i*n vitro* en un entorno controlado.
- De nuestros estudios se deduce que las variedades con un enraizamiento deficiente presentan alguna o varias de estas características: (1) un retraso en la iniciación de las raíces adventicias, (2) un número reducido de primordios radiculares, (3) una tasa de crecimiento reducida de las raíces adventicias primarias y/o secundarias.
- Hemos evaluado qué parámetros morfométricos, fisiológicos y ambientales afectaban a la capacidad de enraizamiento adventicio de los esquejes en las variedades "*2101-02 MFR*" y "*2003 R 8*" durante una temporada de producción de esquejes.
- Nuestros resultados indican que las diferencias existentes entre las auxinas y las citoquininas en la base del esqueje en el momento de la recolección y durante las primeras etapas del enraizamiento contribuye a explicar las diferencias en el enraizamiento de esquejes y el efecto del almacenamiento en frío.
- Mediante un abordaje basado en la secuenciación de última generación, hemos iniciado la caracterización de la expresión génica y los cambios fisiológicos en la base del esqueje durante las primeras etapas del enraizamiento en las variedades "*2101-02 MFR*" y "*2003 R 8*".

- Los resultados que hemos obtenido nos permitirán identificar algunos los genes implicados en la formación de raíces adventicias en clavel y la búsqueda de marcadores moleculares asociados a las respuestas de enraizamiento entre variedades para su uso en los programas de mejora genética que se están llevando a cabo en la empresa.

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Capítulo 3

Publicaciones

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Artículo 1

Quantitative analysis of adventitious root growth phenotypes in carnation stem cuttings

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RESEARCH ARTICLE

Quantitative Analysis of Adventitious Root Growth Phenotypes in Carnation Stem Cuttings

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Abstract

Carnation is one of the most important species on the worldwide market of cut flowers. Commercial carnation cultivars are vegetatively propagated from terminal stem cuttings that undergo a rooting and acclimation process. For some of the new cultivars that are being developed by ornamental breeders, poor adventitious root (AR) formation limits its commercial scaling-up, due to a significant increase in the production costs. We have initiated a genetical-genomics approach to determine the molecular basis of the differences found between carnation cultivars during adventitious rooting. The detailed characterization of AR formation in several carnation cultivars differing in their rooting losses has been performed (i) during commercial production at a breeders' rooting station and (ii) on a defined media in a controlled environment. Our study reveals the phenotypic signatures that distinguishes the bad-rooting cultivars and provides the appropriate set-up for the molecular identification of the genes involved in AR development in this species.

Introduction

Carnation (*Dianthus caryophyllus* L.) is, after rose, the most important species on the worldwide market of cut flowers, with a yearly sales volume of almost 200 million plants [1]. Current ornamental breeding and production depends largely on rapid multiplication of elite clones, production of healthy and disease-free plants and faster introduction of novel cultivars.

Commercial carnation cultivars are propagated from terminal stem cuttings with 4–6 pairs of leaves [2]. Once separated from the mother plant, the cuttings must remain deprived of the root during cold storage [3, 4]. Rooting is a very sensitive and highly energy-demanding process affected by complex interactions between sucrose and hormone levels [5, 6]. Rooted cuttings are then transferred to hardening chambers before transplanting them to production fields. The production of young plantlets is frequently hampered by minimal adventitious root (AR) formation from stem cuttings, which has a strong genetic dependency and which leads to

production losses in certain carnation cultivars [7, 8]. The problem has been partly overcome by optimizing the storage of fresh cuttings at low temperature [4, 9]. Insufficient rooting extends the production time, causes waste of resources and leads to production losses in certain cultivars. For some of the new cultivars that are being developed by ornamental breeders, poor AR formation limits its commercial scaling-up, due to a significant increase in the production costs. Hence production economics and ecology would tremendously benefit from improved growth characteristics of stem cuttings as well as from reduced high-cost and energy-demanding hardening treatments.

In our attempt to determine the genetic basis of AR development in carnation, we selected several good-rooting and bad-rooting cultivars for a detailed characterization of their root system over time. To maximize the contribution of endogenous (genetic) factors responsible for the differences in AR formation between cultivars, phenotyping was performed on fresh stem cuttings without the aid of exogenous auxin. The entire root system developed at the base of the stem cutting was studied at regular time-intervals using a non-invasive imaging method on a controlled environment. In addition, phenotyping of AR formation was also performed on a breeders' rooting station which allowed us to define a qualitative scale of non-overlapping stages for visual assignment of rooting performance. Morphological and ecophysiological data from the shoot region of the stem cutting was also scored. Our results indicate that most of the differences observed between bad-rooting and good-rooting cultivars are caused by a delay in AR initiation from the base of the stem cutting, a reduced number of root primordia, and/or a slow growth rate of primary and secondary ARs in the bad-rooting cultivars. This study will set the bases for the molecular identification of the genes involved in AR formation in this species that will help to establish a marker-assisted selection approach to select for improved AR performance in current carnation breeding programs.

Materials and Methods

Plant material

From stored data about rooting losses scored in 132 commercial carnation cultivars that were vegetatively propagated at Barberet & Blanc (http://www.barberet.es) between 2011 and 2013, we selected 10 cultivars for further studies (S1 Table). Stem cuttings from the cultivars used in this work are available upon request. Due to organizational issues, about 110 stem cuttings were pinched from several mother plants of each cultivar by skilled operators at noon on 29th April 2013 (batch 1) and 20th May 2013 (batch 2). All the mother plants had been grown in the same glasshouse under environmental conditions at 37°34′50′′ N, 1°46′35′′ W and 395 altitude (Puerto Lumbreras, Murcia, Spain). Stem cutting lengths and fresh weights were measured to discard the outliers in each cultivar (Table 1). Ninety of the most representative stem cuttings per cultivar were kept for further analyses: (i) stem cutting ecophysiology and morphometric analysis, (ii) adventitious rooting in soil plugs and (iii) *in vitro* adventitious rooting.

Stem cutting ecophysiology

Some ecophysiological stem cutting and leaf traits were measured as described in [10]. Ten cuttings and five full-developed leaves per cultivar were used for the measurements. Cuttings and leaves were rehydrated by full immersion in tap water for 24h and later they were gently dried with soft-paper before being used for the different determinations. Cutting water saturated weight (CWSW) was estimated as the cutting weight after full rehydration. Then cuttings were kept in a chamber at 4°C and 75% relative humidity (RH) with no further rehydration, and their weight loses were daily monitored. Cuticular evaporation (Ecut) was estimated by measuring the water loses of rehydrated cuttings after one day of being transferred to the chamber,

Table 1. Gross morphology and some ecophysiological traits of unrooted carnation stem cuttings.

Cultivar code	Cutting length (cm)[a]	Cutting fresh weight (g)[a]	Cutting water content (g)[b]	Cuticular evaporation (g/day) [b]	Leaf area (cm^2)	Grade of succulence (g/cm^2)	Specific leaf area (cm^2/g)	Leaf dry matter content (g/g)
13-78-1 MFC	12.70 ± 1.06	1.53 ± 0.28	1.55 ± 0.22 D	0.20 ± 0.03 E	4.60 ± 0.50 C	0.024 ± 0.002 BCD	147.97 ± 8.30 ABC	0.126 ± 0.008 AB
189 R	15.81 ± 1.25	2.32 ± 0.42	2.30 ± 0.27 BC	0.38 ± 0.09 AB	6.21 ± 1.91 BC	0.024 ± 0.003 BCD	172.33 ± 16.26 A	0.110 ± 0.010 CD
2000 MFJ 7	14.19 ± 0.90	2.06 ± 0.33	2.03 ± 0.23 CD	0.50 ± 0.09 A	5.05 ± 0.94 C	0.022 ± 0.001 D	167.68 ± 12.03 AB	0.120 ± 0.009 ABC
2003 R 8	16.12 ± 1.26	2.93 ± 0.59	2.74 ± 0.31 B	0.36 ± 0.10 BC	5.00 ± 1.20 C	0.026 ± 0.001 ABC	145.57 ± 13.44 ABC	0.117 ± 0.007 BCD
2007 R 32	17.92 ± 1.76	2.50 ± 0.61	2.42 ± 0.34 BC	0.47 ± 0.11 AB	6.40 ± 0.62 BC	0.023 ± 0.002 CD	163.84 ± 15.46 AB	0.119 ± 0.007 BCD
2101–02 MFR	19.29 ± 1.32	2.78 ± 0.47	2.74 ± 0.34 B	0.23 ± 0.11 CDE	7.91 ± 0.86 AB	0.026 ± 0.001 ABC	121.95 ± 3.95 C	0.136 ± 0.006 A
2441–7 R	15.96 ± 1.24	3.83 ± 0.84	4.47 ± 0.59 A	0.36 ± 0.12 BCD	5.75 ± 0.83 BC	0.029 ± 0.003 A	144.10 ± 10.82 BC	0.107 ± 0.007 CD
3002 P	16.35 ± 1.64	2.37 ± 0.39	2.45 ± 0.30 BC	0.38 ± 0.10 AB	5.40 ± 0.67 BC	0.027 ± 0.001 AB	163.73 ± 15.08 AB	0.103 ± 0.007 C
N 576 B	20.70 ± 1.40	4.17 ± 0.85	3.91 ± 0.84 A	0.23 ± 0.06 DE	10.11 ± 2.34 A	0.030 ± 0.003 A	126.30 ± 14.15 C	0.118 ± 0.010 CD
R 667 FJ FOR	15.86 ± 1.15	2.58 ± 0.42	2.65 ± 0.21 B	0.44 ± 0.07 AB	4.83 ± 0.95 C	0.024 ± 0.002 BCD	160.91 ± 12.26 AB	0.115 ± 0.005 BCD

A minimum of [a]100 or [b]10 stem cutting samples were analyzed. Different letters indicate significant differences ($P < 0.01$) between the cultivars.

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and considered as a linear function. Finally, for cutting dry weight (CDW) determination, the cuttings were kept in an oven at 60°C for 24 hours. To estimate leaf area (LA), images of individual leaves were obtained using a flat-bed scanner (300 dpi). Scanned images were then batch-processed with ImageJ software with available plug-in macros [11]. Leaf water saturated weight (LWSW), leaf dry weight (LDW) and leaf water content (LWC) were obtained following the same procedure described above for the cuttings. Grade of succulence (GS) is the ratio between leaf water content and two-sided leaf area, $GS = LWC/2\ xLA$ expressed as g/cm^2. Specific leaf area (SLA) is the one-sided leaf area divided by the leaf dry weight, $SLA = LA/LDW$ expressed as cm^2/g. Leaf dry matter content (LDMC) is the ratio between leaf dry weight and leaf water saturated weight, $LDMC = LDW/LWSW$.

Adventitious rooting in soil plugs

For each cultivar, 30 freshly-harvested stem cuttings were immediately submerged for 15 h in an aqueous fungicide solution (1 g l^{-1} benomyl) and without exogenous auxin treatment, at 15 ± 2°C and 80% RH in dim light (4 μmol m^{-2} s^{-1} of average photosynthetic photon flux density [PPFD]). Afterwards, stem cuttings were individually planted in peat-perlite (90–10 v/v) substrate trays of 294 truncated pyramid plugs (2.5 × 2.5 × 4.0 cm; 16 cm^3) under glasshouse conditions at Barberet & Blanc's rooting station, as described previously [3]. Water, fertilizers and adequate phytosanitary treatments were periodically applied by skilled operators according to standard procedures for homogeneous production of commercial rooted cuttings.

In vitro adventitious rooting

Thirty stem cuttings of each cultivar were immediately wrapped in plastic bags after pinching and were stored in a cold chamber at 5 ± 2°C, 60% RH and complete darkness until they were planted (1 month). Prior to planting, the base of the stem cuttings was washed in 70% (v/v) ethanol for 30 s, surface-sterilized in 3% (w/v) sodium hypochlorite for 10 min, and rinsed thoroughly with sterile distilled water (5 times). Each stem cutting was planted on a 40 ml glass scintillation vial (Fig 1A) containing 35 ml of half-strength Murashige and Skoog (MS) basal salt medium (Duchefa, The Netherlands), 50 mg l^{-1} ampicillin (Duchefa, The Netherlands), and 0.5 g l^{-1} 2-(*N*-morpholino)ethanesulfonic acid (MES; Duchefa, The Netherlands), pH 5.8. Plant cultures were grown on a Panasonic MLR-352 growth chamber set at 22 ± 2°C, 70% RH and a 16:8 (light:dark) photoperiod with average PPFD of 40 μmol m^{-2} s^{-1}.

Sample collection and image analysis

For scoring adventitious rooting at the rooting station, a minimum of three cuttings per cultivar were imaged 13 days after planting (DAP). All the remaining stem cuttings (up to 30) were also imaged 20 and 27 DAP. Stem cutting pictures were taken from the four sides of the soil plug using a Canon 60D camera with a Canon EF-S 17-85mm f/4-5.6 IS USM lens at a resolution of 5184 × 3466 pixels, and saved as an RGB color image in jpeg format. To minimize variations due to sunlight quality during the day, a portable photographic bench was used and the images were taken at the glasshouse between 11 am—1 pm (Fig 1B). For the morphometric analysis of stem cutting images (Fig 1C), individualized vegetative (2800 × 2800 pixels) or soil plug (550 × 1100 pixels) regions were batch-imported into the Gia Roots software [12]. After scale calibration and grayscale conversion, stem cutting or soil plug images were segmented using the global thresholding method (Fig 1D). Eventually, threshold was manually adjusted at each image batch (cultivar and time) to maximize object identification (leaves or roots). *In vitro* adventitious rooting was imaged periodically at 13, 15, 17, 20, 22, 24, 27 and 29 DAP. Pictures were taken using a Sony Cyber-shot DSC-H3 camera (Sony Corporation, Tokyo, Japan)

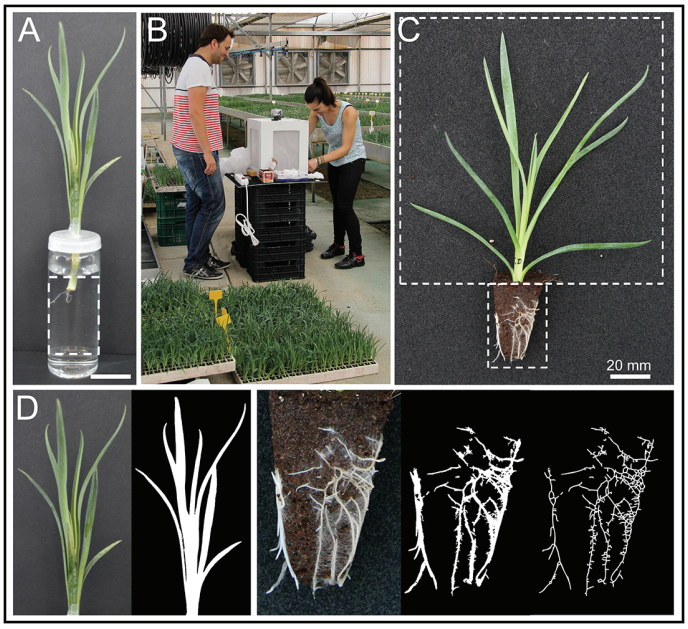

Fig 1. Morphological characterization of carnation stem cutting phenotypes. (A) A representative image of a stem cutting grown *in vitro* for 17 days. (B) Portable photographic bench used for image acquisition of stem cuttings grown in soil plugs at the rooting station. (C) A representative image of a stem cutting grown in soil plugs for 27 days. Dotted lines indicate the area of the image used for the morphometric analysis. (D) Image segmentation files obtained with the Gia Roots software, as described in Materials and Methods.

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at a resolution of 3264 × 2448 pixels, and saved as an RGB color image in jpeg format. A defined region in each image (1000 × 1100 pixels) containing the rooting region was used for image segmentation with the Gia Roots software as described above. All 19 root system architectural traits established previously were initially selected and were computed directly from the image mask or from the skeleton of the image mask as described elsewhere [12]. Raw measurements were exported to Excel spreadsheets for data analysis. Raw data files are available upon request.

Statistical analysis

Descriptive statistics (mean, standard deviation [SD], maximum and minimum, etc.) were calculated for samples taken at each stem cutting by using the StatGraphics Centurion XV software (StatPoint Technologies, Inc. Warrenton, VA USA) and SPSS 21.0.0 (SPSS Inc., Chicago, IL, USA) programs. Data outliers were identified based on aberrant SD values and excluded for posterior analyses. One-sample Kolmogorov-Smirnov tests [13] were performed to analyze the goodness-of-fit between the distribution of the data and a given theoretical distribution as previously described [14]. The differences between the data groups were analyzed by t test ($P < 0.05$) when only two groups were compared. To compare the data for a given variable, we performed multiple testing analyses with the ANOVA F-test or the Fisher's LSD (Least Significant Differences) methods [15]. Non-parametric tests were used when necessary. In that case median was used instead of mean. The differences between the data groups were analyzed by Mann-Whitney U test ($P < 0.05$) when only two groups were compared. In the other cases, data were subjected to Kruskal-Wallis test ($P < 0.05$). Correlations were studied using Pearson product-moment correlation coefficient (Pearson's r) [16]. Principal component analysis was used to reduce the dimensions of our sets of variables as previously described [14].

Results

Stem cutting losses during adventitious rooting differ between carnation cultivars

Rooted stem cuttings of commercial quality are characterized by the presence of 30 − 40 well developed roots of 1 − 9 cm length and by a healthy and homogeneous shoot apex at one month after planting (Fig 1C). Rooted cuttings of insufficient quality are manually removed before commercialization, which increases labor costs. Stem cutting losses on a collection of 132 commercial carnation cultivars grown at the Barberet & Blanc's rooting station between 2011 and 2013 ranged between 0.83 ± 0.89% and 13.54 ± 8.59% (S1 Fig), with significant lower values in spray cultivars (3.93 ± 2.25%) than in standard cultivars (4.72 ± 1.97%).

To initiate a quantitative description of rooting performance in carnation stem cuttings, we chose eight cultivars displaying extreme and contrasting values of rooting losses and two cultivars representative of the mean behavior for this trait within the studied population (S1 Table). We found that lengths and fresh weights of the stem cuttings collected for the rooting experiment were positively correlated (r = 0.687; $P < 0.005$), and that both parameters differed considerably among the studied cultivars, ranging between those in the *13-78-1 MFC* and the *N 576 B* cultivars (Table 1). Also, we observed a strong and significant effect of batch and cultivar type (spray or standard) on the size of the stem cuttings collected. At this point, we could not rule out whether the stem cutting size differences observed between batches are due to differences between mother plant age or due to glasshouse conditions during their growth.

Morphometric characterization of the stem cutting phenotype in representative carnation cultivars

For the quantitative phenotyping of stem cutting morphology, we measured nine parameters in the four sides of each stem cutting at 20 and 27 DAP (Table 2). We found significant correlations for most of the parameters measured (Fig 2A) although r^2 values between any two variables rarely exceed 0.65 (S2 Table). For each parameter we performed multifactorial ANOVA considering cultivar type (spray or standard), rooting performance, batch experiment and DAP. CL was found significantly influenced by batch experiment and was hardly modified for a given cultivar (S2A Fig). ALS was mainly influenced by cultivar type (S2B Fig) thus spray cultivars displayed narrower leaves (2.74 ± 0.02 mm) than standard cultivars (3.26 ± 0.02 mm). In the studied population, CA variation range was narrow (38.81 cm^2) compared to that of CCA (250.50 cm^2) (Fig 2B). Hence, stem cuttings with similar CA values presented striking differences in their CCA values (Fig 2C), which could be explained by changes in the insertion angle of older leaves during the experiment (see below).

Differences in CW, CP, CA, CCA and LN values differed significantly in most cultivars between 20 and 27 DAP, with the exception of cultivars *2441–7 R* and *2003 R 8*, which otherwise rooted poorly and no stem cutting data was collected for these two cultivars at 20 DAP (see next section). The overall differences in CW observed between 20 and 27 DAP (Fig 3A) are likely caused by the changes in the insertion angle of the outer (older) leaves of the stem cutting during the experiment (Fig 3C and 3D). On average, stem cuttings held 1.39 ± 0.40 more leaves at 27 DAP compared to those at 20 DAP. Taking CP as an unbiased descriptor of stem cutting size, differences in CP between 27 and 20 DAP for a given sample were considered an indirect estimate of stem cutting growth. Consequently, CP values increased between 13.1% in *2000 MFJ 7* to 31.3% in *3002 P* (Fig 3B). Stem cutting growth was mainly restricted to the younger leaves. Similar trends were also observed for CA and CCA (S2C and S2D Fig). We

Table 2. Morphometric and ecophysiological parameters studied.

Stem cutting morphometry	**Symbol**	**Calculated in Gia Roots as**	**Unit**
Cutting length	CL	Network depth (Ndepth)	cm
Cutting width	CW	Network width (Nwidth)	cm
Average leaf section	ALS	Average root width (Width)	mm
Cutting area	CA	Network area (NwA)	cm^2
Convex cutting area	CCA	Convex area (ConvA)	cm^2
Cutting perimeter	CP	Perimeter (Perim)	cm
Cutting solidity	CS	Network solidity	cm^2/cm^2
Cutting width-to-length ratio	CWL	Network width to depth ratio	cm/cm
Leaf number	LN	nd	
Stem cutting ecophysiology		**Estimated as**	**Unit**
Cutting water content	CWC	CWSW-CDW	g
Cuticular evaporation	Ecut	This paper	g/day
Leaf area	LA	This paper	cm^2
Grade of succulence	GS	LWC/2×LA	g/cm^2
Specific leaf area	SLA	LA/LDW	cm^2/g
Leaf dry matter content	LDMC	LDW/LWSW	g/g
Root system parameters	**Symbol**	**Calculated in Gia Roots as**	**Unit**
Rooting stage	RSG	nd	
Average root diameter	ARD	Average root width (Width)	mm
Root depth	RD	Network depth (Ndepth)	cm
Root width	RW	Network width (Nwidth)	cm
Root length	RL	Network length (Nlen)	cm
Root area	RA	Network area (NwA)	cm^2
Convex root area	CRA	Convex area (ConvA)	cm^2
Root perimeter	RP	Perimeter (Perim)	cm
Root width-to-depth ratio	RWD	Network width to depth ratio	cm/cm
Root length distribution	RLD	Network length distribution (Ldist)	
Root solidity	RS	Network solidity	cm^2/cm^2
Maximum number of roots	MXR	Maximum number of roots (MaxR)	

Nd: not determined.

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concluded that stem cutting morphology and stem cutting growth were heterogeneous among the selected carnation cultivars and significantly changed during rooting.

Stem cutting ecophysiology in cultivated carnation

Once detached from the mother plant, stem cuttings quickly start losing water. Hence, we studied some of their ecophysiological traits related to water economy. Differences in measured traits were observed between the studied cultivars (Table 1). Cutting water content (CWC) values ranged from 1.55 ± 0.22 g (*13-78-1 MFC*) to 4.47 ± 0.59 g (*2441–7 R*). Water-loss rate, represented by cuticular evaporation (E_{cut}), was highest in the *2000 MFJ 7* cultivar (0.50 ± 0.09 g/day), while the lowest rate was observed in the *13-78-1 MFC* cultivar (0.20 ± 0.03 g/day). Graphical representation of specific leaf area (SLA) and grade of succulence (GS) for the studied carnation cultivars are shown in Fig 4A as compared with other ornamental species. While xerophytic species displayed low SLA and high GS values (*C. ovata*), all carnation cultivars studied so far clustered together with intermediate SLA values and low GS. A principal component (PC) analysis on

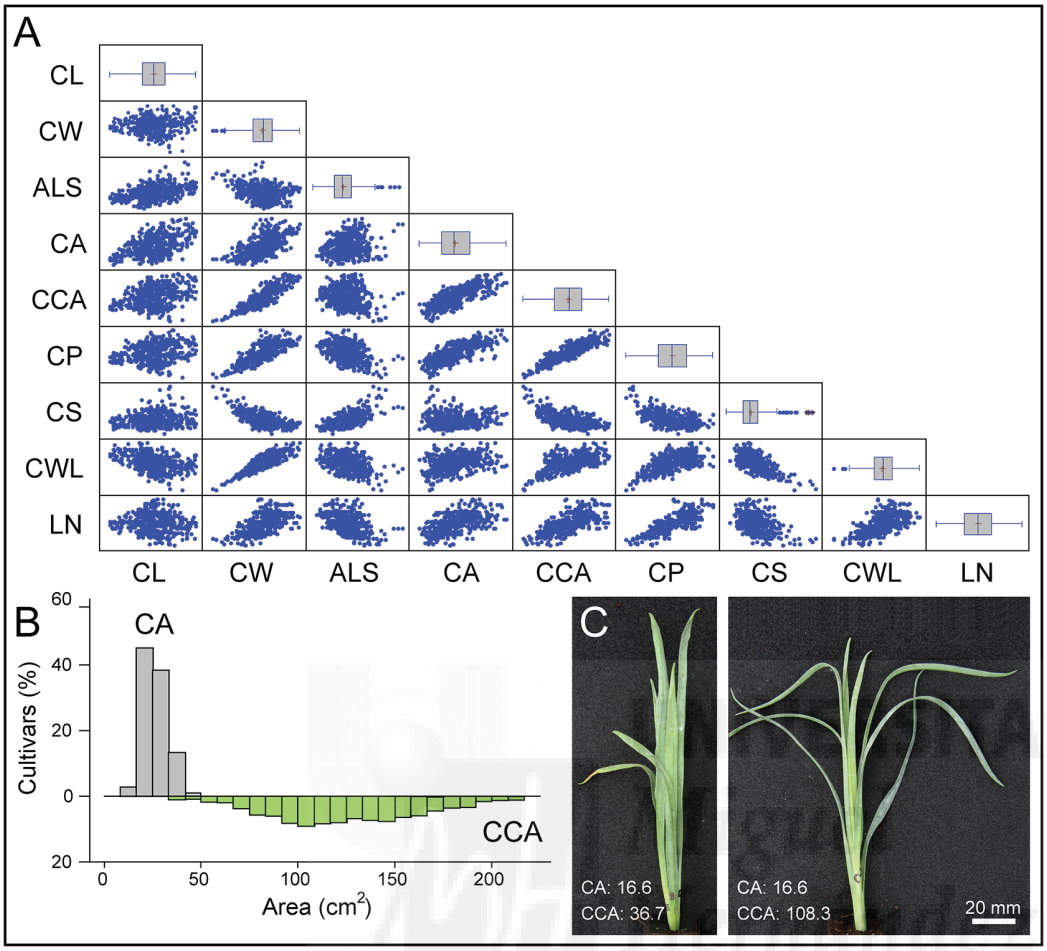

Fig 2. Stem cutting morphology. (A) Scatter plots of vegetative stem cutting parameters as defined in Table 2. Box-plots of each parameter are also represented. (B) Histograms for CA and CCA parameters. (C) Two stem cutting images displaying similar CA values but that differ in their CCA values (in cm^2). Images were obtained as described in Materials and Methods at 20 DAP.

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11 ecophysiological parameters was performed (Fig 4B). The first two components explained the 83.7% of the variance. PC1 seems to be related to water performance, showing a positive relationship with water retention (CWC, LWC, LWSW) and a negative relationship with water evaporation (SLA and Ecut), while PC2 is related to the water/dry weight ratio (LDMC).

Adventitious rooting in cultivated carnation

Previous experiments showed that under the assay conditions at Barberet & Blanc's rooting station almost no adventitious roots emerged in the base of the stem cuttings before 9 DAP, regardless of cultivar, auxin treatment or cold-storage period [3, 17]. Hence, sample images to characterize adventitious rooting in the selected cultivars were taken from freshly harvested stem cuttings grown for 13, 20 and 27 DAP in soil plugs without auxin treatment to maximize the differences between cultivars due to endogenous factors (see Materials and Methods).

Based on previous knowledge of skilled personnel at the Barberet & Blanc's rooting station, we visually defined seven rooting stages representing the different adventitious root phenotypes observed (Fig 5A). In stages 1 and 2, the stem cuttings were easily isolated from the soil

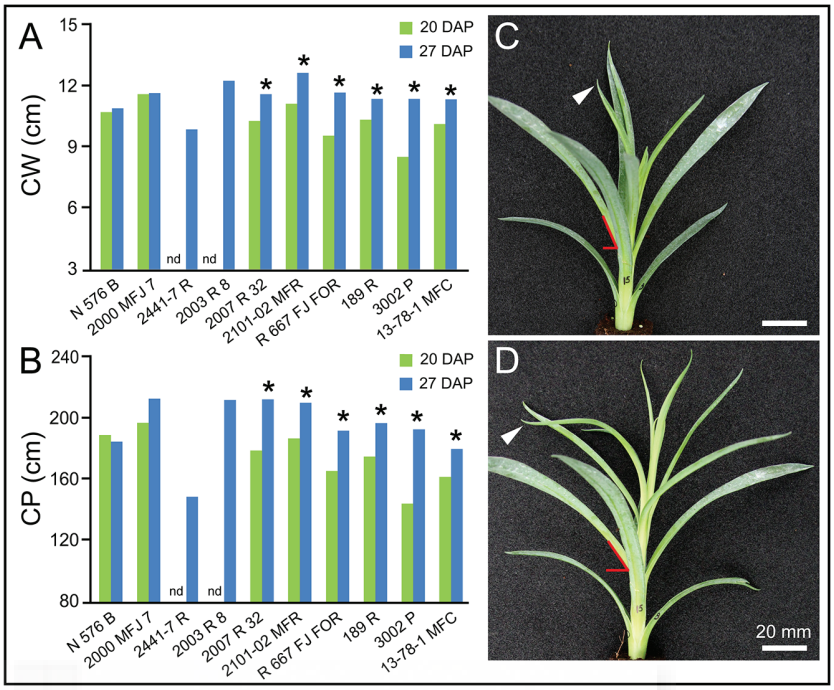

Fig 3. Stem cutting morphology of carnation cultivars. Average stem cutting (A) width and (B) perimeter values are shown for the studied cultivars. Asterisks indicate significant differences ($P < 0.05$) over time for a given cultivar. Nd: not determined. (C-D) A representative stem cutting imaged (C) 20 and (D) 27 DAP. White arrowhead points to the same leaf. Red lines are drawn to highlight leaf angles. Images were obtained as described in Materials and Methods.

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plug and no further morphometric analysis was conducted on these cuttings. Images for the morphometric analysis of the root system were obtained from stage 3 onwards. Stage 7 was assigned to stem cutting images where more than 40% of the soil plug was covered by roots and that represented stem cuttings of commercial quality. The cultivars studied here showed remarkable differences in rooting performance at different DAP as estimated by the RSG parameter (Fig 5B). At 13 DAP, only the *13-78-1 MFC* cultivar displayed differentially enhanced rooting performance. At 20 DAP, the cultivars clustered in five groups with *2441–7 R* and *13-78-1 MFC* as the ones displaying the most contrasting rooting performance. At 27 DAP, the differences in rooting performance between most of the studied cultivars diminished. Interestingly, some cultivars that initially showed poor rooting performance (such as *N 576 B* and *2003 R 8*) behaved as good-rooting cultivars at the end of the experiment (Fig 5B–5E).

In addition, some of the root system parameters measured were used for the quantification of adventitious rooting. RSG and ARD were estimated as the average of the data from the four soil plug images taken for each stem cutting sample. Whereas RA, CRA and RP were the sum of the data measured from the four soil plug images of each stem cutting sample. We found a positive and highly significant correlation between RA and RP, as well as between RSG and either RA or RP (Fig 6A and S2 Table). Root system area, estimated by RA, displayed a 10-fold variation ranging from an average value of 1.54 ± 1.23 cm^2 in stem cuttings scored as RSG = 4 to 14.95 ± 3.75 cm^2 in stem cuttings scored as RSG = 7 (Fig 6B). Similar analyses were performed for RP and ARD (S3A and S3B Fig). Multifactorial ANOVA tests for each parameter as regards cultivar type, rooting performance, batch experiment and DAP were performed as described above. RGS, RA and RP were significantly influenced by DAP and by cultivar type.

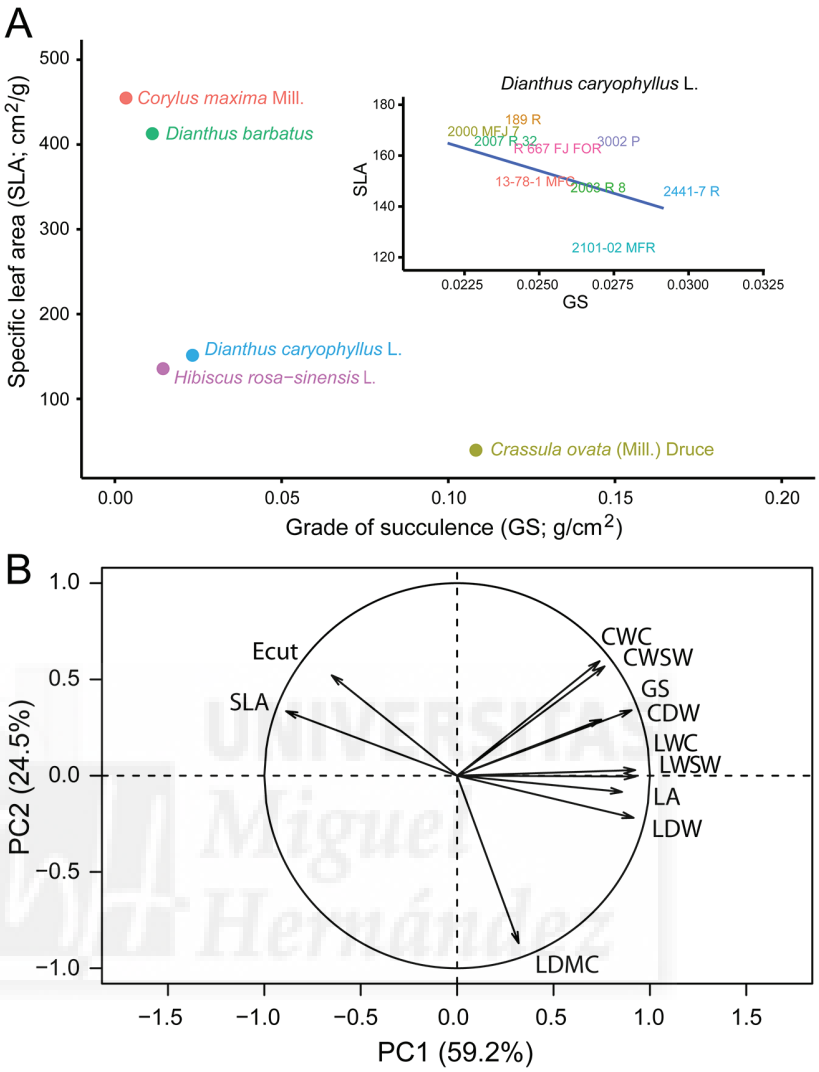

Fig 4. Stem cutting ecophysiology. (A) Representative distribution of species according to SLA and GS average values. Insert: Distribution of the *D. caryophyllus* cultivars studied in this work. (B) Principal component analysis of ecophysiological parameters. Graphical representation of PC1 and PC2 are shown.

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ARD was mainly dependent on DAP. A residual batch effect was also found for each of these parameters. Unexpectedly, the rooting performance of the studied cultivars during their commercial production was not associated with the observed differences in adventitious rooting in our experiment, which could be explained by the lack of the exogenous auxin treatment in the later.

RP, RA and CRA values differed significantly in all cultivars between 20 and 27 DAP (Fig 6C and S3C and S3D Figs), with the exception of those cultivars without rooting data at 20 DAP (*2441–7 R* and *2003 R 8*). RA was used here as an estimator or rooting performance. At 20 DAP, the studied cultivars clustered in four groups (Fig 6C), with average RA values ranging from 0.56 ± 0.55 cm^2 in *2000 MFJ 7* to 3.85 ± 2.52 cm^2 in *13-78-1 MFC*. One week later, five groups were observed (Fig 6C and Table 3). Average RA values at 27 DAP ranged between 3.39 ± 3.05 cm^2 in *2441–7 R* and 15.32 ± 4.53 cm^2 in *13-78-1 MFC*. Similar results were found

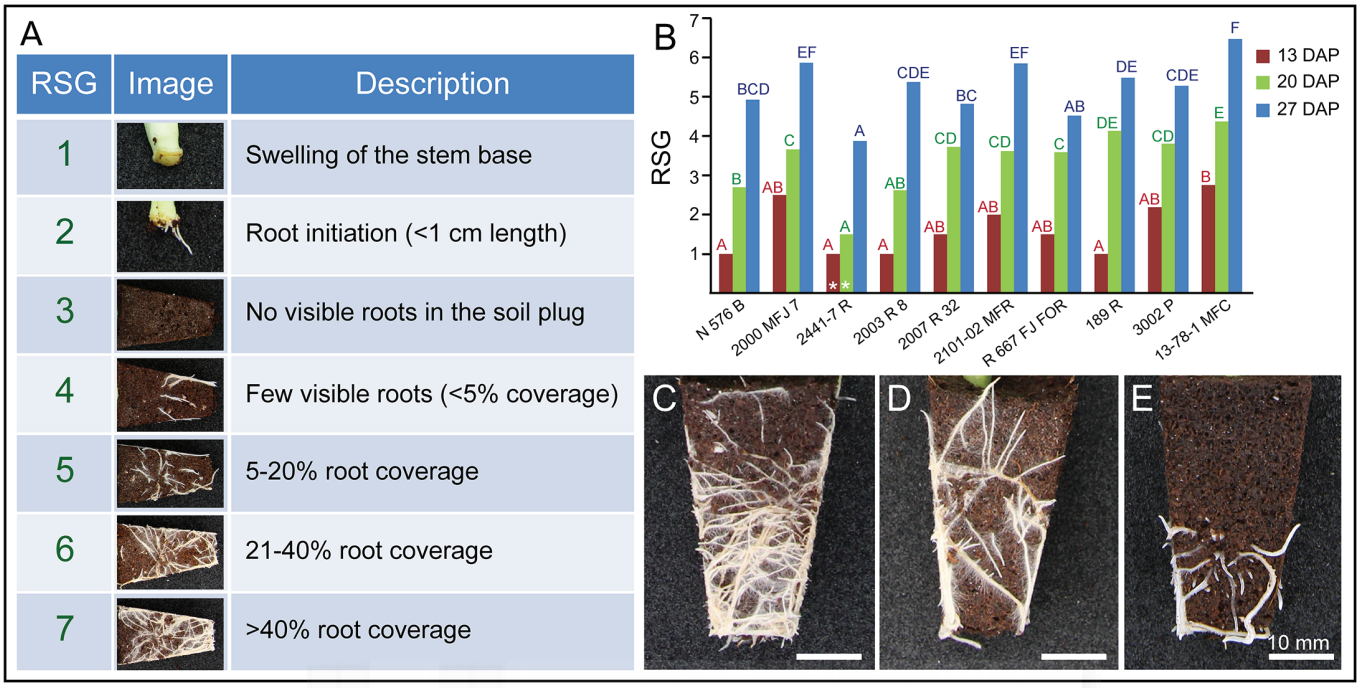

Fig 5. Qualitative description of adventitious rooting in carnation stem cuttings grown in soil plugs. (A) Rooting stages (RSG) based on visual assignment. Only samples from stage 3 onwards were used for later image analysis. (B) Graphic representation of average RSG values in different carnation cultivars over time. White asterisks highlight non-significant differences between the indicated time points for a given cultivar. Different letters indicate significant differences ($P < 0.01$) over cultivars for a given time. Representative soil plug images of adventitious rooting in (C) a good-rooting cultivar (*13-78-1 MFC*), (D) an intermediate-rooting cultivar (*2007 R 32*) and (E) a bad-rooting cultivar (*2441–7 R*). Images were obtained as described in Materials and Methods at 27 DAP.

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for RP values (S3C Fig). Root diameter was estimated by mean ARD data and ranged between 0.49 ± 0.10 mm in *2101–02 MFR* at 20 DAP and 0.71 ± 0.10 mm in *3002 P* at 27 DAP (Fig 6D and Table 3). For each cultivar, rooting speed was estimated as the average of the daily increase in RA between 27 and 20 DAP (expressed in cm^2/day). We found a 4-fold variation in rooting speed, the extremes represented by *2441–7 R* (0.12 ± 0.11 cm^2/day) and *13-78-1 MFC* (0.42 ± 0.15 cm^2/day) (Table 3).

Taken together, we categorized the studied cultivars regarding rooting performance in four groups (Table 3): (i) bad-rooting cultivars (*2441–7 R* and *R 667 FJ FOR*), (ii) poor-rooting cultivars (*N 576 B*, *189 R* and *3002 P*), (iii) intermediate-rooting cultivars (*2003 R 8*, *2007 R 32* and *2101–02 MFR*), and (iv) good-rooting cultivars (*2000 MFJ 7*, and *13-78-1 MFC*).

Correlations between morphometric, ecophysiological and adventitious rooting traits in cultivated carnation

We wondered whether we could predict rooting performance based on a given morphometric parameter of the vegetative stem cutting. We found low correlations for some stem cutting and root parameters (S2 Table) that prevented us for root performance assignment based on stem cutting morphology alone. Next, we performed PC analysis on nine parameters: CW, logCP, logCA, CCA, logALS, ARD, RA, CRA and RP (see Materials and Methods). Three PCs accounted for 88.2% of the variation among the studied samples. PC1 explained 56.2% of the total variance. PC2 and PC3 accounted for 18.6 and 13.4% of the variance, respectively (S3A Table). Consistently with the low correlation found between vegetative and rooting parameters,

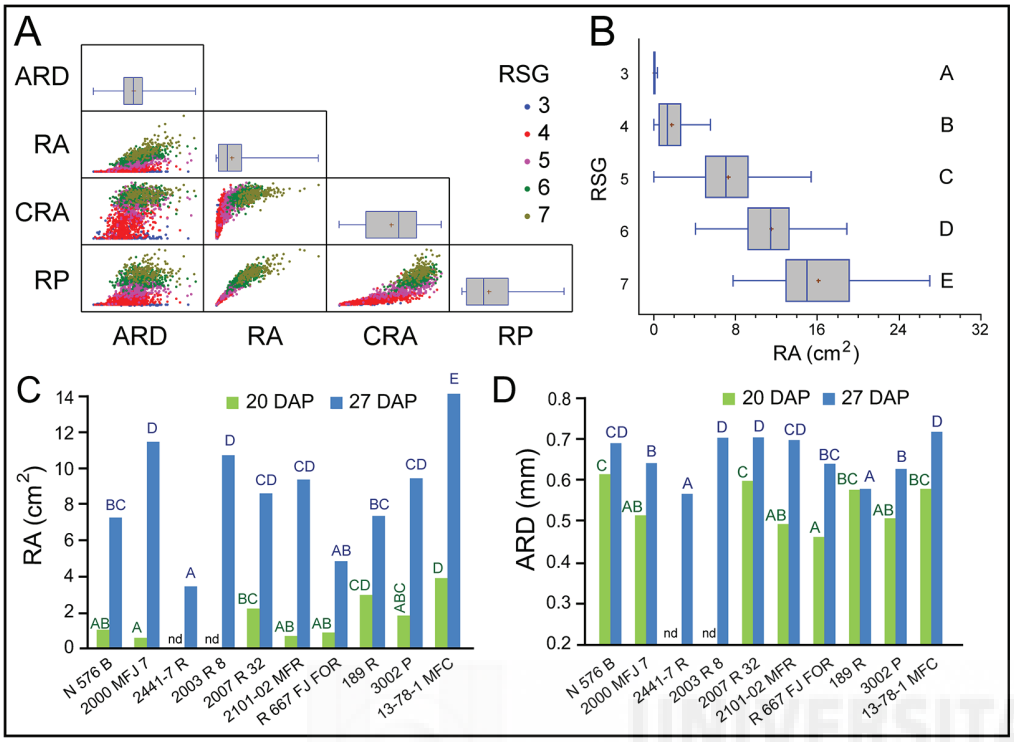

Fig 6. Quantitative description of adventitious rooting in carnation stem cuttings grown in soil plugs. (A) Scatter plots of some root system parameters as defined in Table 2. Data are color-clustered based on their RSG values. Box-plots of each parameter are also represented. (B) Box-plots of RA according to RSG values. Different letters indicate significant differences ($P < 0.01$) over RSG values. Graphic representation of average (C) RA and (D) ARD values in different carnation cultivars over time. Different letters indicate significant differences ($P < 0.01$) over cultivars for a given time. Nd: not determined.

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PC2 clearly separates vegetative and rooting traits, while PC3 is mainly influenced by leaf width, estimated by ALS (S4A Fig and S3B Table). These results are consistent with the good-rooting behavior observed for spray cultivars (S4B Fig), which otherwise display smaller stem cutting size than those of standard cultivars (S4C Fig). Interestingly, PC1 was positively

Table 3. Some parameters describing adventitious rooting in carnation stem cuttings grown in soil plugs.

Cultivar code[a]	RSG	RA (cm^2)	ARD (mm)	Rooting speed (cm^2/day)	Rooting performance
13-78-1 MFC	6.47 ± 0.57 F	15.32 ± 4.53 E	0.72 ± 0.13 D	0.42 ± 0.15 E	Good
189 R	5.50 ± 0.81 DE	7.27 ± 3.29 BC	0.58 ± 0.09 A	0.17 ± 0.07 AB	Poor
2000 MFJ 7	5.89 ± 0.45 EF	11.36 ± 3.03D	0.64 ± 0.10 B	0.39 ± 0.10 DE	Good
2003 R 8	5.43 ± 0.66 CDE	10.62 ± 3.83 D	0.70 ± 0.09 D	0.38 ± 0.14 DE	Intermediate
2007 R 32	4.88 ± 0.94 BC	8.52 ± 5.83 CD	0.70 ± 0.15 D	0.23.± 0.14 BC	Intermediate
2101–02 MFR	5.85 ± 1.22 EF	9.28 ± 4.91 CD	0.69 ± 0.11 CD	0.33 ± 0.16 CD	Intermediate
2441–7 R	4.14 ± 0.65 A	3.39 ± 3.05 A	0.56 ± 0.11 A	0.12 ± 0.11 A	Bad
3002 P	5.51 ± 1.01 CDE	9.35 ± 4.79 CD	0.64 ± 0.13 B	0.23 ± 0.14 BC	Poor
N 576 B	4.95 ± 1.00 BCD	7.18 ± 4.63 BC	0.69 ± 0.13 CD	0.25 ± 0.16 BC	Poor
R 667 FJ FOR	4.66 ± 0.98 AB	4.78 ± 4.25 AB	0.65 ± 0.13 BC	0.14 ± 0.11 A	Bad

[a]A minimum of 25 stem cuttings were analyzed, except for *2441–7 R* (n = 16). Average RSG, RA and ARD correspond to those measured at 27 DAP. Different letters indicate significant differences ($P < 0.01$) between the cultivars.

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dependent on overall size parameters of both the vegetative and the rooting part of the stem cutting, suggesting that, at least in part, the rooting performance is positively influenced by vegetative stem cutting size.

Also, a limited number of significant correlations were found between rooting parameters and ecophysiological traits (S2 Table). A highly significant and negative correlation was found between RA (or RSG) and CWC, while rooting speed was positively correlated with those variables measuring water and dry matter content ratio, such as LDMC ($r^2 = 0.65$). In addition, two PCs accounted for 85.3% of the variation among the studied samples regarding rooting parameters and ecophysiological traits (S5A Fig). The cultivars studied were nicely scattered along these two PCs as it was shown in the factor map diagram (S5B Fig).

Time course experiment of adventitious rooting in cultivated carnation

To provide some understanding into the observed differences in adventitious rooting between the studied cultivars, we morphometrically characterized root system architectural traits in the base of the stem cuttings of six of these cultivars between 13 and 29 DAP to environmentally-controlled *in vitro* conditions and without exogenous auxin treatment (see Materials and Methods). For each stem cutting, we gathered quantitative data of 19 previously stablished root system architectural traits [12]. We first reduced the number of studied parameters by partial correlation analysis. Then, iterative PC analysis (see Materials and Methods) allowed us to select for further studies the most relevant parameters, which were related to root network size (RL, RA, RD, RW, ARD and MXR) or root network distribution (RWD, RLD, and RS). Three PCs accounted for 84.7% of the observed variation. PC1 explained 55.3% of the total variance. PC2 and PC3 accounted for 18.1 and 11.3% of the variance, respectively (S4 Table). To visualize the effects of PC1, PC2 and PC3 (S6 Fig) on root architecture, representative images are depicted in Fig 7, where the PC values vary plus or minus two SDs from the mean. PC1 mostly

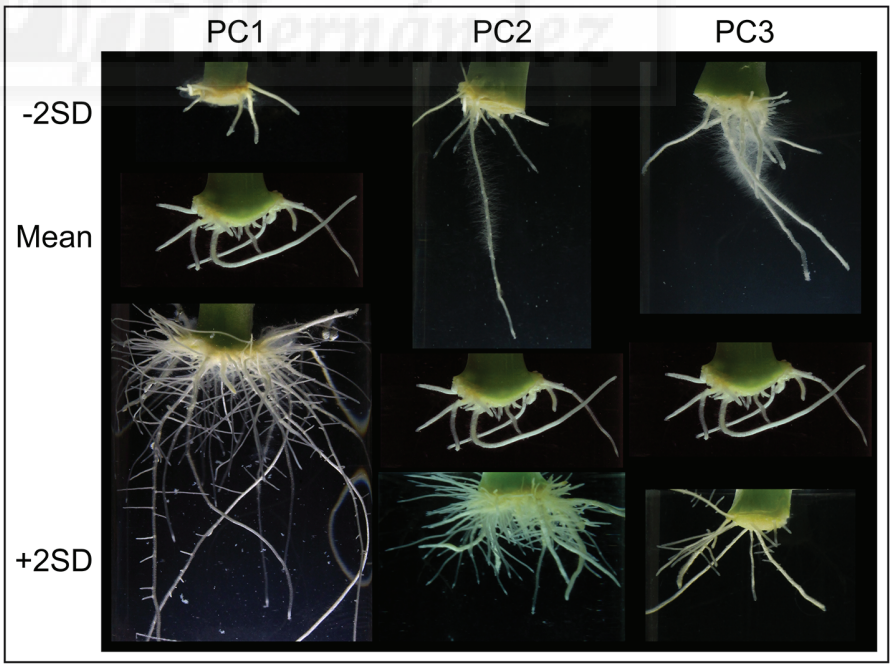

Fig 7. Variation in root system architecture among carnation stem cuttings grown *in vitro*. For each PC, a representative image corresponding to minus or plus two times the standard deviation (–2SD and +2SD) over the mean is shown.

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accounted for differences in the size of the root system whereas PC2 and PC3 affected specific attributes of the spatial distribution of the root network.

We found highly significant and positive correlations for most of the parameters studied measuring root network size except ARD (S7A Fig and S5 Table). Multifactorial ANOVA tests indicated that all the studied traits were strongly dependent on cultivar genotype and DAP (data not shown). For each cultivar, we estimated root initiation as the time when the roots were first observed in the stem cutting base. Consistent with our results obtained on soil plugs (see above), the *2003 R 8* and *2441–7 R* cultivars displayed a significant delay in the initiation of adventitious roots compared to that of the other cultivars studied (Fig 8A). Interestingly, ARD values were significantly increased along the experiment in all the studied cultivars and remained low in some cultivars with bad-rooting performance, such as *2441–7 R* (Fig 8B and Table 4). Root growth was estimated by the concomitant increase in RA along the experiment. The differences in RA values between any two cultivars were apparent after 20 DAP (Fig 8C). Similar results were found for the total length of the root network (S7B Fig). In addition, the cultivars differed in the number of emerged roots from the stem cutting base, as estimated by the MXR parameter (Fig 8D). Therefore, the largest root system developed by the *2101–02 MFR* cultivar is likely due to its higher number of roots and faster growth than other spray cultivars, such as *2000 MFJ 7* (Fig 9). On the other hand, standard cultivars such as *2003 R 8*, and *2441–7 R* displayed smaller root systems because of a delay in their root emergence and their slow root growth (data not shown). Another important parameter that characterizes root system architecture is maximum root depth as it is known that deeper rooting improves water and nutrient capture in different environments [18, 19]. In general, spray cultivars display deeper and wider root systems than standard cultivars at 29 DAP (Fig 8E and 8F, and Table 4). However, the *N 567 B* standard cultivar displayed a good-rooting behavior as it produced a higher number of roots and longer than those of other standard cultivars (Fig 8). Three additional parameters (RWD, RLD and RS) accounted for the spatial distribution of the root network. RWD and RLD values were relatively constant for most of the studied genotypes along the experiment (S7C and S7D Fig). In contrast, RS values decreased during rooting, which reflected the transition between a compact root system at 15 DAP to a wider and less dense root system at 29 DAP (Fig 9 and S7E Fig).

In our *in vitro* rooting experiment, two groups of cultivars displayed contrasting rooting-performance. On the one hand, the *2101–02 MFR* spray cultivar displayed a good-rooting behavior. On the other hand, the *2003 R 8* and *2441–7 R* standard cultivars showed a bad-rooting behavior. Interestingly, one standard cultivar (*N 576 B*) and one spray cultivar (*2000 MFJ 7*) displayed similar rooting behavior which was achieved by potential different mechanisms (Fig 9).

Discussion

The wide variation range of stem cutting losses observed during commercial production at the Barberet & Blanc's rooting station was strongly dependent on the cultivar type. As such, most of the cultivars with high stem cutting losses (>5%) were of the standard type, while those with the lowest stem cutting losses were of the spray type. Besides, only 12 cultivars displayed stem cutting losses larger than 7%, a result which is consistent with this trait being under strong negative selection during the development of new cultivars.

Stem cuttings are periodically pinched from adult mother plants by skilled operators based on their external attributes, named size, color, leaf number and morphology, and are kept cold for long-term storage. For the studied cultivars in this work, our results are consistent with the fact that stem cuttings in spray cultivars are usually smaller than those of standard cultivars,

irrespectively of the environmental variables, suggesting that these differences are genetically encoded. Four morphological parameters of the stem cuttings (CP, ALS, LN and the CCA/CA ratio) were shown to be informative for cultivar discrimination and the differences in CP between any two given time points allowed us to measure stem cutting growth during rooting. Stem cutting growth was mainly restricted to the younger leaves [2], while the changes in older leaves during rooting were limited to their insertion angle and more folding of their lamina along the midrib. As a diversity of crop plants has been observed to alter leaf angle in response to water deficit [20], the reported changes in leaf angle could be caused by the dehydration of older leaves occurring during the early steps of rooting.

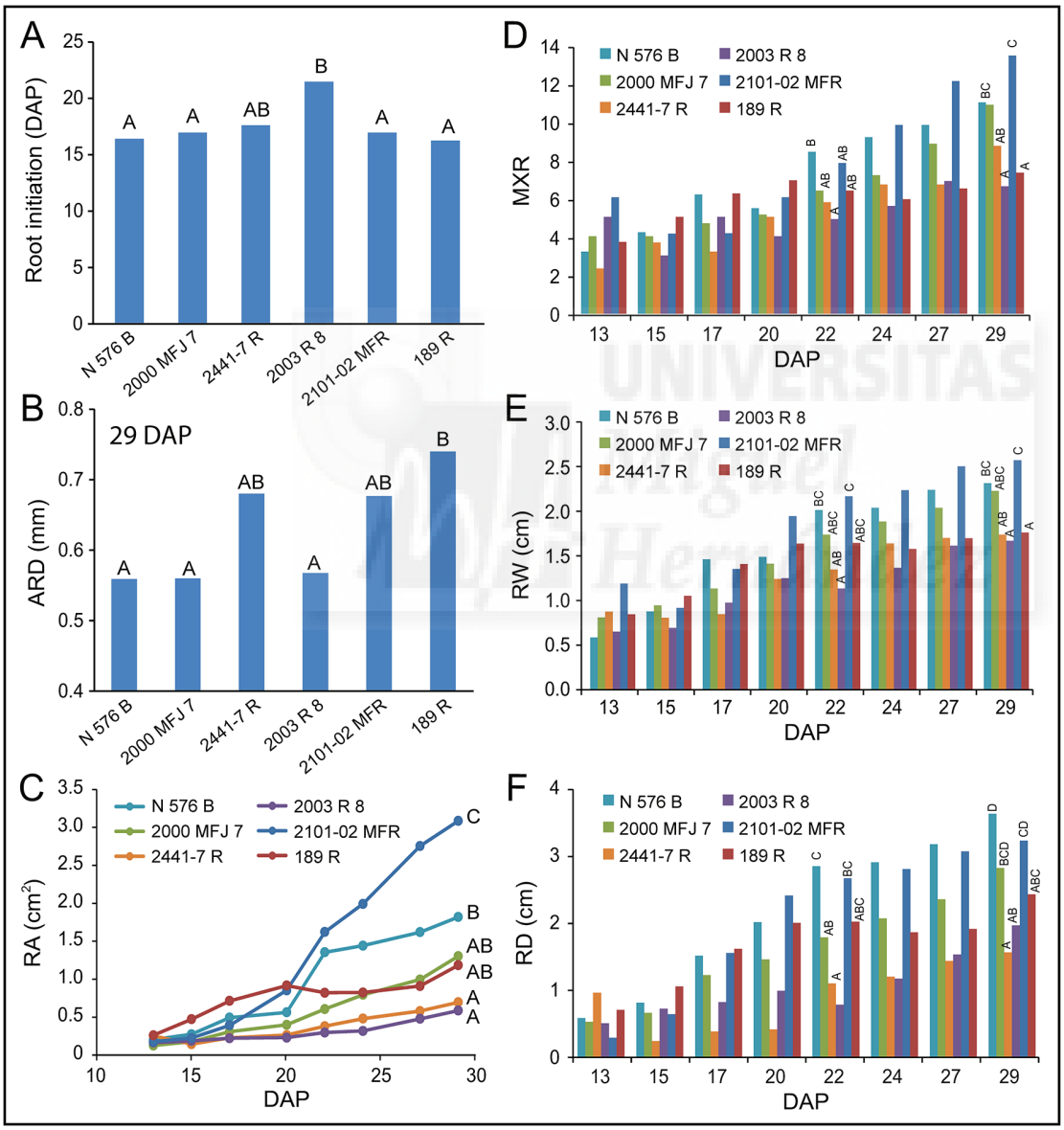

Fig 8. Quantitative description of adventitious rooting in carnation stem cuttings grown *in vitro*. (A) Root initiation and average root diameter (B) values are shown for the studied cultivars. Asterisks indicate significant differences ($P < 0.05$) over time for a given cultivar. (C-F) Time-course analysis of some root network parameters: (C) area, (D) root number, (F) depth, and (F) width. Different letters indicate significant differences ($P < 0.01$) between the cultivars for a given time (13, 22 or 29 DAP).

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Table 4. Some parameters describing adventitious rooting in carnation stem cuttings grown *in vitro*.

Cultivar code[a]	RA (cm^2)	ARD (mm)	MXR	RD (mm)	Rooting performance
189 R	1.22 ± 1.01AB	0.76 ± 0.18 B	7.27 ± 3.83 A	2.43 ± 1.29 ABC	Intermediate
2000 MFJ 7	1.34 ± 0.76 AB	0.57 ± 0.18 A	10.77 ± 3.94 BC	2.83 ± 1.33 BCD	Intermediate
2003 R 8	0.61 ± 0.40 A	0.57 ± 0.19 A	6.57 ± 2.74 A	1.97 ± 1.19 AB	Bad
2101–02 MFR	3.16 ± 1.68 C	0.69 ± 0.16 AB	13.32 ± 4.98 C	3.16 ± 1.68 CD	Good
2441–7 R	0.72 ± 0.52 A	0.69 ± 0.25 AB	8.67 ± 3.94 AB	1.57 ± 1.15 A	Bad
N 576 B	1.87 ± 0.92 B	0.57 ± 0.16 A	10.90 ± 3.78 BC	3.64 ± 0.94 D	Good

[a]A minimum of 15 stem cuttings were analyzed, except for *2441–7 R* (n = 5). Average RA, ARD, MXR and RD correspond to those measured at 29 DAP. Different letters indicate significant differences ($P < 0.01$) between the cultivars.

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We observed statistically-significant differences amongst some ecophysiological traits for the studied cultivars (Table 1). It's been shown previously that high SLA implies a greater surface to volume ratio in leaves and therefore a higher water loss, whereas high photosynthetic rates is linked to high transpiration and lower drought tolerance [21]. The studied carnation cultivars displayed lower SLA and a slightly higher GS values than those of their close relative from temperate regions, *D. barbatus*, suggesting adaptation to a Mediterranean habitat characterized by high-light and dry climate.

For commercial production of rooted cuttings at the breeders' rooting station, cold-stored cuttings are usually treated with exogenous auxin prior to planting them to soil plugs. To better describe the differences in adventitious rooting that are caused by endogenous (genetic) factors, we planted freshly-harvested cuttings from ten cultivars differing in their commercial rooting losses directly on soil plugs without the addition of exogenous auxin. As expected, the studied cultivars showed remarkable differences in quantitative rooting parameters such as total root area (RA) between cultivars and at different times after planting. Rooting speed, estimated by the observed change in RA, was one of the best indicators of the rooting performance in the studied population. Interestingly, the average root diameter (ARD) was found negatively correlated with rooting performance, and the poor-rooting cultivars always displayed thinner roots than the good-rooting cultivars. To support a quick assignment of rooting performance for the

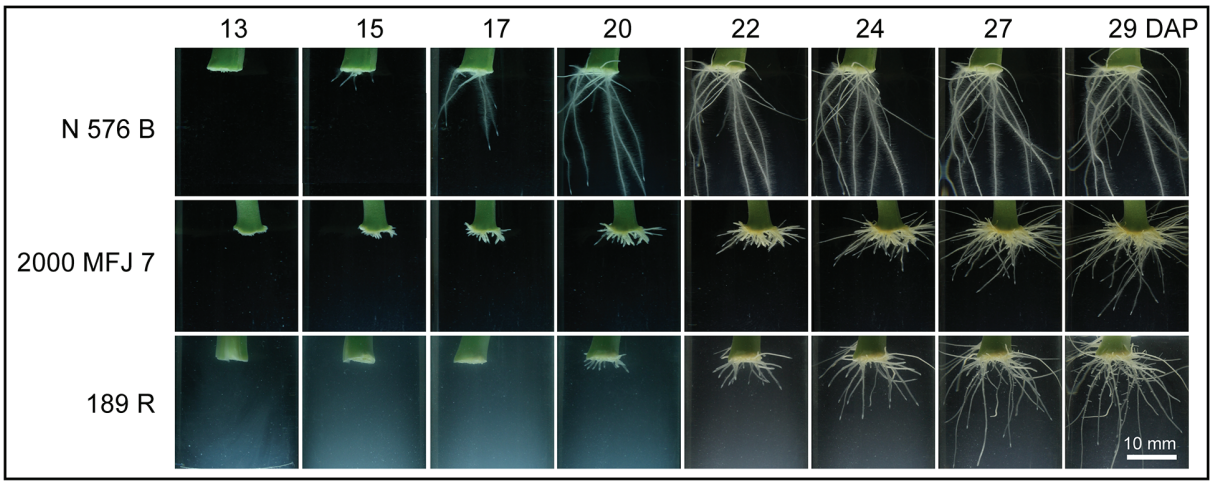

Fig 9. Time-series of adventitious rooting in some carnation cultivars grown *in vitro*. A representative stem cutting was imaged between 13 and 29 DAP for each cultivar.

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new carnation cultivars being bred, we defined a qualitative scale based on seven non-overlapping rooting stages (RSG; Fig 5A). We found a strong and positive correlation between some root system size parameters (RA, RP) and RSG, which validated RSG for the fast determination of rooting performance *in situ*. A trained person is able to visually assign RSG to soil plug images at a rate of about 800 images per hour. Thus, the RSG parameter could be easily scored on the same stem cutting at different time-points after planting. To increase discrimination power of this parameter, intermediate rooting stages could be added.

Based on the data obtained from stem cuttings grown in soil plugs, we assigned the ten studied cultivars to four groups that differed in their rooting performance from bad-rooting cultivars to good-rooting cultivars. Curiously, *R 667 FJ FOR* was initially selected because of its low rooting losses during commercial production but it behaved as a bad-rooting cultivar when cold-storage and exogenous auxin treatment were not applied.

From our PC analysis on the variation found in stem cutting morphology (shoot and root traits), we concluded that two different mechanisms could account for the observed differences. On the one hand, PC1 values were dependent on the size of both the shoot region and of the root region, suggesting that common genes are contributing to both traits. On the other hand, PC2 clearly separates vegetative and root traits. Consistent with our results, a recent study in wheat using a double-haploid mapping population found a significant and positive correlation between plant height and some components of root architecture [22]. Indeed, a few QTLs for root and shoot traits were found co-localized in this work. This indicates that there are common genes, or at least closely linked genes, contributing to both traits. Consistent with our results too, other genes only influence root traits or plant height [22].

Many studies have been carried out to highlight the relationship between leaf and root parameters during drought adaptation [23, 24] but not much is known about the association between leaf and root traits in relation to adventitious rooting. On the one hand, we found that rooting performance in carnation cuttings was negatively correlated with their water content, suggesting that a drought stress signal might induce AR formation in these species. On the other hand, rooting speed was found positively correlated with the water/dry weight ratio, so cultivars accumulating higher (sugar) resources might be able to surpass the restriction from water excess over AR formation. Interestingly, the studied cultivars were perfectly separated by only two PCs including a small number of rooting and ecophysiological parameters (S5 Fig). Further experiments are required to narrow down this observation and to shed some light into the complex link between water stress, nutrient content and AR formation.

In addition, we performed a time-course analysis of adventitious rooting in carnation stem cuttings by following a novel *in vitro* approach using transparent agar tubes in a controlled environment. Similar approaches have been used for the study of root traits in other crops, such as maize [25] or rice [26, 27]. Quantitative data describing the root system architecture was obtained for six carnation cultivars from 13 to 29 DAP using a previously stablished root analysis software [12]. Although this software was initially designed for the analysis of primary and lateral roots, we demonstrated its application for the quantitative analysis of ARs. Three PCs including nine parameters accounted for most of the variation found in adventitious rooting, which was mainly triggered by differences in the overall size of the root system (Fig 7). Among the parameters measured, total root length and root depth are quite significant as they determine capture of water and nutrients by plants, and are targets for crop improvement [28]. Indeed, Kirkegaard *et al.* (2007) demonstrated that a relatively small increase in rooting depth in wheat could provide a significant yield increase in this species [29]. Consistent with these results, the good-rooting carnation cultivars *N 576 B* (standard) and *2010–02 MFR* (spray) displayed the deeper root system among the studied ones. The latter cultivar also showed higher number of roots and faster growth than other spray cultivars. Standard cultivars, such as *2003*

R 8, displayed smaller root systems mainly due to a delay in root emergence and slow root growth. Interestingly, the bad rooting behavior of *2003 R 8* observed in our experiments was partly rescued by exogenous auxin application [30], which might explain the differences in rooting performance during commercial production.

By the detailed study of the time-series of adventitious rooting in several carnation cultivars described in this work, we derived general principles that might account for the differences in rooting performance observed between other carnation cultivars. The bad-rooting cultivars are characterized by one or several of the following attributes: (i) a delay in root initiation from the stem cutting base, (ii) a reduced number of AR primordia, (iii) a slow elongation rate of primary ARs, and (iv) slow initiation and elongation rates of secondary ARs. A novel semi-automated image analysis platform is being implemented in our lab (S. Tormos-Moltó and J.M. Pérez-Pérez, unpublished) which will allow the continuous monitoring of AR growth from carnation stem cuttings over a long-term period (>1 month). Using this platform, we will interrogate the extensive germplasm collection at Barberet & Blanc for carnation cultivars displaying specific alterations in their AR system.

To get some insight into the genetic influence in shoot and/or root traits in carnation stem cuttings, a similar approach to that described elsewhere [22], needs to be undertaken. To this end, two carnation cultivars extensively differing in both their stem cutting morphology and their rooting performance, and for which some genetic information is already known, will need to be crossed and their descendants studied at the genetic and the phenotypic levels. The molecular signature of the different stages of adventitious rooting in the two contrasting cultivars *2101–02 MFR* and *2003 R 8* that have been studied in this work will be presented elsewhere [30], which makes them appropriate parents of the mapping population required for QTL analysis. In addition, a combined approach for non-targeted metabolite and hormone profiling in carnation cultivars displaying contrasting AR formation responses will shed some light in the biochemical signatures of this process. By these additional approaches that have been initiated in our lab, we intend to contribute to the basic understanding of the molecular events leading to the complex developmental response of AR formation which will help to establish a marker-assisted selection approach to select for improved adventitious rooting in this and other ornamental species.

Supporting Information

S1 Fig. Distribution of average rooting losses among carnation cultivars. The data from the studied population (n = 132 cultivars) fit the log-normal distribution. Values indicate average ± SD (in %).
(TIF)

S2 Fig. Graphic representation of some stem cutting parameters in different carnation cultivars over time. Asterisks indicate significant differences ($P < 0.05$) over time for a given cultivar. Nd: not determined.
(TIF)

S3 Fig. Graphic representation of some rooting parameters in different carnation cultivars grown in soil plugs. (A-B) Box-plots of RP (A) and ARD (B) data according to RSG values. Different letters indicate significant differences ($P < 0.01$) between RSG values. (C-D) Graphic representation of average RP (C) and CRA (D) values in different carnation cultivars over time. Different letters indicate significant differences ($P < 0.01$) between the cultivars for a given time. Nd: not determined.
(TIF)

S4 Fig. Principal component analysis of stem cutting morphology and adventitious rooting parameters. (A) Graphical representation of PC1 and PC2. (B-C) Representative images of two good-rooting stem cuttings of a spray cultivar (B) and a standard cultivar (C) displaying contrasting stem cutting morphology and similar rooting performance. Images were obtained as described in Materials and Methods at 27 DAP.
(TIF)

S5 Fig. Principal component analysis of stem cutting ecophysiology and adventitious rooting parameters. (A) Principal component analysis of rooting and ecophysiological parameters. Graphical representation of PC1 and PC2 are shown. (B) Individual factor map for PC analysis of rooting and ecophysiological parameters. Cultivars have been color-coded as regards their rooting performance (red: bad; orange: poor; blue: intermediate; green: good).
(TIF)

S6 Fig. Principal component analysis of adventitious rooting parameters measured in carnation stem cuttings grown *in vitro*. Representation of PC1, PC2 and PC3 in a tridimensional space. Root system parameters are represented as defined in Table 2.
(TIF)

S7 Fig. Quantitative description of adventitious rooting in carnation stem cuttings grown *in vitro*. (A) Scatter plots of some adventitious rooting parameters as defined in Table 2. Boxplots of each parameter are also represented. Graphic representation of RL (B), RLD (C), RWD (D) and RS (E) values in different carnation cultivars over time. Different letters indicate significant differences ($P < 0.01$) between the cultivars for a given time.
(TIF)

S1 Table. Carnation cultivars studied in this work.
(PDF)

S2 Table. Linear correlation matrix of average stem cutting parameters measured.
(PDF)

S3 Table. Principal component analysis of root system parameters in carnation stem cuttings grown in soil plugs.
(PDF)

S4 Table. Principal component analysis of root system parameters in carnation stem cuttings grown *in vitro*.
(PDF)

S5 Table. Linear correlation matrix of root system parameters measuredin carnation stem cuttings grown *in vitro*.
(PDF)

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Author Contributions

Conceived and designed the experiments: MA JMPP. Performed the experiments: VB JV AC. Analyzed the data: JV AC MA JMPP. Contributed reagents/materials/analysis tools: EAC. Wrote the paper: AC MA JMPP.

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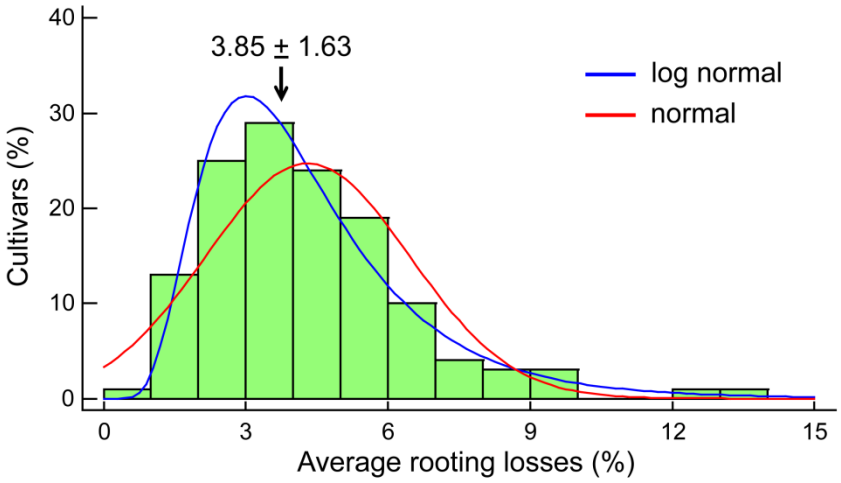

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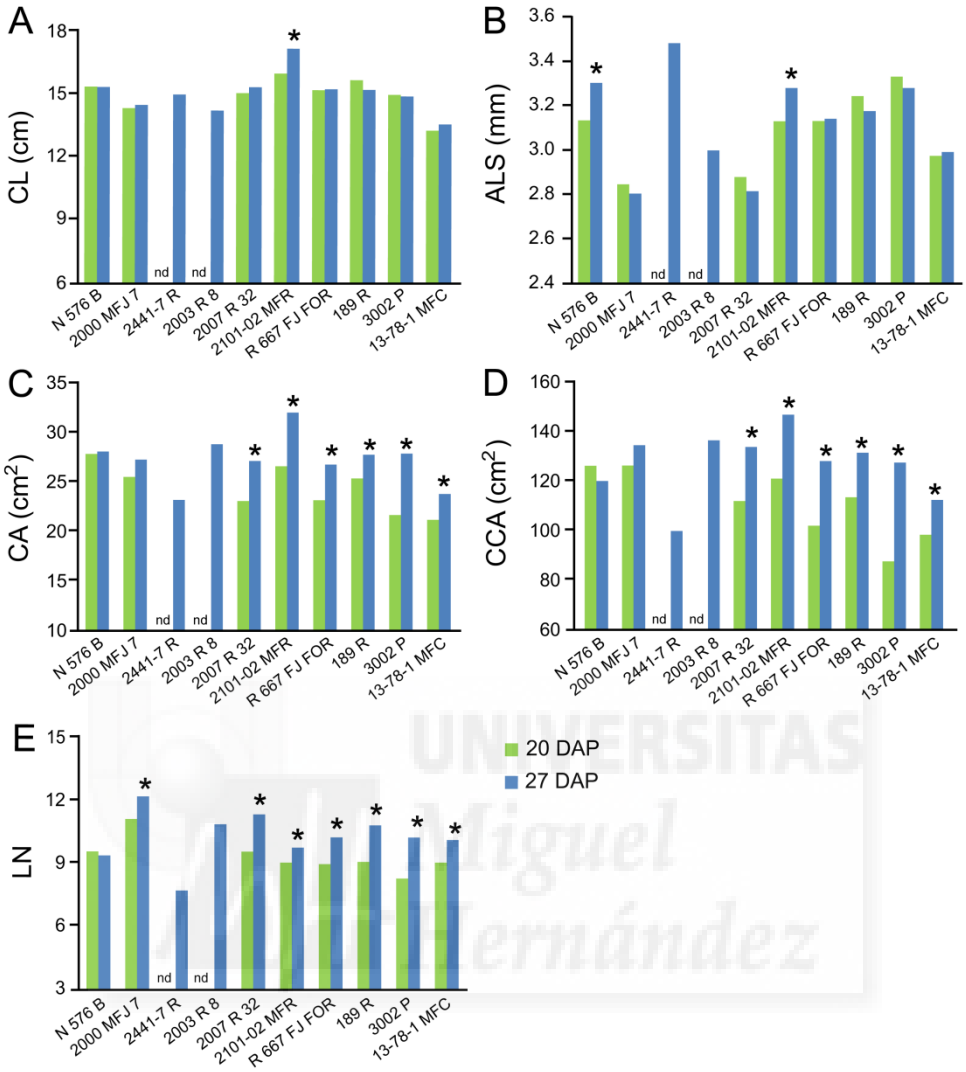

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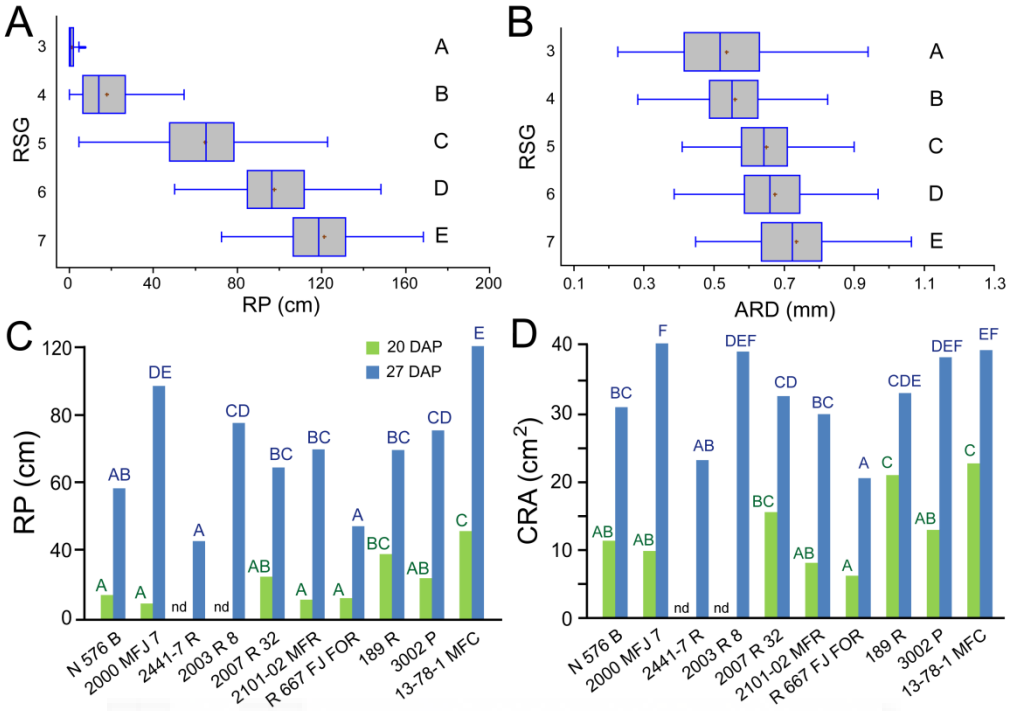
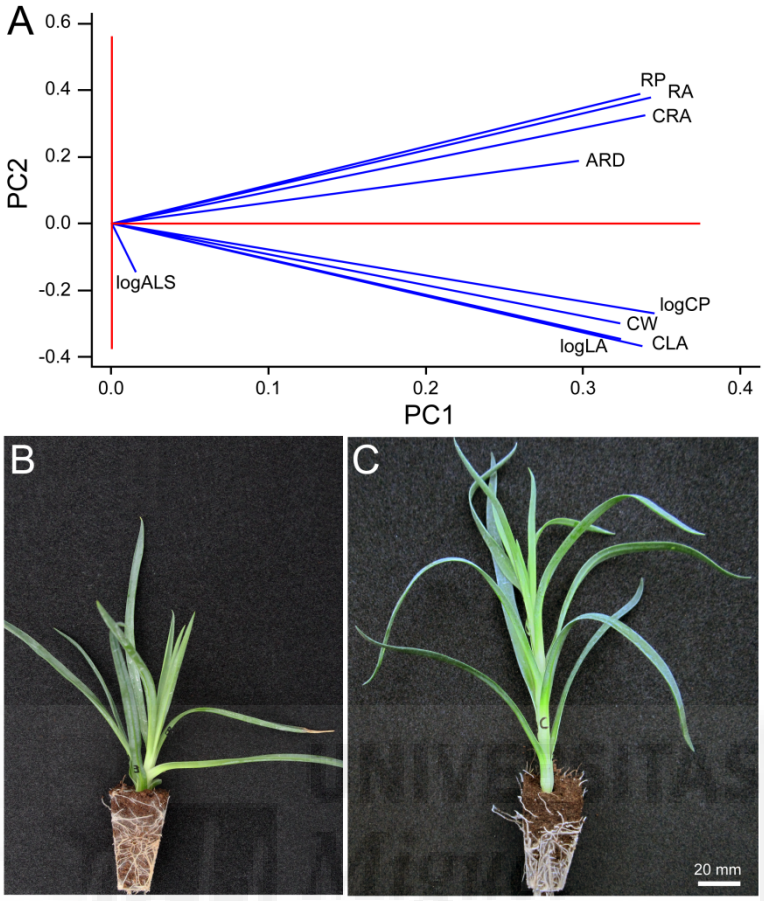
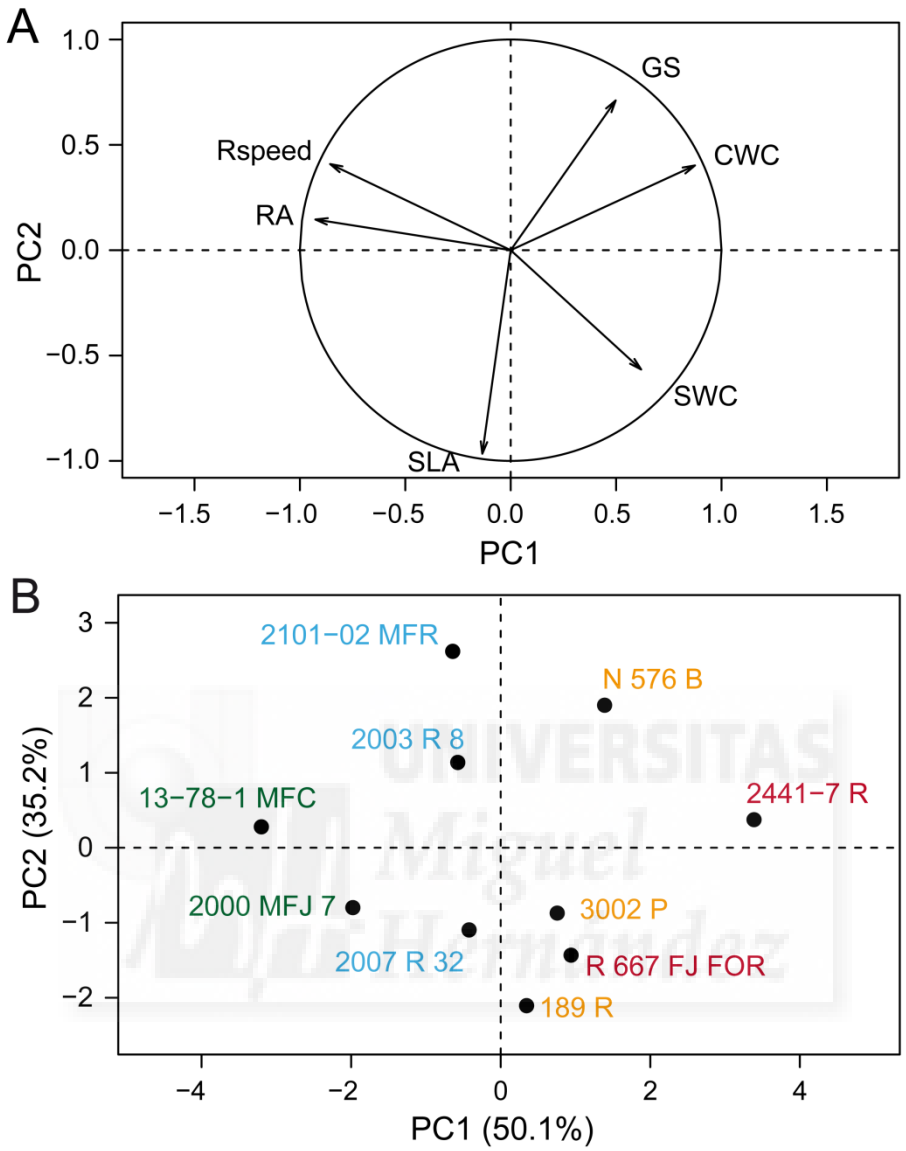


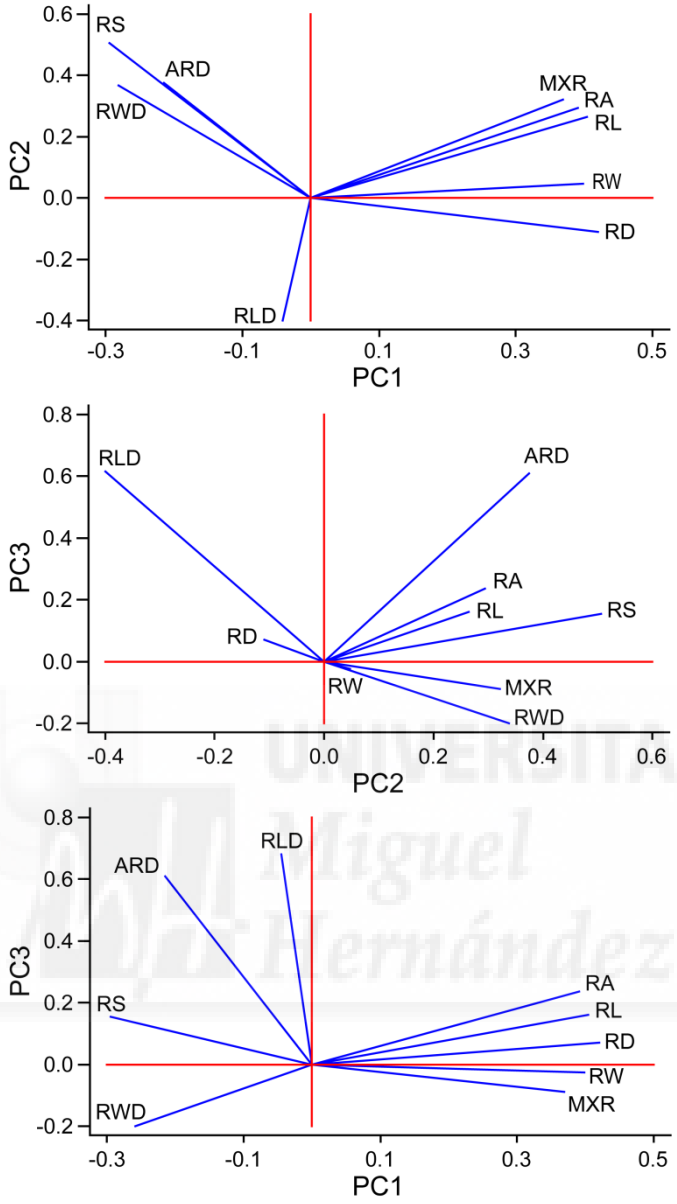
S3 Fig. Graphic representation of some rooting parameters in different carnation cultivars grown in soil plugs. (A-B) Box-plots of RP (A) and ARD (B) data according to RSG values. Different letters indicate significant differences ($P < 0.01$) between RSG values. (C-D) Graphic representation of average RP (C) and CRA (D) values in different carnation cultivars over time. Different letters indicate significant differences ($P < 0.01$) between the cultivars for a given time. Nd: not determined.



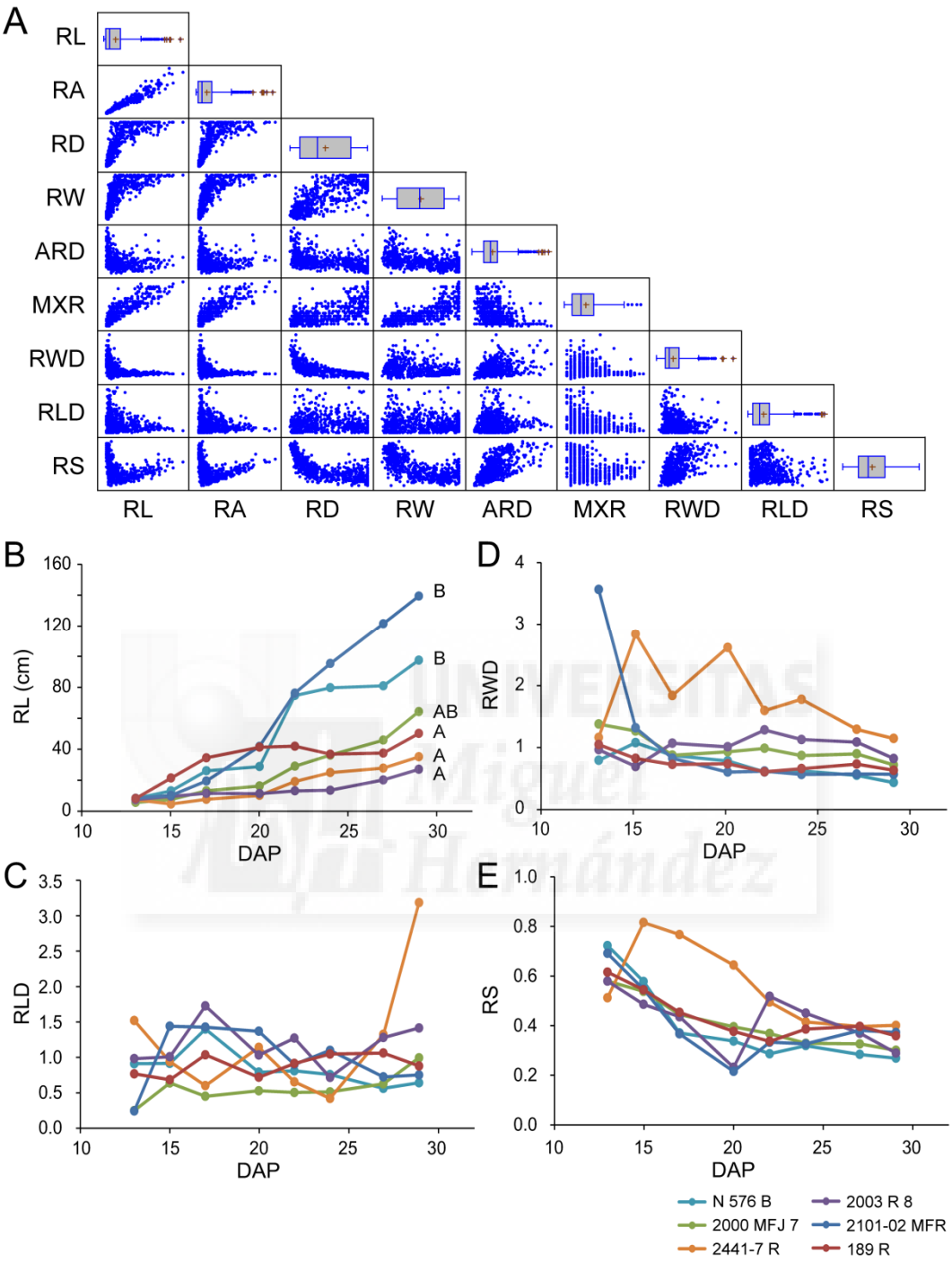
S4 Fig. Principal component analysis of stem cutting morphology and adventitious rooting parameters. (A) Graphical representation of PC1 and PC2. (B-C) Representative images of two good-rooting stem cuttings of a spray cultivar (B) and a standard cultivar (C) displaying contrasting stem cutting morphology and similar rooting performance. Images were obtained as described in Materials and Methods at 27 DAP.



S5 Fig. Principal component analysis of stem cutting ecophysiology and adventitious rooting parameters. (A) Principal component analysis of rooting and ecophysiological parameters. Graphical representation of PC1 and PC2 are shown. (B) Individual factor map for PC analysis of rooting and ecophysiological parameters. Cultivars have been color-coded as regards their rooting performance (red: bad; orange: poor; blue: intermediate; green: good).



S6 Fig. Principal component analysis of adventitious rooting parameters measured in carnation stem cuttings grown *in vitro*. Representation of PC1, PC2 and PC3 in a tridimensional space. Root system parameters are represented as defined in Table 2.



S7 Fig. Quantitative description of adventitious rooting in carnation stem cuttings grown *in vitro*. (A) Scatter plots of some adventitious rooting parameters as defined in Table 2. Box-plots of each parameter are also represented. Graphic representation of RL (B), RLD (C), RWD (D) and RS (E) values in different carnation cultivars over time. Different letters indicate significant differences ($P < 0.01$) between the cultivars for a given time.

Table S1.- Carnation cultivars studied in this work

Cultivar code	Cultivar type	Average rooting losses (%)[a]	Commercial rooting performance[a]	Batch	*In vitro* tested
13-78-1 MFC	Spray	0.83 ± 0.89	Good-rooting	1	No
189 R	Standard	2.60 ± 1.73	Good-rooting	1	Yes
2000 MFJ 7	Spray	3.11 ± 0.80	Intermediate	2	Yes
2003 R 8	Standard	7.29 ± 2.46	Bad-rooting	2	Yes
2007 R 32	Standard	9.03 ± 5.21	Bad-rooting	1	No
2101-02 MFR	Spray	2.02 ± 1.18	Good-rooting	2	Yes
2441-7 R	Standard	8.59 ± 1.11	Bad-rooting	2	Yes
3002 P	Standard	6.29 ± 0.39	Bad-rooting	1	No
N 576 B	Standard	3.78 ± 1.17	Intermediate	2	Yes
R 667 FJ FOR	Standard	1.85 ± 0.71	Good-rooting	1	No

[a]According to empirical data obtained at the breeders' rooting station before the present study.

Table S2.- Linear correlation matrix of average stem cutting parameters measured

	CW	ALS	CA	CCA	CP	CS	CWL	LN	RSG	ARD	RA	CRA	RP
CL	0.268	0.259	0.601	0.564	0.456	*-0.086*	-0.311	*0.003*	*-0.003*	0.146	*-0.005*	*-0.038*	*-0.044*
CW		*-0.009*	0.641	**0.845**	0.729	-0.610	**0.826**	0.412	0.451	0.413	0.418	0.441	0.406
ALS			0.396	0.028	-0.226	0.450	-0.154	-0.311	*-0.033*	*0.087*	*-0.046*	*0.008*	*-0.060*
CA				0.800	0.782	*-0.070*	0.284	0.419	0.397	0.437	0.399	0.442	0.381
CCA					**0.856**	-0.609	0.499	0.480	0.406	0.426	0.405	0.420	0.384
CP						-0.425	0.451	0.643	0.455	0.423	0.463	0.468	0.452
CS							-0.546	-0.305	-0.200	-0.165	-0.184	-0.187	-0.182
CWL								0.404	0.452	0.331	0.422	0.458	0.431
LN									0.433	0.296	0.448	0.493	0.480
RSG										0.559	**0.897**	**0.837**	**0.920**
ARD											0.694	0.595	0.590
RA												**0.865**	**0.973**
CRA													**0.909**

	Ecut	LA	GS	SLA	LDMC	RSG	ARD	RA
CWC	*-0.147*	*0.513*	**0.849**	*-0.501*	*-0.288*	-0.793	-0.343	-0.757
Ecut		*-0.462*	*-0.504*	0.780	*-0.448*	*-0.342*	*-0.176*	*-0.287*
LA			*0.556*	-0.643	*0.262*	*-0.188*	*0.245*	*-0.264*
GS				-0.659	*-0.250*	*-0.235*	*0.211*	*-0.231*
SLA					*-0.560*	*0.013*	*-0.061*	*0.015*
LDMC						*0.504*	*0.125*	*0.495*

Non-significant correlations ($P > 0.05$) are shown in italics. r values larger than 0.81 ($r^2 \sim 0.65$) are indicated in bold.

Table S3.- Principal component analysis of root system parameters in carnation stem cuttings grown in soil plugs

3A.- Principal component analysis

Principal component	Eigenvalue	Variance
1	5.0565	56.183
2	1.6777	18.641
3	1.2076	13.418
4	0.4855	5.394
5	0.3170	3.522
6	0.1392	1.546
7	0.0978	1.086
8	0.0112	0.125

3B.- Eigenvectors for the three main principal components (PCs)

Parameters	PC1	PC2	PC3
CW	0.345265	-0.319970	-0.131645
logCP	0.368628	-0.287599	-0.297825
logCA	0.345895	-0.370170	0.263357
CCA	0.360629	-0.392486	-0.097157
logALS	0.016021	-0.152633	0.885513
ARD	0.317331	0.201794	0.164968
RA	0.366201	0.406235	0.030007
CRA	0.362496	0.348536	0.053740
RP	0.358813	0.416310	0.006068

Table S4.- Principal component analysis of root system parameters in carnation stem cuttings grown *in vitro*

4A.- Principal component analysis

Principal component	Eigenvalue	Variance
1	4.9735	55.262
2	1.6308	18.120
3	1.0161	11.290
4	0.6503	7.225
5	0.4266	4.740
6	0.1195	1.328
7	0.0879	0.976
8	0.0700	0.778

4B.- Eigenvectors for the three main principal components (PCs)

Parameters	PC1	PC2	PC3
RL	0.403784	0.263924	0.161014
RA	0.390869	0.293961	0.236238
RD	0.420354	-0.109710	0.071432
RW	0.397987	0.046778	-0.024799
ARD	-0.213881	0.374525	0.609607
MXR	0.368666	0.320827	-0.088581
RWD	-0.280960	0.367182	-0.217851
RLD	-0.044941	-0.442112	0.679758
RS	-0.294060	0.506184	0.153467

Table S5.- Linear correlation matrix of root system parameters measured in carnation stem cuttings grown *in vitro*

	RA	RD	RW	ARD	MXR	RWD	RLD	RS
RL	**0.971**	0.788	0.754	-0.224	**0.867**	-0.437	-0.117	-0.300
RA		0.763	0.755	-0.145	**0.836**	-0.420	-0.108	-0.261
RD			0.803	-0.426	0.659	-0.696	*-0.022*	-0.680
RW				-0.362	0.756	-0.328	*-0.038*	-0.631
ARD					-0.342	0.357	*0.021*	0.623
MXR						-0.241	-0.132	-0.265
RWD							-0.109	0.573
RLD								-0.157

Non-significant correlations ($P > 0.05$) are shown in italics. r values larger than 0.81 ($r^2 \sim 0.65$) are indicated in bold.

Artículo 2

Multiple factors influence adventitious rooting in carnation (*Dianthus caryophyllus* L.) stem cuttings

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ORIGINAL PAPER

Multiple factors influence adventitious rooting in carnation (*Dianthus caryophyllus* L.) stem cuttings

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Abstract Efficient propagation of uniform starting material is a critical requirement for mass production of most ornamental plants, including carnation. For some elite cultivars, the production of young plantlets is limited by poor adventitious root formation from stem cuttings. We previously characterized the molecular signature during adventitious rooting in two carnation cultivars, *2101-02 MFR* and *2003 R 8*, which were selected because of their contrasting rooting performance. To determine additional factors that contribute to the differences observed in adventitious rooting during the commercial scaling-up of this species, we characterized rooting performance and endogenous hormone levels in stem cuttings of these two cultivars during one production season. We found that stem cutting production declined during the harvest season in a cultivar-dependent manner. In addition, the initiation of adventitious roots in the stem cutting base depended on its endogenous auxin and cytokinin levels at harvest time, while their subsequent growth and development was mainly influenced by the physiological status of the mother plant at harvest time and of the stem cutting during the rooting process.

Keywords Adventitious roots · Auxin/cytokinin ratio · ABA · Ornamental plants

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Introduction

As most commercial carnation (*Dianthus caryophyllus* L.) cultivars are of hybrid nature (Sheela 2008), an efficient propagation method for uniform starting material is a critical requirement for mass-production of planting stock. Commercial carnation cultivars are propagated from terminal stem cuttings with 4–6 pairs of mature leaves that are periodically collected from elite mother plants (Agulló-Antón et al. 2014; Garrido et al. 2002). Adventitious root formation in carnation stem cuttings is affected by complex interactions between environmental and endogenous cues (Garrido et al. 2002; Guerrero et al. 1999). In addition, the large variation in adventitious root formation in this species is also genotype-dependent (Birlanga et al. 2015). Auxin is a well-known trigger for adventitious root formation in several species (de Klerk et al. 1999), and low levels of exogenously-added auxin have been found to promote rooting in carnation stem cuttings (Garrido et al. 1998). As opposed to auxins, cytokinins (CKs) are negative regulators of adventitious root formation in many species (de Klerk et al. 2001). In the commercial production of rooted carnation plants, cuttings must frequently be stored at low temperatures for several weeks to match production with demand. The duration of the storage period modifies the rooting performance of different cultivars, suggesting that some changes might occur in endogenous auxin levels and/or auxin sensitivity during cold storage (Agulló-Antón

et al. 2011; Garrido et al. 1996). In carnation stem cuttings, auxins are mainly produced in the leaves and are transported basipetally to roots (Garrido et al. 2002). Endogenous indole-3-acetic acid (IAA) levels transiently accumulate in the stem cutting base shortly after its excision from the mother plant (Agulló-Antón et al. 2014; Garrido et al. 2002), while extended cold-storage periods of the stem cuttings reduce the rates of root formation and growth in an auxin-dependent manner (Garrido et al. 1996). Endogenous *trans*-zeatin (TZ) levels are low in the stem cutting base after its excision and steadily increase during cold storage (Agulló-Antón et al. 2014).

In addition, a decline in adventitious rooting capacity routinely occurs during maturation in woody species (Abarca and Díaz-Sala 2009; Wendling et al. 2014). The maturation status of a plant can be described in terms of ontogenetic stage, and physiological and chronological ages (Rasmussen et al. 2015). Physiological age may also depend on environmental and growth conditions and stress responses of the plant. Although it has been proposed that auxin inactivation may be part of the reason for the decline in adventitious root formation with age, several lines of evidence suggest that other signals might contribute to the maturation-related loss of adventitious rooting competence (Greenwood et al. 2001; Rasmussen et al. 2015).

To get some insight into the different factors that contribute to adventitious root formation in carnation stem cuttings during commercial production, we evaluated a number of morphometric and physiological parameters in stem cuttings collected at regular intervals during a 4-month period from mature (>1 year-old) mother plants grown at a single location. To account for the genotype-dependency on the studied traits, we selected two cultivars, *2102-01 MFR* and *2003 R 8*, showing contrasting rooting performance in a previous experiment (Birlanga et al. 2015). We characterized rooting performance and endogenous hormone levels in stem cuttings of these two cultivars during one production season. Our results are consistent with the hypothesis that the initiation of adventitious roots from stem cuttings is mostly dependent on their endogenous auxin/CK ratio, while their subsequent growth is also influenced by the physiological status of the stem cuttings at their harvest time and during the rooting process, which could be indirectly estimated by their endogenous ABA levels at planting time.

Materials and methods

Plant material and sample collection

Two carnation cultivars (*2101-02 MFR* and *2003 R 8*) were selected for further studies at Barberet & Blanc (http://www.barberet.es) because of their contrasting rooting performance (Birlanga et al. 2015). All the mother plants were grown in the same location in the greenhouse and under environmental conditions at 37°34×50″N, 1°46×35″W and 395 m altitude (Puerto Lumbreras, Murcia, Spain). Water, fertilizers and adequate phytosanitary treatments were periodically applied, as described previously (Birlanga et al. 2015; Jawaharlal et al. 2009). For each cultivar, all stem cuttings with commercial quality were single-pinched from 36 mature (>1 year-old) mother plants by skilled operators every 15 days from 7th April 2014 to 30th July 2014 (Figure S1A). Stem cutting lengths and fresh weights were then measured in a sample of 100 randomly-collected cuttings of each cultivar.

Adventitious rooting in soil plugs

For adventitious rooting, fresh and cold-stored stem cuttings were used (Figure S1A). For the cold treatment, over 50 stem cuttings of each cultivar were immediately wrapped in plastic bags after pinching and were stored in a cold chamber at 5 ± 2 °C, 60 % relative humidity (RH) and complete darkness for 15 days before they were planted. Fifty cold-stored (harvested at 380, 410 and 440 days after mother plant planting) or fresh (harvested at 395, 425 and 455 days after mother plant planting) stem cuttings were submerged for 15 h in an aqueous fungicide solution (1 g L^{-1} benomyl) and without exogenous auxin treatment, at 15 ± 2 °C and 80 % RH in dim light [4 µmol m^{-2} s^{-1} of average photosynthetic photon flux density (PPFD)]. Afterwards, stem cuttings were individually planted in peat/perlite (90/10 v/v) substrate trays at Barberet & Blanc's rooting station, as described previously (Birlanga et al. 2015).

For scoring adventitious rooting, a minimum of 50 cuttings per cultivar were imaged 30 days after planting (DAP). Stem cutting pictures were taken from one side of the soil plug (Figure S1B) using a portable photographic bench as described elsewhere (Birlanga et al. 2015). In addition, the soil plug was carefully removed by washing it with high-pressure tap water and the entire root system was scanned (Figure S1C) at a resolution of 800 dpi and 24-bit colour on an Epson Perfection V330 Photo scanner (Seiko Epson Corporation, Nagano, Japan), and saved as an RGB colour image in jpeg format.

Image analysis

For the morphometric analysis of rooted stem cutting images (Figure S1C), we individualized vegetative roots at the same scale and batch-imported them into the Gia Roots software (Galkovskyi et al. 2012). After scale calibration and grayscale conversion, the root images were segmented using the global thresholding method (Figure S1D–F).

Eventually, threshold values were manually adjusted at each image to maximize object identification. Twelve root system architectural traits were initially selected and were computed directly from the image mask or from the skeleton of the image mask as described previously (Birlanga et al. 2015). Raw measurements were exported to Excel spreadsheets for data analysis.

Phytohormone extraction and analysis

Samples for hormone analysis were collected from cold-stored and fresh cuttings prior to transplanting them to soil plugs (Figure S1A). At least two biological replicates, each consisting of ten stem cutting bases (~5 mm long), were collected per cultivar, and treatment, and were frozen in liquid N_2.

Phytohormones were extracted and analysed as previously described (Großkinsky et al. 2014). Briefly, 100 mg of frozen tissue from each sample was extracted twice with 1 mL of 80 % methanol/water, centrifuged at 20,000*g* for 15 min at 4 °C, the supernatant was passed through a C18 cartridge, and the samples were collected in a 5-mL tube for speed-Vac evaporation to dryness. The residue was resuspended in 1 mL 20 % methanol/water. 10 µL of filtrated extract was injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 µg L^{-1}) and corrected for 10 µg L^{-1} deuterated internal standards (Ghanem et al. 2011). Recovery percentages ranged between 92 and 95 %.

Statistical analyses

Descriptive statistics [mean, standard deviation (SD), maximum and minimum, etc.] were calculated for samples taken at each stem cutting by using the StatGraphics Centurion XV software (StatPoint Technologies, Inc. Warrenton, VA USA) and SPSS 21.0.0 (SPSS Inc., Chicago, IL, USA) programs. Data outliers were identified based on aberrant SD values and excluded for posterior analyses. One-sample Kolmogorov–Smirnov tests were performed to analyse the goodness-of-fit between the distribution of the data and a given theoretical distribution as previously described (Chacón et al. 2013). The differences between the data groups were analysed by *t* test ($P \leq 0.05$) when only two groups were compared. To compare the data for a given variable, we performed multiple testing analyses with a three-way ANOVA test (cultivar × collection date × treatment) and the Fisher's LSD (Least Significant Differences) methods ($P \leq 0.01$). Correlations were studied using Pearson product-moment correlation coefficient (Pearson's r). Multiple comparisons between various sets of variables were performed by canonical correlation (Hotelling 1936) and multiple regression (Pearson 1908) analyses. Hypothesis testing for binomial distributions were performed for the different conditions tested as described elsewhere (Chacón et al. 2013).

Results

Stem cutting production depends on mother plant age

For commercial stem cutting production, fresh stem cuttings are harvested at regular time-intervals during several months from adult mother plants. We wondered whether stem cutting production was influenced by mother plant age and varied during the production season. To this end, we measured during a ~4-month period (7th April to 30th July) the stem cutting production and quality from 1-year-old mother plants of two genotypes, *2003 R 8* and *2101-02 MFR*, showing contrasting performance during adventitious rooting of stem cuttings (Birlanga et al. 2015). Stem cutting production, estimated as the average number of harvested stem cuttings per mother plant, diminished over time for both genotypes, on an almost linear scale (Fig. 1a). Stem cuttings are harvested by skilled operators according to their morphological characteristics. In line with this practice, we did not observe significant variation of stem cutting height during the experiment or between cultivars (Fig. 1b). We measured stem cutting weight as an indirect estimate of stem cutting quality. Stem cutting weight also diminished across the collection dates in both cultivars, but more strongly in the *2003 R 8* cultivar (Fig. 1c).

Rooting performance is influenced by genotype, physiological status and cold-storage conditions of stem cuttings

During commercial production, freshly-harvested stem cuttings are kept cold for long storage to match production with demand. It was previously shown that the duration of the cold storage period of stem cuttings influenced subsequent rooting in a cultivar-dependent manner (Garrido et al. 1998, 1996). Our experimental design was planned to interrogate whether (a) the age of the mother plant and time of the season at which stem cuttings are collected or (b) the effect of the cold treatment, affected rooting performance in the two cultivars under study. To minimize the effect of the environment during rooting, fresh- and cold-stored stem cuttings were planted in the same greenhouse conditions.

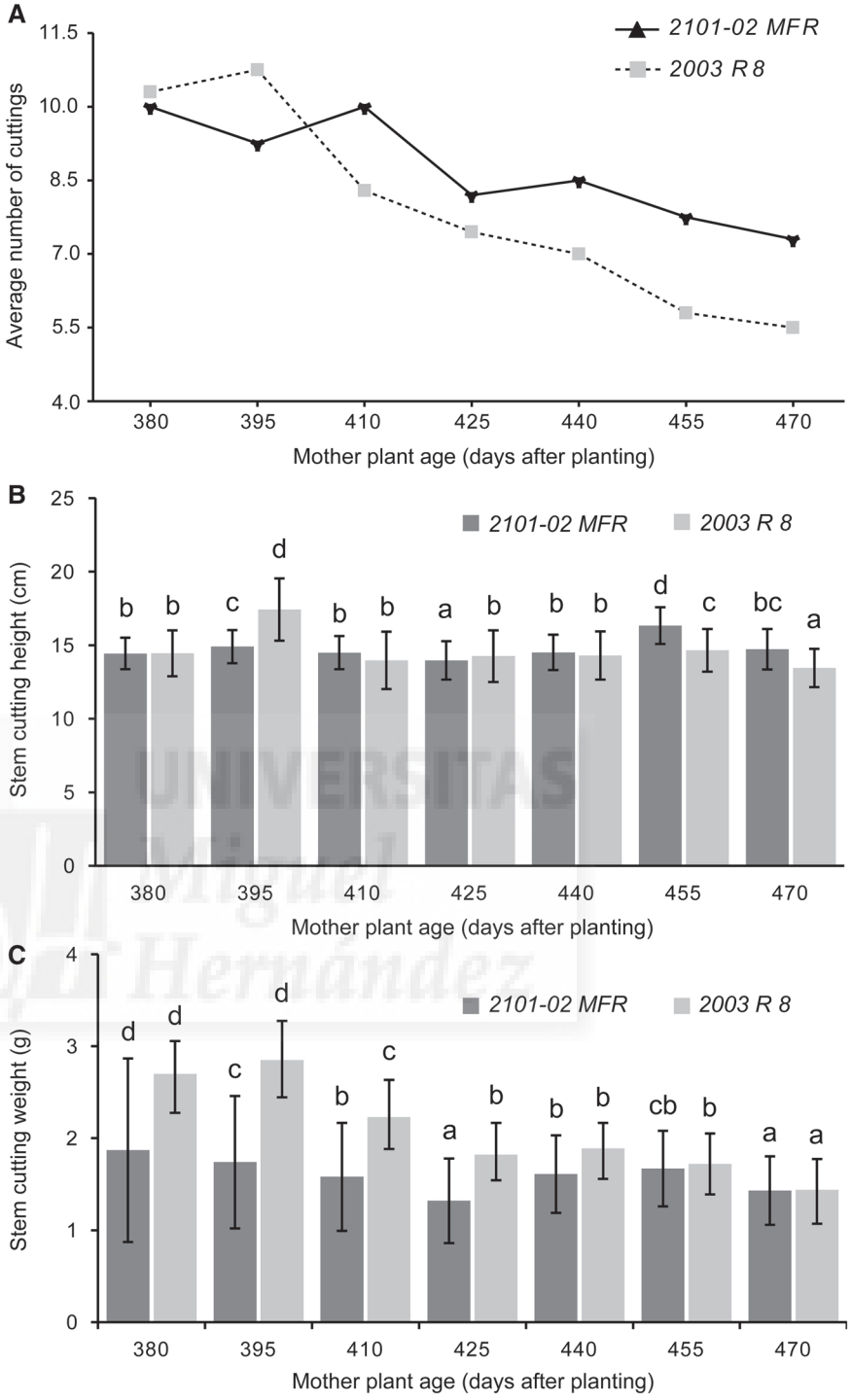

Fig. 1 Time course of cutting production in carnation cultivars. **a** Average number of cuttings with commercial quality collected per mother plant at regular time-intervals in *2101-02 MFR* and *2003 R 8* cultivars. Stem cutting heights (**b**) and weights (**c**) were measured in a minimum of 80 samples per collecting date. Average values ± a standard deviation values are shown. *Different letters* indicate significant differences (LSD; $P \leq 0.01$) over time

The percentage of cuttings with visible roots at 30 days after planting was significantly higher ($P < 0.001$) in *2101-02 MFR* than in *2003 R 8* (Fig. 2a). We did not observe significant variation for the percentage of rooted cuttings in *2101-02 MFR* as regards collection date ($P = 0.813$) or treatment ($P = 0.157$), although cold-stored cuttings in *2101-02 MFR* collected at later time of the season showed lower rooting percentages than those collected earlier. On the contrary, the percentage of rooted cuttings in *2003 R 8* was significantly influenced by collection date ($P = 0.008$), and slightly by the cold treatment. Mother plant age and time of the season negatively influenced the rooting percentage in stem cuttings of the *2003 R 8* cultivar, irrespectively of the cold treatment.

We next measured the area and length of the scanned root system in rooted cuttings at 30 days after planting. The variation found in root area was attributed to genotype,

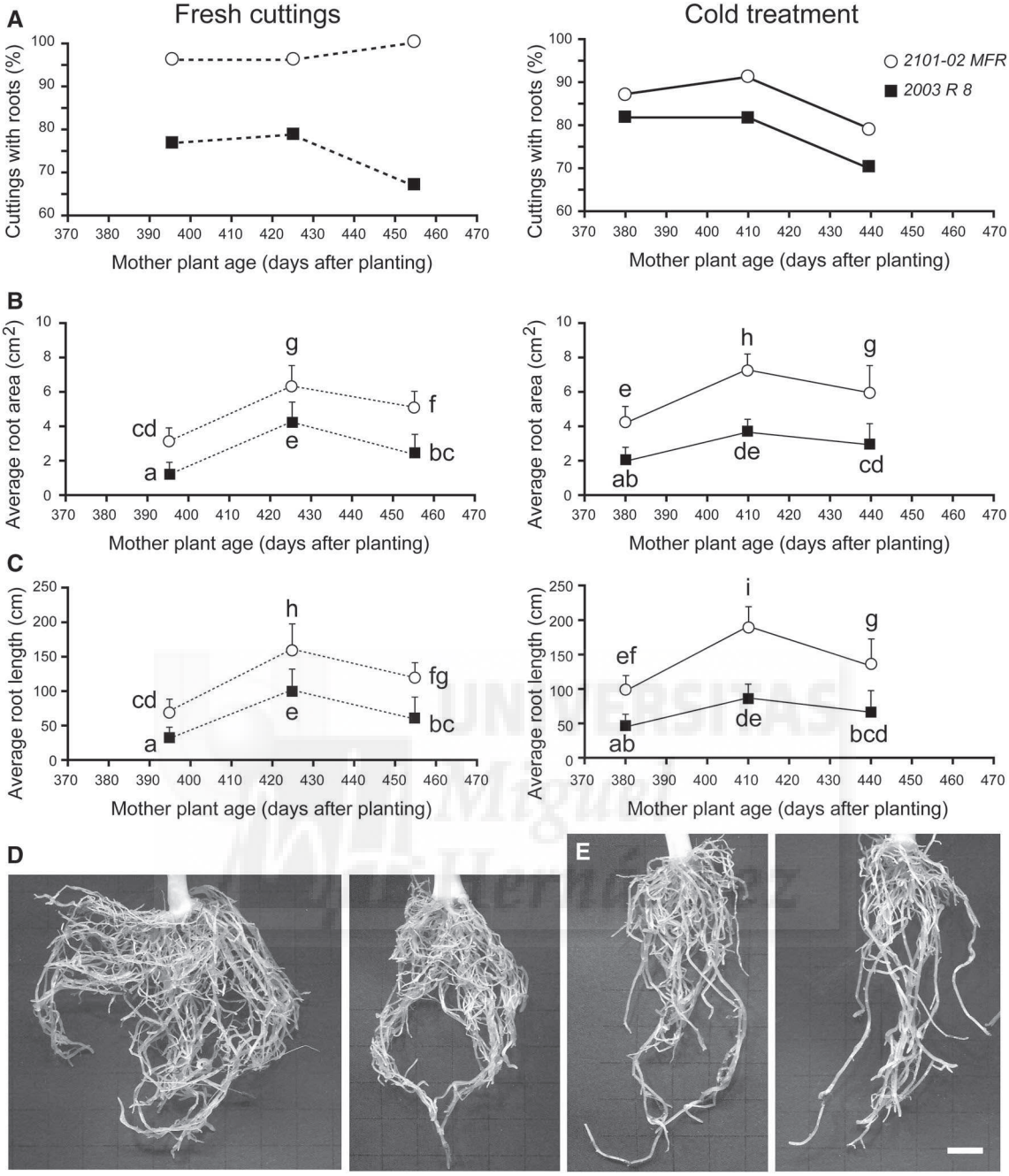

Fig. 2 Quantitative description of adventitious rooting in carnation cultivars. **a** The percentage of cuttings with roots, **b** the area and **c** the length of the scanned root system at 30 days after planting in rooted stem cuttings. *Dashed lines* represent rooting data from fresh cuttings. *Closed lines* represent rooting data from cold-stored cuttings. Average values ± a standard deviation values are shown. *Different letters* indicate significant differences (LSD; $P \leq 0.01$) over time. **d** Representative images at the end of the experiment of *2101-02 MFR* cuttings collected at 410 (*left* cold-stored) and 425 (*right* fresh) days after planting. **e** Representative images at the end of the experiment of *2003 R 8* cuttings collected at 410 (*left* cold-stored) and 425 (*right* fresh) days after planting. *Scale bars* 5 mm

then to mother plant age and time of the season, and the least to the cold treatment. A two-fold variation in root area and root length was found between *2101-02 MFR* (5.35 ± 0.07 cm^2) and *2003 R 8* (2.75 ± 0.08 cm^2). Unexpectedly, from the three rooting experiments performed from fresh cuttings collected at different mother plant ages (395, 425 and 455 days after planting), the second experiment gave the highest values in both root area and root length. As all stem cuttings were grown in the same greenhouse, which was equipped with a fog system to control excessive heat, we gathered additional meteorological data to identify the hidden environmental variation that might

account for the observed differences in rooting performance between this experiment and the other two experiments (Figure S2). Additional research will be required to assess whether variations in air or soil temperature during rooting might account for the observed differences in rooting performance.

As regards the *2101-02 MFR* cultivar, we found a significant effect of the mother plant age/time of the season ($P < 0.001$) and the cold treatment ($P < 0.001$) on rooting area (Fig. 2b). Similar results were found for root length in this cultivar (Fig. 2c). Indeed, the cold-treatment positively improved rooting performance of the *2101-02 MFR* cultivar by increasing root length by about 20% (Fig. 2c). In contrast, rooting area (and root length) in the *2003 R 8* cultivar was not significantly affected by the treatment ($P = 0.089$). Hence, poor rooting growth of the *2003 R 8* cultivar could not be restored by a 2-week cold treatment, while in the *2101-02 MFR* cultivar there was a positive effect of the cold treatment on root growth (Fig. 2d, e).

Endogenous auxin/cytokinin levels correlate with adventitious root initiation

We measured endogenous auxin and CK levels in the stem cutting base. On the one hand, we found higher IAA levels in freshly harvested stem cuttings which were strongly reduced after the cold treatment in both cultivars ($P < 0.001$; Fig. 3a). On the other hand, endogenous *trans*-zeatin (TZ) levels were influenced both by genotype ($P = 0.001$) and by the treatment ($P < 0.001$). Lower CK levels were found in freshly harvested stem cuttings in both cultivars and significantly increased after the cold treatment, but mostly in the

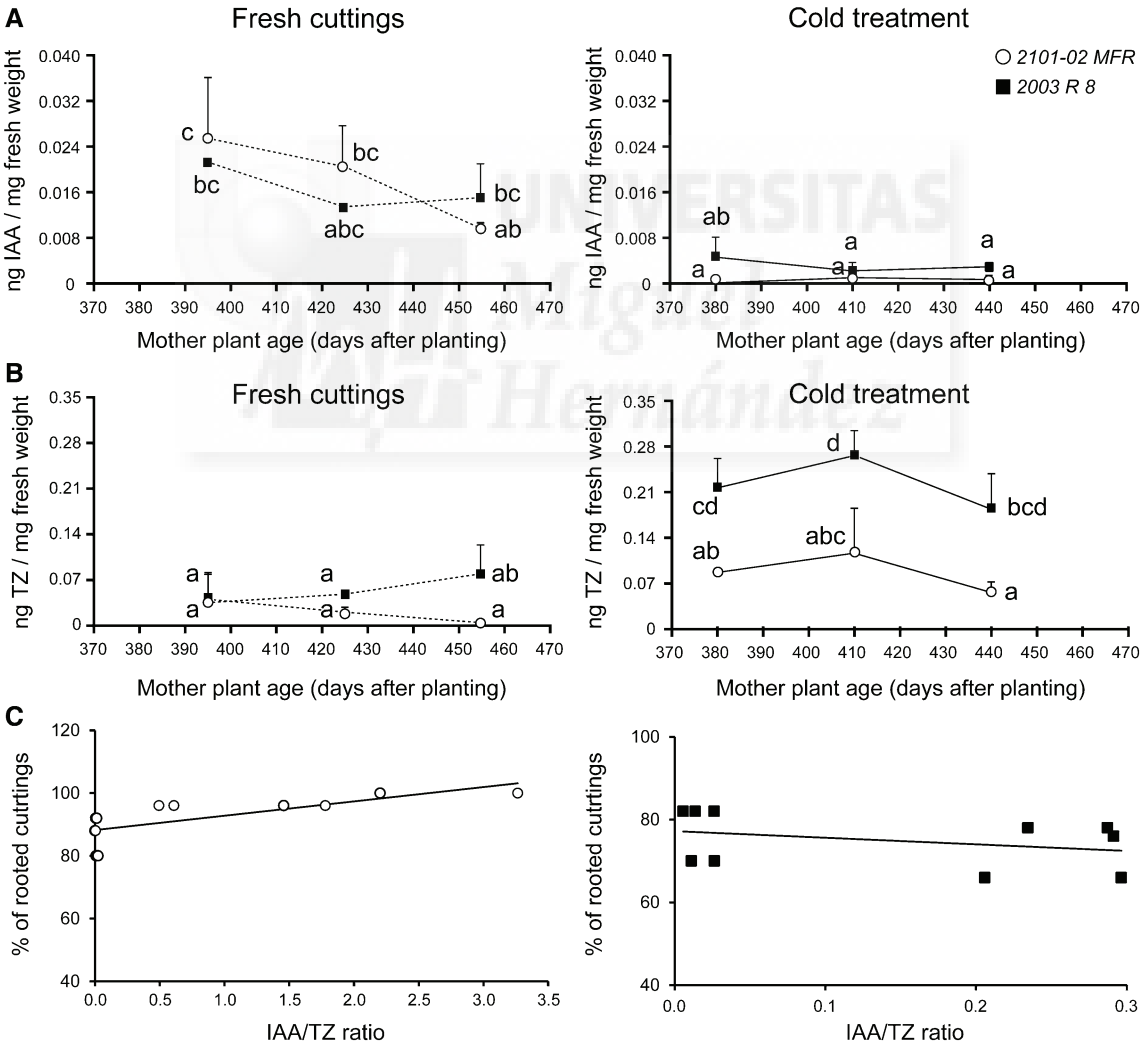

Fig. 3 Morphogenetic hormone levels in the stem cutting base in carnation cultivars. **a** Indole-3-acetic acid (IAA), **b** *trans*-zeatin (TZ), and **c** auxin to cytokinin ratio (IAA/TZ). *Dashed lines* represent data from fresh cuttings. *Closed lines* represent data from cold-stored cuttings. Average values ± a standard deviation values are shown. *Different letters* indicate significant differences (LSD; $P \leq 0.01$)

2003 R 8 cultivar (Fig. 3b). We found a strong and significant correlation (R = 0.644; P = 0.024) between the percentage of rooted cuttings and the endogenous auxin/CK ratio only in the *2101-02 MFR* cultivar (Fig. 3c). The endogenous auxin/CK ratio was highest in fresh cuttings of *2101-02 MFR* (1.634) and the lowest auxin/CK ratio was found in cold-stored cuttings of the *2101-02 MFR* cultivar (0.010).

The reduction in auxin levels observed during cold storage might be caused by enhanced auxin degradation, reduced auxin transport to the stem cutting base or both, as previously suggested (Garrido et al. 2003). We measured the levels of two IAA derivatives, 2-oxindole-3-acetic acid (OxIAA) and indole-3-acetyl-L-aspartic acid (IAA-Asp), which are known forms of biologically-inactive IAA (Korasick et al. 2013). On the one hand, OxIAA levels were low across the entire experiment and were only slightly increased after the cold treatment (P = 0.073; Figure S3). On the other hand, we found a significant effect of the collection date (P < 0.001) and the cold treatment (P < 0.001) on endogenous IAA-Asp levels, irrespectively of the genotype. IAA-Asp was higher in cold-stored stem cuttings at earlier collection dates (Figure S3). We found a highly significant correlation between OxIAA and IAA-Asp levels (R = 0.484; P = 0.017), as well as between IAA and IAA-Asp levels in both cultivars (R = −0.485; P = 0.016).

Stress-related hormones might affect adventitious root growth and development

Cutting excision from the mother plant leads to numerous stresses caused by the interruption of water and nutrient uptake, the altered transport of phytohormones and the activation of wound responses (Delessert et al. 2004). We measured endogenous 1-aminocyclopropane-1-carboxylic acid (ACC) in the stem cutting base as an indirect estimate of ethylene concentration (Agulló-Antón et al. 2014). On the one hand, low ACC levels were found in the stem cutting base of freshly harvested cuttings (Fig. 4a), in agreement with quick ethylene biosynthesis upon wounding (Yu et al. 1998). On the other hand, ACC levels were significantly increased in cold stored cuttings in both cultivars (P < 0.001; Fig. 4a), suggestive of a cold-inhibition of ACC oxidase (ACO) or an up-regulation of ACC synthase (ACS) during storage (Yu et al. 1998). Endogenous ACC levels in cold-stored cuttings were dependent on the genotype (P = 0.003) and were influenced by the collection date (P = 0.009). In addition, we found a positive correlation (R = 0.566; P = 0.055) between ACC levels in cold-stored cuttings of both cultivars and with their root area but not with the percentage of rooted cuttings.

Another hormone that has been connected to the wound response is jasmonic acid (JA) (Ahkami et al. 2009). Endogenous JA levels peak transiently in the stem cutting base just after excision, which has been proposed to play a positive role in adventitious root formation in some species (Ahkami et al. 2009; Rasmussen et al. 2015). We found that JA levels in the stem cutting base were significantly enhanced in fresh cuttings of both genotypes compared to cold-stored ones (P value <0.001; Fig. 4b). Also, significant differences in JA were found between collection dates (P = 0.021), which could not be explained by mother plant age alone as fresh cuttings from younger plants contain lower JA levels (Fig. 4b). In all cases, the JA levels returned to steady-state levels after the cold treatment, confirming a quick JA turnover (Widemann et al. 2013). In contrast with a positive role for JA in adventitious root formation in carnation stem cuttings, endogenous JA levels were not correlated with root initiation (P value = 0.647) or with root area in rooted stem cuttings (P = 0.062).

Endogenous salicylic acid (SA) levels were higher in the fresh cuttings than in the cold-stored ones (P = 0.002; Fig. 4c), but in a manner that was affected by the collection date (P = 0.004). Indeed, JA and SA levels were strongly correlated in the stem cutting base for both cultivars (R = 0.721; P < 0.001), which is consistent with the role of these two hormones in the response to wounding at the site of damage (JA) and systemically (SA) (Savatin et al. 2014).

In the *2101-02 MFR* cultivar we found higher levels of ABA in cold-stored cuttings than in fresh cuttings (P < 0.001), while significant differences were not found in ABA levels in the *2003 R 8* cultivar after the cold treatment (Fig. 4d). These results indicate that the *2101-02 MFR* cultivar is more sensitive to cold stress than the *2003 R 8* cultivar. Similarly to that found for JA and SA, there were significant differences in ABA levels between the collection dates (P < 0.001). Interestingly, ABA and JA (or SA) levels were found negatively correlated in our studies (R = −0.507; P = 0.012).

We finally questioned whether a linear combination of some of the hormonal parameters quantified in the stem cutting base before rooting could explain the differences observed on the rooting performance (percentage of rooted stem cuttings and root area in these stem cuttings) between the studied cultivars at the end of the experiment. Thus, multiple regression analysis was performed between the average values for percentage of rooted cuttings and hormone levels. The most plausible model indicated a low correlation (R = 0.396; P = 0.068) between the percentage of cuttings with roots and the auxin/CK ratio (percentage of rooted cuttings = 81.032 + 3.978 auxin/CK ratio). Surprisingly, a strong and highly significant correlation (R = 0.710; P = 0.001) was found between the rooting area at the end of the experiment and the initial levels of TZ and ABA (rooting area = 3.332 − 8.241 TZ + 87.367 ABA levels).

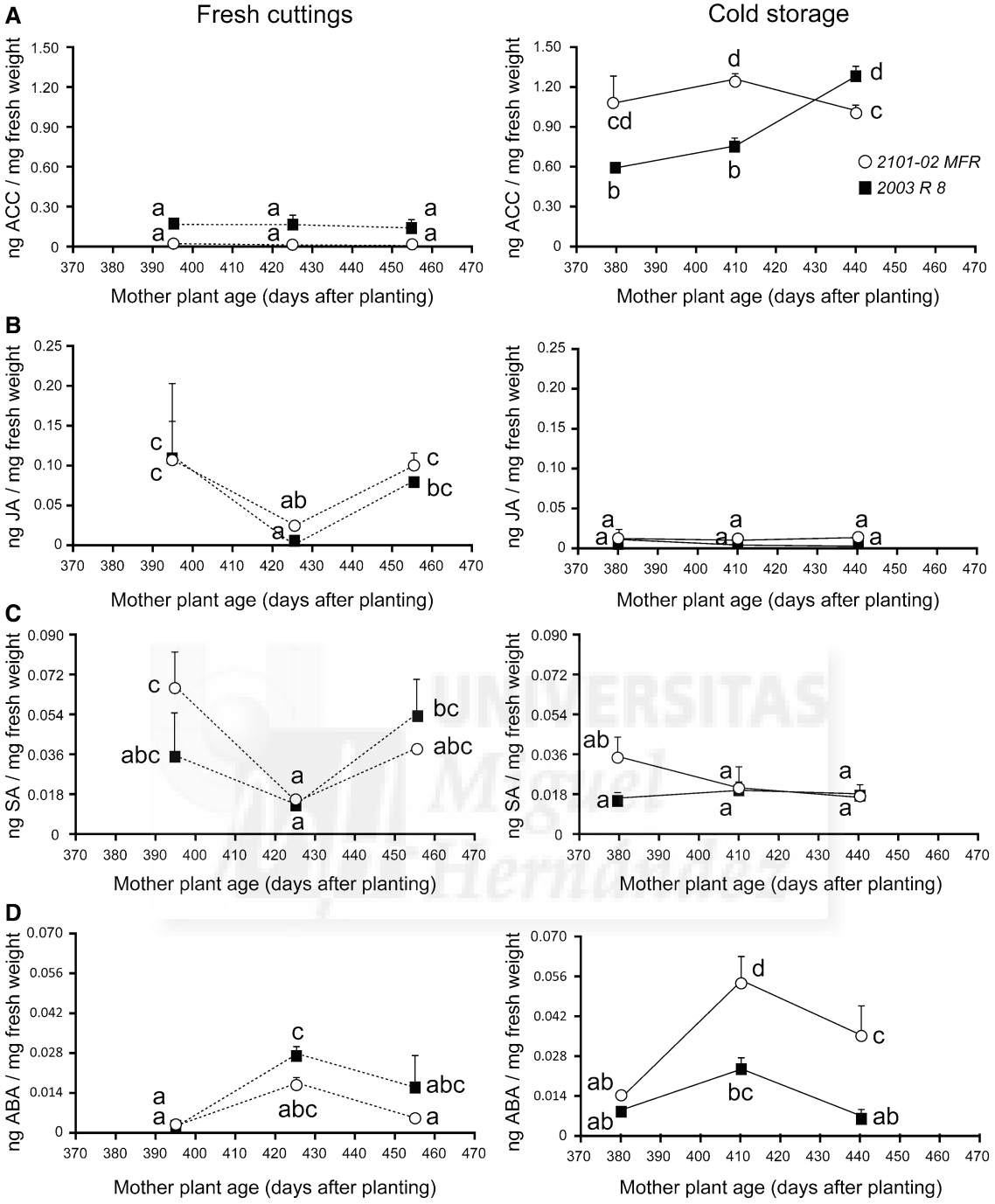

Fig. 4 Stress hormone levels in the stem cutting base in carnation cultivars. **a** The ethylene precursor, ACC, **b** jasmonic acid (JA), **c** salicylic acid (SA), and **d** abscisic acid (ABA). *Dashed lines* represent data from fresh cuttings. *Closed lines* represent data from cold-stored cuttings. Average values ± a standard deviation values are shown. *Different letters* indicate significant differences (LSD; $P \leq 0.01$)

Discussion

We found that stem cutting production (i.e. number and quality of stem cuttings per mother plant harvested at a given time) was negatively influenced by the chronological age of the mother plants in both studied cultivars, *2101-02 MFR* and *2003 R 8*, an observation that perfectly fits with the current production practice at Barberet & Blanc of discarding mother plants older than 16 months, as they negatively influence stem cutting production and quality. Different reports have shown that physiologically younger propagation material is more suitable for

successful adventitious rooting (Osterc and Štampar 2011; Rasmussen et al. 2015). As older mother plants are further exposed to pathogen attach and other environmental insults, such as high temperatures, we could not discard that the observed rooting decline during the production season might be caused by differences in the physiological status of the mother plants. Adventitious root induction in carnation stem cuttings is promoted by high auxin levels and low cytokinin levels in the rooting zone shortly after excision (Agulló-Antón et al. 2014). Recent studies performed in pea suggest that a significant reduction in the IAA pool predates the juvenile to mature switch of stem cuttings which ultimately results in a decline in adventitious root formation in the latter (Rasmussen et al. 2015). Osterc et al. (2009) demonstrated that juvenile cuttings of *Prunus subhirtella* "Autumnalis" contained higher concentrations of free IAA in the root emergence zone at the time of severance than mature cuttings. In addition, higher concentrations of inactive IAA conjugates, such as IAA-Asp were found in mature cuttings (Osterc and Štampar 2011). Consistent with these results, we found higher levels of endogenous IAA in fresh stem cuttings at earlier collection dates. However, endogenous levels of inactive auxins (OxIAA and IAA-Asp) did not significantly differ in fresh stem cuttings but were increased in cold-stored stem cuttings of both genotypes at earlier collection dates. Hence, auxin homeostasis in the rooting zone through the regulation of auxin signaling, polar auxin transport and/or auxin metabolism, might directly contribute to the different rooting success between juvenile and mature cuttings, as well as between fresh cuttings and cold-stored cuttings.

As indicators of the rooting performance during commercial production, we used the percentage of cuttings with roots and the size of the root system (area and length) at 30 days after planting of rooted stem cuttings. Consistent with previous results (Birlanga et al. 2015; Villacorta-Martín et al. 2015), the poor rooting performance of *2003 R 8* might be explained by its delay in root emergence and slow root growth compared to those in the *2101-02 MFR* cultivar. In addition, stem cuttings harvested from chronologically older stock plants in *2003 R 8* displayed lower root initiation rates and contained lower auxin/CK ratio in their stem cutting base.

Surprisingly, the highest values in root area and root length in the studied cultivars were found in the second rooting experiment performed between 425 and 455 days after planting. We reasoned that the high rooting performance observed in this experiment might not be related to chronological mother plant age, but to the environmental conditions on which their stem cuttings were rooted (*i.e.* their physiological status during rooting). A few mineral nutrients are able to influence adventitious rooting, both root number (Ca, N, Zn) and root length (P, Fe, Mn) in microcuttings of *Eucalyptus globulus* (Schwambach et al. 2005). In addition, an adequate supply of carbohydrates to the rooting zone is required to promote root initiation in carnation stem cuttings (Agulló-Antón et al. 2014). As irrigation, fertilization and phytosanitary treatments were applied equally in all the experiments, environmental variations during the growth of the stem cuttings in the different experiments might explain the observed variation. Indeed, the levels of some stress hormones, such as ABA, JA and SA were significantly lower in fresh cuttings of the second experiment, which might indirectly correlate with their superior plant performance. Additional experiments are required to narrow down which one of the environmental variables (soil temperature, air temperature, water use efficiency, etc.) lie beneath the observed differences in adventitious rooting.

Although it has long been known that cold storage is a good procedure for preserving carnation cuttings before rooting, several studies indicate that the rooting response to cold storage in this species depends on the cultivar (Garrido et al. 1996, 1998). On the one hand, the cold treatment only slightly improved root initiation in the *2003 R 8* cultivar but significantly reduced the percentage of rooted cuttings in the *2101-02 MFR* cultivar. On the other hand, there was a positive effect of the cold storage on the size of the root system only in the *2101-02 MFR* cultivar. Despite the contrasting effects of cold storage in the two cultivars under study, rooting performance is generally improved. Taken together, our results indicate that root initiation and root growth are differentially influenced by the physiological status of the stem cuttings and by the cold treatment in a cultivar-dependent manner. After cold storage, TZ levels increased in both cultivars, but more significantly in *2003 R 8*. CKs are known repressors of root growth (Dello Ioio et al. 2008), which could explain why the cold treatment enhanced root growth in the *2101-02 MFR* cultivar but not in *2003 R* with higher TZ levels. However, the differences in the auxin/CK ratio alone are not sufficient to explain the different rooting behaviour of cold-stored cuttings of the two cultivars under study.

In carnation stem cuttings, both the mechanical wounding and the water imbalance caused by the excision of the stem cuttings from the mother plant altered the endogenous levels of the hormones regulating stress responses, particularly JA, ABA and SA (Agulló-Antón et al. 2014). Among those, transient JA levels have been directly linked to adventitious root induction in some species, such as petunia (Ahkami et al. 2009) and tobacco (Fattorini et al. 2009). As we did not find a clear correlation between endogenous JA levels in the stem cutting base with root initiation or with rooting area and exogenously-added JA did not significantly improve rooting performance in the *2003 R 8* cultivar (J. Villanova and J.M Pérez-Pérez, data not shown), we

hypothesized that JA levels alone are not directly influencing adventitious root formation in carnation stem cuttings. Consistently with a strong and highly significant correlation between rooting area and endogenous ABA levels, high ABA levels were found in the good-rooting *2101-02 MFR* cultivar after the cold treatment. High temperatures, such as those experienced at the Barberet & Blanc rooting station during our experiments, might increase water loss through transpiration and indirectly trigger an adaptive water stress response mediated by ABA (Hong et al. 2013). It is well known that high temperatures induced the expression of some ABA biosynthetic genes, such as *ABA1/ZEP* and *NCED9* (Toh et al. 2008), and ABA signalling is required for the emergence of the lateral roots through its interaction with polar auxin transporters (Shkolnik-Inbar and Bar-Zvi 2010). Alternatively, the high ABA levels induced during cold storage might act as a trigger to enhance carbon allocation at the stem cutting base by modifying phloem loading and/or sugar transporters expression, as it has been recently suggested in grapevine plants (Murcia et al. 2016).

Taken together, our data are consistent with the hypothesis that in carnation the initiation of adventitious roots in the stem cutting base after wounding is dependent on the endogenous auxin/CK ratio (Agulló-Antón et al. 2014; Villacorta-Martín et al. 2015), while their subsequent growth and development is also influenced by the physiological status of the stem cuttings at their harvest time and during the rooting process, which could be indirectly estimated by their endogenous ABA levels.

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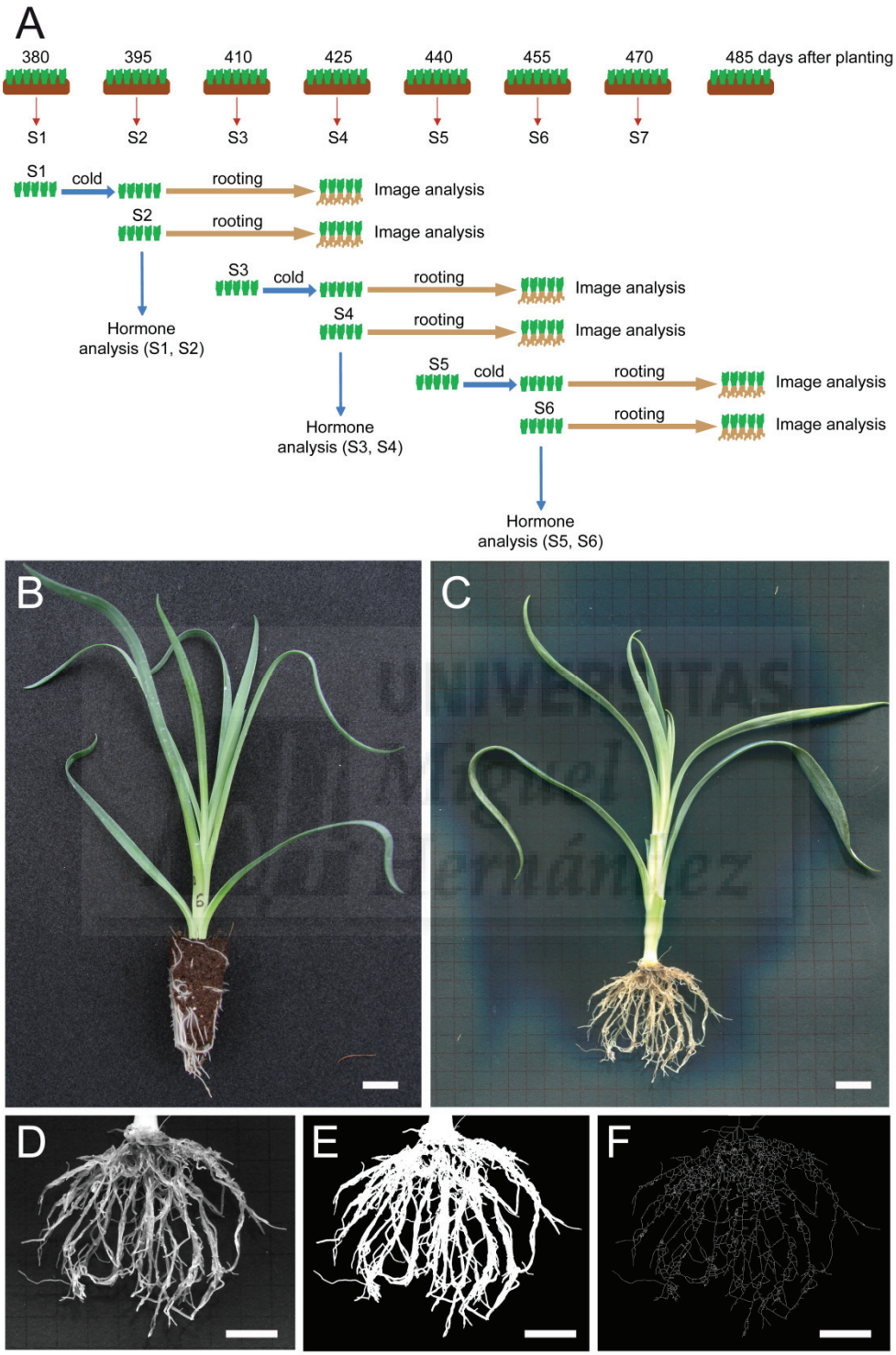
A
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cold
rooting
Image analysis
S4
rooting
Image analysis
Hormone analysis (S3, S4)
S5
cold
rooting
Image analysis
S6
rooting
Image analysis
Hormone analysis (S5, S6)
B
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Figure S1.– Adventitious rooting morphology of carnation stem cuttings. (A) Experimental design for adventitious rooting evaluation in cultivated carnation. For each cultivar, all stem cuttings with commercial quality from 36 mother plants were collected every 15 days from 365 to 485 days after planting. Stem cutting samples collected at 380 (S1), 410 (S3) and 440 (S5) days after planting were cold-treated for 15 days. Stem cutting samples for hormone analysis were taken just before rooting at 395 (S1 and S2), 425 (S3 and S4) and 455 (S5 and S6) days after planting. Three consecutive rooting experiments were performed: from 395 to 425 (S1 and S2), from 425 to 455 (S3 and S4) and 455 to 485 (S5 and S6) days after planting. (B) A representative image of a stem cutting grown in soil plugs for 30 days. (C) The stem cutting shown in B after removal of the soil substrate with high-pressure tap water. Notice that some leaves have been cut off. (D) A magnification of the root system from C that will be used for the morphometric analysis. (E, F) Image segmentation files obtained with the Gia Roots software. Scale bars: 10 mm.

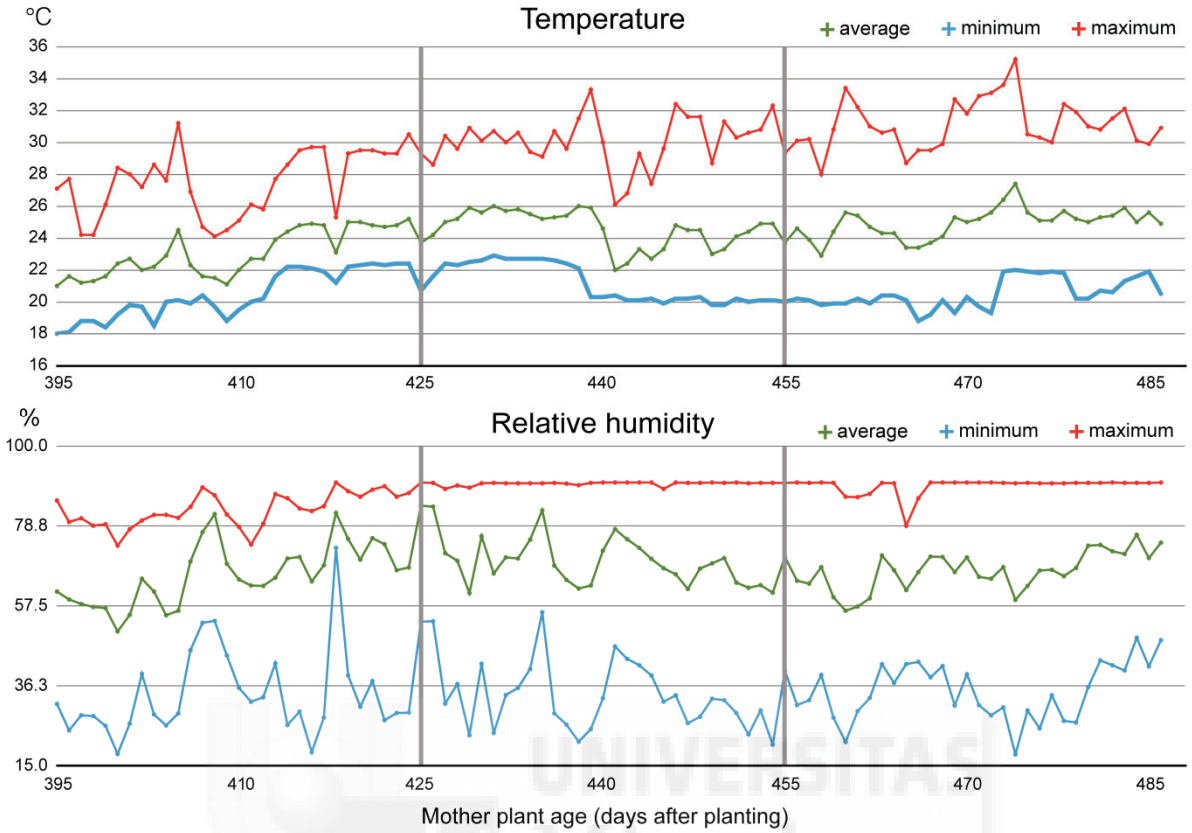

Figure S2.– Environmental parameters at the greenhouse during production of carnation stem cuttings. Average, minimum and maximum temperature (°C) and relative humidity (%) data collected at the greenhouse during the entire experiment on a daily basis.

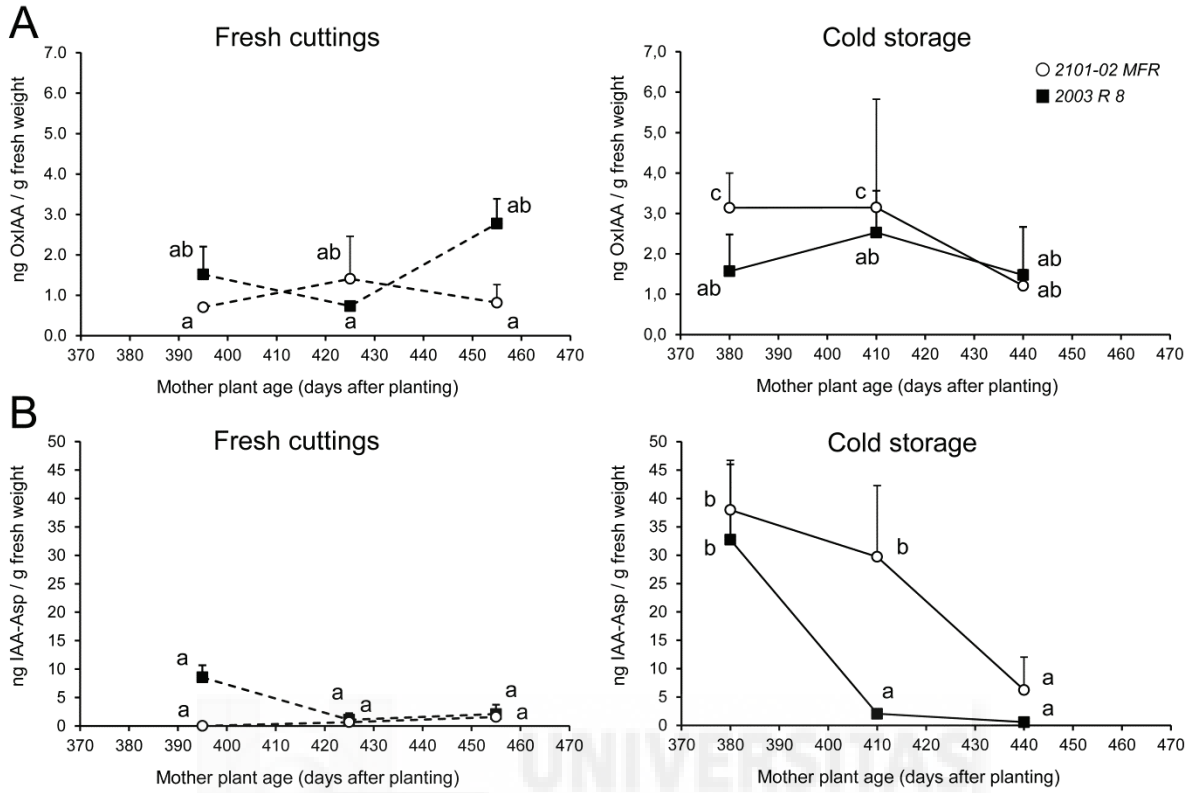

Figure S3.– Levels of IAA catabolites in the stem cutting base in carnation cultivars. (A) 2-oxindole-3-acetic acid (OxIAA), and (B) indole-3-acetyl-L-aspartic acid (IAA-Asp). Dashed lines represent data from fresh cuttings. Closed lines represent data from cold-stored cuttings. Average values ± a standard deviation values are shown. Different letters indicate significant differences (LSD; $P < 0.01$).

Artículo 3

Gene expression profiling during adventitious root formation in carnation stem cuttings

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Abstract

Background: Adventitious root (AR) formation is a critical step in vegetative propagation of most ornamental plants, such as carnation. AR formation from stem cuttings is usually divided into several stages according to physiological and metabolic markers. Auxin is often applied exogenously to promote the development of ARs on stem cuttings of difficult-to-root genotypes.

Results: By whole transcriptome sequencing, we identified the genes involved in AR formation in carnation cuttings and in response to exogenous auxin. Their expression profiles have been analysed through RNA-Seq during a time-course experiment in the stem cutting base of two cultivars with contrasting efficiencies of AR formation. We explored the kinetics of root primordia formation in these two cultivars and in response to exogenously-applied auxin through detailed histological and physiological analyses.

Conclusions: Our results provide, for the first time, a number of molecular, histological and physiological markers that characterize the different stages of AR formation in this species and that could be used to monitor adventitious rooting on a wide collection of carnation germplasm with the aim to identify the best-rooting cultivars for breeding purposes.

Keywords: RNA-Seq, Time-series analysis, Differential expression profiling, Regulatory network analysis, *Dianthus caryophyllus*

Background

In horticulture and forestry, vegetative propagation is widely used for the multiplication of plants with optimal phenotypes obtained in breeding programs or selected from natural populations. Adventitious root (AR) formation is a critical step in vegetative propagation: substantial losses can occur because cuttings do not form roots or they form poor quality root systems. A conservative estimation quantifies the burden of inadequate rooting treatments on US $50 million per year only in The Netherlands.

ARs are distinct from lateral roots in that they form from any tissue that is not a root, such as leaves and stems, naturally or in response to altered environments [1, 2]. AR formation from stem cuttings is usually divided into several stages according to physiological and metabolic markers: *i)* dedifferentiation, during which cells become competent to respond to the rhizogenic signal (auxin), *ii)* cell division (or induction phase), and *iii)* root primordia outgrowth from the stem [3]. Several plant hormones are known to control AR formation, of which auxin is a central player [4]. Auxin is often applied exogenously to promote the development of ARs on stem cuttings of difficult-to-root genotypes [1, 3]. In many species, high auxin levels in the basal region of the cuttings are required for the competent cells in the cambium to resume proliferation and to start the root-specific developmental program [5, 6]. Consistently with a positive role for auxin in AR formation, Arabidopsis mutants overproducing auxin spontaneously develop ARs on the hypocotyl [7–9]. Auxin and cytokinins are known to play a crucial role in many aspects of plant development, often acting antagonistically. A negative role for cytokinins in AR formation has been proposed as mutants defective in cytokinin biosynthesis or perception displayed increased production of ARs, whereas enhanced cytokinin biosynthesis has the opposite effect [10–12]. Moreover, interrelationships between auxin and carbohydrate metabolism during adventitious rooting have been investigated by the application of exogenous auxins and by monitoring of carbohydrate levels, carbon translocation and activities

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of key enzymes involved in sugar metabolism in the rooting zone [13–15].

Various molecular approaches have been employed to study AR development in Arabidopsis and other model plants [1]. In Arabidopsis, it was shown that the balance between AUXIN RESPONSE FACTOR17 (ARF17), a negative regulator of adventitious rooting, and ARF6 and ARF8, positive regulators of AR formation, as well as the maintenance of the homeostasis of their regulatory microRNAs (miRNAs), plays a critical role in AR formation [16, 17]. Additionally, the proteomic analysis of mutants affected in AR formation led to the identification of 11 proteins, including three auxin-inducible GRETCHEN HAGEN (GH3)-like proteins, whose expression was altered [18]. In turn, these GH3-like proteins are required for fine-tuning AR initiation in the hypocotyl, through modulating jasmonic acid homeostasis [19]. These results suggest that the early stages of AR formation need to be tightly regulated at the physiological and the genetic level and that improving rooting performance of economically important genotypes requires identifying the molecular components of the hormonal crosstalk that regulates AR formation in non-model species. As an alternative strategy to identify genes involved in AR formation, a number of studies have been conducted to characterize the gene expression profiles in the stem cutting base of different species during rooting [13, 20–25]. Based on these studies, some of the molecular events occurring during AR formation have been outlined. In our study, we aimed to characterize gene expression and functional changes occurring in the stem cutting base during the early stages of adventitious rooting in two carnation cultivars, *2003 R 8* and *2101–02 MFR*, which have been selected because of their contrasting rooting performance [26]. Our results will allow the identification of the genes involved in AR formation in this species, which will contribute to our basic understanding of the molecular events leading to this complex developmental response.

Methods

Plant material and growth conditions

Stem cuttings were pinched from several mother plants of the *2003 R 8* and *2101–02 MFR* cultivars by skilled operators at noon on 2nd December 2013 (–23 h). About 500 stem cuttings of each cultivar were wrapped in plastic bags after pinching and were sent refrigerated and in complete darkness to the laboratory (–15 h). Next, the stem cutting bases were submerged for 15 h in a 100 ml-water solution containing either mock or an auxin cocktail (1.5 μM indole-3-butyric acid [IBA; Duchefa, The Netherlands] and 1 μM α-naphtalene acetic acid [NAA; Duchefa, The Netherlands]). After the treatment, the cuttings were individually planted in 70-well trays containing moistened perlite plugs (4.5 × 4.5 × 4.5 cm; 90 cm^3), and their basal regions were collected at 0, 6, 24 and 54 h after planting (hAP) in a walk-in growth chamber that was set at 22 ± 2°C, 70 % relative humidity and under continuous fluorescent light with an average photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}. Three biological replicates, each consisting of fifteen stem cutting bases (~5 mm long), were collected per cultivar, treatment and time point, and were immediately frozen in liquid N_2. To minimize variation due to subtle environmental differences within the growth chamber, an incomplete block experimental design was used. The experimental design used for sample collection is shown in Fig. 1a.

RNA isolation, library construction and Illumina sequencing

For each sample, total RNA from ~120 mg of powdered stem cutting base tissue that was kept at –65 °C was extracted using Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, USA). The RNA integrity was confirmed using the 2100 Bioanalyzer (Agilent Technologies, USA). External RNA Controls Consortium RNA Spike-In mixes (Life technologies, USA) were used to assess the sensitivity and dynamic range of the experiment. The samples were prepared for sequencing using the TruSeq RNA Sample Preparation Kit v2 (Illumina, USA) . Illumina 100 bp paired-end sequencing on the HiSeq2000 was carried out by Macrogen, Korea. The raw Illumina reads were preprocessed using our in-house quality control pipeline. The 3'ends with a quality score below 20 were trimmed.

Reference genome: feature re-annotation and functional annotation

We used the carnation reference genome assembly released by [27]. We extended the available gene prediction using transcript sequence evidence. To this end, we assembled a comprehensive transcriptome with RNA sequencing (RNA-Seq) data comprising the sum of eight different tissues and cultivars. Each of the transcriptome assemblies was done using a genome-guided hybrid approach with Trinity [28]. Next, we leveraged this information by first aligning the transcripts to the annotated genome. The best alignments between transcript and genome annotation (those spanning at least 90 % of the transcript length) were selected and subsequently clustered in groups based on a minimum overlap of 30 % between alignments. These clusters were used as evidence to update the existing feature annotation with PASA [29]. To obtain a functional annotation,

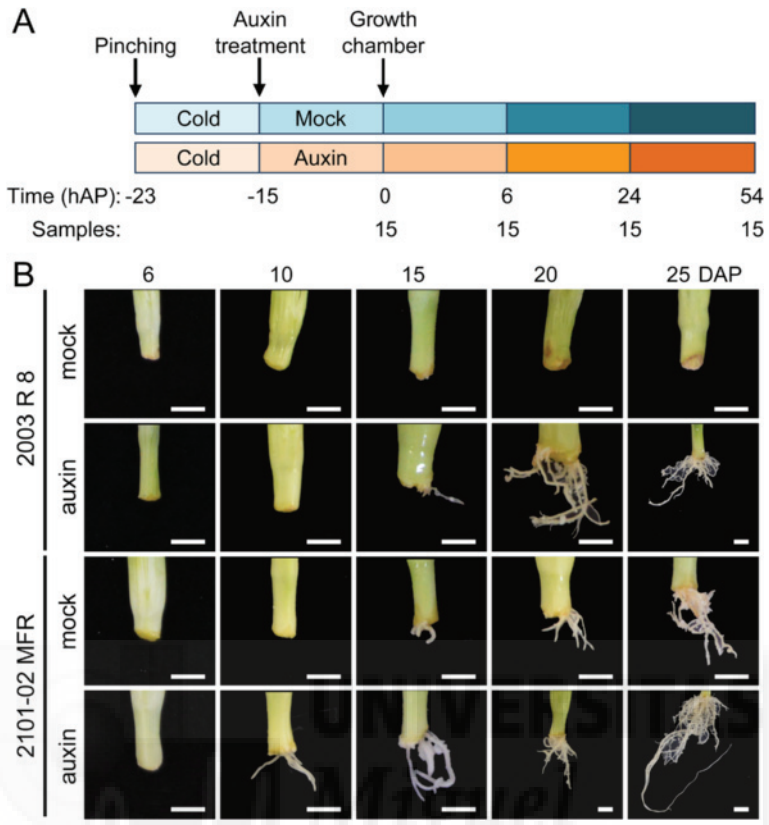

Fig. 1 Experimental design and time-series analysis of AR formation in two carnation cultivars. **a** Schematic representation of the experimental design followed in our time-course experiment. hAP: hours after planting. **b** Representative images of the basal stem of carnation cuttings of the *2003 R 8* and *2101–02 MFR* cultivars between 6 and 25 days after planting (DAP). Scale bars: 5 mm

open reading frames (ORFs) were inferred from the updated, evidence-based gene models using Transdecoder [29]. We then blasted these ORFs against a database comprising all the complete proteins from core-eudicots with a Gene Ontology (GO) [30] annotation (approximately 200,000 proteins). The use of a relatively small and highly informative set of proteins as a database increases power (smaller search-space, smaller e-values) and minimizes the chance for noisy alignments with non-homologous or non-annotated proteins. The ORFs were also compared to model profiles from the Pfam domain database [31] using HMMER [32]. The output from these sequence comparisons was integrated using ARGOT2 [33] to assign one (or several) GO annotation to each ORF. Beside this functional annotation, and in order to include information that is mainly available in model species, we mapped carnation genes to their putative *Arabidopsis thaliana* (Arabidopsis) orthologues from the The Arabidopsis Information Resource (TAIR) database [34] by means of: *i)* reciprocal best-hits between carnation and Arabidopsis proteomes, and *ii)* one-way best-hits (OBH) of carnation to the Arabidopsis proteome.

Exploratory data analysis and differential expression tests

Prior to the differential expression analysis with the DESeq2 package [35], we assessed the overall similarity between samples in order to check that it fitted the expectation from the experimental design. We calculated the Euclidean distance between samples using regularized-log transformed expression values to avoid that a few highly variable genes dominated the distance measure. We also used principal-component analysis (PCA) to examine the similarity between samples according to the components that explain most of the variance in the data as shown in Additional file 1: Figure S1.

Using the DESeq2 package we fitted generalized linear models of gene expression. The significance of the coefficient of the fitted models was inferred using a Wald test. To increase power, we filtered out genes with zero counts in all the samples, which reduces the burden of a strong multiple test correction [36]. This reduced the

data to 37,849 genes. Biological replicates were considered for each time point, as previously described [37]. Functional enrichment was tested with topGO [38] for the lists of resulting differentially expressed genes (DEG).

Gene expression analysis by quantitative reverse-transcription PCR

The selection of candidate genes for their experimental validation by quantitative reverse-transcription PCR (qRT-PCR) was based on the following criteria: *i)* high relative expression level in the RNA-Seq experiment at 0 hAP, *ii)* the function of its putative Arabidopsis ortholog was related to root growth and development, and *iii)* a dynamic expression range across the evaluated period. Six genes fulfilling these criteria were chosen for qRT-PCR analysis: *Dca5879*, *Dca23172*, *Dca29160*, *Dca30890*, *Dca40234* and *Dca43825*. For primer design, small amplicons (90 to 140 bp) were chosen within the first third of the cDNA sequences. Whenever possible, forward and reverse primers bind to different exons and the reverse primer was designed to hybridize with two consecutive exons to avoid amplification of genomic DNA.

The first strand cDNA was synthesized from 1 μg of purified RNA using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, USA). The resulting cDNA was diluted by adding 40 μl of sterile distilled water. Fourteen μl reactions were prepared with 7 μl of the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), 5 μM of specific primer pairs (Additional file 2: Table S1) and 1 μl of cDNA. PCR amplifications were carried out in 96-well optical reaction plates on a Step One Plus Real-Time PCR System (Applied Biosystems, USA). Two independent RNA isolates and three technical replicates were used per cultivar, treatment and time point assayed. The thermal cycling program started with a step of 10 s at 95 °C, followed by 40 cycles (15 s at 95 °C and 60 s at 60 °C), and the melt curve (from 60° C to 95°C, with increments of 0.3°C every 5 s). Dissociation kinetics and agarose gel loading of the amplified products confirmed their specificity.

Primer pair validation and relative quantification of gene expression levels were performed by using the $2^{-\Delta\Delta CT}$ method [39]. The *Dca17200* gene (the putative homolog of the Arabidopsis housekeeping gene *ACTIN2*; *AT3G18780*) was chosen for normalization of the assayed genes as its expression was constant among the different cultivars, treatments and time points studied. All samples were compared to the expression level of the control treatment (mock) at the zero-time point (0 hAP). The average of fold-change values were used for graphic representation.

Time-course analysis

To cluster genes according to their time-course profile, we reformatted cross-sectional data where each sample corresponds to cuttings from different plants (*i.e.,* destructive sampling) as longitudinal data. To this purpose, the normalized counts of replicated samples at different time points were paired, producing complete time courses. To handle the missing data of one of the mock replicates at 0 hAP in the cultivar *2003 R 8*, missing values were imputed averaging the normalized counts from the other two replicates at the same time point and cultivar. Gene clustering and GO enrichment analysis within clusters was performed in STEM [40] using default parameters (STEM Clustering Method). In order to increase the signal-to-noise ratio, we filtered out genes with a log expression difference across time points smaller than 1.25 and correlation between replicates smaller than 0.75.

Light microscopy

For each cultivar and treatment, ~5 mm long segments from the base of the stem cuttings were sectioned at different time points (0, 6, 24 and 54 hAP). Samples were fixed in a FAA/Triton solution (1.85 % v/v formaldehyde, 45 % ethanol, 5% acetic acid, and 1 % Triton X-100) for 8 h on a light vacuum (400 mbar) until the tissue sank. Samples were then kept in the FAA/Triton solution for 3 days at 4 °C. The fixed tissue was rinsed 3 times in 0.1 M sodium phosphate buffer (pH 7.2) before dehydrating in a graded ethanol series (70, 80, 90 and 96 % ethanol, 60 min each step). Dehydrated samples were then embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Germany) according to the manufacturer's instructions with slight modifications, as follows. Samples were immersed in the pre-infiltration solution (50 % v/v resin and 50 % ethanol) for 2.5 h. Then, stem cutting samples remained 4 h in the infiltration solution on a light vacuum at room temperature and polymerized for 20 h at 4 °C. Thin sections of 7 μm-thickness were cut using a tungsten microtom knife (MICROM International GmbH, Germany) on a HS 350 S rotary microtome (MICROM International GmbH). Sections were stained either with 0.05 % weight/volume (W/V) toluidine blue (Sigma-Aldrich) or 0.05 % W/V ruthenium red (Sigma-Aldrich) in water and mounted in Eukitt (Chem-Lab NV, Belgium). Samples were observed using a bright-field Motic BA210 microscope (Motic Spain, Spain) and selected images were captured with a built-in Moticam 580INT documentation station (Motic Spain) and processed with Adobe Photoshop CS3.

Phytohormone extraction and analysis

Phytohormones were extracted and analysed according to [41]. Briefly, ~100 mg of frozen tissue from the same batches used for the RNA-Seq experiment were extracted

twice with 1 ml of methanol/water 80 %, centrifuged at 20,000 *g* for 15 min. at 4 °C, the supernatant was passed through a C18 cartridge, and the samples were collected in a 5-ml tube for speed-Vac evaporation to dryness. The residue was resuspended in 1 ml methanol/water 20 %. Ten μl of filtrated extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific) using a heated electrospray ionization interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific). For quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50, and 100 μg l^{-1}) and corrected for 10 μg l^{-1} deuterated internal standards. Recovery percentages ranged between 92 and 95 %.

Transcription factor analysis

To find out which transcription factor (TF) families are likely to play a more important role along the experimental process, we analysed their enrichment among genes annotated with the function "sequence-specific DNA binding transcription factor activity" (GO:0003700). In correspondence with the filtering criteria for the time-course analysis, we excluded genes with a log expression difference across time smaller than 1.25 and correlation between replicates smaller than 0.75. Carnation genes with predicted transcription factor activity were classified in families via their putative Arabidopsis orthologs, based on the OBH method (see functional annotation section). The family classification of these orthologs was obtained from the Database of Arabidopsis Transcription Factors [42]. Genes mapped to TF families were further categorised as upregulated or downregulated according to their profiles in the time-course analysis at 54 hAP. For each category, a Fisher exact test was done to assess significant enrichment. P-values were adjusted for multiple testing (Benjamini-Hochberg).

Results

Sequencing and transcriptome assembly supports the discovery of novel genes expressed in the stem cutting base

In a recent study [26] we characterized AR formation in a collection of 10 carnation cultivars. The *2003 R 8* and the *2101–02 MFR* cultivars have been chosen for further studies due to their differences in rooting performance and in their differential response to a mild auxin treatment during rooting (Fig. 1b). The bad-rooting behaviour of the *2003 R 8* cultivar, which was mostly caused by a delay in AR initiation, was partially restored by exogenous auxin application.

Several cDNA libraries prepared from stem cutting bases of mock-treated and auxin-treated samples at particular time points during adventitious rooting (0, 6, 24 and 54 hAP) were sequenced (see Methods; Fig. 1a). As a result, 3,683 million of raw reads were obtained. The amount of expression data generated in our study had the potential to transform the boundaries and extent of previous feature annotations in the carnation genome [27]. Genome-guided assemblies were performed with the purpose of serving as sequence evidence for a genome re-annotation. Thus, our updated, evidence-based annotation comprised 59,396 transcripts, corresponding to 57,641 genes, with an average length of 2,856 bp (Table 1). We were able to merge exons from genes that had been previously predicted as separate coding sequences [27]; these merges resulted in a more complete or contiguous annotation for 394 genes and their corresponding transcripts (see Additional file 3). In order to quantify the improvements of the new annotation in relation to the former annotation, we compared the proportion of reads mapping to each of them and also generated a number of descriptive statistics (Table 1).

Time-dependent comparison of the auxin treatment identifies 1,286 differential expressed genes (DEGs) in response to the auxin stimulus

We then tested the effects in gene expression of factors like cultivar, treatment and time point using different models and contrasts (Table 2). Three questions were addressed: *i)* for which genes does the cultivar factor have a significant effect? (Table 2, test 1), *ii)* which genes change their expression over each pair of time points? (while accounting for cultivar-specific effects; Table 2, test 2–13) and *iii)* how does the auxin treatment affect gene expression distinctively at each time point for each cultivar? (Table 2, test 14–21). To reduce the complexity of the model, in this last case, we made subsets of samples belonging to each cultivar and estimated the parameters separately.

In all cases, expression models were fitted to our time-course study by treating each time point as a different "experimental group", even though the inherent ordering and spacing provided by time points is ignored then.

To investigate the dependencies between treatments and time, explicitly addressing the question of when a gene is differentially expressed, we modeled the interaction between time and treatment as a covariate. Of 57,641 genes, we filtered out genes that were not

Table 1 Comparison between the previously published annotation and the updated genome annotations

Feature	Yagi et al. [27]	Evidence-based annotation
Gene count	56,137	57,641
Transcript count	56,382	59,396
Average transcript length	2,742	2,856
Median transcript length	2,065	2,125

Table 2 Differential expression tests

Test	Data subset	Model formula	Contrast tested	DEGs
1	All	C + Ti + C : Ti	2003 R 8 *vs.* 2101–02 MFR	23,029
2	All	C + Ti + C : Ti	2003 R 8:Ti 1 *vs.*2003 R 8:Ti 2	3,820
3	All	C + Ti + C : Ti	2003 R 8:Ti 1 *vs.* 2003 R 8:Ti 3	4,645
4	All	C + Ti + C : Ti	2003 R 8:Ti 1 *vs.* 2003 R 8:Ti 4	5,880
5	All	C + Ti + C : Ti	2003 R 8:Ti 2 *vs.* 2003 R 8:Ti 3	2,828
6	All	C + Ti + C : Ti	2003 R 8:Ti 2 *vs.* 2003 R 8:Ti 4	2,983
7	All	C + Ti + C : Ti	2003 R 8:Ti 3 *vs.* 2003 R 8:Ti 4	690
8	All	C + Ti + C : Ti	2101-02 MFR:Ti 1 *vs.* 2101–02 MFR:Ti 2	11,536
9	All	C + Ti + C : Ti	2101-02 MFR:Ti 1 *vs.* 2101–02 MFR:Ti 3	12,694
10	All	C + Ti + C : Ti	2101-02 MFR:Ti 1 *vs.* 2101–02 MFR:Ti 4	12,129
11	All	C + Ti + C : Ti	2101-02 MFR:Ti 2 *vs.* 2101–02 MFR:Ti 3	9,336
12	All	C + Ti + C : Ti	2101-02 MFR:Ti 2 *vs.* 2101–02 MFR:Ti 4	9,125
13	All	C + Ti + C : Ti	2101-02 MFR:Ti 3 *vs.* 2101–02 MFR:Ti 4	3,430
14	2003 R 8	Ti + Ti : Tr	Ti 1:Aux *vs.* Ti 1:Mock	86
15	2003 R 8	Ti + Ti : Tr	Ti 2:Aux *vs.* Ti 2:Mock	1
16	2003 R 8	Ti + Ti : Tr	Ti 3:Aux *vs.* Ti 3:Mock	
17	2003 R 8	Ti + Ti : Tr	Ti 4:Aux *vs.* Ti 4:Mock	6
18	2101-02 MFR	Ti + Ti : Tr	Ti 1:Aux *vs.* Ti 1:Mock	1,188
19	2101-02 MFR	Ti + Ti : Tr	Ti 2:Aux *vs.* Ti 2:Mock	21
20	2101-02 MFR	Ti + Ti : Tr	Ti 3:Aux *vs.* Ti 3:Mock	1
21	2101-02 MFR	Ti + Ti : Tr	Ti 4:Aux *vs.* Ti 4:Mock	

Datasets were fitted to the corresponding formula (C = cultivar, Ti = time point, Tr = treatment; additive effects are represented by "+"; ":" represents interaction). After model fitting, selected factors were tested (Ti 1 = 0 hAP, Ti 2 = 6 hAP, Ti 3 = 24 hAP, Ti 4 = 54 hAP). DEG: Differential expressed genes; *P-adj.* < 0.05 (Benjamini-Hochberg correction)

expressed (0 counts), resulting in a total of 37,936 genes tested for the subset of cultivar *2101–02 MFR* and 37,849 for the cultivar *2003 R 8* subset. The factorial analysis identified a total of 1,286 distinct genes as differentially expressed between auxin-induced and control cuttings over different time points (Table 2, test 14–21). Most auxin-related expression changes took place in the initial time points (0 hAP *vs.* 6 hAP). Among them, DEGs of *2101–02 MFR* were associated (Fisher exact test) to functions like photosynthesis (GO:0015979; P <0.001) and chlorophyll binding (GO:0016168; P <0.001). As for the same comparison in the cultivar with poor rooting performance, *2003 R 8*, the functions associated to DEG were translational initiation factor activity (GO:0003743, $P = 0.0025$), and negative regulation of signal transduction (GO:0009968, $P = 0.0024$), among others.

Clustering of time-course expression profiles reveals co-expression of functionally related genes

To get some insight into the specific pathways regulated at different time points during AR formation in the two cultivars studied, we performed a GO-enrichment analysis for the sets of DEGs shown in Fig. 2a. In the *2003 R 8* cultivar, the GO categories "protein amino acid phosphorylation" (GO:0006468; 105 genes; $P < 0.001$) and "transmembrane transport" (GO:0055085; 50 genes; $P < 0.001$) were specifically and significantly enriched at 6–24 and 24–54 hAP, respectively. Interestingly, the "auxin-activated signalling pathway" (GO:0009734; 11 genes; $P < 0.001$) category was found significantly enriched among DEGs shared between 0–24 hAP in this cultivar. Conversely, in the *2101–02 MFR* cultivar, the GO category "hormone-mediated signalling pathway" (GO:0009755; 58 genes; $P < 0.001$) was specifically enriched at 0–6 hAP. Moreover, the "auxin-activated signalling pathway" (GO:0009734; 34 genes; $P < 0.001$) and "cell cycle" (GO:0007049; 79 genes; $P < 0.001$) categories were found significantly enriched for DEGs shared between 0 and 24 hAP. The GO categories "glucose catabolic process" (GO: 0006007; 41 genes; $P < 0.001$), and "cellulose biosynthetic process" (GO:0030244; 22 genes; $P = 0.002$) were significantly enriched specifically at 6–24 hAP, whereas at 24–54 hAP the most significant GO-enrichment was found for genes assigned to the "response to stress" (GO:0006950; 46 genes; $P = 0.007$) category.

To obtain a more general view of the functions involved in the early stages of AR formation, we transformed the GO functional annotation into its cut-down

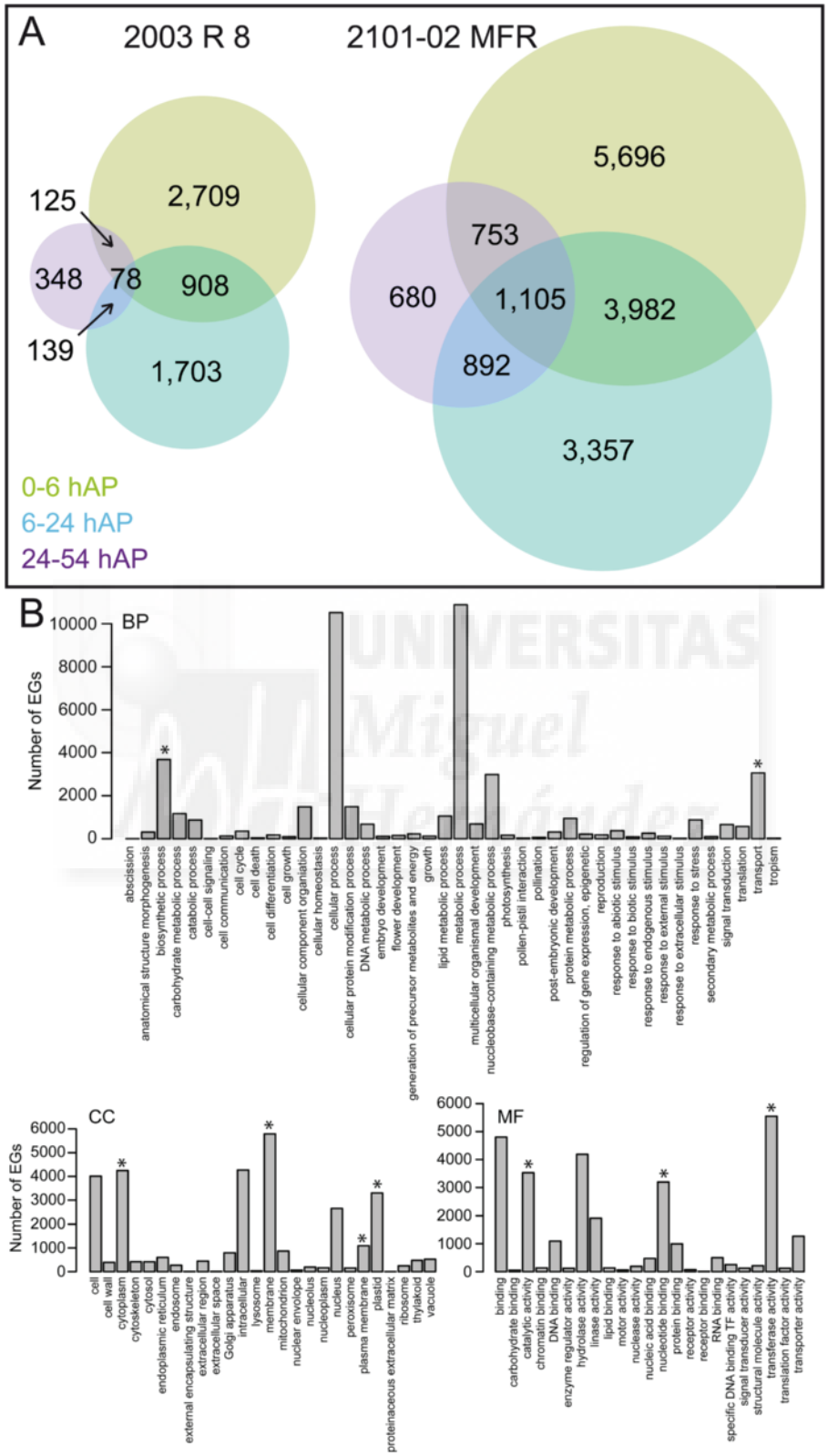

Fig. 2 (See legend on next page.)

(See figure on previous page.)
Fig. 2 Differentially expressed genes (DEGs) over time during AR formation. **a** Venn diagram illustrating DEGs ($P < 0.05$, Benjamini-Hochberg correction) in the stem cutting base of the *2003 R 8* and *2101–02 MFR* cultivars over time. **b** Gene ontology (GO) classification of genes whose expression changes more than 1.25 log-fold over the time-course with respect to biological process (BP), cellular component (CC) and molecular function (MF). Asterisks indicate significant enrichment of genes ($P < 0.05$)

version (Plant GO slim) for the 14,554 DEGs in the *2101–02 MFR* cultivar between 0 hAP and 6 hAP (Fig. 2b). The Biological Process (BP) classification of DEGs highlighted a significant enrichment ($P < 0.001$) for the following GO categories: "biosynthetic process" (GO:0009058) and "transport" (GO:0006810). Among the Cellular Component (CC) categories, "plastid" (GO:0009536) and "plasma membrane" (GO:0005886) were the most significantly enriched ones. In terms of Molecular Function (MF), a significant enrichment was found for genes at the categories "transferase activity" (GO:0016740) and "nucleotide binding" (GO:0000166). These results indicated that large expression changes are taking place at transcriptome level in the stem cutting base during the initial stages of AR formation in this cultivar.

Validation of expression of some of the genes detected during AR formation

The reliability of our transcriptome profiling dataset was validated by examining the expression of selected genes by using qRT-PCR and by comparing them to the normalized data obtained in the RNA-Seq analysis (see Methods). We found highly significant and positive correlations between qRT-PCR and RNA-Seq results for both cultivars in all time points and treatments (Fig. 3a-b). Additional statistical analysis revealed that the variation observed between qRT-PCR and RNA-Seq results depended largely on the expression levels of the studied genes (Additional file 4: Figure S2). Thus, for genes with very low or very high numbers of RNA-Seq reads, the qRT-PCR validation was less accurate. Representative examples of the results obtained for genes with contrasting expression profiles are shown in Fig. 3c-f. While the expression levels of *Dca5879* were only varying over time (Fig. 3c-d), those of *Dca29160* were also depending on cultivar and the auxin treatment (Fig. 3e-f).

Gene set enrichment analysis in the *2003 R 8* cultivar

As the number of DEGs between auxin-treated and mock-treated samples in the *2003 R 8* cultivar was scarce (see above), we did not distinguish between treatments in this cultivar when performing a GO-enrichment analysis using STEM (see Methods). The expression of 7,341 genes was found to be specifically regulated during AR formation in the *2003 R 8* cultivar and 4,599 of these genes were clustered along different expression profiles and further classified into four major groups based on their expression pattern between 0–6 hAP and 6–54 hAP: DownDown (DD), DownUp (DU), UpDown (UD), and UpUp (UU) (Fig. 4a).

1,286 genes were gradually repressed (DD group) during AR formation in these conditions. Some of the most significantly-enriched GO categories within this group were "response to auxin" (GO:0009733; 15 genes; $P < 0.001$) and "ion transport" (GO:0006811; 46 genes; $P < 0.001$). We assigned 1,381 additional genes to the DU group. About two-thirds of these genes showed an early repression and later became upregulated above their expression at 0 hAP (labelled as "a" in Fig. 4a). The remaining genes in this group, which were quickly downregulated and whose levels were more-or-less restored to initial levels at later time points (labelled as "b" in Fig. 4a), showed enrichment in "photosynthetic membrane" (GO:0034357; 30 genes; $P < 0.001$) encoding genes. Some of the most significantly-enriched GO categories within the DU group as a whole were "cell wall organization or biogenesis" (GO:0071554; 51 genes; $P < 0.001$), "cytoskeleton" (GO:0005856; 46 genes; $P < 0.001$), and "cellular carbohydrate metabolic process" (GO:0044262; 63 genes; $P < 0.001$). Another 790 genes showing a biphasic response were classified into the UD group. Finally, 1,142 genes within the UU group were ranked for GO enrichment: "cell division" (GO:0051301; 32 genes; $P < 0.001$), "microtubule" (GO:0005874; 52 genes; $P < 0.001$), and "cell wall organization or biogenesis" (28 genes; $P < 0.001$) among others. Interestingly, the expression of most genes included within the "cell division" and "cell wall organization or biogenesis" categories peaked after 6 hAP in agreement with the timing of cell cycle reactivation in the cambium observed for this cultivar, as it is shown later.

Expression profiling in the *2101–02 MFR* cultivar and in the response to auxin

12,525 genes were identified in the *2101–02 MFR* cultivar as being specifically expressed during AR formation without exogenous auxin treatment (Fig. 4b). 3,586 genes were assigned to the DD group where one of the most significantly-enriched GO categories was "protein serine/threonine kinase activity" (GO:0004674; 175 genes [13.8 % of the protein serine/threonine kinase encoding genes with dynamic expression profiles (EGs)]; $P < 0.001$). 1,737 genes showing a biphasic response were classified into the DU group. Those upregulated at later time points (labelled as "a" in

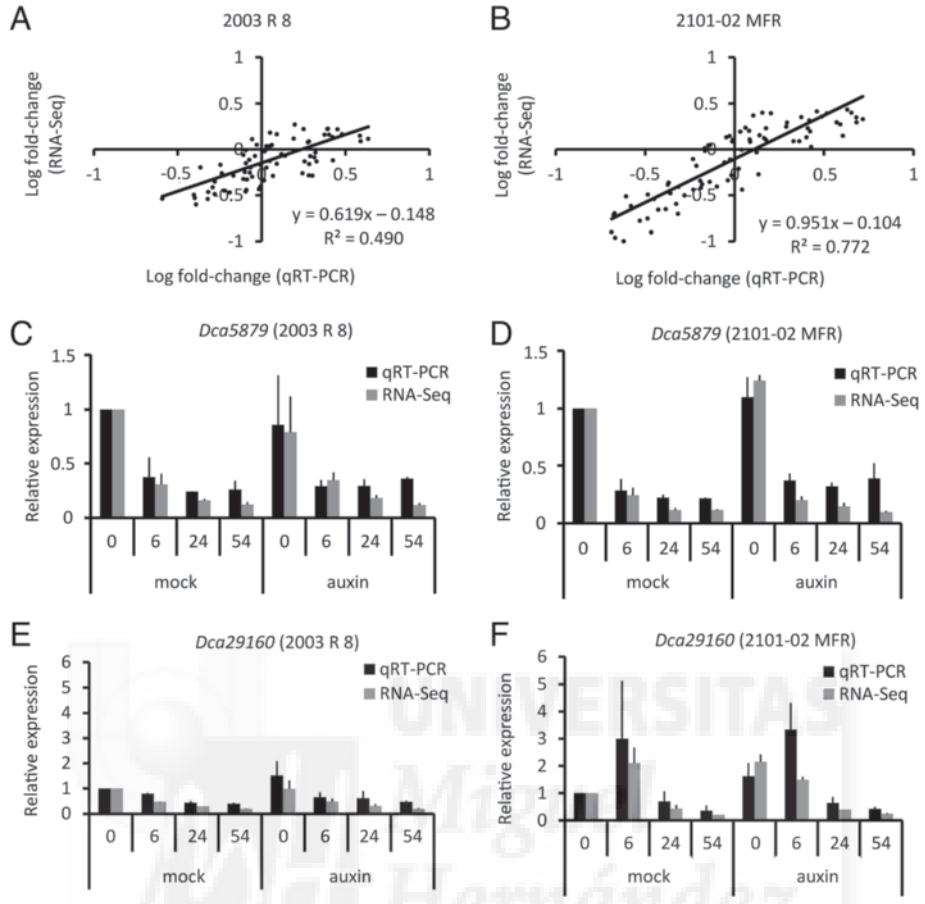

Fig. 3 qRT-PCR validation of RNA-Seq results. **a**-**b** Correlation analysis of qRT-PCR and RNA-Seq data from selected genes in the *2003 R 8* (**a**) and *2101–02 MFR* (**b**) cultivars. Each dot represents the relative expression data for a given gene and a given sample. **c**-**f** Bars represent the mean of the relative expression level of qRT-PCR (black) or RNA-Seq (grey) data relative to mock-treated samples at 0 hAP. Error bars indicate the standard deviation for the mean data shown

Fig. 4b) encoded proteins enriched in "cytoskeleton" (GO:0005856; 41 genes; $P < 0.001$) and "cell division" (17 genes; $P < 0.001$). Similarly to that found previously for the *2003 R 8* cultivar, the "photosynthetic membrane" (35 genes; $P < 0.001$) category was found enriched among genes whose expression levels were restored to basal levels (labelled as "b" in Fig. 4b). Among the biphasic genes that were assigned to the UD group (1,385), one of the significantly-enriched GO categories found was "carbohydrate derivative metabolic process" (GO:1901135; 52 genes; $P = 0.002$). Finally, 2,103 genes were included within the UU group, where the most significantly-enriched GO categories were "cellular carbohydrate metabolic process" (69 genes; $P < 0.001$) and "cell wall organization or biogenesis" (54 genes; $P < 0.001$). In addition, we found specific GO-enriched categories in profile P29 (Fig. 4b). On the one hand, enriched genes upregulated after 6 hAP (P29) encoded proteins related to "microtubule" (38 genes; $P < 0.001$), "cell division" (24 genes; $P < 0.001$), and "regulation of cell cycle" (GO:0051726; 23 genes; $P < 0.001$). On the other hand, genes encoding putative chromatin-related functions such as "histone H3 lysine 9 methylation" (GO:0051567; 14 genes; $P < 0.001$), or "DNA packaging" (GO:0006323; 14 genes; $P < 0.001$) were also found significantly enriched.

Additionally, the expression of 9,645 genes was found specifically altered during AR formation in the *2101–02 MFR* cultivar after exogenous auxin treatment and 5,568 of these genes were significantly clustered to different expression profiles and grouped as described above (data not shown). We found a substantial overlap between EGs of auxin-treated and mock-treated samples (61.2 % for the auxin-treated EGs and 79.5 % for the mock-treated EGs). Consistently, no significant differences in the overall trends of EGs were found between auxin-

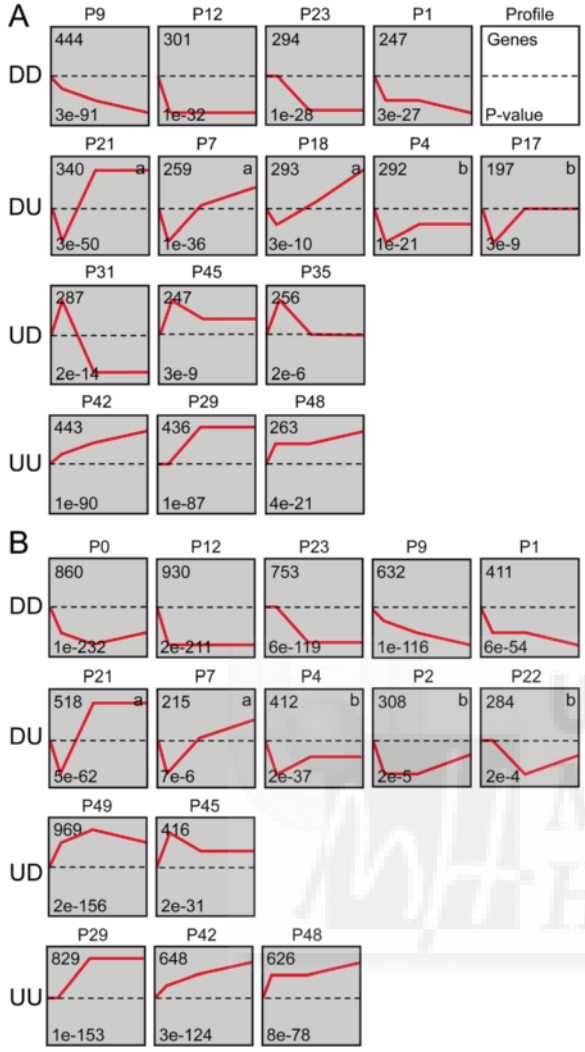

Fig. 4 Model profiles identified during AR formation in carnation cultivars. **a** A combined auxin- and mock-treated-dataset is shown for the *2003 R 8* cultivar. **b** The mock-treated dataset for the *2101–02 MFR* cultivar. Profiles were classified into four groups: DownDown (DD), DownUp (DU), UpDown (UD), and UpUp (UU) and further ordered based on their P-value (bottom left-hand corner). The number of genes assigned to each profile is shown in the top left-hand corner

and mock-treated samples for the *2101–02 MFR* cultivar (Additional file 5: Figure S3). However, for a small number of genes assigned to specific profiles in mock-treated samples, we found some changes in their expression profiles after the auxin treatment. About half of the EGs-encoding proteins belonging to "microtubule", "cell division" and "histone H3 lysine 9 methylation" were reciprocally assigned to profiles P29 (UU) or P21 (DU) in mock-treated and in auxin-treated samples, respectively. We also found that the expression levels of most genes assigned to the "cellular carbohydrate metabolic process" category were complementary at earlier time points in auxin-treated *vs*. mock treated samples.

Comparative transcriptome profiling of AR formation between carnation cultivars for selected GO categories

To identify genes whose expression correlates with the different stages of AR formation and that could be used as markers, we selected EGs belonging to the GO categories "cell division" and "response to auxin" from the different samples studied (see above). We then built heat map representations from log expression data for all these genes (Fig. 5). On the one hand, the expression of some genes encoding proteins related to cell division were clustered together along the time point series with higher expression at earlier time points, independently of cultivar and treatment (Fig. 5a). Interestingly, genes encoding mitotic cyclins (A-type and B-type) showed a peak of expression between 24 hAP and 54 hAP (Fig. 5a), which is in light with the cellular changes observed in the stem cutting base during AR rooting (see next section). Noteworthy, the majority of these genes moderately respond to the auxin treatment by increasing their expression levels at earlier time points (Fig. 5a). On the other hand, a small number of genes displayed contrasting expression profiles between cultivars. Examples for the latter are the *Dca37619* and *Dca642* genes, which are respectively upregulated and downregulated in *2003 R 8* compared to the *2101–02 MFR* cultivar (Fig. 5a).

Considering the expression profiles of genes assigned to the "response to auxin" category, the effect of the auxin treatment was quite small irrespective of the cultivar, and was mainly restricted to earlier time points (Fig. 5b). However, we found striking differences in the expression of a few of these genes between cultivars, such as *Dca1208* and *Dca39239*, which makes them candidates for further studies to analyze their role in the differential response in auxin-mediated AR initiation between these two cultivars.

Cellular changes in the stem cutting base during AR formation reflects the effect of the auxin signal

In a previous study we found that cell divisions within the cambial region of the stem cutting base took place between 12 hAP and 24 hAP in a good-rooting cultivar used as a reference [43]. We next characterized the cellular changes occurring within the cambium region in the stem cutting base of the *2003 R 8* and *2101–02 MFR* cultivars both in mock- and auxin-treated samples to understand the differential responses observed in these two cultivars during AR formation. Although the cambial ring of the *2003 R 8* cultivar displayed a very organized cellular pattern at 0 hAP, we found that some regions within the cambium displayed subtle tissue disorganization (Fig. 6a and Additional file 6: Figure S4). Interestingly, we observed an

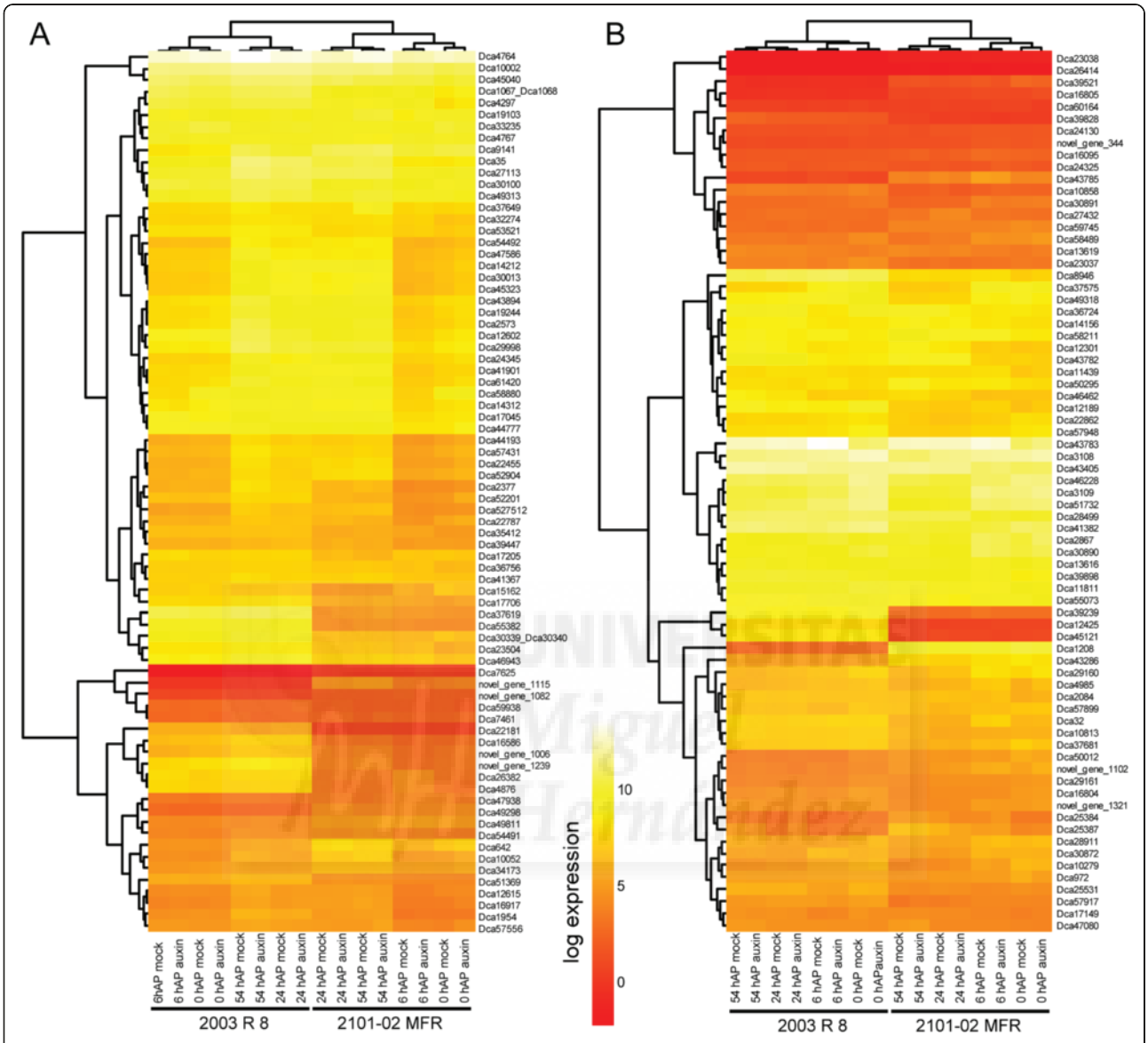

Fig. 5 Analysis of expression of transcripts related to cell division (**a**) and response to auxin (**b**) during AR formation. Heat map drawing and clustering was done as described in Methods

increase in the number of disorganized regions within the cambial ring at later time points, which could reflect local activation of cell divisions (Fig. 6b). In addition, stem cutting bases of the *2003 R 8* cultivar treated with auxin contained an increased number of these disorganized regions already at 0 hAP (Fig. 6c and 6d). In the *2101–02 MFR* cultivar, we observed a higher frequency of small cell clusters within the cambial ring at 0 hAP, which at later time points developed as large clusters of meristematic cells with a disorganized internal structure (Fig. 6f). In most cases, these cell clusters appeared juxtaposed but physically isolated by collapsed neighbouring cells (arrowheads in Fig. 6f). In auxin-treated samples however, cell clusters became apparent already at 0 hAP (Fig. 6g), which is indicative of an early activation of cell division in the *2101–02 MFR* cultivar. At 54 hAP, cell clusters were clearly evident and their numbers were higher than in mock-treated samples (Fig. 6h).

To confirm our observations, we estimated some cellular parameters in the two contrasting regions identified within the cambium (Additional file 6: Figure S4; see Methods). In the *2003 R 8* cultivar we observed that the cell division rates significantly differed between organized and disorganized regions at the different time points studied, which seemed not to be affected by the auxin treatment (Additional file 6: Figure S4B). These results suggested that auxin act as a trigger for cell division within a certain population of responsive cambial cells. On the

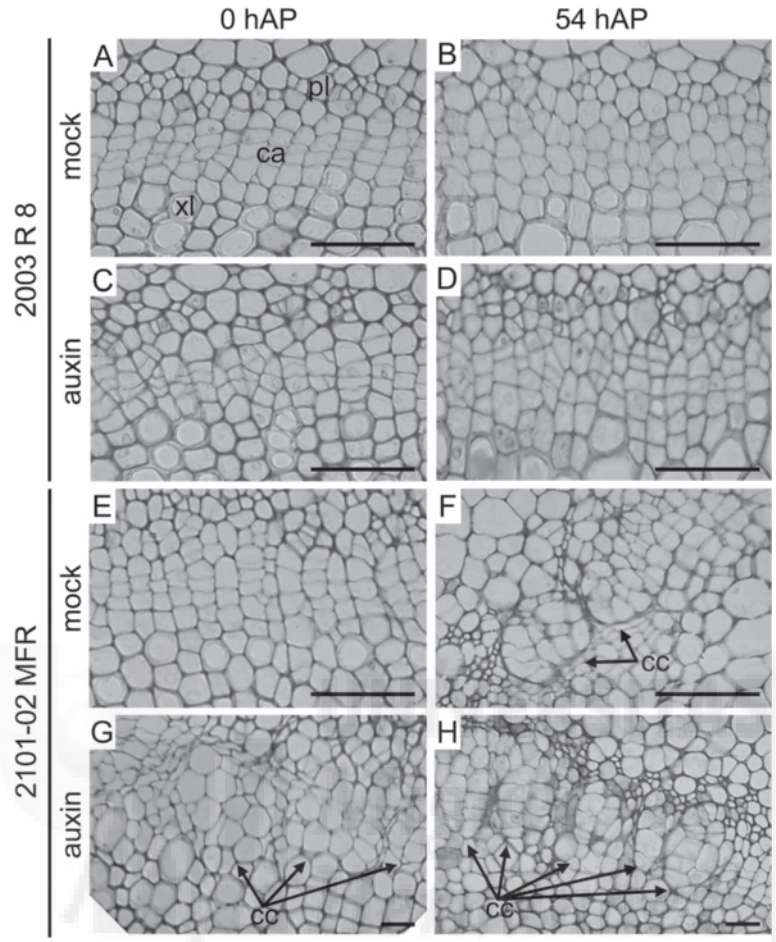

Fig. 6 Morphological changes in the ultrastructure of the basal stem of carnation cuttings during adventitious rooting. Light micrographs were taken from cross-sections of stem cutting basal regions in the *2003 R 8* (**a**-**d**) and *2101–02 MFR* (**e**-**h**) cultivars, either treated with mock (**a**-**b**, **e**-**f**) or with auxin (**c**-**d**, **g**-**h**) at different time intervals (0 and 54 hAP). ca, cambium; cc, cell clusters; pl, phloem; xl, xylem. Scale bars: 50 μm

other hand, we observed a significant increase in the number of cells within the cambium at later time points for the *2101–02 MFR* cultivar, both in organized and disorganized regions (Additional file 6: Figure S4C), which is indicative of a broad activation of cell division within the cambium, as has been previously described for a good-rooting reference cultivar [43]. Interestingly, the division rate at a given time point was found unchanged irrespectively of the auxin treatment (Additional file 6: Figure S4B). Taken together, these results suggested that auxin acts by promoting divisions of quiescent cambial cells rather than by increasing the number of divisions of already dividing cells, the former producing a net increase in the number of cell clusters within the cambial ring.

Morphogenetic hormone levels in the stem cutting base during AR formation are correlated with rooting performance

Several plant hormones play a crucial role in controlling AR formation, with auxin and cytokinin playing opposite roles [1, 44]. In addition, wounding stimulates ethylene biosynthesis which is known to positively influence AR formation in some species [45, 46]. We found high levels of endogenous indole-3-acetic acid (IAA) only in the *2101–02 MFR* cultivar at 0 hAP, which were quickly downregulated to basal levels (Fig. 7), as previously described [43]. In addition, we found very low levels of *trans*-zeatin (tZ) in the stem cutting base of the *2101–02 MFR* cultivar. In contrast, in the *2003 R 8* cultivar, tZ levels steadily increased during the time-course experiment (Fig. 7). Hence, the endogenous auxin/cytokinin ratio estimated as the proportion between IAA and tZ levels was much higher in the *2101–02 MFR* cultivar than in the *2003 R 8* cultivar for all time points, with the highest ratio found at 0 hAP. In addition, the levels of the 1-aminocyclopropane-1-carboxylic acid (ACC) ethylene precursor were higher in the *2101–02 MFR* cultivar than in the *2003 R 8* cultivar (Fig. 7), which might reflect higher endogenous ethylene production in the *2003 R 8* cultivar.

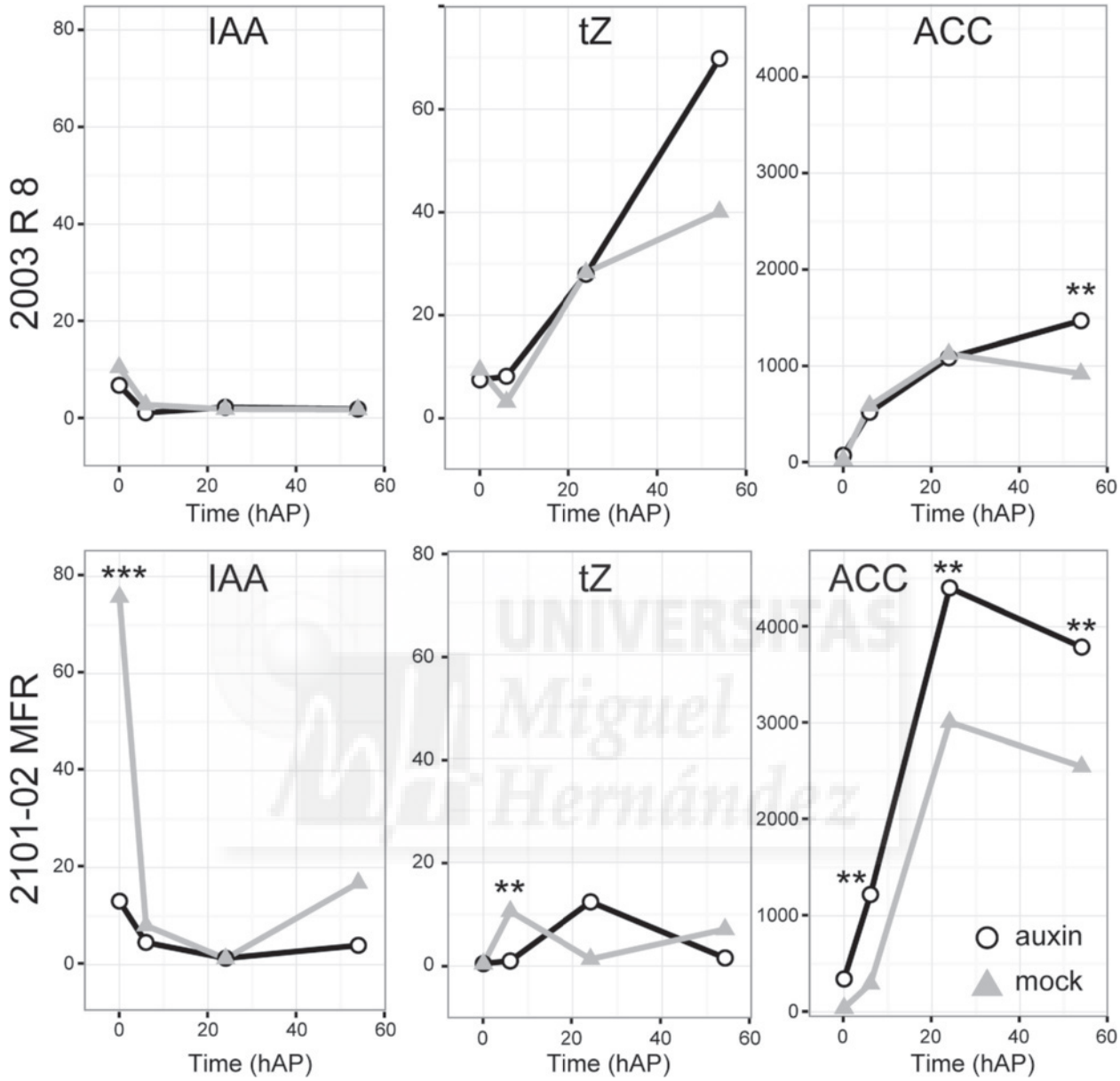

Fig. 7 Changes in the concentration of some phytohormones in the basal stem of carnation cuttings during adventitious rooting. Data for indole-3-acetic acid (IAA), *trans*-zeatin (tZ) and the ethylene precursor ACC are shown in ng/g fresh weight. Time refers to hours after planting (0 hAP). Asterisks indicate significant differences ($P < 0.05$) between treatments for a given sampling time

TF analysis

By modulating gene transcription at specific times and during specific processes, TFs and their regulatory networks have important roles in development and stress response. A number of transcripts that show significant expression changes during the time-course experiment performed with STEM, encoded putative transcription factors that belonged to 19 transcription factor families (http://planttfdb.cbi.pku.edu.cn/; Fig. 8). To identify the transcription factors that might regulate the differential rooting responses observed between *2101–02 MFR* and *2003 R 8* cultivars, we performed an enrichment analysis using Fisher's *T*-test (see Methods). In the *2003 R 8* cultivar, a number of genes encoding C2C2-GATA transcription factors, such as *Dca7186* and *Dca56796*, were significantly enriched and showed a clear downregulation of their expression over time (Fig. 8). The WRKY transcription factor family was among the most highly downregulated transcription factor genes irrespective of cultivar and treatment (Fig. 8). Of the 14 carnation genes encoding putative WRKY proteins that were shared between cultivars and treatments, 10 were

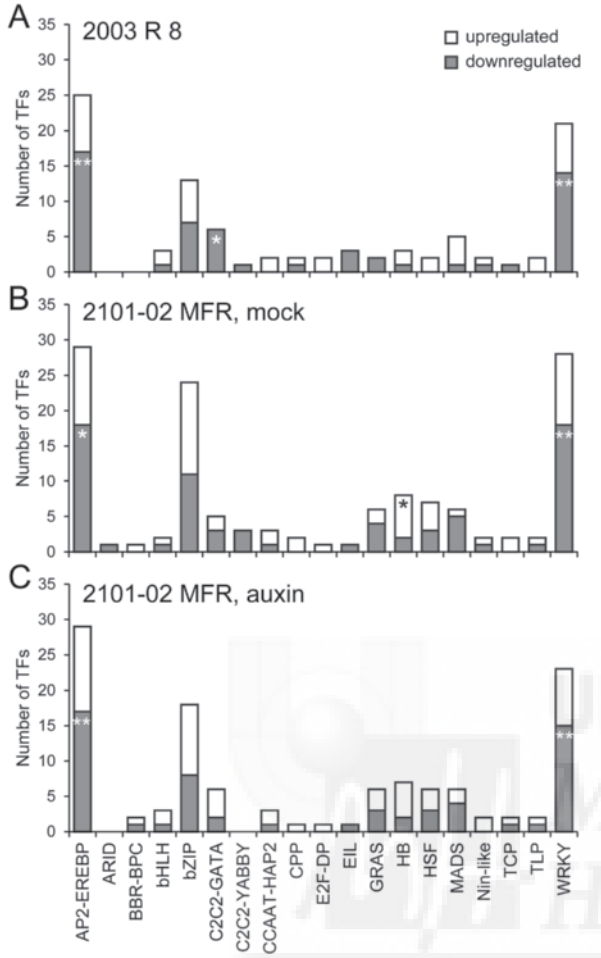

Fig. 8 Expression of TF genes during AR formation. The number of upregulated (white bars) and downregulated (grey bars) TFs is indicated for each family in *2003 R 8* (**a**) and *2101–02 MFR* mock-treated (**b**) or auxin-treated (**c**) samples. Asterisks indicate significant enrichment of genes ($P < 0.05$) within each TF family

downregulated over the time-course experiment. Interestingly, the expression of *Dca28099*, the putative carnation ortholog of the Arabidopsis *PLETHORA5* gene [47] was found upregulated only in the *2101–02 MFR* cultivar after auxin treatment, which emphasizes its value as an early marker for adventitious root formation in this species.

Discussion

We followed a next-generation sequencing approach to characterize the gene expression profiles in the stem base of two cultivars with contrasting efficiencies of AR formation and in response to exogenous auxin treatment. It was found that the most significant expression differences were driven by the cultivar, less by the time after planting, and the least by the auxin treatment.

Whereas ARs arise directly from cambial tissues in easy-to-root species such as poplar, callus formation precedes AR initiation in difficult-to-root species such as *Pinus* spp. or *Eucalyptus grandis* [23, 48]. Our histological analysis during root-primordia initiation in two carnation cultivars confirmed that some cambial cells located between the phloem and xylem activate formative (periclinal) divisions in response to the endogenous auxin signal. Next, several clusters of meristematic cells arise along the cambial ring which will later give rise to organized root primordia, as it has been shown previously in the Master reference cultivar [43]. The differences in the rooting ability of the *2003 R 8* and *2101–02 MFR* cultivars are due to a delay in the early activation of cell divisions in the former. Exogenous auxin treatment had a similar effect on both cultivars: it accelerated the activation of cell division and it caused a higher number of initials within the cambium. As a result, the rate and the number of ARs increased by the auxin treatment in both cultivars [26], which is in agreement with the inductive effect across plant species of exogenously applied auxins [4]. In addition, the analysis of morphogenetic hormone levels in the two carnation cultivars studied indicated that the bad-rooting behavior of *2003 R 8* was directly correlated with the low ratio of auxin *vs.* cytokinin levels found in this cultivar.

Consistent with transcriptome data from other species [13, 46], several genes encoding known regulators of auxin response were found differentially regulated during the early stages of AR formation (0 hAP and 6 hAP) in both cultivars. Several EGs encoding putative Aux/IAA corepressors showed specific upregulation 6 hAP in the *2101–02 MFR* cultivar and they were found to be transiently induced by the auxin treatment in both cultivars (Fig. 5b). Among them were *Dca28911*, *Dca30890* and *Dca58489*, putative homologs of *SHY2* (also known as *IAA3*) [49], *MASSUGU2* (*IAA19*) [50] and *SOLITARY ROOT* (*IAA14*) [51]. In addition, some other genes encoding Aux/IAA proteins, such as *Dca39239* and *Dca39521*, showed divergent expression levels in the two cultivars studied (Fig. 5b), which suggested that a differential auxin response could explain the differences between good-rooting and bad-rooting cultivars in this species. However, as both cultivars are able to initiate AR formation in response to exogenous auxin, we believe that the auxin response in the *2003 R 8* cultivar is fully functional and the rooting differences observed are due to differential auxin homeostasis between the two cultivars studied. We also found that exogenous auxin treatment did not significantly affect the expression of the EGs encoding putative ARF transcription factors, which also showed similar expression profiles in these two cultivars (Fig. 5b). Our gene expression profiling results are a starting point to identify which auxin response modules involving specific Aux/IAA corepressors and ARF transcription factors are controlling the early steps of AR development in carnation stem cuttings (Fig. 9b).

As previously found during AR development in *Pinus contorta* hypocotyls [21], several integral components of the photosynthetic machinery were downregulated during the initial stages of adventitious rooting and up to 54 hAP (Fig. 9a). This clearly shows that cells within the stem cutting base transiently lack their potential to function as photosynthetic cells, which we believe might be linked to the establishment of a new sink within the stem cutting base, as has been described in petunia cuttings [14]. In line with this hypothesis, we found that the expression of genes encoding sucrose degradation enzymes, such as vacuolar invertase (*Dca8627* and *Dca54544*) and cell-wall localized invertase (*Dca51558* and *Dca59840*), showed a biphasic response during adventitious rooting, coinciding with the onset of the induction phase. In addition, *Dca4507* encoding a homolog of the Arabidopsis SUCROSE SYNTHASE4 [52] was found upregulated after 6 hAP. Our previous results [15, 43] indicated a high energy requirement during rooting in the base of the stem, which was provided by an increase of sucrolytic enzymes during the early phases of rooting. With this study, we confirm that the burst of sucrolytic enzymatic activity observed previously is regulated at the transcript level.

Both the histological analysis and the transcript profiling presented in this work confirmed that the timing for the activation of cell division in the cambial initials depended on the cultivar and it was accelerated by the exogenous auxin treatment. In plants, D-type and A3-type cyclins have been implicated in the G1-to-S transition [53, 54] while subgroups of A-type and B-type cyclins act in the G2-to-M transition [55, 56]. A number of genes encoding mitotic A-type (*Dca24345* and *Dca44777*) and B-type cyclins (*Dca14212*, *Dca43894* and *Dca44193*) were clearly upregulated in both cultivars from 24 hAP onwards and their early expression was slightly higher in the auxin-treated samples. These results are in agreement with those found in Arabidopsis, where the cyclin-dependent kinase activity required for mitosis is regulated by redundant genes encoding CYCLINA2 and CYCLINB [55, 57].

Another of the functional groups that showed differences in their expression levels during rooting were those encoding transcripts related to microtubules (MTs) and MT-associated proteins, such as kinesins. MTs play essential roles in cell division and cell elongation [58] and they indirectly might regulate morphogenesis [59]. Several genes encoding MT-associated proteins showed a biphasic response along the time-course experiment with a clear downregulation at earlier stages and a concomitant upregulation at later time points (Fig. 9a and data not shown).

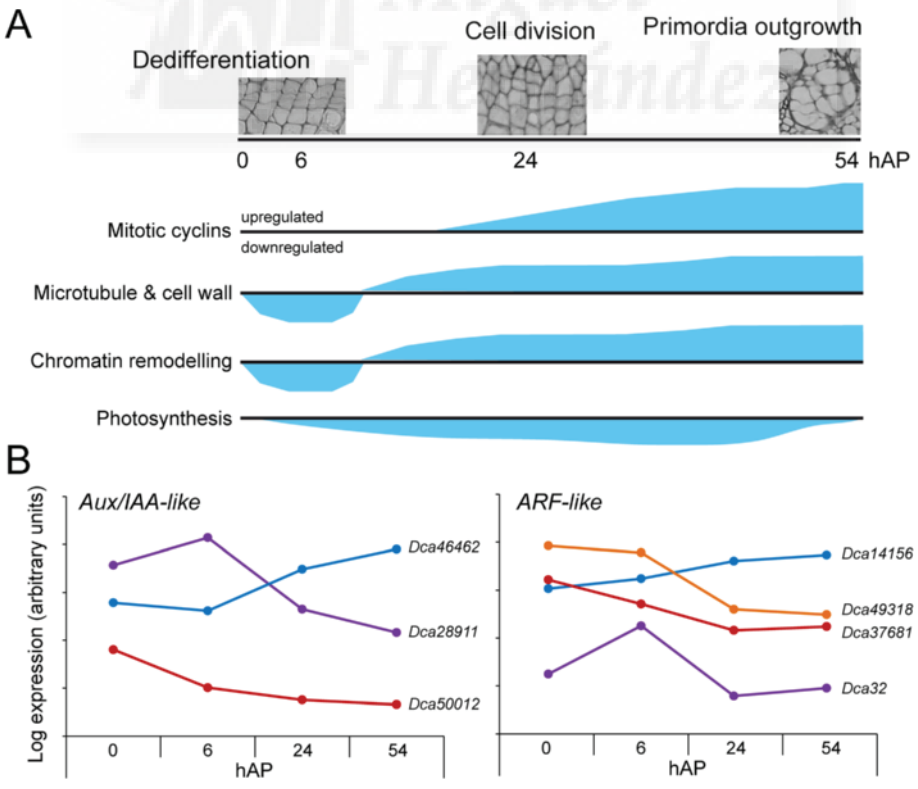

Fig. 9 A proposed model of AR formation in carnation stem cuttings derived from transcriptome profiling. **a** Some cellular and molecular events observed during AR formation over the time-course experiment. **b** Representative expression profiles of several genes encoding putative auxin regulators that showed differential expression over the time-course experiment

Among those, several kinesin-encoding genes (*Dca24841*, *Dca27864* and *Dca45361*) were found highly expressed from 24 hAP onwards, coinciding with the activation of cell division. Differential remodelling of MTs has been observed previously in juvenile cuttings compared to mature cuttings in *Eucalyptus grandis* [23]. If MT dynamics also plays a role in AR formation in carnation, we expect that subtle perturbations of MTs might improve the rooting success of the bad-rooting carnation cultivars, as it was previously shown for mature *E. grandis* cuttings [23].

Interestingly, we found several genes encoding specific histone variants (*Dca5695*, *Dca16479* and *Dca21788*) that displayed a biphasic expression profile during adventitious rooting consisting of a slight downregulation during the initial stages (up to 6 hAP) and a concomitant increase in their expression levels afterwards. A similar expression profile was found for *Dca58880*, encoding an homolog of the KRYPTONITE (KYP, also known as SUVH4) histone H3 lysine 9 methyltransferase [60]. Recent studies in the *Arabidopsis thaliana* model indicated that H3.3 (whose putative carnation homolog encoding gene was *Dca21788*) was associated with active genes and showed a positive correlation with their expression levels, suggesting that H3 variant replacement may contribute to enable reprogramming at developmental transitions [61]. In addition, the functional loss of KYP resulted in altered expression of developmental regulators, such as *WUSCHEL*, and defects in callus formation during hormone-mediated dedifferentiation [62, 63]. Our results suggest that extensive chromatin remodelling is taking place in the stem cutting base in carnation cuttings prior to the activation of cell division. Whether these chromatin regulators are regulated by the inductive (auxin) signal remains to be elucidated.

Conclusions

With this work we initiated a multidimensional approach to characterize AR formation in the stem cutting base of a series of carnation cultivars with contrasting rooting performance. Our results allowed us to precisely define the different stages during AR formation and to identify a number of molecular, histological and physiological markers. These will allow us to monitor adventitious rooting in a wide collection of carnation germplasm and to select the good-rooting cultivars for breeding purposes.

Availability of supporting data

RNA-Seq data supporting this study are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3698.

Additional files

Additional file 1: Figure S1. Exploratory data analysis. (A) Heat map representation of the Euclidean distance between samples. The colour code in the histogram goes from white (lowest correlation values) to dark blue (highest correlation values). (B) PCA analysis. (TIFF 2974 kb)

Additional file 2: Table S1. Oligonucleotides used in this work. (DOC 30 kb)

Additional file 3: Data file for evidence-based gene models used in this work. (7Z 1766 kb)

Additional file 4: Figure S2. Validation of RNA-seq results by qRT-PCR. The relative expression of six genes was studied in the *2003 R 8* (A) and *2101–02 MFR* (B) cultivars. Each dot represents the relative expression data for a given sample. (TIFF 918 kb)

Additional file 5: Figure S3. Model profiles comparison between mock-treated and auxin-treated samples in the *2101–02 MFR* cultivar. A profile to the immediate left of a yellow bar is from mock-treated samples. A profile to the right of the yellow bar is from the auxin-treated experiment, and has a significant intersection (in terms of the genes assigned to them) with the profile to the left of the yellow bar in its row. The number of genes and the P-value of the intersections are shown in the bottom left-hand. DD, DU, UD and UU are defined in Figure 4. The number on the right, indicates the overlap (in %) between genes assigned to each profile in mock- and auxin-treated samples. Profiles are coloured by default. (TIFF 3893 kb)

Additional file 6: Figure S4. Cellular parameters in the cambial cells during AR formation. (A) A representative cross-section image of the stem cutting base used to quantify some cellular parameters within the cambium. Squares represent the area measured for disorganized and organized regions. (B) Division rate of the cambial cells in the studied cultivars. Treatments and time-points are represented by coloured bars (white: mock 0 hAP; light grey: mock 54 hAP; dark grey: auxin 0 hAP; black: auxin 54 h AP). Different letters indicate significant differences ($P < 0.005$) between regions. Error bars indicate the standard deviation (SD) for the mean data shown. (C) Number of cambial cells per mm^2 in the studied cultivars. Organized and disorganized regions within the cambium are represented as lined and dotted bars, respectively. Asterisks indicate significant differences ($P < 0.005$) between regions at a given time-point. Different letters indicate significant differences ($P < 0.005$) between samples. (TIFF 6084 kb)

Abbreviations

ACC: 1-aminocyclopropane-1-carboxylic acid; AR: Adventitious root; Arabidopsis: *Arabidopsis thaliana*; ARF: AUXIN RESPONSE FACTOR; bp: base pair; BP: Biological process; CC: Cellular component; cDNA: complementary DNA; DAP: Days after planting; DD: DownDown; DEG: Differentially expressed gene; DNA: Deoxyribonucleic acid; DU: DownUp; EGs: Genes with dynamic expression profiles; GO: Gene ontology; GRETCHEN HAGEN3: GH3; hAP: hours after planting; IAA: indole-3-acetic acid; IBA: indole-3-butyric acid; MF: molecular function; miRNAs: microRNAs; MT: microtubules; NAA: α-naphtalene acetic acid; OBH: one-way best-hits; ORF: open reading frame; PCA: principal-component analysis; qRT-PCR: quantitative reverse-transcription PCR; RNA: ribonucleic acid; RNA-Seq: RNA sequencing; TAIR: The Arabidopsis Information Resource; TF: transcription factor; tZ: *trans*-zeatin; UD: UpDown; UU: UpUp; W/V: Weight/volume.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CVM carried out statistical and bioinformatic analyses and helped drafting the manuscript. ABSG performed the validation of the experiments and the histology labwork and participated in drafting the manuscript, JV carried out the treatments in the lab and participated in drafting the manuscript, AC and MA designed and performed the phytohormone analyses, MvdR carried out the library preparations and participated in the coordination of the study, JdH and PP participated in the conception, design and coordination of the study, JMPP conceived the study, collaborated in the time-series analysis and drafted the manuscript. All authors read and approved the final manuscript.

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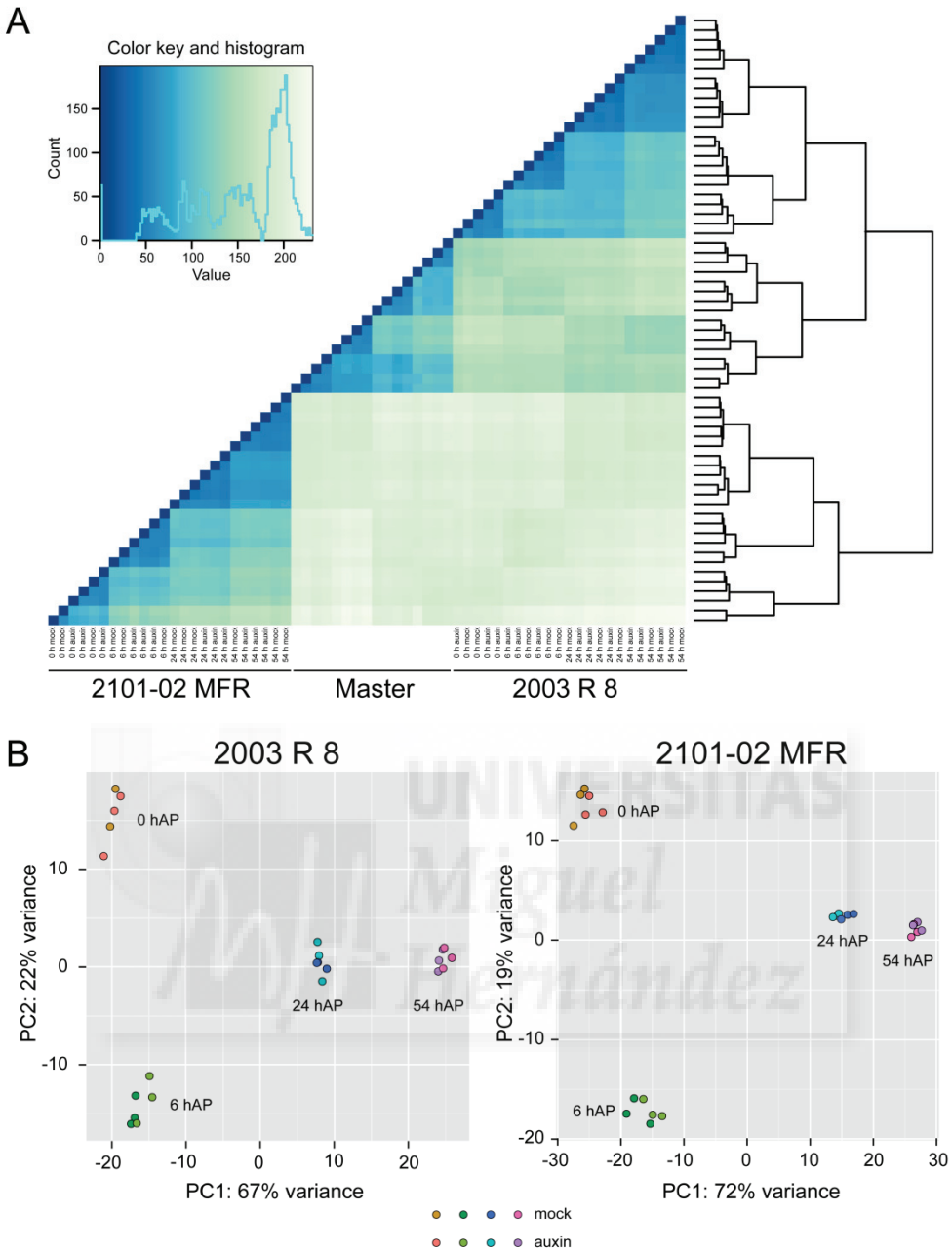

Figure S1.- Exploratory data analysis. (A) Heat map representation of the Euclidean distance between samples. The colour code in the histogram goes from white (lowest correlation values) to dark blue (highest correlation values). (B) PCA analysis.

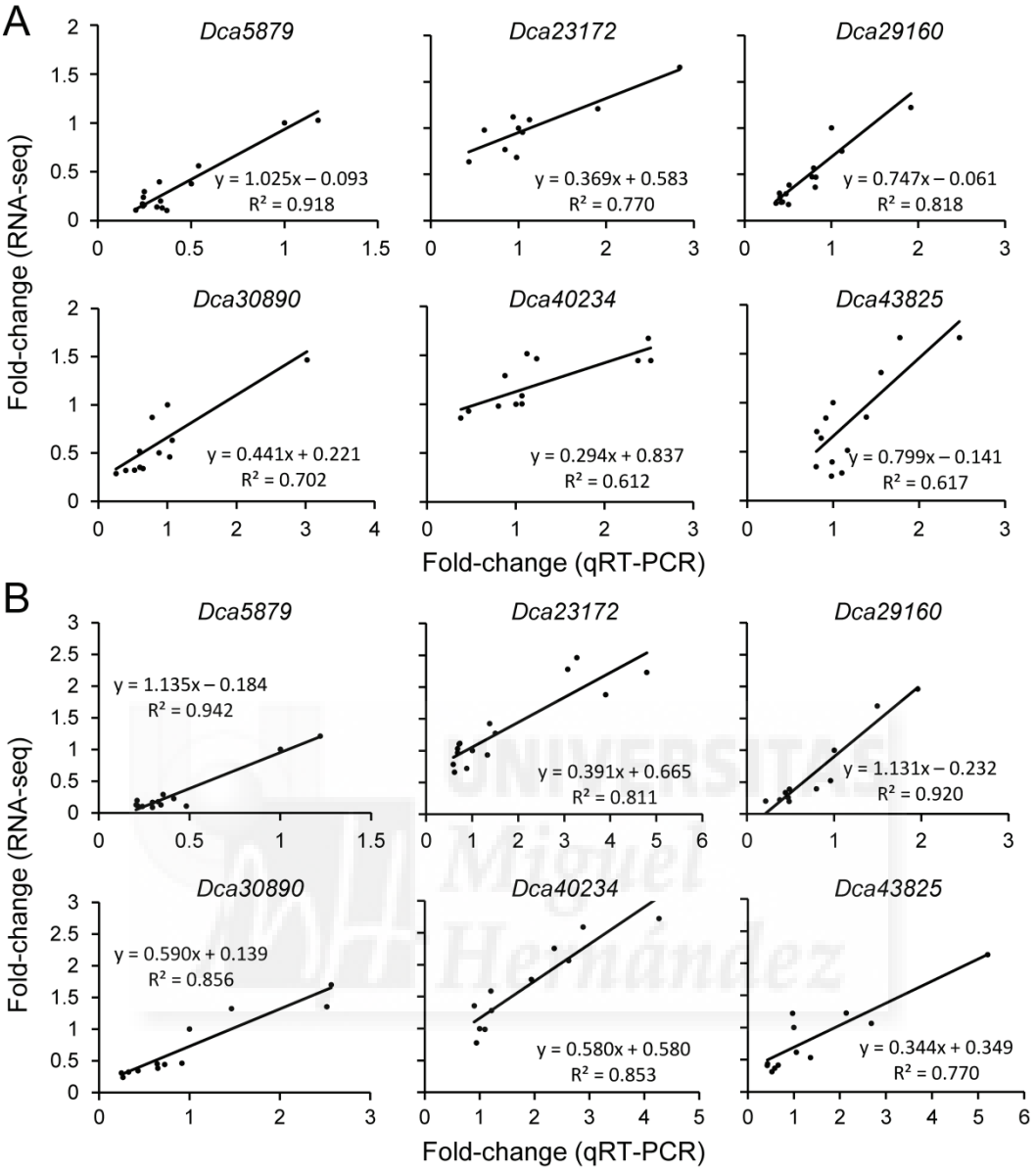

Figure S2.- Validation of RNA-seq results by qRT-PCR. The relative expression of six genes was studied in the *2003 R 8* (A) and *2101-02 MFR* (B) cultivars. Each dot represents the relative expression data for a given sample.

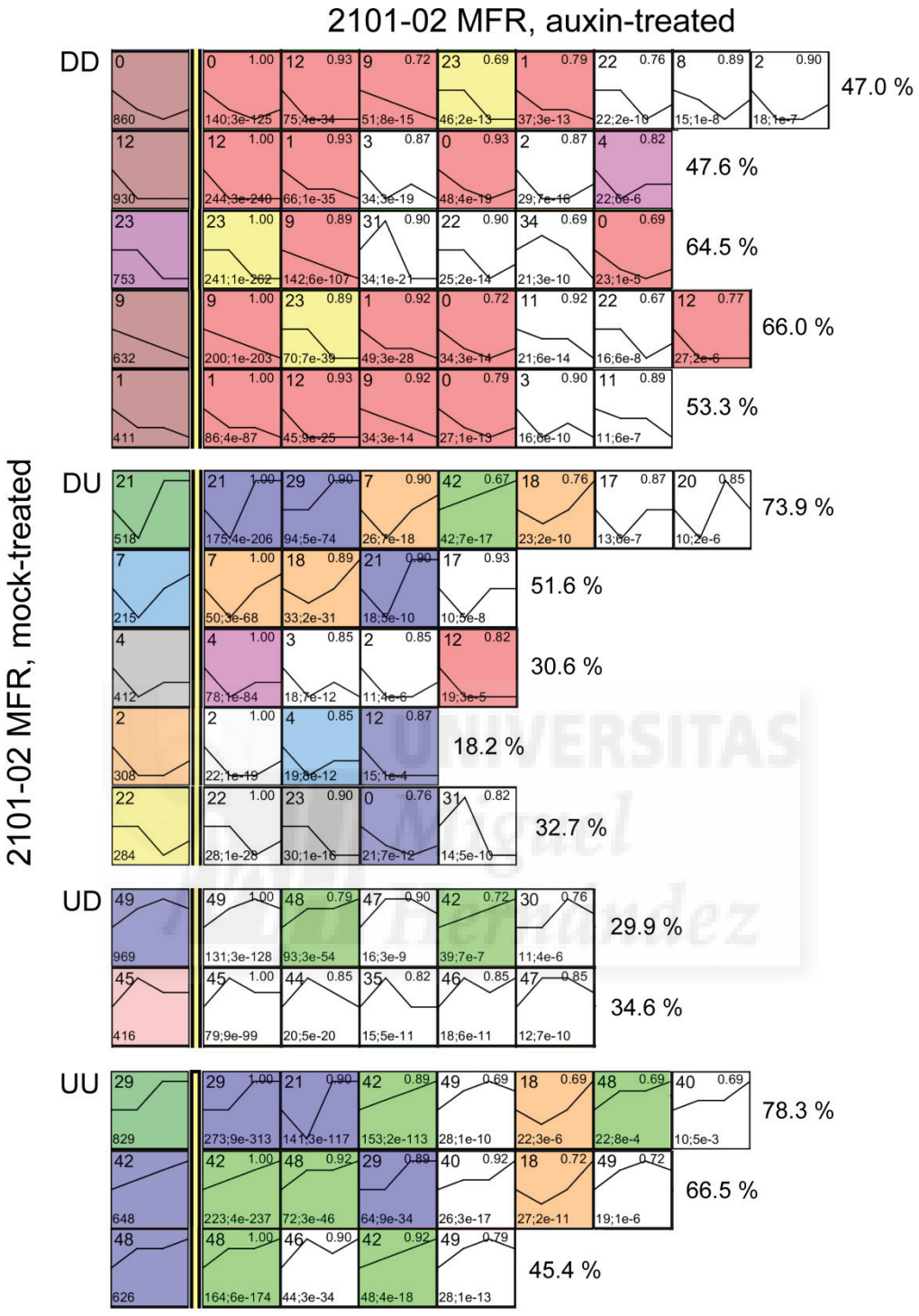

Figure S3.- Model profiles comparison between mock-treated and auxin-treated samples in the *2101-02 MFR* cultivar. A profile to the immediate left of a yellow bar is from mock-treated samples. A profile to the right of the yellow bar is from the auxin-treated experiment, and has a significant intersection (in terms of the genes assigned to them) with the profile to the left of the yellow bar in its row. The number of genes and the p-value of the intersections are shown in the bottom left-hand. DD, DU, UD and UU are defined in Figure 4. The number on the right, indicates the overlap (in %) between genes assigned to each profile in mock- and auxin-treated samples. Profiles are coloured by default.

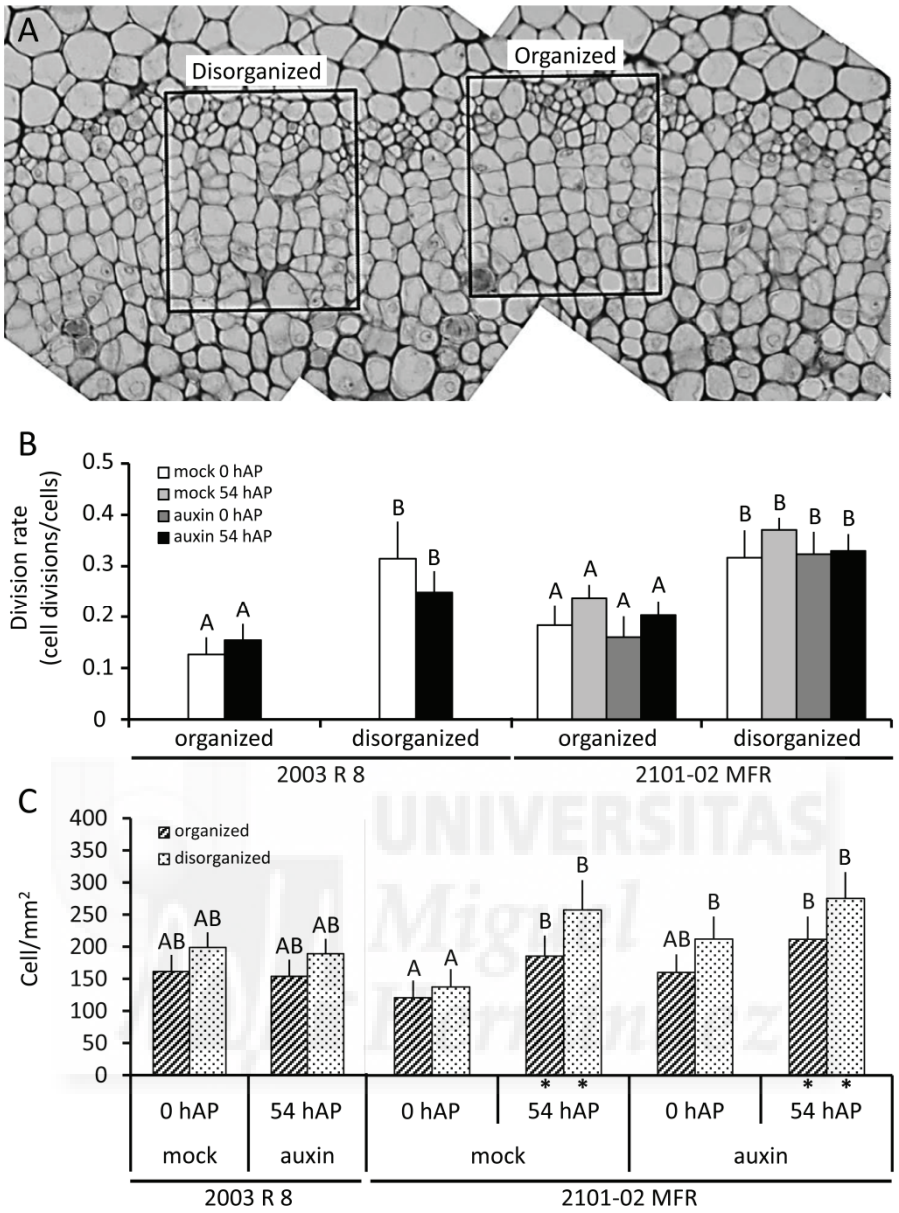

Figure S4.- Cellular parameters in the cambial cells during AR formation. (A) A representative cross-section image of the stem cutting base used to quantify some cellular parameters within the cambium. Squares represent the area measured for disorganized and organized regions. (B) Division rate of the cambial cells in the studied cultivars. Treatments and time-points are represented by coloured bars (white: mock 0 hAP; light grey: mock 54 hAP; dark grey: auxin 0 hAP; black: auxin 54 h AP). Different letters indicate significant differences ($P < 0.005$) between regions. Error bars indicate the standard deviation (SD) for the mean data shown. (C) Number of cambial cells per mm^2 in the studied cultivars. Organized and disorganized regions within the cambium are represented as lined and dotted bars, respectively. Asterisks indicate significant differences ($P < 0.005$) between regions at a given time-point. Different letters indicate significant differences ($P < 0.005$) between samples.

Table S1.- Oligonucleotides used in this work

Gene locus	Oligonucleotide sequences (5' to 3')		Product (bp)
Dca5879	TTTCGAGAAACTAGTAGAACAGGCG	GAAGACTGTGTTCTAGCGACAAGCC	111
Dca23172	CATTGATCAGCTTTCATTAAGGTTC	CGGTGTTTGCTGTAATTAGAGGCGG	122
Dca29160	AGAGCATTACCTGATACGCATGACG	GGCCACCCGACTATTTGTGCCTTGG	113
Dca30890	TCATTTTGTTGCTTCTTTTGAGAAG	CATCACCAACAAGGAGCCAATCTCC	131
Dca40234	GATGGACTTACCTGTTATAATGCTG	GCTGCCATATGTTTTTAAGCTCCTC	102
Dca43825	TTGCCACAATTTAGAAATGCAGTAG	CACCACTCATTACTATGCGACTTGG	94
Dca17200	CCAAGCAGCATGAAGATTAAGG	CCTTTGAGATCCACATCTGCTG	115

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Capítulo 4

Comunicaciones a Congresos

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7th International Symposium on Root Development, Weimar (Alemania), septiembre 2014

Pérez-Pérez JM, **Villanova J**, Cano A, Cano EA, van de Rhee M, Passarinho P, Acosta M. Genetical genomics of adventitious root formation in carnation cuttings. Comunicación oral, presentada por J.M. Pérez-Pérez

XIV Congreso Hispano-Luso de Fisiología Vegetal, Toledo, junio 2015

Pérez-Pérez JM, **Villanova J**, Sánchez-García AB, Villacorta-Martín C., Cano A, Cano EA, Passarinho P, Acosta M. Genetical genomics of adventitious root formation in carnation cuttings. Comunicación oral, presentada por J.M. Pérez-Pérez

Jornada "Innovación e impacto económico de las variedades vegetales" Fruit Attraction, Madrid, octubre 2015

Pérez-Pérez JM, **Villanova J**, Sánchez-García AB, Cano A, Acosta M. Desarrollo de herramientas moleculares para la mejora genética en ornamentales. Comunicación oral, presentada por J.M. Pérez-Pérez

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Genetical genomics of adventitious root formation in carnation cuttings

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Carnation is, after rose, the most important species on the worldwide market of cut flowers. The production of young plantlets is frequently hampered by minimal adventitious root (AR) formation from stem cuttings, which has a strong genetic dependency and which leads to production losses in certain carnation varieties (Agulló-Antón et al. 2011).

Over the past decade, modern technologies, such as genomics, high-throughput sequencing and phenotyping, expression profiling and bioinformatics have revolutionized breeding for elite varieties in vegetable and agricultural crops. Among cut flowers, carnation is especially amenable to these approaches as its genome size is rather small (Yagi et al. 2013). We started to characterize the phenotypic and genotypic variation present on a core collection of commercial carnation varieties. Genetic variation discovery among carnation cultivars with contrasting rooting phenotypes can then be used for the identification of trait-linked markers through genome-wide association studies (GWAS). In this way the genetic determinants responsible for the induction of AR formation in carnation stem cuttings will be identified, and linked molecular markers will be allocated.

Agulló-Antón MA, et al. (2011). Auxins or sugars: what makes the difference in the adventitious rooting of stored carnation cuttings? *J. Plant Growth Regul.* **30**, 100-113.

Yagi M, et al. (2013). Sequence analysis of the genome of carnation (*Dianthus caryophyllus* L.). *DNA Res*. doi: 10.1093/dnares/dst053.

Work funded by Eurostars-EUREKA (CARNOMICS E!6834) and MINECO/FEDER (AGL2012-33610).

Keywords: *Dianthus caryophyllus*, adventitious root formation, hormonal crosstalk, phenotyping, genome-wide association (GWA).

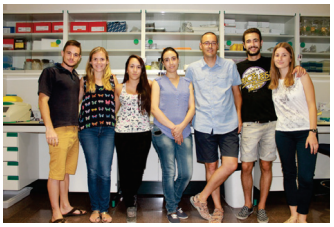
Genetical genomics of AR formation in carnation cuttings

José Manuel Pérez-Pérez

Weimar, 18/09/2014

www.arolab.es
laboratory of adventitious rooting and organogenesis

Joan Villanova
Amparo Primo
Virginia Birlanga



Carnomics

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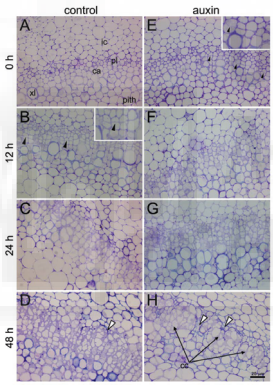
Barberet & Blanc®

AR formation in carnation cuttings

Dianthus caryophyllus L. (cv. Master)

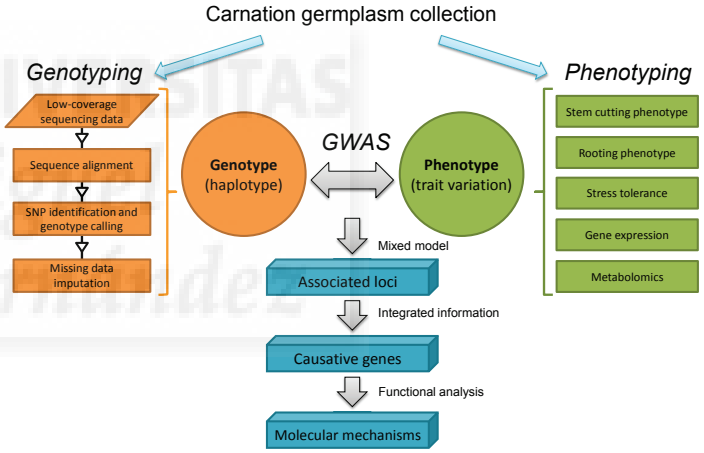
- Carnation plants are vegetatively propagated from excised cuttings



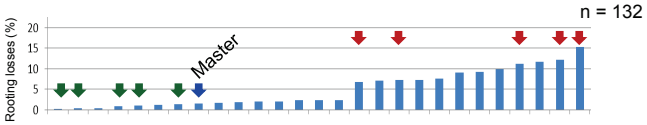
- Basipetal auxin transport causes IAA accumulation at the base of the cutting, which triggers AR formation
- Exogenous auxin treatment accelerates AR formation

Garrido *et al.* 2002; Acosta *et al.* 2009; Agulló-Antón *et al.* 2014; Cano *et al.* 2014

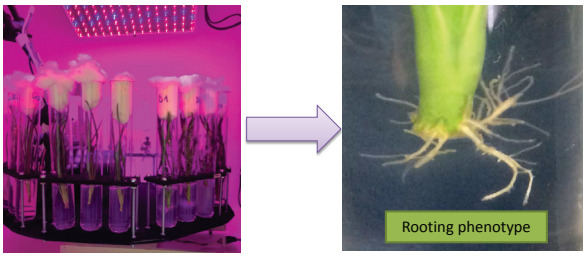
Dissecting AR formation: tools needed



HTP phenotypic annotation



- 5 good-rooting and 5 bad-rooting varieties were studied



HTP phenotypic annotation

Good-rooting

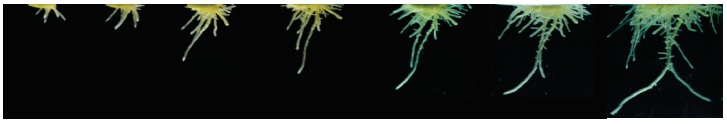
Bad-rooting

13 d 15 d 18 d 20 d 22 d 25 d 27 d

- Qualitative observations
  - Delay in root initiation
  - Reduced number of AR primordia
  - Slow elongation rate of primary ARs
  - Slow elongation rate of lateral ARs

HTP phenotypic annotation

Good-rooting



Bad-rooting

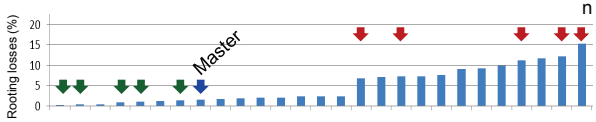
13 d 15 d 18 d 20 d 22 d 25 d 27 d

- Quantitative observations

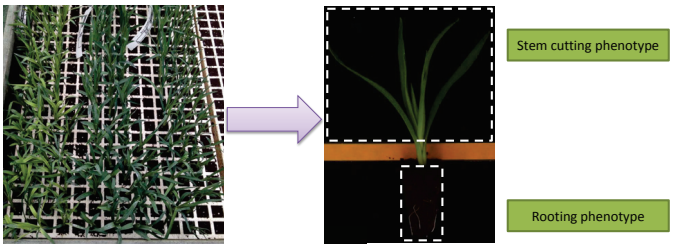
Measured feature	Good-rooting	Bad-rooting
Average Root Width (Diameter)	2.18 ± 1.45	3.88 ± 1.69
Maximum Number of Roots	5.10 ± 3.96	3.30 ± 5.03
Network Bushiness	4.10 ± 3.21	1.77 ± 1.47
Network Length	114.69 ± 51.00	73.58 ± 51.91
Network Perimeter	37.87 ± 34.64	26.42 ± 49.82
Network Surface Area	700.09 ± 508.75	663.88 ± 262.15
Specific Root Length	0.22 ± 0.16	0.12 ± 0.21

HTP phenotypic annotation

n = 132



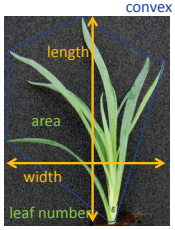
- 5 good-rooting and 5 bad-rooting varieties were studied



HTP phenotypic annotation

Stem cutting phenotype

convex area



Some eco-physiological parameters (SLA, GS, LDMC,…)

Rooting phenotype

Stage	Image
1	
2	
3	
4	
5	
6	

Raw image

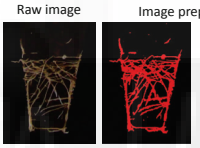


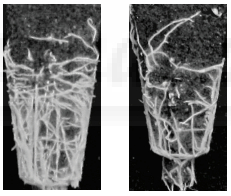
Image preparation

- Total root length
- Total root area
- Average root diameter
- Network length distribution
- Network solidity
- Estimated root number

HTP phenotypic annotation: results

- We found non-significant correlations between morphological stem characteristics and rooting parameters
- Spray cultivars produce more roots that standard cultivars
- Some standard good-rooting cultivars rooted poorly without exogenous auxin treatment

Galaxia Duero



- Galaxia roots initiate earlier and grow faster
- Duero display a delay in root initiation, which is rescued by auxin treatment

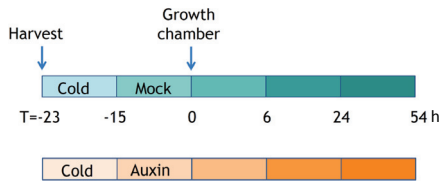
Development of molecular tools

Genome annotation (transcripts)	Tomato	cv. Francesco (Yagi *et al.* 2013)	cv. Master (CARNOMICS)
Count	34,675	56,382	39,506
Avg. length (bp)	3,161	2,741	4,484
Median length (bp)	2,045	2,065	3,242
Total length (kb)	109,6	154,6	177,1
Avg. coding length (bp)	1,032	-	1,429

- NGS (Illumina HiSeq2000) from different libraries (PE300, PE400, 3 and 5 kb MP, and 40 kb fosmids)
- Covered about 80% of the 706 Mb carnation genome (estimated by k-mer analysis)
- We are combining RNA-seq data to improve current gene models

Expression profiling during AR formation

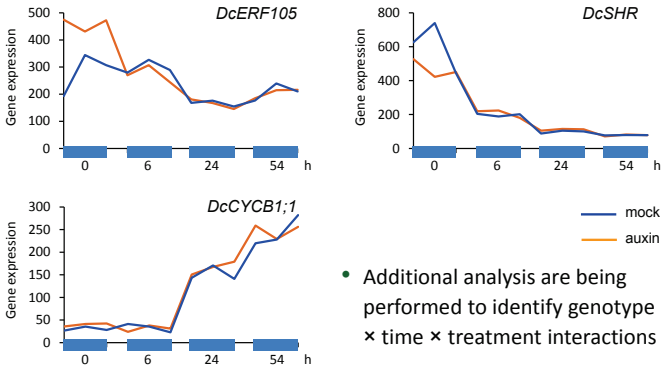
Experimental design



- 3 varieties: Galaxia, Duero, Master
- 3 biological replicates
- 15 stem bases per replicate
- 8 sequencing lanes

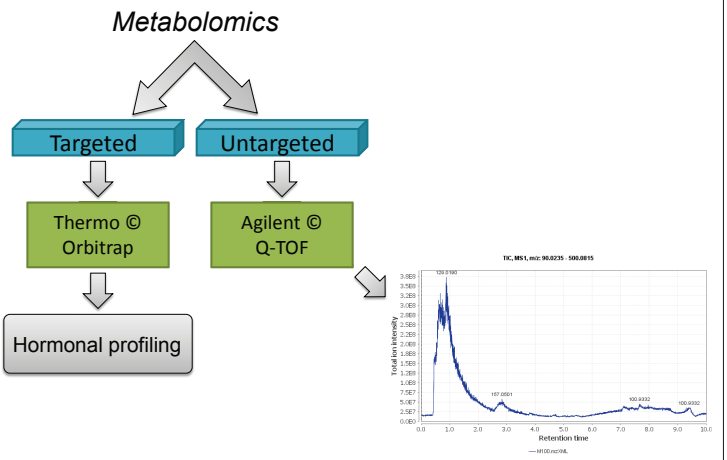
Expression profiling during AR formation

- A large proportion of genes (~24%) were differentially expressed during AR formation

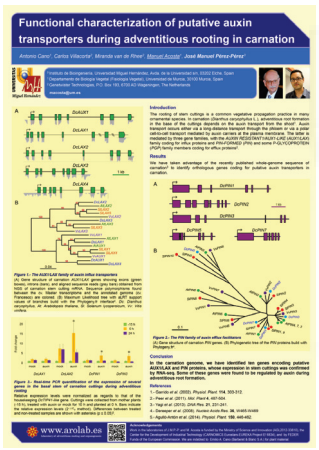


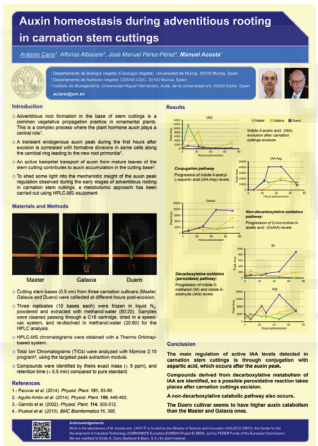
- Additional analysis are being performed to identify genotype × time × treatment interactions

Metabolic profiling during AR formation



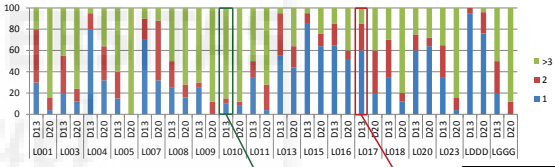
Key role of the auxin pathway in AR formation



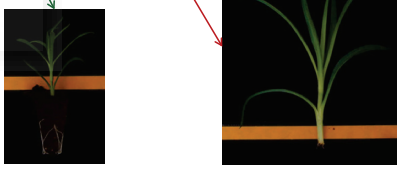


A GWAS platform for molecular breeding

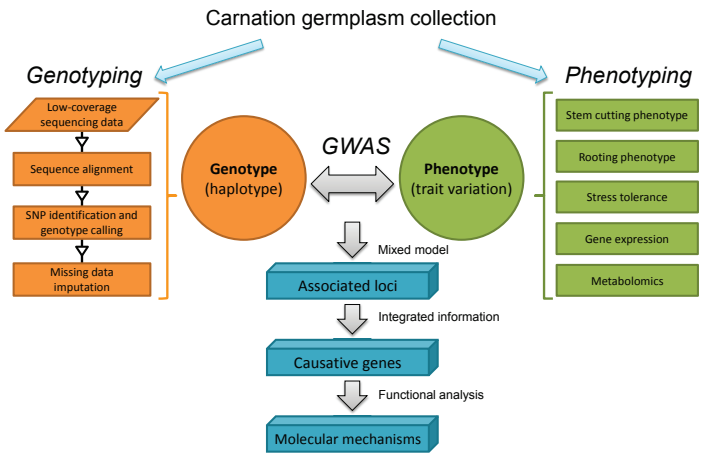
- A Galaxia × Duero mapping population was built (n = 146 lines)



- Substantial differences among lines
- Precocity, root growth speed, total root length, etc.



Genome-wide dissection of AR formation



Genetical genomics of adventitious root formation in carnation cuttings

José Manuel Pérez-Pérez[1], Joan Villanova[1,#], Ana Belén Sánchez-García[1,#], Carlos Villacorta-Martín[2#], Antonio Cano[3], Emilio A. Cano[4], Paul Passarinho[2], Manuel Acosta[3]

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[2]*Genetwister Technologies, P.O. Box 193, 6700 AD Wageningen, The Netherlands*
[3]*Departamento de Biología Vegetal (Fisiología Vegetal), Universidad de Murcia, Campus de Espinardo, 30100 Murcia, Spain*
[4]*Barberet & Blanc, Apdo. Correos 38, 30890 Puerto Lumbreras, Spain*
[#]*These authors contributed equally*
jmperez@umh.es

The economic value of ornamental plants has increased significantly worldwide and is increasing annually by 8 to 10%. Cultivated carnation (Dianthus caryophyllus L.) is one of the most important species on the worldwide market of cut flowers. The production of young plantlets is frequently hampered by minimal adventitious root (AR) formation from stem cuttings, which has a strong genetic dependency and which leads to production losses in certain carnation varieties (Agulló-Antón et al. 2011).

Over the past decade, modern technologies, such as genomics, high-throughput sequencing and phenotyping, expression profiling and bioinformatics have revolutionized breeding for elite varieties in vegetable and agricultural crops. Among cut flowers, carnation is especially amenable to these approaches as its genome size is rather small (Yagi et al. 2014).

We have characterized the phenotypic variation present on a core collection of commercial carnation varieties. Gene expression and metabolite profiling in the stem base of two cultivars with contrasting effects on AR formation have been analyzed. The identification of the genes involved in AR formation in this species will contribute to our basic understanding of the molecular events leading to this complex developmental response and to the eventual manipulation of AR formation in other recalcitrant, ornamental species.

Agulló-Antón et al. (2011). J. Plant Growth Regul., 30: 100-113

Yagi et al. (2014). DNA Res., 21: 231-241

Work funded by Eurostars-EUREKA (CARNOMICS E!6834) and MINECO/FEDER (AGL2012-33610)

Genetical genomics of AR formation in carnation cuttings

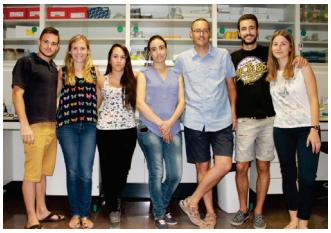
José Manuel Pérez-Pérez

Toledo, 16/06/2015

FISIOLOGÍA VEGETAL TOLEDO 2015 14 - 17 DE JUNIO

www.arolab.es
laboratory of adventitious rooting and organogenesis

Joan Villanova
Ana Belén Sánchez-García
Virginia Birlanga



Carnomics

Genetwister

Dr. Paul Passarinho
Dr. Miranda van de Rhee
Dr. Jorn de Haan
Carlos Villacorta
Genetwister Technologies B.V.
Wageningen, The Netherlands

Prof. Manuel Acosta
Dr. Antonio Cano
Department of Plant Biology
University of Murcia, Spain

Dr. Emilio A. Cano

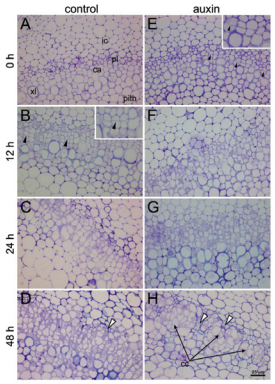
Barberet & Blanc®

AR formation in carnation cuttings

Dianthus caryophyllus L. (cv. Master)

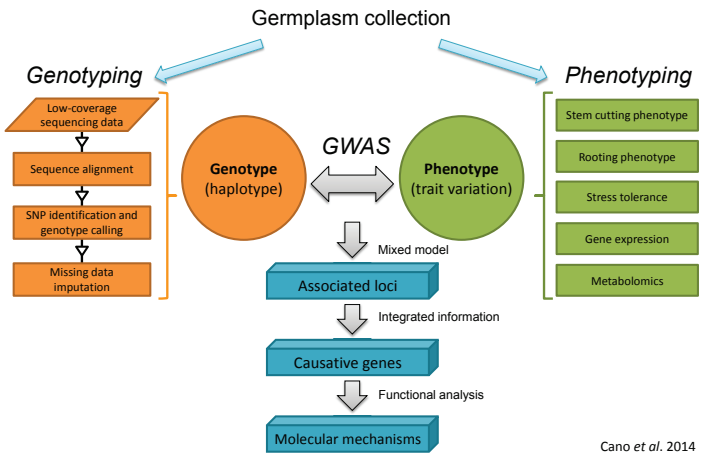
- Carnation plants are vegetatively propagated from excised cuttings

- Basipetal auxin transport causes IAA accumulation in the base of the cuttings, which triggers AR formation
- Exogenous auxin treatment accelerates AR formation



Garrido *et al.* 2002; Acosta *et al.* 2009; Agulló-Antón *et al.* 2014

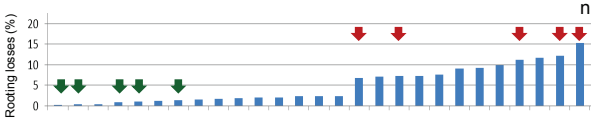
Dissecting AR formation: tools needed



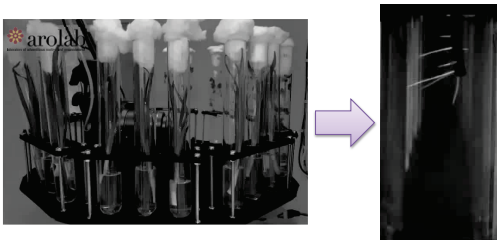
Cano *et al.* 2014

HTP phenotypic annotation

n = 132

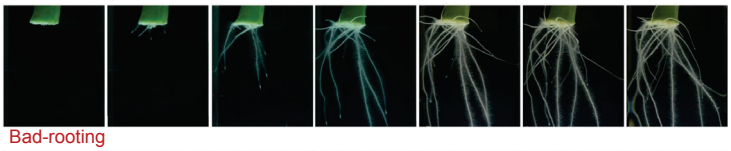


- 5 good-rooting and 5 bad-rooting varieties were studied

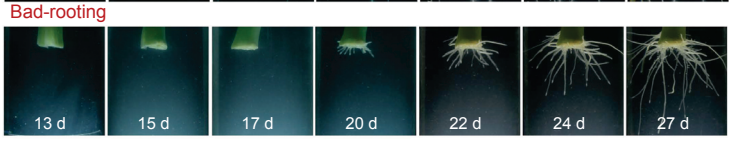


HTP phenotypic annotation

Good-rooting



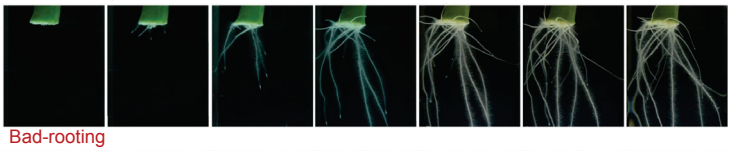
Bad-rooting



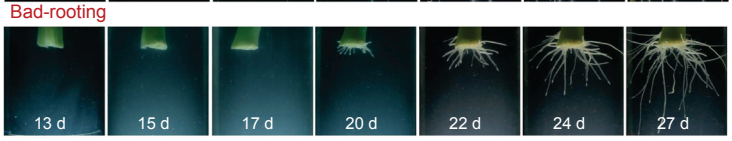
- Qualitative observations
  - Delay in root initiation
  - Reduced number of AR primordia
  - Slow elongation rate of primary ARs
  - Slow elongation rate of lateral ARs

HTP phenotypic annotation

Good-rooting



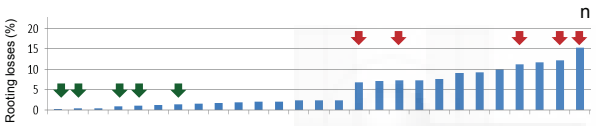
Bad-rooting



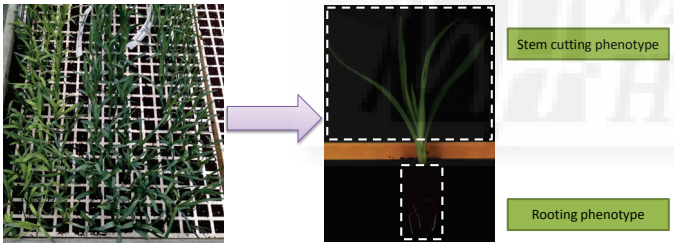
- Quantitative observations

Measured feature	Good-rooting	Bad-rooting
Average Root Width (Diameter)	2.18 ± 1.45	3.88 ± 1.69
Maximum Number of Roots	5.10 ± 3.96	3.30 ± 5.03
Network Bushiness	4.10 ± 3.21	1.77 ± 1.47
Network Length	114.69 ± 51.00	73.58 ± 51.91
Network Perimeter	37.87 ± 34.64	26.42 ± 49.82
Network Surface Area	700.09 ± 508.75	663.88 ± 262.15
Specific Root Length	0.22 ± 0.16	0.12 ± 0.21

HTP phenotypic annotation

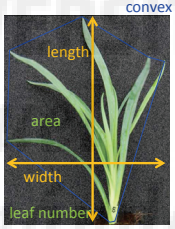


- 5 good-rooting and 5 bad-rooting varieties were studied

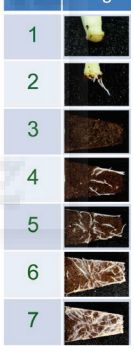


HTP phenotypic annotation

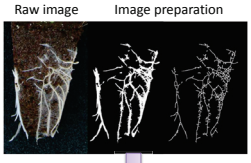
Stem cutting phenotype



Some eco-physiological parameters (SLA, GS, LDMC,…)



Rooting phenotype

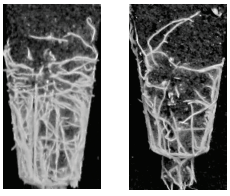


- Total root length
- Total root area
- Average root diameter
- Network length distribution
- Network solidity
- Estimated root number

HTP phenotypic annotation: results

- We found non-significant correlations between morphological stem characteristics and rooting parameters
- Spray cultivars produce more roots that standard cultivars
- Some standard good-rooting cultivars rooted poorly without exogenous auxin treatment

2101-02 MFR 2003 R 8



27 days

- In 2101-02 MFR, ARs initiate earlier and grow faster
- 2003 R 8 display a delay in AR initiation, which is rescued by exogenous auxin treatment

Development of molecular tools

- NGS (Illumina HiSeq2000) from different libraries (PE300, PE400, 3 and 5 kb MP, and 40 kb fosmids)
- Covered about 80% of the 706 Mb carnation genome (estimated by k-mer analysis)
- We combined RNA-seq data to improve current gene models

Genome annotation (transcripts)	Tomato	cv. Francesco (Yagi *et al.* 2014)
Count	34,675	56,382
Avg. length (bp)	3,161	2,742
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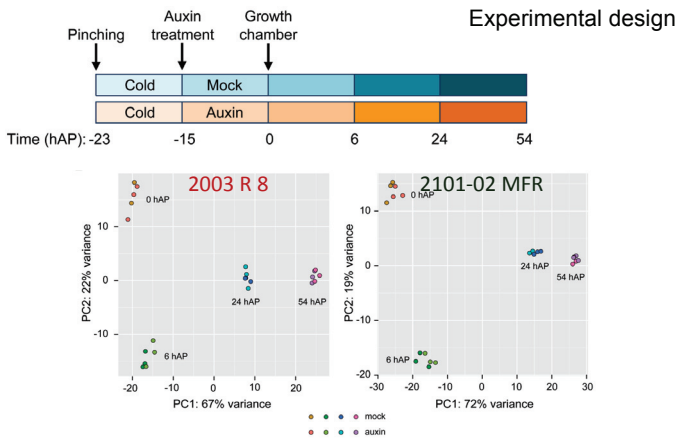
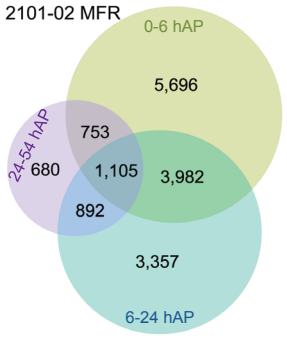
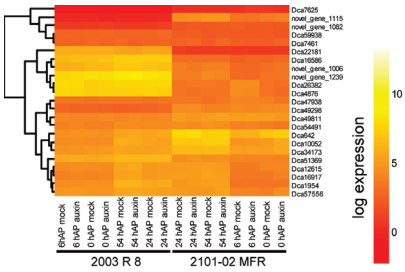
Expression profiling during AR formation



Expression profiling during AR formation

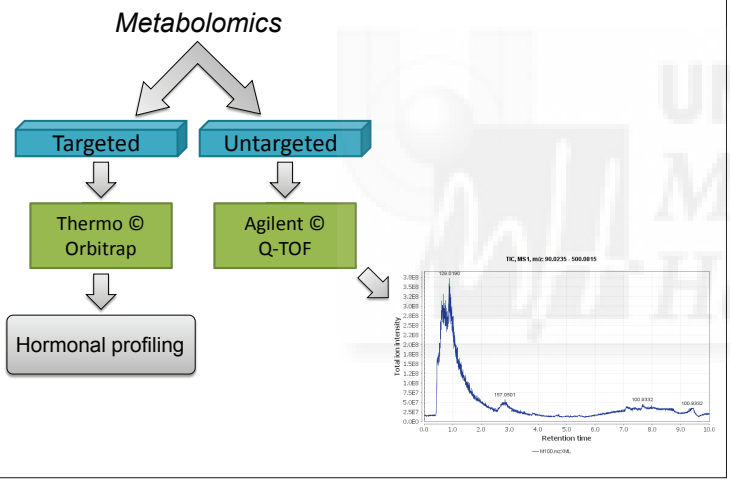
- A large proportion of genes were differentially expressed during AR formation



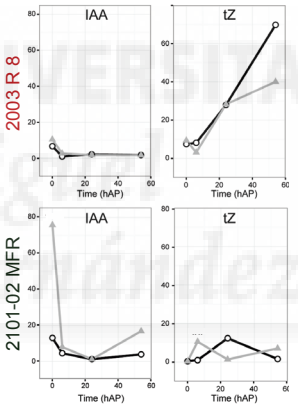


- Genes encoding cell-division related proteins showed higher expression at earlier tine points

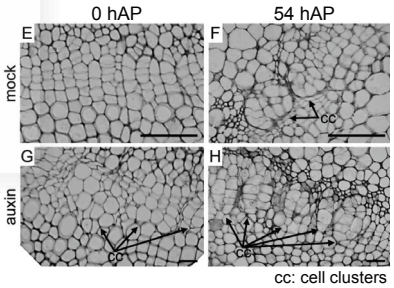
Metabolic profiling during AR formation



Key role of the auxin pathway in AR formation

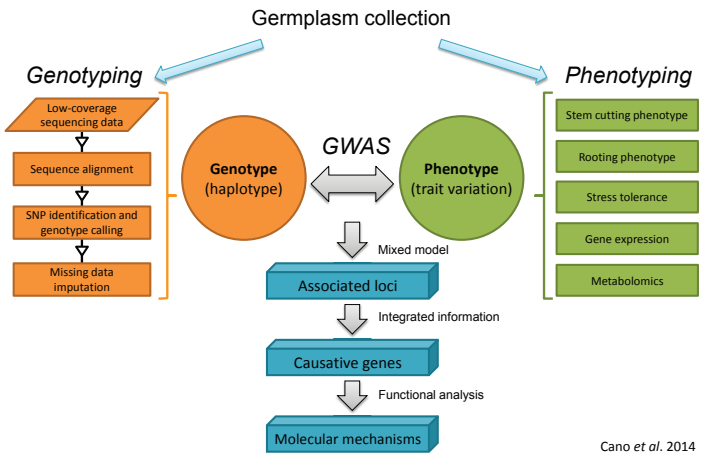


- Auxin might act as a trigger for cell division within a certain population of responsive cambial cells



cc: cell clusters

Genome-wide dissection of AR formation



Cano *et al.* 2014

Jornada BIOVEGEN

"Innovación e impacto económico de las variedades vegetales"

biovegen
plataforma tecnológica
de biotecnología vegetal

fruit attraction
FERIA INTERNACIONAL DEL SECTOR DE FRUTAS Y HORTALIZAS

Programa de la Jornada

10:30 h. **APERTURA**

10:40 h. **Economía agraria e innovación**
José Manuel Silva Rodríguez. Ex director general de Agricultura. Ex director general de Investigación. *Comisión Europea*

11:10 h. **Importancia de la Innovación varietal**
Manuel Talón Cubillo. *Instituto Valenciano de Investigaciones Agrarias (IVIA)*

11:40 h. **Innovación en cultivos emergentes**
Almendro - **María José Rubio-Cabetas**. *Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA)*
Albaricoque – **David Ruiz González**. *Centro de Edafología y Biología Aplicada del Segura (CEBAS CSIC Murcia).*
Ornamentales – **José Manuel Pérez Pérez**. *Universidad Miguel Hernández de Elche (UMH)*
Pistacho – Esaú Martínez Burgos. *Centro Investigación Agroambiental "El Chaparrillo" (IRIAF-Castilla La Mancha)*

12:40 h. **Protección y defensa de obtenciones vegetales**
Esther Esteban Rodrigo. *Oficina Española de Variedades Vegetales (OEVV)*

13:00 h. **Marco público de apoyo a la innovación varietal**
Europa: H2020 - **Marta Conde Vidal**. *Centro para el Desarrollo Tecnológico Industrial (CDTI)*
España: Estrategia Española de Bioeconomía – **Manuel Lainez Andrés.** *Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)*

13:20 h. **BIOVEGEN como catalizador del desarrollo de proyectos**
Gonzaga Ruiz de Gauna Gutiérrez. *Plataforma Tecnológica de Biotecnología Vegetal BIOVEGEN*

13:30 h. **CLAUSURA**

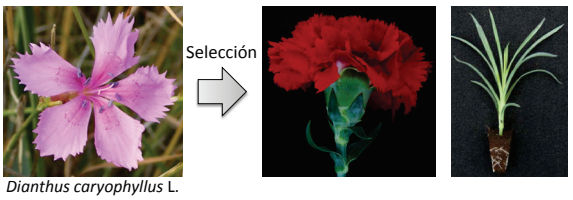
Desarrollo de herramientas moleculares para la mejora genética en ornamentales

José Manuel Pérez-Pérez
Profesor Titular, Área de Genética

Instituto de Bioingeniería
Universidad Miguel Hernández

Madrid, 30/10/2015

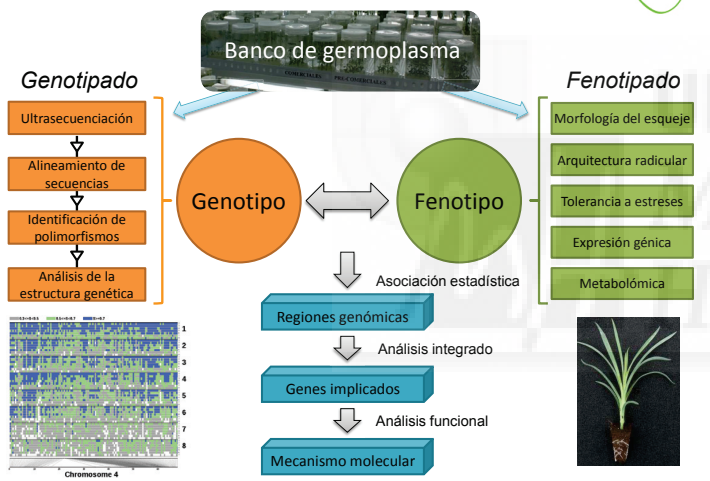
La mejora genética del clavel cultivado: Carnomics



Dianthus caryophyllus L.

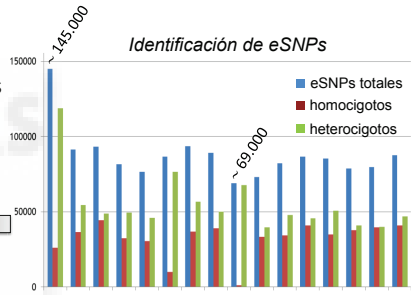
- La propagación del clavel se realiza de forma vegetativa a partir de esquejes
- El escalado industrial de algunas variedades de clavel se encuentra limitado por su baja capacidad de enraizamiento

La mejora genética del clavel cultivado: Carnomics



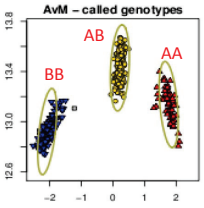
Transcriptómica comparada en 16 variedades

- Anotación funcional de genes
- Identificación de polimorfismos (eSNPs)



Selección de 50.000 eSNPs (en unos 16.300 cóntigos)

Diseño de una matriz de ADN para el genotipado masivo de eSNPs



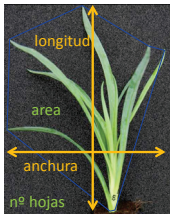
Secuenciación del genoma de una variedad de referencia

- Ultrasecuenciación Illumina HiSeq2000 de librerías con distinto tamaño de inserto (300 pb, 400 pb, 3 y 5 kb)
- Se ha podido ensamblar el 72% del genoma de la variedad Master (706 Mb) a pesar de su elevada heterocigosidad
- La anotación funcional de los genes se ha realizado a partir de los datos de RNA-seq de distintos tejidos (raíces, tallos, hojas y flores)

Genome annotation (transcripts)	Tomato	cv. Francesco (Yagi *et al.* 2014)	cv. Master (CARNOMICS)
Count	34,675	56,382	59,396
Avg. length (bp)	3,162	2,742	2,856
Median length (bp)	2,045	2,065	2,125
Total length (Mb)	109,6	154,6	177,1
Avg. coding length (bp)	1,032	-	1,429

Caracterización fenotípica de variedades comerciales

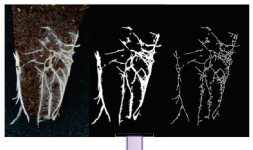
Morfología del esqueje



Parámetros ecofisiológicos (SLA, GS, LDMC,…)

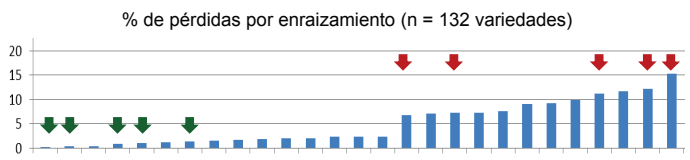
Arquitectura radicular

RSG	Image
1	
2	
3	
4	
5	
6	
7	

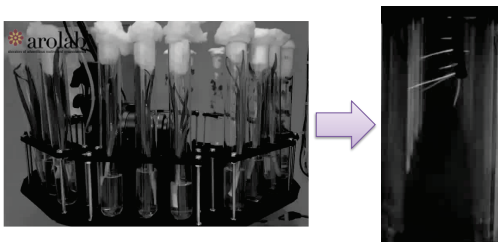


- Longitud
- Área
- Diámetro promedio
- Nº de raíces
- Densidad

Caracterización morfológica del crecimiento radicular

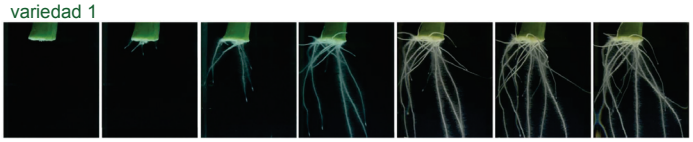


- Se seleccionaron diez variedades de los extremos de la distribución (5 buenas enraizantes y 5 malas enraizantes) para su análisis detallado

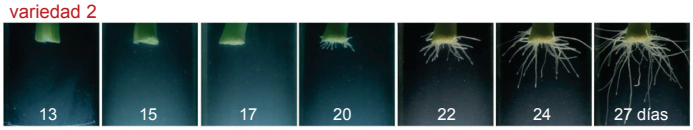


Caracterización morfológica del crecimiento radicular

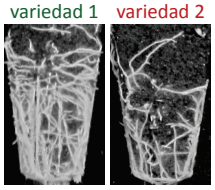
variedad 1



variedad 2



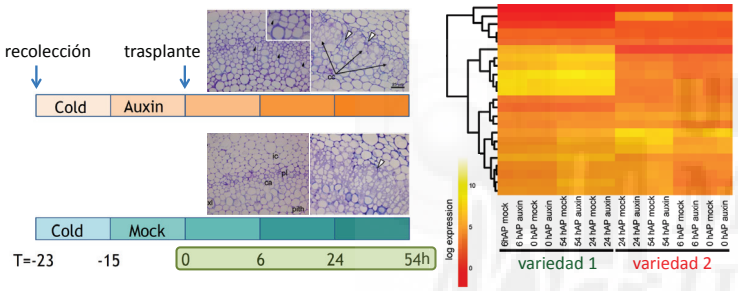
variedad 1 variedad 2



40 días

- Las raíces de la variedad 1 inician su desarrollo tempranamente y crecen más rápido que las de la variedad 2

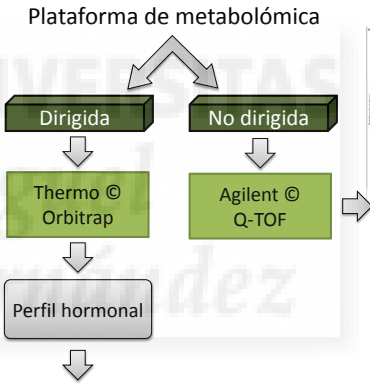
Perfil de expresión génica durante el enraizamiento

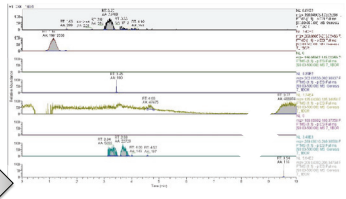


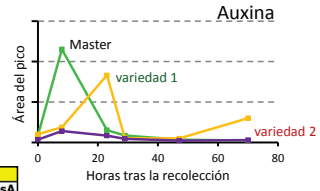
- Mediante ultrasecuenciación, hemos estudiado la expresión génica en estas dos variedades que difirieron en su capacidad de enraizamiento y en su respuesta a las auxinas

Hemos identificado varias decenas de genes cuya expresión temprana está correlacionada con la capacidad de enraizamiento

Análisis de metabolitos durante el enraizamiento



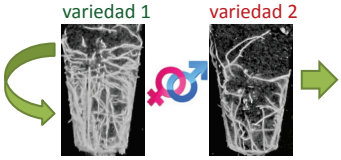




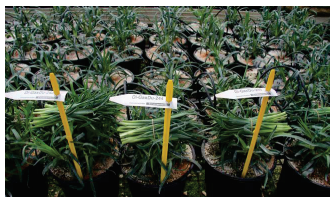
Phytohormones (ng/g FW)										
ACC	tZ	ZR	IPA	GA1	GA3	GA4	IAA	ABA	SalA	JasA
554.8	975.0	6177.6	6.9	-	20.5	19.2	38.3	19.8	15.5	128.7
41.6	321.6	-	7.8	-	17.0	-	-	-	19.8	61.7

Estudios de asociación a genoma completo del enraizamiento de esquejes

variedad 1 variedad 2



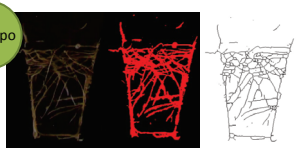
40 días



195 líneas estudiadas

Autofecundación	Cruzamiento
47 líneas	148 líneas

Fenotipo

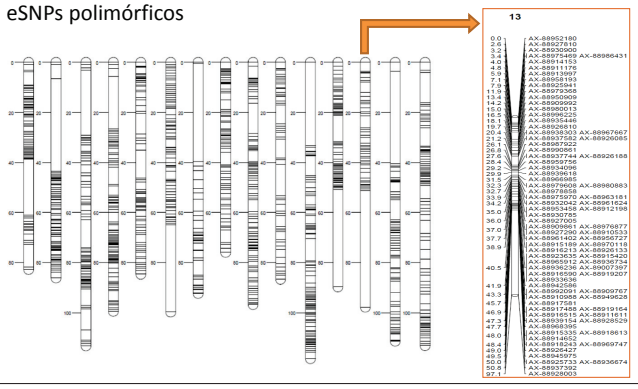


20 días

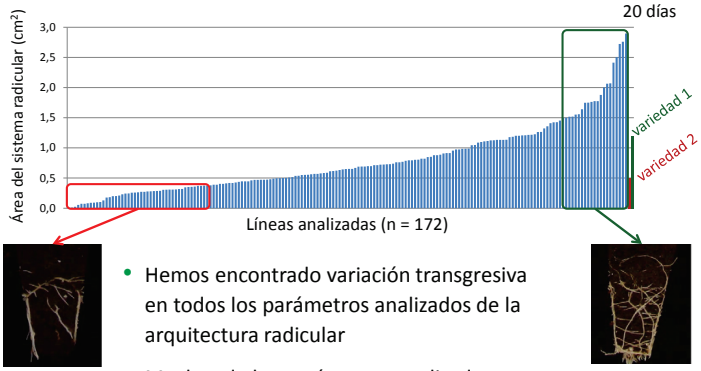
Genotipo

Mapa genético de clavel de alta densidad

- Se seleccionaron 130 individuos obtenidos del cruzamiento anterior (variedad 1 x variedad 2)
- Se determinó la posición en el mapa genético de clavel de casi 2.000 eSNPs polimórficos



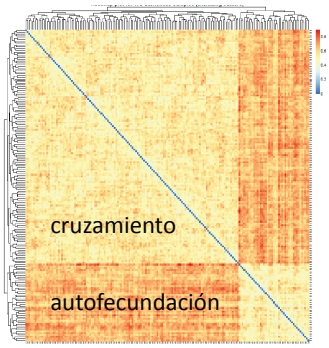
Estudios de asociación a genoma completo del enraizamiento de esquejes

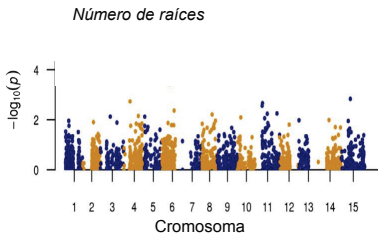


- Hemos encontrado variación transgresiva en todos los parámetros analizados de la arquitectura radicular
- Muchos de los parámetros analizados presentaron baja heredabilidad (<0,30)

Estudios de asociación a genoma completo del enraizamiento de esquejes

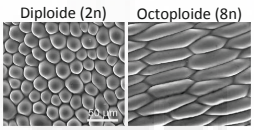
172 líneas ~ 24.000 eSNPs ⟺ Arquitectura radicular





Se encontraron 7 eSNPs asociados con el buen enraizamiento de esquejes

Estudio de la variabilidad morfológica en las flores del clavel cultivado



- Nuestros resultados indican que el tamaño de las flores está determinado por el tamaño, el número y la poliploidía de sus pétalos

Hemos realizado el análisis morfométrico detallado de flores y pétalos en 235 variedades de clavel

Análisis genético de la morfología floral en el clavel

El análisis de marcadores moleculares en esta población nos permitirá identificar regiones del genoma responsables de la variabilidad observada

Conclusiones:

- Se ha ensamblado y anotado el genoma de una variedad de referencia
- Se ha estudiado el transcriptoma en 16 variedades
- Se ha construido un mapa genético de alta densidad
- Se ha diseñado una matriz de ADN para el genotipado de variedades
- Se ha caracterizado el transcriptoma y el metaboloma en la base del esqueje en dos variedades que difieren en su capacidad de enraizamiento
- Se ha realizado un análisis de GWAS para identificar marcadores asociados con el buen enraizamiento
- Se ha caracterizado la variabilidad morfológica floral en una colección extensa de variedades de clavel

Las herramientas moleculares desarrolladas en este proyecto acelerarán la obtención de las nuevas variedades de clavel

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