Programa de Doctorado en Bioingeniería
Universidad Miguel Hernández de Elche

Funciones de *VCC* y las citoquininas en la morfogénesis del margen y la simetría bilateral de las hojas de Arabidopsis

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Funciones de *VCC* y las citoquininas en la morfogénesis del margen y la simetría bilateral de las hojas de Arabidopsis

Trabajo realizado por el Graduado Sergio Navarro Cartagena, en la Unidad de Genética del Instituto de Bioingeniería de la Universidad Miguel Hernández de Elche, para optar al grado de Doctor.

Elche, 7 de diciembre de 2022

La presente Tesis Doctoral, titulada “Funciones de *VCC* y las citoquininas en la morfogénesis del margen y la simetría bilateral de las hojas de Arabidopsis”, se presenta bajo la modalidad de **tesis por compendio** de la siguiente **publicación**:

Navarro-Cartagena, S., y Micol, J.L. (2022). Is auxin enough? Cytokinins and margin patterning in simple leaves. *Trends in Plant Science*, en prensa.

JOSÉ LUIS MICOL MOLINA, Catedrático de Genética de la Universidad Miguel Hernández de Elche (UMH)

HAGO CONSTAR:

Que el presente trabajo ha sido realizado bajo mi dirección y recoge fielmente la labor desarrollada por el Graduado Sergio Navarro Cartagena para optar al grado de Doctor. Las investigaciones reflejadas en esta Tesis se han desarrollado íntegramente en la Unidad de Genética del Instituto de Bioingeniería de la UMH, según los términos y condiciones definidos en el Plan de Investigación del doctorando, y cumpliendo los objetivos inicialmente previstos de forma satisfactoria y lo establecido en el Código de Buenas Prácticas de la UMH.

José Luis Micol Molina

Elche, 7 de diciembre de 2022

PIEDAD NIEVES DE AZA MOYA, Coordinadora del Programa de Doctorado en Bioingeniería de la Universidad Miguel Hernández de Elche por Resolución Rectoral 0169/17, de 1 de febrero de 2017

HACE CONSTAR:

Que da su conformidad a la presentación de la Tesis Doctoral de Don Sergio Navarro Cartagena, titulada “Funciones de *VCC* y las citoquininas en la morfogénesis del margen y la simetría bilateral de las hojas de Arabidopsis”, que se ha desarrollado en el Programa de Doctorado en Bioingeniería bajo la dirección del profesor José Luis Micol Molina.

Lo que firmo en Elche, a instancias del interesado y a los efectos oportunos, a siete de diciembre de dos mil veintidós.

Profesora PIEDAD NIEVES DE AZA MOYA
Coordinadora del Programa de Doctorado en Bioingeniería

A mis padres Pedro y Marisa y mi hermano Carlos.

A mi familia y mis amigos.

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I.- PREFACIO

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Siguiendo la normativa de la Universidad Miguel Hernández de Elche para la "Presentación de Tesis Doctorales por compendio de publicaciones", este documento se ha dividido en las partes siguientes:

I.- Este *Prefacio*.

II.- Un *Resumen* en español.

III.- Un *Summary* en inglés.

IV.- Una *Introducción*, en la que se presenta el tema de la Tesis y los antecedentes y objetivos del trabajo realizado.

V.- Un resumen de los *Materiales y métodos* de las publicaciones de la Tesis.

VI.- Un resumen de los *Resultados y discusión* de las publicaciones de la Tesis.

VII.- Un resumen de las *Conclusiones y perspectivas* de las publicaciones de la Tesis.

VIII.- Una *Bibliografía de los apartados IV-VI*; algunas de las referencias que incluye se repiten en las bibliografías de los artículos incluidos en esta memoria.

IX.- Un apartado de *Publicaciones,* que incluye las tres siguientes.

Navarro-Cartagena, S., y Micol, J.L. (2022). Is auxin enough? Cytokinins and margin patterning in simple leaves. *Trends in Plant Science*, en prensa [FI: 22,012].

Navarro-Cartagena, S., Wilson-Sánchez, D., y Micol, J.L. Cytokinins play a role in Arabidopsis leaf margin morphogenesis. En preparación.

X.- Un apartado de *Agradecimientos*.

Con el fin de reducir redundancias innecesarias e inconvenientes, se han mencionado en los apartados VI y VII solo los resultados y conclusiones más relevantes obtenidos en esta Tesis. El apartado IX recoge íntegramente el trabajo realizado.

II.- RESUMEN

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La simetría es un aspecto central de los planes corporales de muchos seres vivos pluricelulares, que influye en gran medida en su aptitud biológica. Aunque la mayoría de los cuerpos de los animales presentan simetría bilateral, la de muchos órganos de las plantas es radial, con algunas excepciones como las flores bilaterales de ciertos clados del reino vegetal y la mayoría de las hojas. Se ha recopilado una gran cantidad de información durante los últimos dos siglos sobre los tipos de simetría de las arquitecturas corporales de las plantas y los animales, tanto a nivel del organismo en su conjunto como de sus órganos e histotipos especializados. Sin embargo, se ha obtenido muy poca información de la perturbación experimental de la simetría bilateral y es bien poco lo que se sabe sobre su genética; constituyen excepciones los genes animales y vegetales causantes de la asimetría bilateral (generalmente denominada ruptura de la simetría bilateral) y los responsables de las transiciones entre los patrones de simetría radial y bilateral de las flores, que han ocurrido varias veces a lo largo de la evolución de las plantas.

La abundante información disponible sobre los genes determinantes de la polaridad anteroposterior en los animales, la próximo-distal en las plantas y la dorsoventral en ambos reinos contrasta con la prácticamente inexistente genética de la simetría bilateral. No pocos autores asumen que la simetría bilateral en las plantas y los animales es una consecuencia inexorable de la aparición de la dorsoventralidad en un espacio tridimensional. En esta Tesis se ofrece una visión panorámica de los conocimientos y las incógnitas sobre la simetría en el mundo biológico, con énfasis en la bilateralidad.

La disección genética de un proceso biológico suele tener como punto de partida el aislamiento de mutantes que manifiesten alteraciones en alguna de sus facetas; estos mutantes sirven para identificar y caracterizar los correspondientes genes causales de su fenotipo. Son pocos los mutantes de la brasicácea *Arabidopsis thaliana* (en adelante, Arabidopsis) que muestran un cierto grado de asimetría bilateral en sus hojas. De hecho, en el laboratorio de José Luis Micol se analizó el fenotipo morfológico de la roseta de 20.000 líneas de la colección SALK y se encontraron 706 mutantes foliares viables y fértiles, pero solo uno mostró asimetría bilateral en sentido estricto, sin ninguna perturbación obvia de la dorsoventralidad. Se denominó *desigual1-1* (*deal1-1*) a este mutante, cuya anotación indicaba que era portador de una inserción de ADN-T en el gen At2g32280. Durante el desarrollo de dicho trabajo, otros autores denominaron *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*) al gen At2g32280, nomenclatura que se sigue en esta Tesis. VCC pertenece a la subfamilia DESIGUAL (DEAL) de la familia de proteínas que contienen el

Domain of Unknown Function 1218 (DUF1218), que es específico de las plantas. Los alelos de insuficiencia de función de *VCC* causan, con penetrancia incompleta y expresividad variable, asimetría bilateral en las hojas de la roseta, debida a la posición, tamaño y número aleatorios de las protrusiones y senos del margen foliar.

La intercomunicación entre la auxina y las citoquininas modula muchos aspectos del desarrollo de las plantas, como la formación de los óvulos y las raíces laterales, entre otros. Se ha demostrado que las citoquininas favorecen la complejidad foliar durante el desarrollo de las hojas compuestas de la solanácea *Solanum lycopersicum* y la brasicácea *Cardamine hirsuta*. Sin embargo, no se ha propuesto papel alguno para estas hormonas en la morfogénesis del margen de las hojas simples. De hecho, se asume que la localización de la auxina basta para explicar las indentaciones de las hojas simples de Arabidopsis. Por otra parte, autores anteriores obtuvieron líneas mutantes y/o transgénicas cuyos fenotipos foliares no estudiaron en detalle, al no ser objeto de su interés; dichos fenotipos sugieren la implicación de las citoquininas en la morfogénesis del margen foliar de las hojas simples. En efecto, la reducción de la biosíntesis o la señalización de las citoquininas, o el incremento de su degradación, reducen la complejidad del margen foliar, haciéndolo más liso que el silvestre. Un ejemplo de ello es el triple mutante *ipt3 ipt5 ipt7* de Arabidopsis, portador de alelos nulos de tres de los genes que codifican isopentenil transferasas (IPT), las enzimas que catalizan la primera etapa de la ruta de biosíntesis de las citoquininas. En consecuencia, consideramos verosímil que las citoquininas jueguen un papel en la morfogénesis del margen en interacción con la auxina, tal como ocurre en otras facetas del desarrollo de las plantas. En esta Tesis comentamos dichos fenotipos aparentemente desapercibidos y discutimos las evidencias que respaldan nuestra hipótesis.

En los márgenes de los primordios foliares de la roseta de Arabidopsis, la localización de la auxina depende del transportador de su eflujo PIN-FORMED1 (PIN1), del factor de transcripción CUP-SHAPED COTYLEDON2 (CUC2), y de la propia hormona. Estos tres factores generan bucles de retroalimentación que crean dominios periódicos y alternos de auxina y CUC2, que especifican las protrusiones y los senos del margen de la hoja expandida, respectivamente. La formación de los máximos de auxina y las protrusiones depende de la dosis de CUC2. En efecto, el alelo hipomorfo *cuc2-3* del gen *CUC2* causa la ausencia de máximos de auxina en el margen del primordio foliar y márgenes lisos en las hojas expandidas. El alelo hipermorfo *cuc2-1D* causa los fenotipos moleculares, histológicos y morfológicos contrarios: la expansión de los dominios de expresión de CUC2 en los primordios foliares y un incremento en el número de protrusiones y senos en la hoja expandida.

El objetivo inicial de esta Tesis fue continuar la caracterización funcional del gen *VCC*. Dado que VCC tiene cuatro dominios transmembrana predichos, realizamos un ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida. Se identificaron así 263 presuntos interactores, codificados por 57 genes, que incluyen a componentes del complejo de elongación de los ácidos grasos, que sintetiza los de cadena muy larga. Dado que estos ácidos grasos inhiben la biosíntesis de las citoquininas, decidimos estudiar los efectos del exceso y el defecto de estas hormonas sobre la morfogénesis del margen foliar de Arabidopsis, mediante abordajes farmacológicos y genéticos: visualizamos dichos efectos en mutantes simples y múltiples en estadios tempranos del desarrollo de la hoja, mediante microscopía confocal de la expresión de transgenes que codifican marcadores fluorescentes en el primordio foliar, y tardíos, mediante análisis morfométrico de sus hojas expandidas.

En esta Tesis se ha sometido a contraste la hipótesis de que las citoquininas participan en la morfogénesis del margen de las hojas simples, en interacción con la auxina, tal como ocurre en otras facetas del desarrollo vegetal. Esta hipótesis se ha confirmado, ya que hemos demostrado (1) que la disminución de los niveles de las citoquininas o de la respuesta a estas hormonas reduce el número de máximos de auxina en los primordios foliares y de las protrusiones en el margen de las hojas expandidas, (2) que los patrones de expresión del gen *CUC2* y del marcador de respuesta a las citoquininas *TCSn::GFP* coinciden en la región basal del limbo de los primordios foliares del acceso silvestre Col-0, y (3) que la respuesta a las citoquininas depende de la actividad de los genes *CUC2* y *ERECTA* (*ER*).

También se demuestra en esta Tesis que (4) la asimetría bilateral de las hojas del mutante *vcc-2* se extrema mediante tratamiento con la citoquinina sintética 6-bencilaminopurina, y se suprime en el cuádruple mutante *vcc-2 ipt3 ipt5 ipt7*, y (5) que la respuesta a las citoquininas, visualizada con el marcador *TCSn::GFP*, es asimétrica entre las mitades izquierda y derecha del limbo de los primordios foliares *vcc-2*.

En conclusión, en esta Tesis se aportan evidencias de que las citoquininas participan en la morfogénesis del margen de las hojas simples de Arabidopsis, y de que en este proceso interaccionan con la auxina. Proponemos además que VCC regula la homeostasis de la auxina y las citoquininas en el primordio foliar, contribuyendo a coordinar el crecimiento de la hoja a lo largo de sus ejes mediolateral y próximo-distal, y al mantenimiento de la simetría bilateral de la hoja.

III.- SUMMARY

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Symmetry is a central aspect of the body plans of many multicellular living beings, which strongly influences their fitness. While most animal bodies exhibit bilateral symmetry, many plant organs have radial symmetry, exceptions being the bilateral flowers of some plant clades and most leaves. A vast amount of information has been gathered during the last two centuries on the types of symmetries observed in the body architecture of plants and animals, at the levels of whole organism, organs and specialized cell types. Much less information has been obtained from experimental perturbation of bilateral symmetry, and little is known on its genetics, exceptions being animal and plant genes causal for bilateral asymmetry (usually called bilateral symmetry breaking), and genes responsible for the symmetry pattern transitions—from radial to bilateral, and vice versa—shown by some plant clades. The wealth of information available on the genes responsible for anteroposterior (or proximodistal) and dorsoventral polarity is in stark contrast with the dearth of knowledge on bilateral symmetry genetics. Not few authors assume that bilateral symmetry in plants and animals is a mere, inexorable consequence of the appearance of dorsoventrality in a three-dimensional space, in other words, of the orthogonal intersection of the anteroposterior and dorsoventral axes. Here, we provide a panoramic view of the knowns and unknowns on the genetics of symmetry, with a focus on bilaterality.

The genetic dissection of a biological process usually involves the isolation of mutants affected in such process, which allows to identify and then characterize the corresponding causal genes. Only a few mutants of the Brassicaceae *Arabidopsis thaliana* (hereafter, Arabidopsis) have been described that show leaf bilateral asymmetry to some extent. In the laboratory of José Luis Micol, 20,000 lines from the SALK collection of Arabidopsis insertional mutants were screened for leaf phenotypes, finding 706 viable and fertile leaf mutants. Only one of these lines exhibited bilateral asymmetry in a strict sense, which was dubbed *desigual1-1* (*deal1-1*) and had been annotated to harbor a T-DNA insertion at the At2g32280 gene. The *deal1-1* mutant did not exhibit any obvious perturbation in dorsoventrality. During the progress of that work, other authors named At2g32280 as *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*), nomenclature that we follow in this Thesis. The VCC protein is a member of the DESIGUAL (DEAL) subfamily of the family of proteins containing the plant-specific Domain of Unknown Function 1218 (DUF1218). Loss-of-function alleles of the *VCC* gene cause, with incomplete penetrance and variable expressivity, bilateral asymmetry in rosette leaves, due to the random position, size and number of marginal protrusions and sinuses.

The interplay between auxin and cytokinins affects many aspects of plant development, including ovule development and lateral root initiation. Moreover, work in *Solanum lycopersicum* (Solanaceae) and *Cardamine hirsuta* (Brassicaceae) has shown that cytokinins favor complexity in the development of compound leaves. Nevertheless, the role of cytokinins in patterning the margins of simple leaves, such as those produced by Arabidopsis, remains unclear, and auxin localization has been considered enough to explain the serration of the simple leaves of Arabidopsis. Observations made by prior authors, which not received much attention, suggest the implication of cytokinins in margin morphogenesis of simple leaves. For example, Arabidopsis mutants or transgenic plants defective in cytokinin biosynthesis or signaling, or with increased cytokinin degradation, have smoother leaf margins than wild type leaves. Such is the case of the Arabidopsis *ipt3 ipt5 ipt7* triple mutant, which carries null alleles of three of the genes encoding isopentenyl transferases (IPT) that catalyze the first step of the cytokinin biosynthesis pathway. Therefore, we hypothesized that cytokinins play a role in leaf margin morphogenesis via crosstalk with auxin, as occurs in other aspects of plant development. Here, we discuss the unnoticed evidence that supports such hypothesis.

In Arabidopsis rosette leaf primordia margins, auxin localization depends on the PIN-FORMED1 (PIN1) auxin efflux carrier, the CUP-SHAPED COTYLEDON2 (CUC2) transcriptional regulator, and auxin itself. These factors generate feedback loops that in turn create periodic, interspersed domains of auxin and CUC2, which specify protrusions and sinuses of the developing leaf, respectively. The formation of auxin maxima and protrusions directly depend on the dose of CUC2; this is shown by the loss-of-function *cuc2-3* allele, which causes the absence of auxin maxima and protrusions, and smooths leaf margins. Plants carrying the *cuc2-1D* gain-of-function allele display the opposite molecular, histological and morphological leaf phenotypes: expanded CUC2 expression domains, and extra lobes and sinuses.

The initial objective of this Thesis was to continue the functional characterization of the *VCC* gene. Since VCC has four predicted transmembrane domains, we conducted a split-ubiquitin yeast two-hybrid membrane-based assay, finding 263 putative interactors, encoded by 57 genes, including components of the fatty acid elongase complex, which synthesizes Very-Long-Chain Fatty Acids (VLCFAs). Given that VLCFAs are known to inhibit cytokinin biosynthesis, we studied the effects of cytokinin excess and depletion, through pharmacological and genetic approaches, on Arabidopsis leaf margin morphogenesis: we visualized in simple and multiple mutant plants such effects in early stages of leaf development, by confocal imaging of the expression of transgenes encoding fluorescent markers in leaf

primordia late stages of leaf development, as well as in late stages, by morphometry of expanded leaves.

The main objective of this Thesis was to test the hypothesis that cytokinins play a role in simple leaf margin morphogenesis, in crosstalk with auxin, as already known for other developmental events. We found (1) that reduced cytokinin content or response causes a reduction in the number of auxin maxima in Arabidopsis leaf primordia margins, as well as in the serration of expanded leaves, (2) that the patterns of expression of the *CUC2* gene and the *TCSn::GFP* cytokinin response marker overlap at the basal region of Col-0 primordia laminae, both of which are excluded from margin lobes, and (3) that cytokinin response in leaf primordia depends upon *CUC2* and *ERECTA* (*ER*) gene activity.

We also found that (4) the bilateral asymmetry phenotype of *vcc-2* leaves is severed by treatment with exogenous cytokinin and suppressed in the *vcc-2 ipt3 ipt5 ipt7* quadruple mutant, and (5) that cytokinin response, visualized by the *TCSn::GFP* marker, is asymmetric between the left and right halves of *vcc-2* leaf primordia laminae.

In conclusion, this Thesis provides evidence that cytokinins play a role in the patterning of the margin of the simple leaves of Arabidopsis, in crosstalk with auxin. In addition, we propose that VCC regulates both auxin and cytokinin homeostasis at the margins of leaf primordia, contributing to coordinate leaf growth along the medio-lateral and proximo-distal axes and maintaining leaf bilateral symmetry.

IV.- INTRODUCCIÓN

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IV.1.- La simetría en el mundo biológico

IV.1.1.- Planes corporales de los seres vivos

La simetría es un aspecto fundamental del plan corporal de los seres vivos, con importantes consecuencias en sus funciones y éxito evolutivo (Moubayidin y Ostergaard, 2015; Genikhovich y Technau, 2017). Se dispone de mucha información sobre los tipos de simetría que exhiben los animales y las plantas, tanto a nivel del organismo en su conjunto como de sus órganos e histotipos (Hudson, 2000; Manuel, 2009; Muñoz-Nortes *et al.*, 2014). Las simetrías de los cuerpos de los organismos pluricelulares se clasifican en función de sus elementos: los planos y ejes de simetría. La rotación de un cuerpo en torno a un eje de simetría rinde la misma imagen en cualquier ángulo. Un plano de simetría divide a un cuerpo en dos mitades iguales, cada una de las cuales es imagen especular de la otra. Un cuerpo es bilateral si presenta un solo plano y ningún eje de simetría, y radial, si presenta un solo eje y n (n-radial) o infinitos (∞-radial) planos de simetría que lo cruzan (Holló, 2015). Se denomina eje de polaridad a aquel a lo largo del cual se observa variación morfológica (Manuel, 2009).

Los cuerpos asimétricos, como los de muchas demosponjas o los placozoos, no presentan planos o ejes de simetría y solo manifiestan un eje de polaridad, que distingue la parte basal, en contacto con el sustrato, de la apical (Figura 1A, en la página 9). Los de simetría esférica o cilíndrica tienen infinitos planos de simetría. Son esféricos los cuerpos de las demosponjas del género *Tethia* y las colonias de las algas del género *Volvox*; estas estructuras tienen además infinitos ejes de simetría, y polaridad radial, desde el centro al exterior de la esfera (Figura 1B). Son estructuras cilíndricas, con simetría ∞-radial, los cuerpos de algunas esponjas calcáreas y el columnar de los pólipos de los cnidarios hidrozoos y cubozoos, que presentan un eje de simetría y dos de polaridad; uno de estos últimos es radial y el otro, oral-aboral y coincidente con el de simetría (Figura 1C).

Las simetrías n-radial y bilateral son las más representadas entre los seres vivos. Los cuerpos u órganos con simetría n-radial poseen una unidad estructural que se repite n veces alrededor de un eje central de simetría, con n planos de simetría. Son ejemplos de simetría n-radial en el reino animal las esponjas calcáreas de organización siconoide y la mayoría de las esponjas silíceas hexactinélidas, así como los ctenóforos, muchos cnidarios y los equinodermos adultos; estos últimos constituyen una singularidad, ya que sus formas larvarias presentan simetría bilateral (Lowe y Wray, 1997). En las plantas, tienen simetría radial órganos como las raíces, el tallo y la mayoría de las flores (Figura 1D).

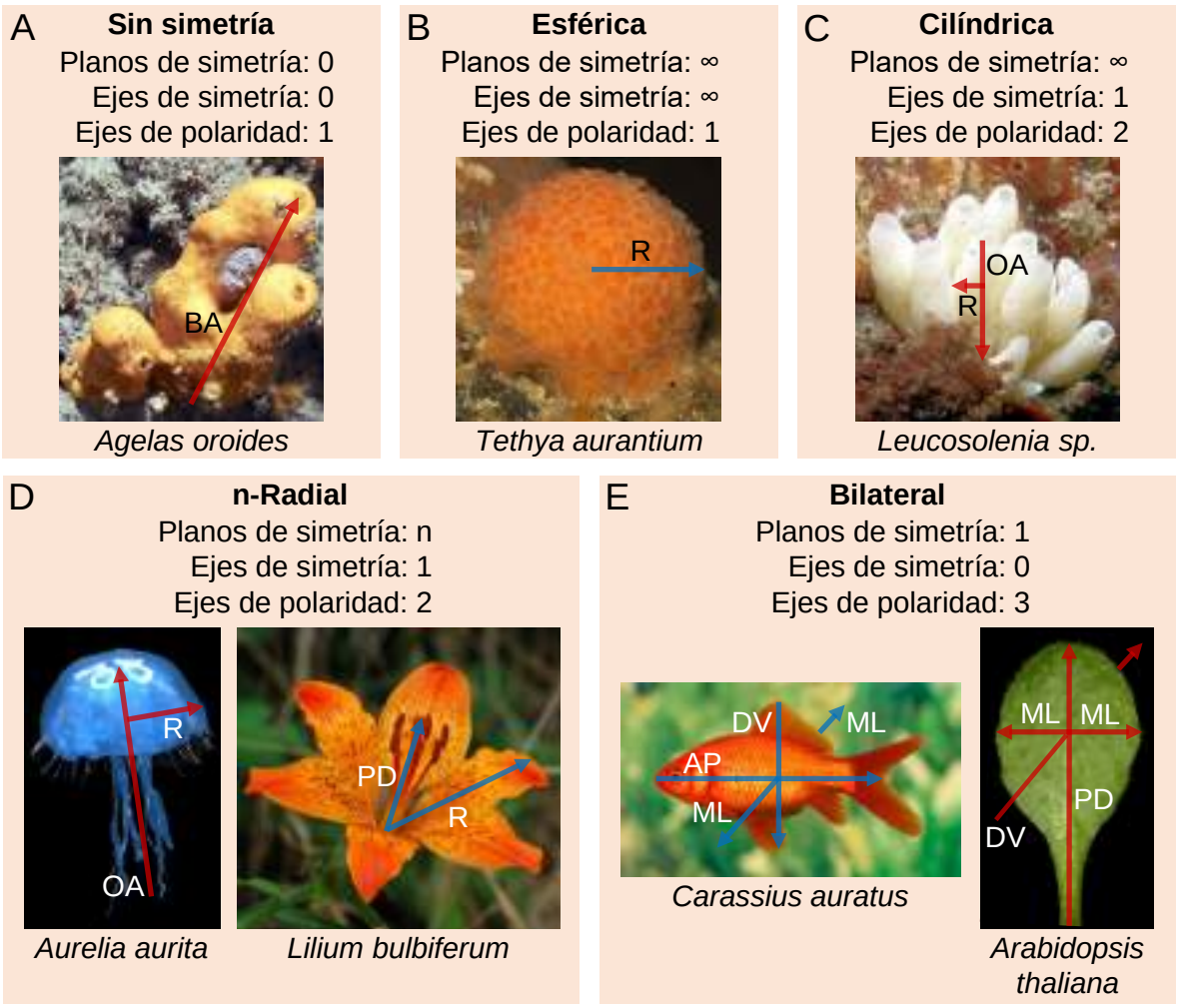

Figura 1.- Ejemplos de algunos de los tipos de simetría en los seres vivos. (A) La demosponja asimétrica *Agelas oroides*. (B) La demosponja esférica *Tethya aurantium*. (C) La esponja calcárea cilíndrica *Leucosolenia sp.* (D) La simetría n-radial del cnidario *Aurelia aurita* o medusa común (n = 4; a la izquierda), y de la flor de la azucena anaranjada *Lilium bulbiferum* (n = 6; a la derecha). (E) La simetría bilateral de la carpa dorada *Carassius auratus* (a la izquierda) y la hoja de *Arabidopsis thaliana* (a la derecha; se muestra su cara dorsal o haz). El número de planos, ejes de simetría y ejes de polaridad se indica en cada caso. Las flechas indican ejes de polaridad: BA (basal-apical), R (radial), OA (oral-aboral), PD (próximo-distal), AP (anteroposterior), DV (dorsoventral) y ML (mediolateral). Las imágenes se han tomado de (A) https://www.sub-vidayfoto.com/indice-alfabetico/agelas-oroides-(schmidt-1864)-/78/, (B) https://www.cibsub.cat/bioespecie_es-tethya_aurantium-32831, (C) https://alchetron.com/Leucosolenia, (D) https://www.turbosquid.com/es/3d-models/3d-model-moon-jellyfish-aurelia-aurita-1354313 y https://commons.wikimedia.org/wiki/File:Liliaceae_-_Lilium_bulbiferum_var._croceum-4.JPG y (E) https://www.malawimascotas.com/producto/cometa-naranja/ y Jover-Gil (2005).

Presentan simetría bilateral los cuerpos de casi todos los animales (Holló, 2015), algunas flores y la mayoría de las hojas de las plantas (Preston y Hileman, 2009). La simetría bilateral se caracteriza por un solo plano de simetría, ningún eje de simetría y tres de polaridad

(Figura 1E): el anteroposterior en los animales y el próximo-distal en las plantas (equivalente al oral-aboral), el dorsoventral (perpendicular al anteroposterior o próximo-distal) y el mediolateral. Este último es análogo al radial de otros tipos de simetría, pero queda restringido a una única dirección como consecuencia de la dorsoventralidad, y se repite de manera especular a ambos lados del plano de simetría (Palmer, 2004). En las hojas de las plantas, el eje dorsoventral se manifiesta externamente en sus caras adaxial o dorsal (el haz) y abaxial o ventral (el envés) (Husbands *et al.*, 2009).

IV.1.2.- La simetría bilateral

IV.1.2.1.- Diferencias entre los planes corporales de los animales y las plantas

Los distintos modos de vida y adaptación al medio de los animales y las plantas están asociados a diferentes modelos de desarrollo. El de los animales es fundamentalmente embrionario, ya que la embriogénesis construye un individuo que presenta el plan corporal del adulto. El de las plantas, cuyo plan corporal es más simple que el de los animales (Alberts *et al.*, 1994), es fundamentalmente postembrionario, lo que les permite adaptarse mejor a los cambios en el ambiente, en particular los relacionados con la disponibilidad de luz y nutrientes. En el reino vegetal, el desarrollo postembrionario se inicia en los meristemos apicales de la raíz y el tallo, que están formados por células indiferenciadas y pluripotentes, a partir de las cuales se generan las partes subterránea y aérea de la planta, respectivamente (Lyndon, 1990; Sachs, 1991; Holwell, 1998).

La simetría bilateral ha aparecido varias veces a lo largo de la evolución de los animales y las plantas. En los animales, un plan corporal con simetría bilateral permite una movilidad más eficiente, que es esencial para su supervivencia (Finnerty, 2003). De hecho, casi todos los animales son bilaterios. Las plantas son sésiles y la mayoría de sus estructuras, como la raíz, el tallo y muchas flores y frutos, presentan simetría radial. Sin embargo, la mayoría de las hojas y algunas flores son bilateralmente simétricas, aparentemente como consecuencia inevitable de la dorsoventralidad que han adquirido durante su evolución. La simetría bilateral optimiza la captación de luz y el intercambio gaseoso en las hojas, y la polinización de las flores (Hudson, 2000; Husbands *et al.*, 2009; Preston y Hileman, 2009; Endress, 2012).

IV.1.2.2.- Origen evolutivo de la simetría bilateral

Aunque la simetría bilateral en los seres vivos está ampliamente extendida en nuestro planeta desde hace cientos de millones de años, no existe un consenso sobre su origen evolutivo en el reino animal. Los cnidarios son metazoos basales que constituyen un grupo

separado de los bilaterios; sin embargo, los de las clases Hydrozoa y Anthozoa tienen estructuras internas con simetría bilateral de función no relacionada con la locomoción, que les sirven para la canalización del agua a través de sus cavidades internas (Finnerty, 2005).

Hace 440-410 millones de años aparecieron las primeras plantas vasculares terrestres, cuyas estructuras derivadas del meristemo apical del tallo eran radiales. Las primeras hojas, que aparecieron hace 410-360 millones de años, fueron micrófilas: simples estructuralmente y sin ramificaciones vasculares. Hace unos 360 millones de años aparecieron las primeras hojas megáfilas, con ramificaciones vasculares y organizadas sobre el tallo con una filotaxia definida, crecimiento determinado, dorsoventralidad y forma laminar. Esta última característica fue crucial para optimizar la función fotosintética, gracias al incremento de la ratio superficie/masa, que facilita el intercambio gaseoso y la captación de la luz solar (Beerling *et al.*, 2001; Cronk, 2001; Osborne *et al.*, 2004; Beerling, 2005; Tomescu, 2009).

Existe una gran variación morfológica entre las flores de las angiospermas, que se clasifican por su simetría en tres clases: radiales o actinomorfas, bilaterales o zigomorfas y asimétricas (Endress, 2001; Almeida y Galego, 2005). Se acepta generalmente que la simetría ancestral es la radial (Endress y Doyle, 2009; Sauquet *et al.*, 2017) y que las flores zigomorfas y asimétricas proceden de las actinomorfas (Endress, 1999; Cubas *et al.*, 2001; Cubas, 2004; Feng *et al.*, 2006), por adaptación a los polinizadores (Fenster *et al.*, 2009; Ushimaru *et al.*, 2009; Ambika Manirajan *et al.*, 2016; Krishna y Keasar, 2018). Las primeras flores zigomorfas aparecieron en el Cretácico superior, 30-40 millones de años después de las primeras flores conocidas (Crane *et al.*, 1995; Crepet, 1996; Soltis *et al.*, 1999). A lo largo de la evolución han ocurrido 23 transiciones de flores radiales a bilaterales en las monocotiledóneas y 46 en las dicotiledóneas (Group, 2009; Citerne *et al.*, 2010). Las flores zigomorfas están presentes en las dicotiledóneas de los clados Asteraceae, Dipsacales, Lamiales y Fabaceae, y el Orchidaceae de las monocotiledóneas (Endress, 2001; Hileman, 2014a; Spencer y Kim, 2018). La transición contraria, de flores bilaterales a radiales, se ha producido en las Antirrhinaceae (Endress, 2001; Endress, 2012) y Lamiaceae (Zhong *et al.*, 2017).

El análisis de algunos mutantes de *Antirrhinum majus* ha revelado que la expresión asimétrica de unos pocos genes es suficiente para determinar la aparición de un eje de polaridad dorsoventral y de flores bilaterales (Busch y Zachgo, 2009; Preston y Hileman, 2009; Rosin y Kramer, 2009; Preston *et al.*, 2011). Los factores de transcripción CYCLOIDEA (CYC) y DICHOTOMA (DICH), de la familia TCP (por TEOSINTE BRANCHED1, CYCLOIDEA y PROLIFERATING CELL FACTOR1 y 2), confieren identidad a la región dorsal de las flores de *Antirrhinum majus* (Green *et al.*, 2010; Kennaway *et al.*, 2011). De hecho, las flores de los dobles mutantes *cyc dich* manifiestan simetría radial y pérdida de identidad dorsal (Luo *et al.*,

1996; Luo *et al.*, 1999; Hileman y Baum, 2003; Martín-Trillo y Cubas, 2010; Hileman, 2014b). El factor de transcripción DIVARICATA (DIV), de la familia MYB, confiere identidad ventral (Galego y Almeida, 2002). RADIALIS (RAD) es otro factor de transcripción que se expresa en el dominio dorsal y restringe la actividad de DIV al ventral (Rose *et al.*, 1999; Corley *et al.*, 2005; Raimundo *et al.*, 2013).

IV.1.2.3.- La asimetría bilateral en la naturaleza

Se ha escrito mucho sobre el origen evolutivo de los ejes de polaridad anteroposterior y dorsoventral, y el de los genes y mecanismos moleculares responsables de su establecimiento (Genikhovich y Technau, 2017; Du *et al.*, 2018). Sin embargo, la información disponible sobre la especificación del eje mediolateral es escasa. No se han encontrado mutantes de *Drosophila melanogaster* con pérdida de la simetría bilateral en alguna de sus estructuras corporales (Smith y Sondhi, 1960; Purnell y Thompson, 1973; Coyne, 1987; Tuinstra *et al.*, 1990). Sin embargo, se ha logrado incrementar la asimetría en el número de quetas esternopleurales entre las mitades izquierda y derecha del notum de este díptero mediante selección directa en poblaciones de laboratorio (Mather, 1953; Thoday, 1958; Polak y Trivers, 1994). No obstante, no se han encontrado genes responsables de la simetría bilateral, razón por la que se asume que no es más que una consecuencia inexorable de la dorsoventralidad (Palmer, 2004). Esto contrasta con la existencia de genes y mecanismos desencadenantes de varios tipos de desviación o pérdida de la simetría bilateral, algunos de los cuales se comentan en este apartado (Bock y Marsh, 1991; Vandenberg y Levin, 2013). Resulta en consecuencia paradójico que no existan genes para establecer o mantener la simetría bilateral, pero sí para desviarse de ella.

Se denomina asimetría fluctuante al conjunto de desviaciones sutiles y aleatorias de la simetría bilateral que causan el ambiente y los polimorfismos genéticos en una población (Palmer, 1996). Por ejemplo, el estrés por calor incrementa la asimetría fluctuante en la mosca *Scathophaga stercoraria* (Hosken *et al.*, 2000) y en las hojas del mutante *sub-2* de Arabidopsis; *sub-2* es un alelo del gen *STRUBBELIG* (*SUB*), también denominado *SCRAMBLED* (*SCM*), que codifica un receptor de tipo quinasa (Lin *et al.*, 2012).

Se denomina asimetría conspicua a las diferencias controladas genéticamente entre las dos mitades de un organismo u órgano. Puede ser direccional, si se manifiesta de la misma forma en todos los individuos de una determinada especie, y aleatoria o antisimetría, si en la población coexisten dos formas especulares (Palmer, 2004). Un ejemplo de la primera, que afecta a la organización corporal interna y ocurre durante el desarrollo embrionario, es el posicionamiento del corazón en los vertebrados, que se encuentra normalmente desplazado

hacia la mitad izquierda del cuerpo (Fishman y Chien, 1997). En este proceso intervienen proteínas de la superfamilia del Transforming Growth Factor Beta (TGF- β): Lefty y Nodal especifican la mitad izquierda, y las Bone Morphogenetic Proteins (BMP), la derecha (Meno *et al.*, 1998; Hamada *et al.*, 2002; Ocaña *et al.*, 2017).

Existen varios ejemplos de antisimetría externamente visible durante el desarrollo postembrionario. El cangrejo *Homarus americanus* tiene dos pinzas equivalentes en tamaño en sus estadios juveniles tempranos, que crecen diferencialmente en función de su uso, de modo que la pinza más utilizada se hipertrofia (Emmel, 1908; Govind y Pearce, 1986; 1989a; Govind, 1989b). Los pleuronectiformes o peces planos son los vertebrados con un mayor nivel de asimetría en su organización corporal y comportamiento, teniendo ambos ojos situados en el mismo lado de la cabeza (Schreiber, 2006; Power *et al.*, 2008; Laudet, 2011; Schreiber, 2013; Shao *et al.*, 2017). Por último, la dirección del enrollamiento de la concha en los moluscos ha suscitado un cierto interés al respecto de la asimetría entre las partes izquierda y derecha del cuerpo de un animal. El gen *Lsdia1*, que está relacionado con la actina, determina la dirección del enrollamiento de la concha del caracol de agua dulce *Lymnaea stagnalis*. Este gen se expresa en los individuos de enrollamiento espiral dextral desde la oviposición hasta la gastrulación, pero no en los sinistrales (Abe y Kuroda, 2019).

Las flores de las plantas pueden presentar dos tipos de asimetría (Endress, 2001). Una de ellas consiste en la disposición asimétrica del estilo y el estigma, que se curvan hacia un lado, en lugar de hallarse en el centro del órgano. Este rasgo ha aparecido por convergencia en familias distantes, como las pontederiáceas y las leguminosas (Wang *et al.*, 1995; Barrett *et al.*, 2000). En las flores con estivación de pétalos retorcidos, la contorsión puede ser levógira (en sentido contrario al de las agujas de un reloj) o dextrógira. Este segundo tipo de asimetría está representado principalmente entre las rósidas y las astéridas (Endress, 2001).

IV.2.- Funciones morfogenéticas de la auxina y las citoquininas

IV.2.1.- La auxina

Experimentos realizados entre 1880 y 1928 demostraron la existencia de una sustancia aparentemente responsable de que la incidencia lateral de luz sobre el ápice de los coleóptilos de las gramíneas causara la curvatura de su base. Dicha sustancia, el ácido indol-3-acético (IAA), fue denominada auxina por el verbo griego *auxano* (crecer) (Haagen-Smit *et al.*, 1946). La auxina regula numerosos procesos fisiológicos de las plantas, como la dominancia apical, los tropismos, la filotaxia, la elongación del tallo, la inducción de la división celular en el cámbium y la iniciación de las raíces (Teale *et al.*, 2006; Abel y Theologis, 2010).

Además del IAA, existen otras auxinas naturales minoritarias como los ácidos indol-3-butírico (IBA), 4-cloroindol-3-acético (4-Cl-IAA o cloroauxina) y fenilacético (PAA) (Casanova-Sáez *et al.*, 2021). El contenido en IAA depende de su biosíntesis e inactivación por conjugación o ciertos procesos catabólicos. Existen dos rutas de biosíntesis del IAA, la principal de las cuales tiene como precursor al L-triptófano. La hidrólisis de los conjugados de IAA también produce auxina activa (Casanova-Sáez *et al.*, 2021). Por otro lado, el contenido en auxina de cada tejido también depende del transporte polar de esta hormona, que ocurre fundamentalmente gracias a los transportadores de su eflujo PIN-FORMED (PIN; Teale *et al.*, 2006; Zažímalová *et al.*, 2010) e influjo AUXIN1/LIKE-AUX1 (AUX/LAX; Péret *et al.*, 2012; Swarup y Péret, 2012). A nivel global, el transporte de la auxina se produce en dirección basípeta, desde el meristemo apical del tallo y las hojas jóvenes hacia las raíces (Abel y Theologis, 2010). El transporte a distancias cortas dentro de un determinado órgano también tiene consecuencias importantes sobre el desarrollo; este es el caso de la formación de las raíces laterales (Benková *et al.*, 2003; Teale *et al.*, 2006).

Los miembros de la familia AUXIN RESPONSE FACTOR (ARF) son 22 en Arabidopsis y se unen a los auxin-responsive elements (ARE) de los promotores de los genes de respuesta primaria a auxina, activando su transcripción (Li *et al.*, 2016). Arabidopsis cuenta con 29 proteínas Aux/IAA, que forman heterodímeros con los ARF e impiden que estos últimos activen la transcripción de sus dianas (Tian *et al.*, 2002). A su vez, la auxina promueve la degradación de las Aux/IAA.

La transducción de la señal de la auxina se inicia con su unión a los miembros de la familia de proteínas F-box, que actúan como receptores en el núcleo (Ljung, 2013): TRANSPORT INHIBITION RESISTANT1 (TIR1), AUXIN SIGNALING F-BOX PROTEIN1 (AFB1), AFB2 y AFB3 (Dharmasiri *et al.*, 2005; Kepinski y Leyser, 2005; Calderón-Villalobos *et al.*, 2010). Estas proteínas F-Box forman parte del complejo SCF (de Skp1, Cullin y F-Box) y tras su unión a la auxina, propician la poliubiquitinación de las Aux/IAA, que quedan así marcadas para su degradación por el proteasoma 26S (Gray *et al.*, 2001). En síntesis, la auxina promueve la degradación de las Aux/IAA, lo que a su vez libera a los ARF, que tras su liberación pueden inducir la transcripción de sus genes diana (Teale *et al.*, 2006).

IV.2.2.- Las citoquininas

Las citoquininas son fitohormonas derivadas de la adenina. Presentan un anillo de 6-aminopurina con una cadena lateral aromática, como en la cinetina y la 6-bencilaminopurina, o isoprenoide, como en la isopenteniladenina (iP), la *trans*-zeatina (tZ), la *cis*-zeatina (cZ) y la dihidrozeatina (DZ). Estas últimas son las citoquininas activas más abundantes (Sakakibara,

2006). Las citoquininas deben su nombre a su capacidad de inducir la citocinesis. La primera citoquinina estudiada fue la cinetina, que promueve en el tabaco el crecimiento del tallo, inhibe el de las raíces y estimula la división celular y la coloración verde de los callos (Miller *et al.*, 1955; Miller *et al.*, 1956). Se estableció poco después que la ratio citoquininas/auxina determina la identidad de los órganos que se desarrollan a partir de callos en cultivos *in vitro* (Skoog y Miller, 1957), que las citoquininas inhiben la senescencia foliar (Richmond y Lang, 1957) y que son antagonistas de la auxina en la dominancia apical (Wickson y Thimann, 1958). Posteriormente, se aislaron la *trans*-zeatina (Miller, 1961) y las restantes citoquininas (Skoog *et al.*, 1965; Mok y Mok, 2001). En las páginas 61-65 se aporta información adicional al respecto del metabolismo y las funciones de estas fitohormonas.

IV.3.- Estructura y función del meristemo apical del tallo

Se distinguen en el meristemo apical del tallo de todas las plantas estudiadas con cierto grado de detalle el centro organizador (organizing center, OC) y las zonas central (central zone, CZ), periférica (peripheral zone, PZ) y medular (rib zone, RZ) (Lopes *et al.*, 2021; Wu *et al.*, 2021). La CZ ocupa el ápice del meristemo, sobre el OC; estas dos regiones mantienen un número constante de células madre pluripotentes (Brand *et al.*, 2000; Schoof *et al.*, 2000) (Figura 2A, en la página 16). Las células originadas en el ápice van desplazando a las precedentes, que al alcanzar la PZ incrementan su tasa de división y contribuyen a la iniciación de los órganos laterales aéreos, como las hojas y las flores (Bar y Ori, 2014; Shi y Vernoux, 2019). Por su parte, las células que son desplazadas hacia la RZ, situada debajo del OC, generan los tejidos internos del tallo (Ha *et al.*, 2010a).

El meristemo apical del tallo de las dicotiledóneas presenta tres capas celulares estratificadas: la epidérmica (L1, por layer 1) y la subepidérmica (L2) conforman la túnica y tienen un grosor de una sola célula, mientras que el de la interna (L3) o corpus es de varias células (Barton, 2010; Lopes *et al.*, 2021). La división de las células de las capas L1 y L2 es periclinal (paralela a la superficie del meristemo) y las de la L3 se dividen en todas las direcciones. Para la iniciación de un primordio, las células de la capa L2 pasan a dividirse anticlinalmente (perpendicularmente a la superficie del meristemo). La capa L2 genera la mayoría de los tejidos de la hoja, la L1, la epidermis, y la L3, los tejidos vasculares (Barton, 2010). Las antes mencionadas CZ y PZ se solapan parcialmente con la L1, la L2 y la L3, mientras que el OC y la RZ se encuentran en la L3. Además, en la parte más apical de la L3 existe una región de solapamiento entre la CZ y el OC (Lopes *et al.*, 2021) (Figura 2A, en la página 16).

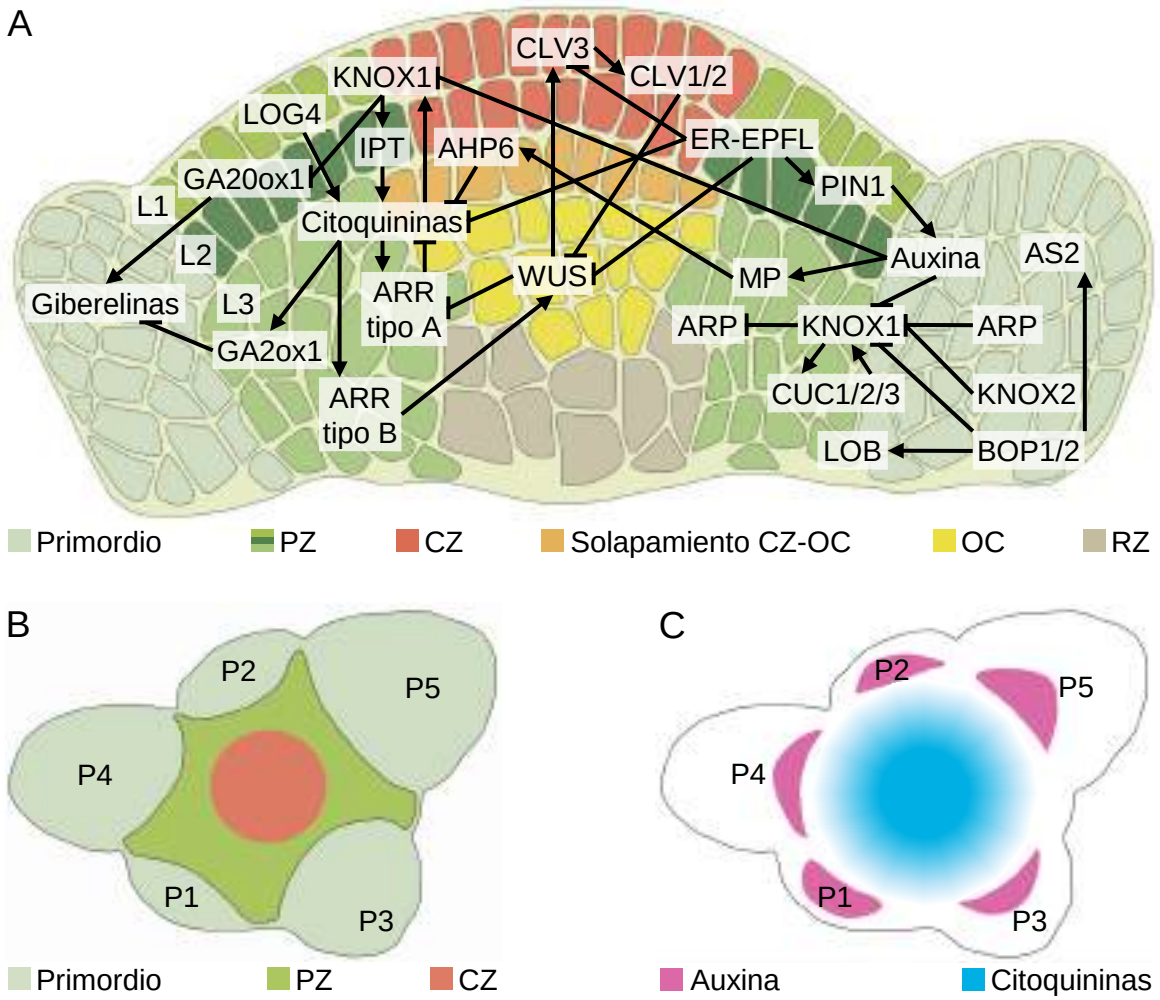

Figura 2.- Estructura del meristemo apical del tallo de Arabidopsis e interacciones entre los factores responsables de su actividad meristemática y de la iniciación foliar. (A) Representación esquemática de un corte transversal del meristemo apical del tallo, con detalles de la regulación de su mantenimiento y de la iniciación foliar. L1, L2 y L3: capas 1 y 2 (túnica), de una célula de grosor, y 3 (corpus) de varias, respectivamente. PZ: zona periférica. CZ: zona central. OC: centro organizador. RZ: zona medular. Los factores que se indican en este esquema se representan sin cursiva porque pueden corresponder a una proteína, al gen que la codifica o a ambos. Las flechas terminadas en punta o con una barra perpendicular indican respectivamente activación o represión. (B y C) Representación esquemática de una vista cenital del meristemo apical del tallo. (B) Se representan la PZ y la CZ, así como varios primordios foliares y su orden de emergencia (P1, P2…). (C) Distribución espacial de la auxina y las citoquininas, que determinan la filotaxia del tallo. Adaptado a partir de (A) Lopes *et al.* (2021) y Wu *et al.* (2021), y (B y C) Schaller *et al.* (2015).

El gen *CLAVATA 3* (*CLV3*; Fletcher *et al.*, 1999) de Arabidopsis se expresa en la CZ, y *WUSCHEL* (*WUS*; Mayer *et al.*, 1998), en el OC. WUS es un factor de transcripción con dominio homeobox que se desplaza del OC a la CZ, reprimiendo la diferenciación y promoviendo en consecuencia el mantenimiento de las células madre (Yadav *et al.*, 2011; Daum *et al.*, 2014). En la CZ, WUS induce la expresión de *CLV3* (Brand *et al.*, 2002), que

codifica un péptido pequeño que se une a diferentes receptores, como CLV1 y CLV2, para reprimir la expresión de *WUS* (Clark *et al.*, 1997; Jeong *et al.*, 1999; Betsuyaku *et al.*, 2011; Hu *et al.*, 2018). De este modo, WUS y CLV3 forman un bucle de retroalimentación negativa que regula el número de células madre en el meristemo apical del tallo (Brand *et al.*, 2000; Schoof *et al.*, 2000) (Figura 2A). La insuficiencia de la función de *CLV3* provoca la desrepresión de *WUS* y la generación de meristemos más grandes que el silvestre, en los que se acumulan células no diferenciadas y producen un número de órganos laterales superior al silvestre (Fletcher *et al.*, 1999). La insuficiencia de la función de *WUS* causa la desaparición del nicho de las células madre y del propio meristemo (Laux *et al.*, 1996).

Son varias las hormonas que juegan un papel importante en la actividad del meristemo apical del tallo. Por ejemplo, la auxina interviene en la iniciación del primordio foliar y contribuye a su desarrollo posterior (Hay *et al.*, 2006), y las citoquininas promueven el desarrollo y la actividad del meristemo (Su *et al.*, 2011), tal como se describe más adelante. Además, la presencia y actividad de las giberelinas es específica del desarrollo foliar (Hay *et al.*, 2002). El transporte polar de la auxina hacia las regiones en las que se iniciarán los primordios foliares conlleva la disminución de los niveles de esta hormona en el OC, que mantiene así su tamaño y estado indiferenciado (Shi *et al.*, 2018).

Los factores de transcripción ARABIDOPSIS RESPONSE REGULATORS (ARR) de tipo B son los efectores últimos de la cascada de señalización de las citoquininas. ARR1, ARR10 y ARR12 activan indirectamente la expresión de *WUS* (Lindsay *et al.*, 2006; Zhang *et al.*, 2017). Por su parte, WUS reprime directamente la transcripción de los genes *ARR5*, *ARR6*, *ARR7* y *ARR15*, que codifican factores de transcripción ARR de tipo A (inhibidores de la respuesta a citoquininas), estableciendo así un bucle de retroalimentación positiva con las citoquininas (Leibfried *et al.*, 2005).

Los factores de transcripción KNOX1, principalmente SHOOT MERISTEMLESS (STM), favorecen un incremento de los niveles de las citoquininas al inducir la expresión de los genes *ISOPENTENYLTRANSFERASE* (*IPT*), que contribuyen a la síntesis de estas hormonas. Las proteínas KNOX1 también causan una disminución de los niveles de las giberelinas, al reprimir la expresión de *GA 20-oxidase 1* (*GA20ox1*) e inducir indirectamente la de *GA2ox1*; estos dos genes participan en la síntesis y degradación de las giberelinas, respectivamente (Hay *et al.*, 2002; Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Hay y Tsiantis, 2010; Scofield *et al.*, 2013; Coudert *et al.*, 2019). El tratamiento con citoquininas o el incremento de su síntesis endógena normalizan parcialmente el fenotipo del mutante *stm*, en el que no se mantiene el nicho de células madre del meristemo apical del tallo (Yanai *et al.*, 2005; Scofield *et al.*, 2013). El incremento de los niveles de citoquininas conlleva el de los

ARNm de dos genes *KNOX1*: *STM* y *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1* (*KNAT1*). Esta última observación sugiere la existencia de una retroalimentación positiva entre la expresión de los genes *KNOX1* y la síntesis y la señalización de las citoquininas (Rupp *et al.*, 1999). El gen *LONELY GUY 4* (*LOG4*) se expresa en la capa L1 y codifica una citoquinina ribósido-5′-monofosfato fosforribohidrolasa que activa las citoquininas, que se difunden formando un gradiente hacia las capas internas del meristemo (Chickarmane *et al.*, 2012) (Figura 2A, en la página 16).

Las proteínas de la familia EPIDERMAL PATTERNING FACTOR/EPF-LIKE (EPF/EPFL; Hara *et al.*, 2009; Takata *et al.*, 2013), que se expresan en la PZ, interaccionan como ligandos con los receptores de la familia ERECTA (ER, ER-LIKE 1 [ERL1] y ERL2; Shpak *et al.*, 2004; Shpak, 2013). Estos últimos se expresan en todo el meristemo, y su interacción con los EPF/EPFL favorece la iniciación de los primordios foliares al inducir la expresión de *PIN1* en la PZ (Chen *et al.*, 2013); a la vez, dicha interacción limita el desarrollo del meristemo apical del tallo al inhibir la expresión de *CLV3* y *WUS* (Kimura *et al.*, 2018; Kosentka *et al.*, 2019; Zhang *et al.*, 2021). En el triple mutante *er erl1 erl2*, el tratamiento con citoquininas incrementa la expresión de *CLV3* y el número de células del meristemo apical del tallo; esta observación sugiere que los receptores ER contrarrestan los efectos del aumento de la concentración de citoquininas (Uchida *et al.*, 2013). En las regiones en las que se acumula la auxina, MONOPTEROS (MP, también denominado ARF5) activa la expresión de *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*), que codifica una proteína inhibidora de la señalización de las citoquininas (Besnard *et al.*, 2014) (Figura 2A, en la página 16). La actividad de la auxina es mayor, y la de las citoquininas, menor, en los primordios foliares que en las células indiferencias del meristemo apical del tallo (Shani *et al.*, 2006; Zürcher *et al.*, 2013; Schaller *et al.*, 2015). Esta alternancia entre la auxina y las citoquininas es necesaria para la conservación de la filotaxia, la disposición regular a lo largo del tallo de los órganos laterales que se inician en el meristemo apical del tallo (Besnard *et al.*, 2014) (Figura 2B y C).

En síntesis, los factores de transcripción KNOX1 y la enzima LOG4 propician la presencia de las citoquininas en el meristemo apical del tallo. La cascada de señalización de las citoquininas regula positivamente a *WUS*, contribuyendo así al mantenimiento del meristemo apical del tallo, al perpetuar el estado indiferenciado y la capacidad de división de sus células. Las citoquininas juegan un doble papel en el desarrollo de los órganos laterales: reprimen la iniciación de los primordios al inhibir la diferenciación de las células madre, y favorecen el mantenimiento de la fuente de estas últimas para el crecimiento no solo apical sino también lateral, que a su vez depende de la acumulación de la auxina (Wu *et al.*, 2021).

IV.4.- El desarrollo de las hojas de las plantas

IV.4.1.- Iniciación del primordio foliar

Se denomina primordio foliar a cada uno de los grupos de células que aparecen en los flancos del meristemo apical del tallo y acaban formando una hoja. La especificación de este grupo de células ocurre en la PZ, en la que algunas células de la capa L2 comienzan a dividirse anticlinalmente (perpendicularmente a la superficie del meristemo), generando la protuberancia que inicia el primordio foliar, rodeado por las capas L1 y L3 (Barton, 2010). La formación de esta protuberancia conlleva un aumento de la elasticidad tisular, asociado a la desmetil-esterificación de las pectinas de las paredes celulares, que ocurre primero en los tejidos subepidérmicos del meristemo y después en la epidermis, durante la evaginación del órgano (Peaucelle *et al.*, 2008; Peaucelle *et al.*, 2011).

La iniciación del primordio foliar también conlleva una acumulación local de auxina, causada por su transporte polar por PIN1 (Reinhardt *et al.*, 2000; Reinhardt *et al.*, 2003; Bayer *et al.*, 2009; Chen *et al.*, 2013). En el primordio foliar, la propia auxina (Hay *et al.*, 2006) y los factores de transcripción ARP (ASYMMETRIC LEAVES 1 [AS1] de Arabidopsis, ROUGH SHEATH 2 [RS2] del maíz y PHANTASTICA [PHAN] de *Antirrhinum majus*; Byrne *et al.*, 2000; Ori *et al.*, 2000; Lin *et al.*, 2003; Hay *et al.*, 2006; Guo *et al.*, 2008; Lodha *et al.*, 2013) reprimen a los genes *KNOX1*, induciendo la pérdida de pluripotencia celular y la iniciación del primordio (Long *et al.*, 1996). Las proteínas KNOX1, a su vez, reprimen a los genes *ARP* en el meristemo apical del tallo (Byrne *et al.*, 2000). Paralelamente, los genes *KNOX* de la clase II (*KNOX2*) se expresan en el primordio foliar y son antagonistas de los *KNOX1*, promoviendo la diferenciación del tejido (Furumizu *et al.*, 2015) (Figura 2A, en la página 16).

Cuando se inicia un primordio foliar se establece en su parte adaxial más cercana al meristemo apical del tallo un dominio constituido por células de división y expansión lenta (Breuil-Broyer *et al.*, 2004; Reddy *et al.*, 2004; Maugarny-Calès y Laufs, 2018), a cuya especificación contribuyen la presencia de los factores de transcripción CUP-SHAPED COTYLEDON 1 (CUC1), CUC2, CUC3 y KNOX1 (Spinelli *et al.*, 2011; Wang *et al.*, 2016; Balkunde *et al.*, 2017) y unos niveles bajos de la auxina y los brasinosteroides (Heisler *et al.*, 2005; Gendron *et al.*, 2012; Maugarny-Calès y Laufs, 2018). En este dominio, el plegamiento del tejido (Hamant *et al.*, 2008) genera un estrés mecánico que induce la expresión de *STM* (Landrein *et al.*, 2015) y *CUC3* (Fal *et al.*, 2016). Por otro lado, las proteínas con dominios BTB-POZ (de BR-C, ttk, y bab, o Pox virus y Zinc finger) BLADE-ON-PETIOLE1 (BOP1) y BOP2 actúan en la región proximal del primordio foliar, activando la expresión de los genes *AS2* y *LOB* (que codifican proteínas con un LATERAL ORGAN BOUNDARIES DOMAIN [LBD], implicadas en la especificación del primordio foliar; Iwakawa *et al.*, 2007; Du *et al.*,

2018), y reprimiendo la de los *KNOX1* (Norberg *et al.*, 2005; Ha *et al.*, 2007; Jun *et al.*, 2010) (Figura 2A).

IV.4.2.- Establecimiento de la dorsoventralidad foliar

Durante el desarrollo del primordio foliar, el establecimiento del eje de polaridad dorsoventral depende de la actividad antagónica de factores de transcripción adaxializantes (PHABULOSA [PHB], PHAVOLUTA [PHV] y REVOLUTA [REV], de la familia HD-ZIPIII; McConnell *et al.*, 2001; Emery *et al.*, 2003; Ramachandran *et al.*, 2017) y abaxializantes (pertenecientes a las familias KANADI [KAN] y YABBY [YAB]; Siegfried *et al.*, 1999; Kerstetter *et al.*, 2001; Eshed *et al.*, 2004; Yamaguchi *et al.*, 2012). Estos factores de transcripción también participan en el establecimiento del eje de polaridad radial del tallo, determinando la disposición relativa de los conductos vasculares (Emery *et al.*, 2003; Campbell y Turner, 2017). Dado que los genes que los codifican ya estaban presentes en los genomas de las plantas ancestrales sin hojas, es razonable suponer que han adquirido posteriormente su papel en el establecimiento de la dorsoventralidad foliar (Boyce, 2010).

La polarización dorsoventral del primordio foliar también depende de la inhibición mutua entre sus dominios adaxial y abaxial. El complejo formado por AS1 y AS2 promueve la identidad adaxial (Lin *et al.*, 2003; Husbands *et al.*, 2015; Machida *et al.*, 2015), mientras que ARF2, ARF3 y ARF4 contribuyen a la abaxial (Pekker *et al.*, 2005; Kelley *et al.*, 2012; Guan *et al.*, 2017). Así mismo, *ARF3* y *ARF4* son reprimidos por un pequeño ARN interferente que actúan en trans (tasiR-ARF), que deriva del procesamiento del transcrito primario del gen *TAS3*, que se expresa en el dominio adaxial del primordio foliar y forma un gradiente hacia el abaxial (Chapman y Carrington, 2007; Kidner y Timmermans, 2007). Por el contrario, los genes *MIR165* y *MIR166* se expresan en el dominio abaxial; sus productos, los microARN miR165 y miR166, forman un gradiente hacia el dominio adaxial, regulando negativamente a los genes HD-ZIPIII (Kidner y Martienssen, 2004; Nogueira *et al.*, 2007; Zhou *et al.*, 2007; Yao *et al.*, 2009), que a su vez inhiben la expresión de *MIR165* y *MIR166* en el dominio adaxial (Bou-Torrent *et al.*, 2012; Reinhart *et al.*, 2013). Además, AS1 y AS2 inducen la expresión de *TAS3* y los HD-ZIPIII (Fu *et al.*, 2007; Husbands *et al.*, 2015) y reprimen la de *ARF3*, *ARF4*, *KAN2*, *YAB5* y *MIR166* (Iwakawa *et al.*, 2007; Iwasaki *et al.*, 2013; Husbands *et al.*, 2015), mientras que KAN1 reprime a *AS2* (Wu *et al.*, 2008) (Figura 3A, en la página 21). En una etapa posterior del desarrollo del primordio, los factores de transcripción WUS-RELATED HOMEOBOX 1 (WOX1) y PRESSED FLOWER (PRS; también denominado WOX3), confieren identidad a un dominio intermedio y al margen foliar, reprimiendo a genes de identidad adaxial y abaxial (Nakata *et al.*, 2012; Nakata y Okada, 2012; Du *et al.*, 2018) (Figura 3B).

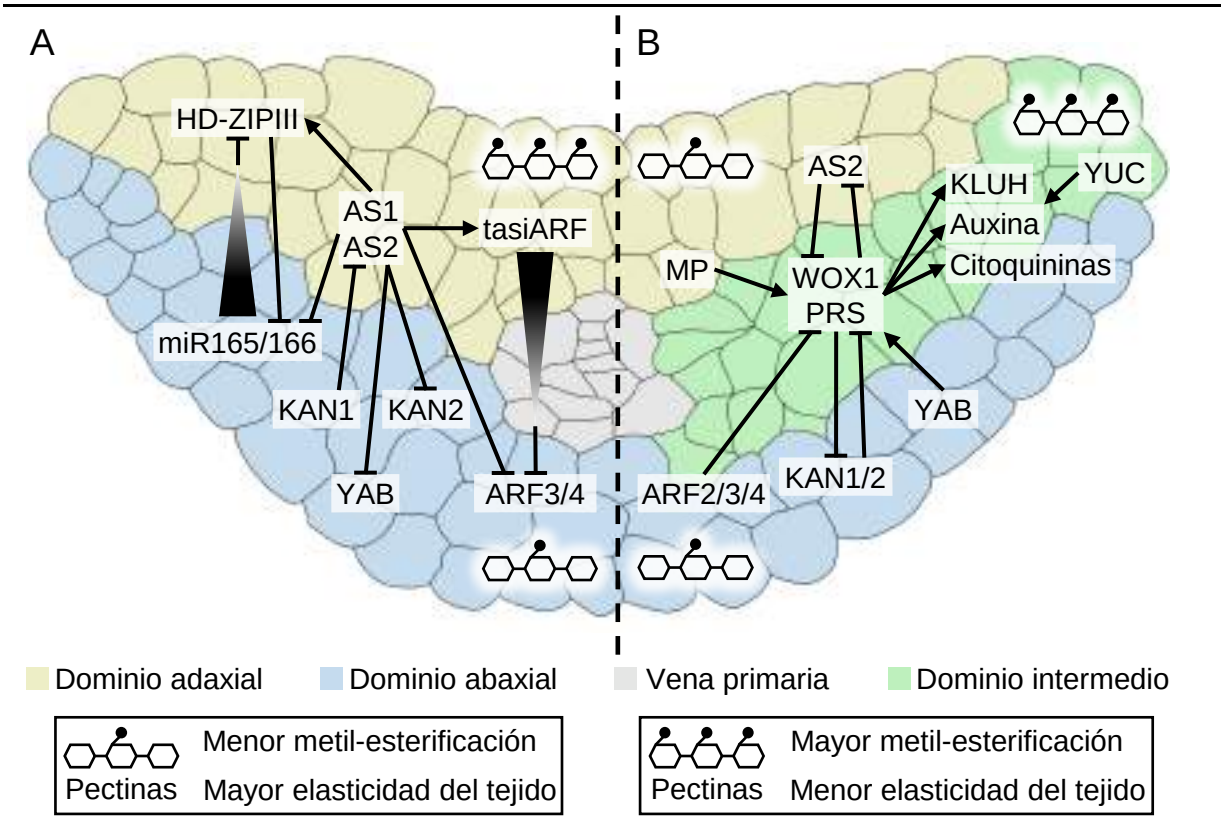

Figura 3.- Establecimiento de la polaridad dorsoventral y expansión del limbo en las hojas de la roseta de Arabidopsis. Se representan esquemáticamente en un corte transversal, perpendicular a la vena primaria, de un primordio foliar, las interacciones (A) entre los dominios adaxial y abaxial, y (B) las que especifican del dominio intermedio y el limbo foliar. Los factores que se indican en este esquema, excepto los miARN, se representan sin cursiva porque pueden corresponder a una proteína, al gen que la codifica o a ambos. Las flechas terminadas en punta indican activación, y las terminadas con una barra, represión. Los triángulos con relleno parcialmente difuminado representan gradientes e indican la dirección en que se forman. Adaptado a partir de Du *et al.* (2018) y Maugarny-Calès y Laufs (2018).

La forma plana de las hojas requiere que la división celular durante su organogénesis sea mayoritariamente perpendicular al eje mediolateral (Du *et al.*, 2018). Se ha demostrado que la perturbación de la red de interacciones que forman el eje dorsoventral aleatoriza la dirección del crecimiento, impidiendo la expansión del limbo. De hecho, en los casos más extremos, la abaxialización o adaxialización de las hojas tiene como consecuencia su radialización, lo que confirma que la coexistencia de las identidades dorsal y ventral es necesaria para la adquisición de la forma laminar de la hoja y, en consecuencia, de su bilateralidad (McConnell *et al.*, 2001; Emery *et al.*, 2003; Kidner y Martienssen, 2004; Fu *et al.*, 2007; Caggiano *et al.*, 2017).

El conocimiento detallado de esta red de interacciones contrasta con lo poco que se sabe sobre los genes a los que regula durante el establecimiento de la dorsoventralidad (Du *et al.*, 2018), que se manifiesta en la generación de dos superficies distintas, las de la

epidermis del haz y el envés (Figura 1E, en la página 9), entre las que se encuentran tejidos también distintos a lo largo del eje dorsoventral. Parece, sin embargo, que la elasticidad de la pared celular, reducida por la metil-esterificación de sus pectinas, tiene un papel relevante en este proceso. En el primordio foliar, las pectinas del dominio abaxial se encuentran desmetil-esterificadas (Figura 3A). Durante el crecimiento de la hoja, la desmetil-esterificación se extiende al dominio adaxial, manteniéndose el dominio intermedio metil-esterificado y con una menor elasticidad (Qi *et al.*, 2017) (Figura 3B).

La mayoría de las células de las epidermis adaxial y abaxial de las hojas de Arabidopsis son pavimentosas y de forma irregular (Becraft, 1999; Zhao y Sack, 1999). El mesófilo en empalizada es el tejido situado bajo la epidermis adaxial y está formado por una o dos capas de células fotosintéticas densamente empaquetadas. El mesófilo lagunar o esponjoso está formado por cuatro capas de células más pequeñas e irregulares, separadas por espacios intercelulares que facilitan la difusión de gases a través de la epidermis abaxial. En la venación, que atraviesa el mesófilo lagunar, el xilema ocupa la parte dorsal, y el floema, la ventral (Turner y Sieburth, 2002; Braybrook y Kuhlemeier, 2010).

IV.4.3.- Determinantes del crecimiento foliar

El eje de polaridad próximo-distal de la hoja, que la recorre desde la base al ápice, atraviesa el peciolo y el limbo (Figura 1E, en la página 9) y se establece al evaginar el primordio foliar. A diferencia de la dorsoventralidad, cuyo establecimiento depende de la compleja red de interacciones descritas en el apartado IV.4.2, en la página 20, es poco lo que se sabe sobre la generación de la polaridad próximo-distal (Du *et al.*, 2018). *BOP1* y *BOP2* están implicados en la especificación del peciolo, ya que su insuficiencia de función causa la aparición ectópica de estructuras del limbo en el peciolo (Ha *et al.*, 2003; Norberg *et al.*, 2005).

La pared de las células vegetales impide su migración. En consecuencia, el crecimiento de las plantas solo depende del ritmo y la orientación de la división de las células y de su expansión final. El primordio foliar incrementa inicialmente su tamaño exclusivamente mediante la proliferación de las células, que pasan después progresivamente a expandirse y diferenciarse. Esta sucesión de procesos ocurre según un gradiente basípeto (empezando por el ápice y terminando por la base del órgano) en Arabidopsis, el tomate (*Solanum lycopersicum*) o el maíz (*Zea mays*) (Donnelly *et al.*, 1999; Nath *et al.*, 2003; Ori *et al.*, 2007; Gonzalez *et al.*, 2012; Nelissen *et al.*, 2012), acrópeto (desde la base al ápice) en *Codiaeum variegatum*, bidireccional en *Syzygium jambos*, o aparentemente sin gradiente en *Coffea arabica* (Das Gupta y Nath, 2015; Maugarny-Calès y Laufs, 2018).

WOX1 y *PRS* se expresan en el dominio intermedio del primordio foliar y promueven

el desarrollo del limbo y el margen foliar en Arabidopsis (Zhang *et al.*, 2020). Las mutaciones de los genes WOX reducen la expansión del limbo en distintas especies (Vandenbussche *et al.*, 2009; Tadege *et al.*, 2011; Nakata *et al.*, 2012). Estos genes son reprimidos por AS2 y activados por MP en el dominio adaxial, y reprimidos por ARF2, ARF3, ARF4, KAN1 y KAN2 y activados por YAB en el abaxial (Nakata *et al.*, 2012; Qi *et al.*, 2014; Guan *et al.*, 2017; Du *et al.*, 2018). A su vez, WOX1 y PRS inducen la expresión de *KLUH*, que codifica la citocromo P450 monooxigenasa CYP78A5, en el margen foliar (Nakata *et al.*, 2012). Concretamente, KLUH se localiza en la base del peciolo y en los senos del margen foliar (Maugarny-Calès *et al.*, 2019) y contribuye a la prolongación de la etapa de proliferación celular; su pérdida o ganancia de función inducen la formación de flores, hojas, óvulos y semillas más pequeños o grandes, respectivamente (Anastasiou *et al.*, 2007; Wang *et al.*, 2008; Adamski *et al.*, 2009; Eriksson *et al.*, 2010). Los genes *STENOFOLIA* (*STF*) de *Medicago truncatula* y *LAM1* de *Nicotiana sylvestris* son ortólogos de *WOX1* e incrementan los niveles de auxina, ya que los mutantes *stf* y *lam1*, cuyas hojas son más delgadas que las silvestres, presentan una menor acumulación de esta hormona. La aplicación exógena de auxina y citoquininas rescata parcialmente el fenotipo del mutante *lam1* (Tadege *et al.*, 2011). Las citoquininas (Riou-Khamlichi *et al.*, 1999; Dewitte *et al.*, 2007; Shani *et al.*, 2010; Bar *et al.*, 2016), las giberelinas (Achard *et al.*, 2009; Gonzalez *et al.*, 2010) y los brasinosteroides (Choe *et al.*, 2001; Gonzalez *et al.*, 2010; Zhiponova *et al.*, 2013) promueven la proliferación celular, contribuyendo así al crecimiento de la hoja. Los genes YUCCA (YUC), que codifican flavina monooxigenasas implicadas en la biosíntesis de auxina, se expresan en el margen foliar y su actividad es necesaria para la expansión del limbo (Wang *et al.*, 2011) (Figura 3B).

Varios factores de transcripción TCP (por TEOSINTE BRANCHED 1, CYCLOIDEA, PROLIFERATING CELL FACTORS) de la clase II son reprimidos por miR319 en la región proximal del limbo de las hojas de la roseta de Arabidopsis (Palatnik *et al.*, 2003). Estos TCP reprimen a los genes *WOX* y los de las ciclinas B (*CYCB*), contribuyendo a la transición de la fase de proliferación a la de diferenciación celular en la región apical del primordio foliar (Alvarez *et al.*, 2016; Bresso *et al.*, 2018). La insuficiencia de función de estos genes TCP de la clase II de Arabidopsis o sus ortólogos prolonga la fase de proliferación celular, generándose hojas o foliolos más grandes y serrados. Por el contrario, su exceso de función adelanta la fase de diferenciación celular, generándose hojas más pequeñas y de margen más simple (Nath *et al.*, 2003; Ori *et al.*, 2007; Efroni *et al.*, 2008). Por otro lado, los factores de transcripción GROWTH-REGULATING FACTOR (GRF) son reprimidos por miR396 en la región apical del limbo, en la que TCP4 induce la expresión de *MIR396* (Jones-Rhoades y Bartel, 2004; Rodriguez *et al.*, 2010; Schommer *et al.*, 2014). GRF y sus interactores GRF-

INTERACTING FACTOR 1 (GIF1, también denominado ANGUSTIFOLIA 3 [AN3]), GIF2 y GIF3 son redundantes, inducen la expresión de varias ciclinas y retrasan la transición de la fase de proliferación a la de diferenciación celular. La insuficiencia y el exceso de función de los GRF tienen consecuencias fenotípicas opuestas a las descritas para los TCP de clase II (Kim *et al.*, 2003; Horiguchi *et al.*, 2005; Lee *et al.*, 2009; Gonzalez *et al.*, 2010; Rodriguez *et al.*, 2010; Debernardi *et al.*, 2014).

IV.4.4.- Diversidad morfológica del margen de las hojas

La forma de las hojas depende de su crecimiento a lo largo de los ejes próximo-distal y mediolateral (Hasson *et al.*, 2010; Kuchen *et al.*, 2012) (Figura 1E, en la página 9). Las hojas se consideran simples cuando presentan un solo limbo, y compuestas cuando tienen varios, denominados foliolos, unidos al mismo raquis (Efroni *et al.*, 2010; Bar y Ori, 2015). El margen del limbo puede ser liso o presentar protrusiones de terminación más o menos abrupta, en cuyo caso recibe la denominación de serrado (con indentaciones o dientes) o lobulado (con lóbulos), respectivamente (Runions *et al.*, 2017).

En los primordios foliares de Arabidopsis y otras plantas, algunas células adquieren actividad meristemática (Hagemann y Gleissberg, 1996; Ichihashi *et al.*, 2011; Ichihashi y Tsukaya, 2015; Alvarez *et al.*, 2016; Tsukaya, 2021). Se inicia así una segunda etapa proliferativa que modula la forma del margen foliar y contribuye en gran medida a la diversidad en la forma de las hojas de distintas especies (Hay y Tsiantis, 2006; Kierzkowski *et al.*, 2019). Algunas especies muestran hojas de distinta morfología en respuesta a determinadas condiciones ambientales (heterofilia; Nakayama *et al.*, 2014) o entre diferentes nudos (heteroblastia; Tsukaya *et al.*, 2000; Rubio-Somoza *et al.*, 2014).

La duración de la segunda etapa de proliferación antes mencionada y su efecto en la modulación del crecimiento dependen de distintos factores que regulan la morfogénesis del margen foliar. En algunas plantas con hojas compuestas, como *Cardamine hirsuta* y el tomate (*Solanum lycopersicum*), la reactivación de la expresión de los genes KNOX1 incrementa la duración de esta etapa permitiendo la generación de formas más complejas (Bharathan *et al.*, 2002; Shani *et al.*, 2009; Hay y Tsiantis, 2010; Piazza *et al.*, 2010; Bar y Ori, 2014; Ichihashi *et al.*, 2014; Chang *et al.*, 2019; Kierzkowski *et al.*, 2019). Para el mantenimiento del meristemo apical del tallo en Arabidopsis (Aida *et al.*, 1999; Belles-Boix *et al.*, 2006) y el desarrollo de las hojas compuestas de *Aquilegia caerulea*, *Solanum lycopersicum*, *Solanum tuberosum*, *Cardamine hirsuta* y *Pisum sativum* (Barkoulas *et al.*, 2008; Blein *et al.*, 2008) se produce una retroalimentación positiva entre los genes KNOX1 y *CUC2*. Sin embargo, en la formación de las hojas simples de Arabidopsis estos genes son reprimidos por los factores de transcripción

CINCINNATA-like TCP (CIN-TCP) y KNOX2 (Furumizu *et al.*, 2015; Challa *et al.*, 2021). Las proteínas CIN-TCP LANCEOLATE (LA) y CLAUSA (CLAU, una proteína MYB) del tomate inducen la diferenciación de las células foliares, reduciendo la respuesta a las citoquininas e incrementando la de las giberelinas (Israeli *et al.*, 2021).

El posicionamiento de los foliolos en el raquis de las hojas compuestas (Koenig *et al.*, 2009) y de las protrusiones en el margen de las hojas simples (Bilsborough *et al.*, 2011) depende de la distribución de la auxina, que a su vez depende de la de proteínas como el transportador PIN1 (Scarpella *et al.*, 2006), los factores de transcripción CUC (Nikovics *et al.*, 2006; Blein *et al.*, 2010; Hasson *et al.*, 2011; Maugarny-Calès *et al.*, 2019; Serra y Perrot-Rechenmann, 2020) y el complejo ligando-receptor EPFL2-ER (Tameshige *et al.*, 2016). La actividad de estas proteínas en la morfogénesis del margen foliar se describe en detalle en las páginas 59-61.

IV.4.5.- Influencias del ambiente sobre la morfología foliar

Existen evidencias de que la forma de las hojas se correlaciona con la adaptación a diferentes condiciones ambientales (Widmer y Ware, 1916; Little *et al.*, 2010; Nicotra *et al.*, 2011; Peppe *et al.*, 2011; Fritz *et al.*, 2018; Maugarny-Calès y Laufs, 2018), como la disponibilidad de agua. Las plantas deben mantener una regulación estricta del intercambio hídrico para mantener la turgencia, el transporte, y otras funciones básicas. Las hojas pueden perder agua a través de los estomas o de la cutícula cerosa, que separa la epidermis de su entorno (Bird y Gray, 2003). Esta cutícula incluye ácidos grasos de cadena muy larga (Very-Long-Chain Fatty Acid [VLCFA]), que además regulan el transporte polar de la auxina (Roudier *et al.*, 2010) e inhiben la biosíntesis de las citoquininas (Nobusawa *et al.*, 2013).

Se conocen algunos casos de modulación de la composición de la cutícula y la forma de las hojas por la temperatura y la humedad. De hecho, la síntesis de los lípidos de la cutícula de las hojas de Arabidopsis varía en función de la disponibilidad de agua (Kosma *et al.*, 2009; Kim *et al.*, 2019). Dos ecotipos de la brasicácea *Eutrema salsugineum*, Yukon y Shandong, de Canadá y China, respectivamente, difieren tanto en la composición de su cutícula como en su respuesta a la pérdida de agua. También difieren en la morfología del margen foliar, que es ligeramente serrado en el ecotipo Yukon y muy lobulado en el Shandong (Xu *et al.*, 2014). *Rorippa aquatica*, una brasicácea cercana a Arabidopsis, es un caso extremo de heterofilia en respuesta a distintas condiciones ambientales. Cuando estas plantas crecen sumergidas a menos de 30°C, o a 15°C y 50 µmol/s·m^2 de intensidad luminosa, o a 20°C y 90 µmol/s·m^2 producen hojas compuestas y pinnadas. Cuando son cultivadas sin inmersión y a 30°C y 50 µmol/s·m^2 producen hojas simples y lobuladas, mientras que a 20°C y 15 µmol/s·m^2, sus hojas

son compuestas, con márgenes relativamente lisos. En las condiciones que favorecen la formación de hojas compuestas y pinnadas en *Rorippa aquatica*, la expresión de *STM* y *CUC3* aumenta en el meristemo apical del tallo y aparece *ex novo* en los primordios foliares, aumenta la síntesis de citoquininas y disminuye la de las giberelinas (Nakayama *et al.*, 2014).

IV.5.- Antecedentes y objetivos

IV.5.1.- Mutantes de Arabidopsis con hojas asimétricas previamente descritos

Se conocen varios mutantes de Arabidopsis que manifiestan alteraciones en la simetría bilateral de sus hojas (Muñoz-Nortes *et al.*, 2014). Algunos de ellos también manifiestan defectos en la polaridad dorsoventral: los mutantes simples *as1*, *as2* y *bop1*, y el doble mutante *bop1 bop2*, en los que aparecen lóbulos en la región basal de la hoja, tanto en el peciolo como en el limbo, distribuidos asimétricamente con respecto a la vena primaria (Byrne *et al.*, 2000; Semiarti *et al.*, 2001; Ha *et al.*, 2007; Ha *et al.*, 2010b). También es este el caso de los mutantes *sub*, que producen hojas asimétricas cuando son cultivados a 30°C (Lin *et al.*, 2012). El gen *SUB* codifica un receptor con actividad tirosina quinasa y regula el ritmo y la orientación de los planos de división celular, así como la transición entre las fases de proliferación, expansión y diferenciación celular (Chevalier *et al.*, 2005).

En otros casos, se ha constatado que la pérdida de la simetría bilateral no está acompañada de alteraciones en la dorsoventralidad: en los mutantes nulos *trn1* y *trn2* se altera el equilibrio entre la proliferación y la diferenciación celular, reduciéndose el número de células de las hojas, cuyos limbos son asimétricos y estrechos. TORNADO 1 (TRN1) es una proteína de función desconocida especifica del reino vegetal, y TRN2, una proteína transmembrana de la familia de las tetraspaninas (Cnops *et al.*, 2000; Cnops *et al.*, 2006).

Por último, existen mutantes con hojas bilateralmente asimétricas, cuya dorsoventralidad no se ha estudiado. El gen *JAGGED* (*JAG*) codifica un factor de transcripción que contiene un dominio de dedos de zinc C2H2, cuya función es prolongar la fase de proliferación celular. Las hojas de los mutantes *jag* muestran asimetría bilateral (Dinneny *et al.*, 2004). Los genes *BARELY ANY MERISTEM 1* (*BAM1*), *BAM2* y *BAM3* codifican receptores con actividad tirosina quinasa, cuyos alelos nulos también alteran la simetría bilateral de las hojas (DeYoung *et al.*, 2006). El gen *TORTIFOLIA 1* (*TOR1*) codifica una proteína específica de las plantas asociada a los microtúbulos; sus mutaciones alteran la orientación de estos últimos y causan un crecimiento helicoidal de las hojas (Buschmann *et al.*, 2004; Buschmann *et al.*, 2009). Por último, el gen *CRINKLED LEAVES 8* (*CLS8*), también denominado *DEFECTIVE IN ORGANELLE DNA DEGRADATION 2* (*DPD2*), codifica la subunidad mayor de la ribonucleótido reductasa, la enzima que cataliza el paso limitante de

la síntesis *de novo* de desoxirribonucleótidos trifosfatados, que se requieren para la replicación y la reparación del ADN. Los mutantes *cls8* (Garton *et al.*, 2007) y *dpd2* (Tang *et al.*, 2012) producen hojas retorcidas que a menudo manifiestan asimetría, aparentemente por la expansión incompleta de una de las mitades de la hoja.

IV.5.2.- Los mutantes *desigual* (*deal*)

La colección de dominio público SALK es la que incluye un mayor número de mutantes insercionales de Arabidopsis. Se obtuvo en el laboratorio de Joe Ecker, en el SALK Institute (Alonso *et al.*, 2003). En una búsqueda a gran escala de mutantes de esta colección que manifestasen alteraciones en la morfología foliar, realizada en el laboratorio de José Luis Micol, se identificaron 706, pero solo uno cuyas hojas se desviaban manifiestamente de la simetría bilateral: la línea SALK_047972, portadora de una inserción de ADN-T en el tercer exón del gen At2g32280, al que se denominó *DESIGUAL1* (*DEAL1*) a la vez que *deal1-1* a su alelo mutante (Wilson-Sánchez *et al.*, 2018). El gen At2g32280 pertenece a una familia de 15 miembros, que codifican proteínas con un dominio DUF1218, de función desconocida y exclusivo de las plantas pluricelulares. Se identificaron 3 parálogos de At2g32280: At4g21310, At1g11500 y At1g05291, a los que se denominó *DEAL2*, *DEAL3* y *DEAL4*, respectivamente. Estos cuatro genes constituyen la subfamilia DEAL (Wilson-Sánchez *et al.*, 2018).

Los alelos mutantes de At2g32280 reducen la complejidad del patrón de venación de los cotiledones, razón por la que se le denominó *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*) en un artículo (Roschzttardtz *et al.*, 2014) que se publicó durante la realización de la búsqueda de mutantes antes mencionada. Estos autores denominaron *vcc-2* a *deal1-1*, nomenclatura que hemos respetado en esta memoria, tal como se hizo en Wilson-Sánchez *et al*. (2018). La forma del margen de las hojas de los mutantes *vcc* es su rasgo fenotípico más característico (Figura 4A-D, en la página 28). No hemos observado ninguna alteración de la dorsoventralidad de las hojas de *vcc-2* (Figura 4E, F). El fenotipo foliar de estos mutantes se manifiesta con penetrancia incompleta y expresividad variable (Figura 4G) en nuestras condiciones de cultivo. VCC se requiere para el mantenimiento de la simetría bilateral en etapas tempranas de la organogénesis foliar (Figura 4H-M), en las que predomina la proliferación celular. En los mutantes *vcc*, los dominios en los que se detectan máximos de concentración de la auxina y CUC2 se distribuyen asimétricamente en el margen del primordio foliar, encontrándose anormalmente separados o próximos entre sí de forma aleatoria (Figura 4L, M). La asimetría bilateral de las hojas de los mutantes *vcc* se debe a la disposición y el tamaño aleatorios de sus lóbulos marginales (Wilson-Sánchez *et al.*, 2018).

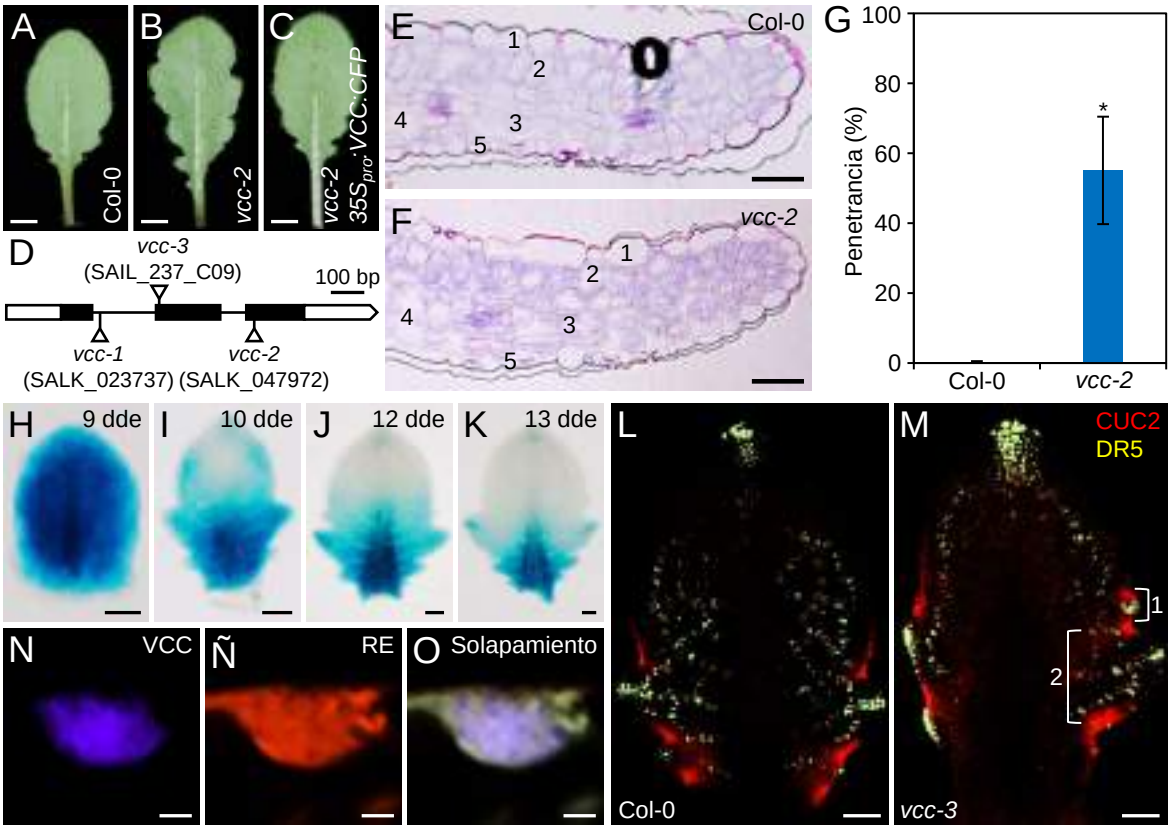

Figura 4.- Caracterización del mutante *vcc-2* realizada antes del comienzo de esta Tesis. (A-D) Fenotipo foliar y naturaleza molecular de las mutaciones *vcc*. (A-C) Hojas del décimo nudo de plantas Col-0, *vcc-2* y *vcc-2 35S$_{pro}$:VCC:CFP*. (D) Estructura del gen *VCC*, con indicación de sus mutaciones. Los exones están representados por rectángulos, los intrones por líneas horizontales, y las inserciones de ADN-T, por triángulos. Los rectángulos blancos representan regiones no traducidas. (E, F) Cortes transversales de hojas de Col-0 y *vcc-2*. 1: epidermis adaxial. 2: mesófilo en empalizada. 3: mesófilo lagunar. 4: espacio aéreo. 5: epidermis abaxial. (G) Penetrancia del fenotipo de asimetría foliar de *vcc-2*. La barra de error indica la desviación estándar, y el asterisco, un valor significativamente distinto del de Col-0 en un test de la U de Mann-Whitney (*$p < 0.01$). (H-O) Tinción GUS de hojas del décimo nudo de plantas *VCC$_{pro}$:GUS*, recolectadas en el momento que se indica en las imágenes en días después de la estratificación (dde). (L, M) Patrones de expresión de los marcadores *CUC2$_{pro}$:CUC2:RFP* (rojo) y *DR5rev$_{pro}$:VENUS* (amarillo verdoso) en primordios de (L) Col-0 y (M) *vcc-3*. Se aprecian en (M) máximos de expresión de *CUC2* más (1) cercanos o (2) lejanos que los de (L). (N-O) Visualización de la expresión de los transgenes *35S$_{pro}$:VCC:CFP* (en azul en N y en cian en O), y *35S$_{pro}$:AtWAK2:YFP:HDEL* (rojo en Ñ y verde en O) en células radiculares. Este último transgén codifica una proteína con la señal de retención en el retículo endoplásmico His-Asp-Glu-Leu (HDEL) en su extremo carboxilo. Barras de escala: (A-C) 2 mm, (E y F) 100 µm, (H-M) 50 µm y (N-O) 2 µm. Las plantas se recolectaron (A-C) 25, (E, F) 20, (L, M) 14 y (N-O) 10 dde. Adaptado de Wilson-Sánchez *et al.* (2018).

VCC presenta cuatro dominios predichos: dos transmembrana y dos solubles. En el laboratorio de J.L. Micol se estableció que VCC se localiza en la membrana del retículo

endoplásmico; se llegó a esta conclusión mediante microscopía confocal de la línea transgénica *35S$_{pro}$:VCC:CFP* en fondo Col-0, y un ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida, realizado con presas de localización conocida en la membrana de distintos orgánulos (Figura 4N-O; Wilson-Sánchez *et al.*, 2018). Sin embargo, otros autores han localizado VCC en la membrana plasmática (Yanagisawa *et al.*, 2021).

IV.5.3.- Objetivos de esta Tesis

El objetivo inicial de esta tesis doctoral fue continuar el estudio previamente iniciado de la función del gen *VCC* mediante abordajes genéticos y moleculares. Nos propusimos inicialmente: (1) realizar una búsqueda de condiciones ambientales o genéticas que incrementasen la penetrancia y la expresividad del fenotipo foliar de los mutantes *vcc*; (2) identificar proteínas interactoras de VCC mediante un ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida; (3) seleccionar entre los presuntos interactores los de mayor interés aparente, tanto por su representación entre los clones positivos identificados en dicho ensayo como por la naturaleza molecular de su función.

Los resultados de nuestro ensayo del doble híbrido de la levadura indicaron que la lista de presuntos interactores de VCC incluía componentes del complejo elongador de ácidos grasos de cadena muy larga, que inhiben la biosíntesis de las citoquininas. Por otra parte, se ha demostrado que las citoquininas modulan la morfología de las hojas simples de monocotiledóneas como el maíz (Muszynski *et al.*, 2020) y que propician el desarrollo de las hojas compuestas del tomate y la brasicácea *Cardamine hirsuta* (Shani *et al.*, 2010; Hajheidari *et al.*, 2019). Además, tal como se detalla en las páginas 65-67, las estirpes mutantes y las líneas transgénicas de Arabidopsis que se han obtenido y estudiado por sus alteraciones en la homeostasis de las citoquininas muestran hojas con márgenes más lisos que el silvestre, rasgo fenotípico que parece haber pasado inadvertido.

Nuestros resultados y los de otros autores mencionados en el párrafo anterior nos condujeron a proponernos también alcanzar los siguientes objetivos sobrevenidos: (4) revisar la literatura sobre la biología de las citoquininas, su interacción con la auxina en distintos procesos del desarrollo vegetal y los efectos fenotípicos de la alteración de su homeostasis en plantas mutantes y/o transgénicas; (5) estudiar el eventual papel de las citoquininas en la morfogénesis del margen foliar, mediante el análisis fenotípico de mutantes previamente aislados por otros autores, cultivados en nuestras condiciones de crecimiento habituales; (6) estudiar la relación entre las citoquininas y las proteínas responsables de la distribución espacial de la auxina a lo largo del margen del primordio foliar, mediante la obtención de

combinaciones mutantes múltiples y la visualización en plantas transgénicas de la expresión de proteínas fluorescentes indicadoras de la presencia y la respuesta a la auxina y las citoquininas; (7) analizar el transcriptoma de *vcc-2* y otros mutantes con alteraciones en la morfogénesis del margen foliar; (8) estudiar los efectos de la variación de los niveles de citoquininas sobre el fenotipo de *vcc-2*, mediante tratamientos farmacológicos y la obtención de combinaciones mutantes múltiples; (9) establecer las eventuales semejanzas y diferencias de la actuación de las citoquininas en el desarrollo de las hojas simples y las compuestas, y (10) proponer una hipótesis sobre el papel de VCC en la iniciación y/o el mantenimiento de la simetría bilateral de las hojas de las plantas.

V.- MATERIALES Y MÉTODOS

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Para la redacción de los apartados I a VII de esta memoria se han seguido las mismas pautas que en Tesis anteriores de los laboratorios de M.R. Ponce y J.L. Micol. En este apartado de Materiales y métodos se reproducen literalmente algunas frases procedentes de dichas Tesis. Se ha preferido usar los acrónimos castellanizados ADN y ARN —de uso común en los medios de comunicación españoles—, en lugar de los recomendados por la International Union of Pure and Applied Chemistry, DNA y RNA, para los ácidos desoxirribonucleico y ribonucleico, respectivamente. Esta elección no está basada en ningún argumento que se considere incontestable; ambas opciones son aceptadas por el *Diccionario de la Lengua Española* (vigesimotercera edición, 2014) de la Real Academia Española (RAE). Tal como recomienda la RAE en su *Ortografía de la lengua española* (2010), en esta memoria no se realiza el plural de las siglas añadiendo al final una s minúscula: se escribe “el ARN” y también “los ARN”.

La nomenclatura que se aplica en esta memoria a genes, mutaciones y fenotipos nuevos se atiene a las pautas propuestas para Arabidopsis por Meinke y Koornneef (1997). No hemos traducido al español muchos de los nombres de genes y proteínas que se mencionan en esta memoria; en estos casos solo hemos usado la cursiva para los genes. Los transgenes se denotan según lo establecido en las instrucciones a los autores de la revista *Plant Cell*. Salvo que se indique lo contrario, las plantas que se describen en este trabajo son homocigóticas para la mutación que se menciona en cada caso.

Las estirpes de Arabidopsis y las condiciones de cultivo empleadas en esta Tesis se describen en la página 81. Hemos realizado tratamientos farmacológicos (página 81), análisis morfométricos (página 82) y de microscopía confocal (página 83) de los mutantes a estudio y un escrutinio de interactores de VCC basado en el ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida (página 81). También hemos secuenciado masivamente ARN y analizado las lecturas obtenidas (página 83).

VI.- RESULTADOS Y DISCUSIÓN

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Una de las publicaciones incluidas en esta tesis es una revisión, en la que detallamos la naturaleza de las observaciones, algunas de ellas hasta ahora inadvertidas, que en nuestra opinión sustentan la hipótesis de la implicación de las citoquininas en la morfogénesis del margen de las hojas simples, como las de la roseta de Arabidopsis.

La segunda publicación incluida en esta Tesis describe una caracterización funcional del gen *VCC*, continuación de la iniciada anteriormente en el laboratorio de J.L. Micol. Las hojas de los mutantes *vcc* son asimétricas respecto a la vena primaria, ya que sus márgenes están deformados, presentando protrusiones y senos de tamaño y distribución irregulares (Wilson-Sánchez *et al.*, 2018).

Con el fin de obtener información sobre la función de VCC, realizamos en primer lugar una búsqueda de interactores basada en el ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida. Varias de las proteínas así identificadas están relacionadas con el metabolismo de los ácidos grasos (página 97), como PASTICCINO2 (PAS2, la 3-hidroxiacil-CoA deshidratasa) y ECERIFERUM10 (CER10, la enoil-CoA reductasa). PAS2 y CER10 forman parte del complejo que sintetiza los ácidos grasos de cadena muy larga, que reprimen a los genes de la biosíntesis de las citoquininas (Nobusawa *et al.*, 2013). Esta observación nos condujo a estudiar la eventual implicación de estas hormonas en la morfogénesis del margen foliar y su interacción con la auxina y VCC.

Hemos usado abordajes genéticos y farmacológicos para estudiar los efectos del incremento y/o la disminución de los niveles de las citoquininas y de su señalización sobre la morfología del margen de las hojas de la roseta de Arabidopsis, tanto en los estadios iniciales de su desarrollo (el primordio foliar) como en los finales (la hoja expendida). Analizamos los triples mutantes *ipt3 ipt5 ipt7* y *arr1 arr10 arr12*, en los que están reducidas la biosíntesis y la señalización de las citoquininas, respectivamente, y las plantas transgénicas *ANT:CKX3*, en las que está incrementada la degradación de estas hormonas. Estas plantas mostraron márgenes foliares más lisos que los del tipo silvestre (páginas 110 y 119). Además, las hojas del triple mutante *ckx1 ckx3-1 ckx5-1*, en el que la degradación de las citoquininas está reducida, resultaron ser más serradas que las silvestres (página 120). Por su parte, el mutante hipermorfo *cuc2-1D* presenta hojas muy lobuladas, como consecuencia del exceso de la función del factor de transcripción CUC2; obtuvimos el cuádruple mutante *cuc2-1D ipt3 ipt5 ipt7*, en el que este fenotipo se suprimió (página 110). El tratamiento con la citoquinina sintética 6-BAP restableció la forma dentada del margen en las hojas *ipt3 ipt5 ipt7* (página 122). Considerados en conjunto, estos resultados indican que las citoquininas se requieren para la

morfogénesis del margen de las hojas simples de Arabidopsis, proceso en el que propician la formación de indentaciones.

Visualizamos mediante microscopía confocal las respuestas a la auxina y las citoquininas durante el desarrollo de los primordios foliares de plantas de diferentes genotipos. La expresión de los marcadores *CUC2$_{pro}$:CUC2:RFP* y *DR5rev$_{pro}$:VENUS* indicó que se forman menos máximos de auxina en los primordios foliares de las plantas *ipt3 ipt5 ipt7* que en los silvestres (página 112). La expresión de *PIN1$_{pro}$:PIN1:GFP* reveló que el transporte de la auxina no está alterado en este mutante triple (página 112). La señal del marcador de respuesta a las citoquininas *TCSn::GFP* fue indetectable en *ipt3 ipt5 ipt7*, inferior a la silvestre en los mutantes hipomorfos *cuc2-3* y *er-3*, y ligeramente mayor en el hipermorfo *cuc2-1D* (página 113). Concluimos que las citoquininas influyen en la formación de los máximos de auxina durante la morfogénesis del margen foliar, y que la respuesta a las citoquininas está regulada por las proteínas CUC2 y ER en este proceso, de forma aún por determinar.

En una secuenciación masiva de ARN extraído de primordios foliares de *cuc2-3* encontramos reprimidos todos los genes desregulados relacionados con la síntesis y la señalización de las citoquininas (página 99), lo que concuerda con la ausencia de respuesta a estas hormonas en este mutante, mencionada en el párrafo anterior. Sin embargo, dichos genes no parecen desregulados en el mutante *cuc2-1D*: el incremento en la expresión de *TCSn::GFP* en *cuc2-1D* no fue tan acusado como su disminución en *cuc2-3*. Estos resultados sugieren que CUC2 modula la actividad de las citoquininas en función de su dosis, aunque de una forma no lineal.

La información bibliográfica que hemos recopilado (páginas 56-75) sugiere, y nuestros resultados (páginas 76-133) confirman, que la función de CUC2 en la morfogénesis del margen foliar está mediada por las citoquininas. En consecuencia, las citoquininas podrían tener un papel conservado, favoreciendo la complejidad de las hojas compuestas de *Solanum lycopersicum* y *Cardamina hirsuta*, así como de las simples de Arabidopsis.

Hemos cultivado el mutante nulo *vcc-2* en presencia de 6-BAP, a fin de establecer la existencia de alguna relación entre VCC y las citoquininas. Este tratamiento farmacológico no tuvo ningún efecto en Col-0 a diferencia de lo descrito por autores anteriores, que constataron un incremento de la complejidad del margen bajo condiciones de cultivo y aplicación de la citoquinina sintética distintas (Steiner *et al.*, 2012; Efroni *et al.*, 2013), pero aumentó la penetrancia y la expresividad de la asimetría foliar de *vcc-2*. Esta observación indica que el exceso de citoquininas agrava los defectos derivados de la insuficiencia de la función de *VCC* (página 114). Los autores mencionados cultivaron sus plantas a 20°C o 22°C y fotoperiodos de 16, 10 u 8 horas de luz, y 8, 14 o 16 de oscuridad, rociando las plantas con disoluciones

de la hormona dos veces por semana, mientras que nuestras condiciones de cultivo fueron 20°C y luz continua, manteniendo las plántulas durante los primeros cinco días posteriores a la estratificación en placas con sustrato carente de hormona, para después transferirlas a macetas con medio de cultivo suplementado con 6-BAP.

Hemos intentado encontrar condiciones ambientales que extremen el fenotipo de los mutantes *vcc*, a fin de facilitar su estudio. El uso de Gelrite como agente gelificante del medio de cultivo, en lugar del agar para plantas, incrementó la penetrancia del fenotipo de los mutantes *vcc*. Ignoramos la razón de este efecto del Gelrite, pero hemos observado que el tipo silvestre Col-0 crece más deprisa, y sus hojas alcanza un tamaño final mayor, en medio de cultivo gelificado con Gelrite que con agar para plantas. Nos parece razonable suponer que este mayor crecimiento incrementa la demanda de la proteína VCC o algún otro factor relacionado con ella, lo que a su vez conlleva que el fenotipo de los mutantes *vcc* se extreme.

La penetrancia completa del fenotipo de asimetría bilateral de las hojas de las plantas *vcc-2* cultivadas en presencia de Gelrite hace muy obvia su supresión en el cuádruple mutante *vcc-2 ipt3 ipt5 ipt7*, que resultó indistinguible del triple mutante *ipt3 ipt5 ipt7* (página 124); esta supresión del fenotipo de *vcc-2* causada por un déficit de citoquininas confirma la existencia de una relación funcional entre VCC y estas hormonas.

La respuesta a las citoquininas en los primordios foliares de *vcc-2*, visualizada mediante el marcador *TCSn::GFP*, se distribuyó asimétricamente entre las mitades izquierda y derecha del limbo, y se manifestó ectópicamente en el margen (página 115). Esta observación sugiere que VCC controla la simetría de la respuesta a las citoquininas entre las dos mitades del limbo foliar y su represión en el margen del primordio foliar. Las citoquininas contribuyen al mantenimiento del estado indiferenciado del meristemo apical del tallo (Su *et al.*, 2011) y a alargar la fase de proliferación del desarrollo foliar, favoreciendo la formación de hojas más complejas (Shani *et al.*, 2010; Israeli *et al.*, 2021). Por tanto, la respuesta ectópica a las citoquininas en el margen de los primordios foliares de *vcc-2* podría deberse a que la fase de proliferación celular se alarga, lo que a su vez explicaría la mayor complejidad de las hojas adultas de *vcc*-2 respecto a las de Col-0 (página 124).

La respuesta a la auxina, visualizada mediante el marcador *DR5rev*$_{pro}$*:GFP*, es asimétrica en plantas *vcc-2* cultivadas en medio con agar para plantas (Wilson-Sánchez *et al.*, 2018). En presencia de Gelrite, sin embargo, también es asimétrica, pero se extiende a lo largo de regiones más amplias del margen foliar (página 116). También hemos observado que el doble mutante *vcc-2 cuc2-3* muestra protrusiones o lóbulos en presencia de Gelrite (página 126) que no se aprecian cuando es cultivado con agar para plantas (Wilson-Sánchez *et al.*, 2018). Estas observaciones confirman la correlación entre el incremento en los defectos de la

homeostasis de la auxina que hemos visualizado en primordios foliares y el de la severidad del fenotipo mutante del margen de las hojas expandidas, en plantas cultivadas en medio de cultivo gelificado con Gelrite.

Hemos realizado una secuenciación masiva del ARN de *vcc-2*. Solo hemos identificado 121 genes desregulados, ninguno de los cuales parece estar relacionado con los ácidos grasos de cadena muy larga, la auxina o las citoquininas (página 128). Carecemos de una explicación verosímil para este resultado.

VII.- CONCLUSIONES Y PERSPECTIVAS

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La mayoría de los animales presentan una simetría aparentemente bilateral; de hecho, esto es fundamentalmente cierto en todos los filos triblásticos. Sin embargo, la simetría bilateral externa en el reino animal no excluye la asimetría en la disposición y/o la forma de algunos órganos internos. En la especie humana, por ejemplo, el hígado y el bazo están situados en la parte derecha del cuerpo, y el corazón, a la izquierda. Se han identificado, tanto en los vertebrados como en los insectos, genes responsables de la generación de estas desviaciones del patrón general de simetría bilateral que les caracteriza (Ocaña *et al.*, 2017; Hamada y Tam, 2020). Sin embargo, no se conocen genes responsables de la generación de la propia simetría bilateral. Resulta paradójico que no existan genes para construir la simetría bilateral, pero sí para desviarse de ella.

En las primeras plantas terrestres, que aparecieron hace 440-410 millones de años, todas las estructuras que se desarrollaban a partir del meristemo apical del tallo eran radiales. Las hojas fueron los primeros órganos laterales aéreos y aparecieron hace 410-360 millones de años, a la vez que descendía la concentración de CO_2 en la atmósfera de nuestro planeta. La adquisición de una estructura laminar y una forma bilateral resultó crucial durante la evolución inicial de las hojas ancestrales para maximizar su ratio superficie/masa y así optimizar su exposición a la luz solar y su función fotosintética.

A lo largo de la evolución de las plantas han ocurrido varias decenas de transiciones entre los patrones radial y bilateral —en ambos sentidos— de la simetría de las flores de varios clados de las monocotiledóneas y las dicotiledóneas. Por lo demás, la genética de la simetría bilateral es igualmente pobre en las plantas y los animales. Se han descrito muy pocas mutaciones que alteren la simetría bilateral de las hojas, pero no su polaridad dorsoventral, como los alelos mutantes del gen *VCC* (*DEAL1*).

Nuestro escrutinio basado en el ensayo del doble híbrido de la levadura para proteínas de membrana por el método de la ubiquitina dividida ha revelado que VCC interacciona con las proteínas PAS2 y CER10, entre otras, y que puede tener, en consecuencia, alguna relación funcional con los ácidos grasos de cadena muy larga. La implicación, previamente descrita, de estos últimos en la regulación de la biosíntesis de citoquininas nos ha impulsado a estudiar la eventual participación de estas hormonas en la morfogénesis del margen foliar. Este proceso se había explicado previamente en función de la actuación de la auxina, cuya localización determinan PIN1, CUC2 y la propia hormona.

Las citoquininas están implicadas en el desarrollo de las hojas compuestas de varias especies, entre ellas *Solanum lycopersicum* y *Cardamine hirsuta*. Los análisis fenotípicos y

de interacciones genéticas que hemos realizado empleando plantas mutantes y/o transgénicas indican que las citoquininas también contribuyen a la formación del patrón del margen de las hojas simples de Arabidopsis. En efecto, las plantas deficitarias en la síntesis o la percepción de las citoquininas muestran márgenes foliares más lisos que los del tipo silvestre. A su vez, el margen de las hojas de las plantas que sufren una degradación deficiente o una percepción incrementada de las citoquininas es más complejo que el silvestre y exhibe indentaciones o lóbulos pronunciados.

Nuestras micrografías confocales del patrón de expresión del marcador *TCSn::GFP* indican que las citoquininas actúan en la base del limbo y los senos marginales del primordio foliar del tipo silvestre de Arabidopsis. Esta región se solapa con la de expresión de *CUC2* y es complementaria a los máximos de respuesta a auxina. Además, hemos comprobado que la respuesta a las citoquininas depende de los niveles de CUC2. Esta observación, junto con la supresión del fenotipo de *cuc2-1D* en las plantas *cuc2-1D ipt3 ipt5 ipt7*, sugiere que las citoquininas actúan aguas abajo de CUC2, un fenómeno que han constatado autores anteriores en el desarrollo de los óvulos. Los factores CUC y las citoquininas también están implicados en la regulación de la iniciación de los órganos laterales a partir del meristemo apical del tallo, por lo que su relación funcional en la hoja constituiría un ejemplo más del reciclaje de mecanismos moleculares en distintos aspectos del desarrollo de las plantas.

Hemos demostrado que VCC está relacionada funcionalmente con las citoquininas. El fenotipo foliar del mutante *vcc-2* se agudiza mediante tratamiento con 6-BAP y se atenúa por el déficit de citoquininas del triple mutante *ipt3 ipt5 ipt7*. Por otra parte, la expresión de *TCSn::GFP* es ectópica y bilateralmente asimétrica en los márgenes de los primordios foliares de *vcc-2*. El fenotipo de *vcc-2* se extrema en presencia de Gelrite, por razones que desconocemos. En estas condiciones, la alteración en la distribución de la respuesta a la auxina a lo largo del margen foliar es más acusada que la observada anteriormente con agar. No tenemos una explicación para la ausencia de genes relacionados con la homeostasis de la auxina o las citoquininas entre los desregulados en *vcc-2*.

La semejanza en el patrón de expresión de los marcadores *TCSv2:3XVENUS* en el tomate y *TCSn::GFP* Arabidopsis sugiere que las citoquininas tienen un papel conservado en la formación de los foliolos de las hojas compuestas y de las protrusiones del margen de las hojas simples. La acumulación de la auxina precede al desarrollo de los órganos laterales a partir de los meristemos radicular y apical del tallo. Las citoquininas contribuyen a la separación y la organización espacial de los máximos de auxina para la iniciación de las raíces laterales y la formación de los óvulos y los primordios de los órganos aéreos. Nuestros resultados revelan la existencia de intercomunicación entre la auxina y las citoquininas en un

nuevo contexto: la morfogénesis del margen foliar. Nuestra observación de que la asimetría bilateral existente entre las dos mitades del primordio foliar de *vcc-2* está acompañada de la distribución aberrante y asimétrica de la respuesta a la auxina y las citoquininas sugiere que VCC juega un importante papel en la coordinación entre estas hormonas para su localización correcta y mutuamente excluyente, probablemente necesaria para el mantenimiento de la simetría bilateral de la hoja. Se requerirán estudios adicionales para dilucidar la función molecular concreta de VCC y determinar en qué compartimentos subcelulares la realiza.

La diversidad de la morfología foliar en el reino vegetal y su compleja regulación están probablemente motivadas por la variedad de las condiciones ambientales a las que se han adaptado las plantas. En respuesta a cambios en tales condiciones, las plantas pueden modular la composición de su cutícula, que incluye ácidos grasos de cadena muy larga, que a su vez regulan el transporte de la auxina y la biosíntesis de las citoquininas. Sería interesante investigar una posible relación entre la regulación hormonal, la composición de la cutícula y la adaptación al clima como elementos moduladores de la morfología foliar, y determinar si VCC juega algún papel en este proceso.

A pesar de que se han buscado sin éxito mutaciones que alteren específicamente la simetría bilateral en especies modelo como *Drosophila melanogaster*, nuestros resultados revelan que, al menos en el reino vegetal, existen genes como *VCC* que parecen regular la simetría bilateral modulando las respuestas a determinadas hormonas.

VIII.- BIBLIOGRAFÍA DE LOS APARTADOS IV-VII

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IX.- PUBLICACIONES

Trends in Plant Science

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Feature Review

Is auxin enough? Cytokinins and margin patterning in simple leaves

Sergio Navarro-Cartagena[1] and José Luis Micol[1,*]

The interplay between auxin and cytokinins affects facets of plant development as different as ovule formation and lateral root initiation. Moreover, cytokinins favor complexity in the development of *Solanum lycopersicum* and *Cardamine hirsuta* compound leaves. Nevertheless, no role has been proposed for cytokinins in patterning the margins of the simple leaves of *Arabidopsis thaliana*, a process that is assumed to be sufficiently explained by auxin localization. Here, we discuss evidence supporting the hypothesis that cytokinins play a role in simple leaf margin morphogenesis via crosstalk with auxin, as occurs in other plant developmental events. Indeed, mutant or transgenic arabidopsis plants defective in cytokinin biosynthesis or signaling, or with increased cytokinin degradation have leaf margins less serrated than the wild type.

Highlights

In simple leaves, like those of *Arabidopsis* thaliana, the positional information provided by auxin has been considered enough to explain leaf serration, which in addition requires the CUP-SHAPED COTYLEDON2 (CUC2) transcription factor.

Cytokinin response and CUC2 localization coincide at the base of arabidopsis leaf primordia, and plants defective in cytokinin activity have leaf margins less serrated than the wild type.

Cytokinins favor leaf complexity in the development of the compound leaves of *Solanum lycopersicum* and *Cardamine hirsuta*.

Auxin and cytokinins crosstalk in different plant developmental processes; we hypothesize that cytokinins also play a role via their interplay with auxin in leaf margin morphogenesis of simple leaves.

Leaf primordia development

Leaves form from the shoot apical meristem (SAM) [1]. Leaf primordia arise as groups of cells that emerge on the flanks of the SAM, at points where auxin accumulates as a consequence of its directional transport by the PIN-FORMED1 (PIN1) auxin efflux carrier [2–4]. Cell proliferation, but not cell expansion, occurs in the first stages of leaf primordia development, and later declines when cells begin to expand and differentiate [5]. In arabidopsis (*Arabidopsis thaliana*) and many other plants, some leaf primordia cells acquire meristematic activity and undergo a second stage of proliferation that shapes the leaf margins [6–10]. The different arrangements of lobes or teeth in simple leaves, and leaflets in compound leaves, confer variation in leaf shape among plant species [11–13] (Box 1). Indeed, leaf margin shape strongly contributes to the shape of the whole leaf.

The maintenance of SAM pluripotency requires the expression of class I *KNOTTED1*-like homeobox-containing (*KNOX1*) genes [14]. Arabidopsis has four *KNOX1* genes: *SHOOT MERISTEMLESS* (*STM*), *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* [*KNAT1*; also called *BREVIPEDICELLUS* (*BP*)], *KNAT2*, and *KNAT6*. These genes encode transcription factors that control hormone homeostasis within the SAM, where they activate cytokinin biosynthesis [15–17] (Figure 1, interactions #1–3). *KNOX1* genes must be locally repressed to allow the shift from indeterminate growth to determinate growth of leaves to occur [18]. The Myb domain transcription factor ASYMMETRIC LEAVES1 (AS1) and the LATERAL ORGAN BOUNDARIES (LOB) domain transcription factor AS2 form the AS1-AS2 repressor complex, which represses *KNOX1* genes, thus allowing this developmental transition [19–23].

In arabidopsis, the AS1-AS2 complex represses *BP*, *KNAT2*, and *KNAT6*; de-repression of these *KNOX1* genes generates leaves with extra lobes or extra serrations [18–23] (Figure 1, #4). The *35S::KNAT1* transgenic line (constitutively expressing *KNAT1* driven by the strong Cauliflower mosaic virus 35S promoter) and the *as2-2* mutant both produce leaves with extra lobes [18].

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Box 1. Gene nomenclature and anatomical terms

Unless otherwise stated, all mutations mentioned here are loss-of-function mutations and all the experimental evidence discussed has been obtained exclusively or fundamentally in *Arabidopsis thaliana* (hereafter referred to as arabidopsis).

Different research communities, working with different species, have developed different nomenclatures for genes, mutations, and constructs, which in addition are not always followed by all members of such communities. Although it may cause confusion to some extent, we have chosen to use the gene, mutation, and construct names coined by each of the original authors whose work we discuss.

Different studies have used different names for the structures at the margins of simple leaves. An oversimplified – but useful for our discussion – way of classifying the margins of simple leaves is to describe them as smooth (entire leaves) or showing protrusions, which may be rounded (lobed leaves) or pointed (toothed or serrated leaves). Since most arabidopsis wild-type accessions (e.g., Col-0) have serrated leaves, we have chosen the term 'serration' to refer to each structural unit of the leaf margin, 'protrusion' for the outermost part of each serration, and 'sinus' for the innermost part.

The BTB-POZ (for BR-C, ttk and bab, or Pox virus and Zinc finger) domain proteins BLADE-ON-PETIOLE1 (BOP1) and BOP2 directly activate *AS2* transcription and negatively regulate *BP*, *KNAT2*, and *KNAT6* expression in the proximal (basal) adaxial region of leaf primordia; in turn, STM represses *BOP1* and *BOP2* expression in the embryonic SAM [24,25]. The *bop1-1* mutant, carrying a dominant-negative allele of *BOP1* [24], and the *bop1-4 bop2-11* double null mutant develop ectopic lamina outgrowths along the petiole [26]. The BEL1-LIKE HOMEODOMAIN (BLH) proteins SAWTOOTH1 (SAW1, also called BLH2) and SAW2 (BLH4) negatively regulate *BP* expression in leaves and act redundantly to limit leaf margin growth, as shown by the increased number and size of leaf serrations in the *saw1 saw2* double mutant, but not in the *saw1* and *saw2* single mutants [27]. In some plants with compound leaves, like *Cardamine hirsuta*, *Lepidium perfoliatum*, *Lepidium hyssopifolium*, and *Neobeckia aquatica* (Brassicaceae), as well as in *Solanum lycopersicum* (Solanaceae), *Cissus congestum* (Vitaceae), and *Daucus carota* (Apiaceae), expression of *KNOX1* genes is reactivated in developing leaf primordia, contributing to the development of leaflets [11,13,17,28–31].

The arabidopsis genome includes 24 genes predicted to encode transcription factors of the TEOSINTE BRANCHED1-CYCLOIDEA-PROLIFERATING CELL FACTOR (TCP) family, whose members regulate the proliferation-differentiation balance in developing leaves. Generally, class I TCP family members promote cell proliferation and class II family members repress cell proliferation [32]. There are two subgroups of class II TCPs: CYCLOIDEA/TEOSINTE BRANCHED1 (CYC/TB1) and CINCINNATA-like (CIN). The CIN-TCP genes *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* are downregulated by a microRNA, miR319 [33] (Figure 1, #5). The *jagged and wavy-Dominant* (*jaw-D*) mutant overexpresses miR319, resulting in overexpression of *BP*, *KNAT2*, and *KNAT6* and increased number and size of leaf serrations. AS2 physically interacts with TCP3, and they bind to similar regions of the *BP* and *KNAT2* promoters; the *as2-1 jaw-D* double mutant exhibits leaves with leaflet-like structures and ectopic expression of *BP*, *KNAT2*, and *KNAT6* [34] (Figure 1, #6).

The KNOX1 and NO APICAL MERISTEM (NAC)-domain CUP-SHAPED COTYLEDON (CUC1, CUC2, and CUC3) transcriptional regulators maintain meristematic activity in the SAM [35,36]. These proteins also promote leaflet formation in developing compound leaves in *Aquilegia caerulea*, *S. lycopersicum*, *Solanum tuberosum*, *C. hirsuta*, and *Pisum sativum* [37,38]. Moreover, CIN-TCPs [39,40] and class II KNOX (KNOX2) proteins promote differentiation in leaves [41]. The *KNOX1* and *CUC* genes undergo positive feedback [36,38,42,43] and TCP4 directly activates the expression of the *KNOX2* genes *KNAT3* and *KNAT4* [44] (Figure 1, #7 and #8). CIN-TCPs and KNOX2 limit meristematic capacity by repressing *KNAT2*, *KNAT6* (Figure 1, #9), and *CUC2* (Figure 1, #10), allowing the formation of simple leaves. Indeed, the simultaneous

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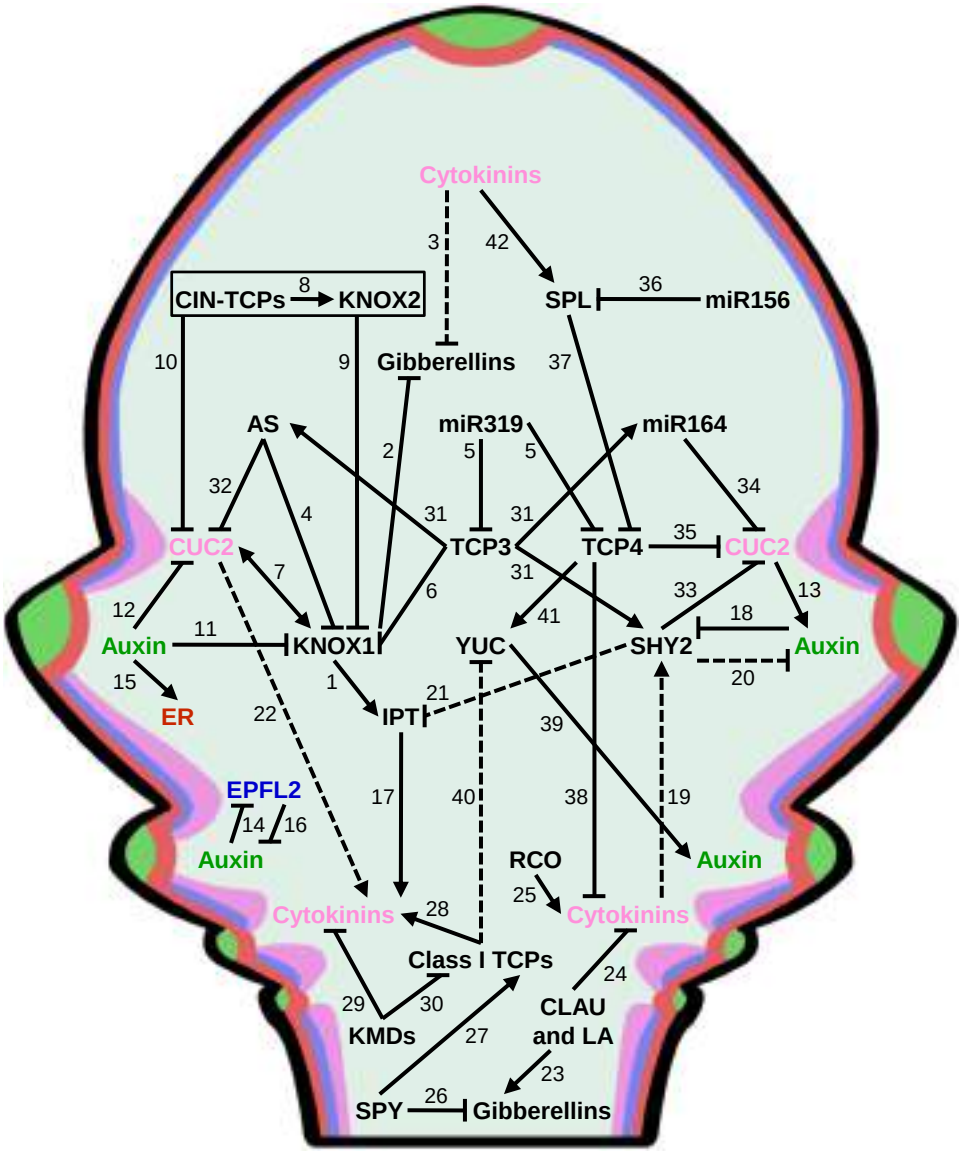

Figure 1. Interactions involving auxin and/or cytokinins in different plant organs, some of which have been shown to occur in leaves. Arrows indicate positive regulation of gene expression or protein activation, and blunt arrows indicate negative gene regulation or protein inhibition. Broken lines indicate a relationship demonstrated in organs other than leaves. Not all genes whose products participate in cytokinin biosynthesis, signaling, or degradation (Boxes 2 and 3) appear in this figure. Factors shown in this figure are represented in Roman font, but may correspond to proteins, genes, or both. Known localization along the leaf margin of auxin response, ER receptors, EPFL2, and CUC2/cytokinin responses are indicated in green, red, blue, and pink, respectively. Black letters are used for other genes, proteins, miRNAs, and gibberellins; these interactions are not represented in the primordium region where they happen. (1) In the SAM, the KNOX1 protein STM positively regulates *IPT5* expression, and STM, BP, and KNAT2 activate *IPT7* [15,16]. (2) In the SAM, KNOX1 reduces gibberellin levels by repressing the *GA 20-oxidase1* (*GA20ox1*) biosynthesis gene and inducing the *GA2ox1* catabolic gene [15,17]. *KNOX1* expression is reactivated in the developing primordia stage that initiates compound leaf development in several species [11,13,17,28–31]. (3) In the SAM, cytokinins activate *GA2ox1* expression [15]. (4) The AS1–AS2 complex represses expression of the *KNOX1* genes *BP*, *KNAT2*, and *KNAT6* [18–22]. (5) miR319 post-transcriptionally represses *TCP3* and *TCP4*, among other *CIN-TCPs* [33]. (6) TCP3 represses *BP*, *KNAT2*, and

(Figure legend continued at the bottom of the next page.)

downregulation of five *CIN-TCP* genes and three *KNOX2* genes causes *KNOX1* and *CUC2* de-repression and indeterminate leaflet formation in arabidopsis [44].

In conclusion, leaf morphogenesis requires a balance between proliferation and differentiation. The KNOX1, CUC, and TCP transcription factors play key roles in this balance. Moreover, KNOX1 proteins activate cytokinin biosynthesis in the SAM and are involved in leaflet formation in plants with compound leaves, and this opens the question of whether there is a connection between cytokinins and simple leaf margin shape.

Factors affecting margin shape in simple leaves

Auxin is the key player in arabidopsis leaf margin patterning. It accumulates in leaf primordia at the protrusions of emerging serrations via polar auxin transport along the leaf primordium margin. At these protrusions, auxin inhibits *BP* expression, as impaired auxin signaling causes de-repression of *BP* and the formation of deeply lobed margins [21] (Figure 1, #11). PIN1, CUC2, and auxin itself create feedback loops that propagate in the developing leaf margin, generating alternating maxima of auxin and CUC2 accumulation, which match protrusion tips and sinuses, respectively [45] (Figure 1, #12 and #13; Figure 2A). CUC2 is required for the formation of auxin maxima and serrations at leaf primordia margins, as plants homozygous for the loss-of-function *cuc2-3* allele lack auxin maxima, showing a diffuse pattern of auxin accumulation, and have smooth, non-serrated leaf margins [46,47]. By contrast, the *cuc2-1D* allele and the *CUC2g-m4* transgene encode *CUC2* mRNAs that are resistant to post-transcriptional repression by miR164. The post-transcriptional de-repression of *CUC2* in *cuc2-1D* and *CUC2g-m4* plants causes development of leaves with extra lobes [46,48].

KNAT6 expression [34]. (7) *KNOX1* and *CUC* genes activate each other's expression in the SAM and compound leaf primordia [36,38,42,43]. (8) TCP4 directly activates expression of the *KNOX2* genes *KNAT3* and *KNAT4* [44]. CIN-TCPs and KNOX2 proteins repress (9) *KNAT2*, *KNAT6*, and (10) *CUC2* [44]. Auxin inhibits (11) *BP* [21] and (12) *CUC2* [45] expression at developing leaf margin protrusions. (13) CUC2 favors PIN1 polarization towards auxin maxima, causing auxin to flow away from CUC2 expression domains [45]. Auxin (14) represses *EPFL2* expression and (15) promotes *ERL2* expression at leaf primordia margin protrusions [60]. (16) EPFL2 interacts with ER family members to inhibit the auxin response in the peripheral cells of the protrusions [60]. (17) IPT family members catalyze the first step of the cytokinin biosynthetic pathway [88]. (18) Auxin stimulates the ubiquitin-mediated proteolysis of SHY2 [111]. At the root meristem, (19) the type-B positive regulator of cytokinin signaling ARR1 [103] activates the expression of *SHY2* [74]; SHY2 inhibits (20) auxin transport and (21) cytokinin biosynthesis by repressing *IPT5* [74]. In addition, SHY2 inhibits the auxin response by binding to ARFs [110]. (22) CUC2 enhances the cytokinin response in *Nicotiana tabacum* BY-2 protoplasts, and increases the levels of active cytokinins by repressing *UGT73C1* and *UGT85A3* expression in pistils [132,133]. CLAU and LA promote the end of the morphogenetic phase in developing tomato leaves, (23) increasing gibberellin responses [137,138] and (24) reducing sensitivity to cytokinins [135,137]. (25) RCO increases endogenous cytokinin levels and the cytokinin response in *Cardamine hirsuta* and in *ChRCOg*-expressing arabidopsis transgenic plants [141]. SPY (26) negatively regulates gibberellin responses [146], and (27) stabilizes the class I TCPs TCP14 and TCP15, (28) which promote cytokinin responses [148,149]. KMDs target (29) type-B ARRs [150] and (30) TCP14 [151] for degradation. (31) TCP3 directly activates *AS1*, *SHY2*, and *MIR164* expression [39]. (32) AS1 and (33) SHY2 inhibit *CUC2* expression [39]. (34) miR164 post-transcriptionally represses *CUC2* [39,46]. (35) TCP4 represses *CUC2* expression [152] and dimerizes with CUC2, inhibiting its activity as a transcriptional regulator [153]. (36) miR156 post-transcriptionally represses *SPL9* [154,155]. (37) SPL9 binds TCP4, interfering with TCP4–CUC2 dimerization [153]. (38) TCP4 induces *ARR16* expression [156]; ARR16, a type-A ARR, negatively regulates cytokinin signaling [101]. (39) YUC family members catalyze a rate-limiting step in tryptophan-dependent auxin biosynthesis [157]. (40) TCP15 represses *YUC1* and *YUC4* expression during gynoecium development [158]. (41) TCP4 directly activates *YUC5* transcription [40,159]. (42) Cytokinins promote vegetative phase transition depending on SPL protein function [169]. Abbreviations: ARF, AUXIN RESPONSE FACTOR; ARR, ARABIDOPSIS RESPONSE REGULATOR; AS, ASYMMETRIC LEAVES; *BP*, *BREVIPEDICELLUS*; CIN, CINCINNATA-like; CLAU, CLAUSA; CUC2; CUP-SHAPED COTYLEDON2; EPFL, EPIDERMAL PATTERNING FACTOR-LIKE; ER, ERECTA; IPT, ISOPENTENYLTRANSFERASE; KMD, KISS ME DEADLY; *KNAT*, *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA;* KNOX, KNOTTED1-like homeobox-containing; LA, LANCEOLATE; PIN1, PIN-FORMED1; RCO, REDUCED COMPLEXITY; SAM, shoot apical meristem; SHY2, SHORT HYPOCOTYL2; *SPL*, *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE*; SPY, SPINDLY; STM, SHOOT MERISTEMLESS; TCP, TEOSINTE BRANCHED1-CYCLOIDEA-PROLIFERATING CELL FACTOR; UGT, uridine diphosphate glycosyltransferase; YUC, YUCCA.

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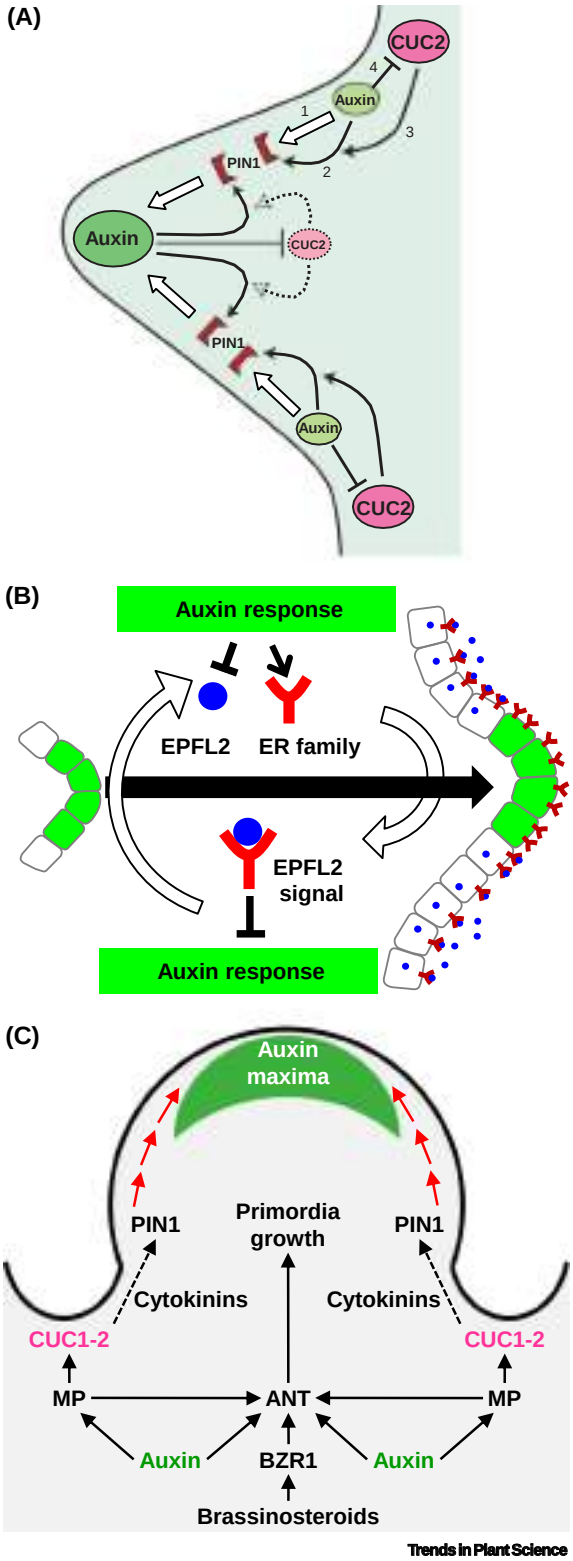


Figure 2. Models for leaf serration development and ovule formation. (A) Model for leaf serration. PIN1 (red wedges) transports auxin in a polar manner (1; white arrows) leading to the formation of auxin maxima and minima (large/intense and small/pale green ovals, respectively). Auxin, in turn, favors PIN1 polarization towards auxin maxima (2; black arrows). CUC2 (large/intense pink ovals) is also required for PIN1 polarization (3; black arrows). Auxin inhibits *CUC2* expression (4; blunt arrows), with much intensity on auxin maxima where the CUC2 minima (small/pale pink oval) have less PIN1 polarization activity (broken arrows). In this way, the protrusions of the serrations match the auxin convergence points and the sinuses match the CUC2 activity maxima. (B) Model for leaf margin morphogenesis that involves cells responding to auxin (green), EPFL2 peptides (blue dots), and ER family receptors (red). In an early stage (large white arrow facing upward), cells responding to auxin favor the expression of genes encoding ER family receptors (small black arrow) and inhibit EPFL2 expression (blunt arrow). In a subsequent stage (small white arrow facing downward), EPFL2 expression is de-repressed in cells with no auxin response (white boxes). EPFL2 interacts with ER receptors suppressing the auxin response. In this way, the protrusions and sinuses of the serrations match the cells responding to auxin and cells expressing EPFL2, respectively. (C) Model for ovule formation. ANT promotes ovule primordia growth, while CUC1 and CUC2 establish the primordia boundaries. Auxin activates *ANT* and *MP* expression. MP is required for *ANT*, *CUC1*, and *CUC2* expression. Brassinosteroids positively regulate the number of ovule primordia, in part by the direct regulation of *ANT* by BZR1. A discussion of the role of brassinosteroids in ovule development is beyond the scope of this review. CUC1 and CUC2 control *PIN1* expression. PIN1 transports auxin (red arrows) leading to the formation of auxin maxima (green-shaded dome). In addition, cytokinins would be required downstream of CUC proteins to promote *PIN1* expression (broken black arrows, which indicate that this regulation may be indirect). Reproduced with minor modifications with permission of the journals and the authors from (A) [45], (B) [60], and (C) [127]. Abbreviations: *ANT, AINTEGUMENTA; BZR1, BRASSINAZOLE-RESISTANT 1; CUC; CUP-SHAPED COTYLEDON; EPFL2, EPIDERMAL PATTERNING FACTOR-LIKE2; ER, ERECTA; MP, MONOPTEROS; PIN1, PIN-FORMED1.*

CUC2 protein levels affect the patterning of serrations (i.e., more CUC2 produces more serrations), but CUC2 is not necessary for outgrowth of the serrations once the pattern has been established [49]. Instead, CUC2 activates the expression of *CUC3* and *KLUH* (encoding cytochrome P450 CYP78A5 monooxygenase). CUC3 and KLUH thus function as CUC2 relays, also localizing at leaf primordia sinuses and regulating auxin maxima formation [49]. In contrast to CUC2, which accumulates in the sinuses and their neighboring cells, CUC3 accumulation is specific to the sinuses [49,50], in a group of cells where it represses cell expansion, thus maintaining differential growth and hence serration outgrowth, as shown by the reduced serration of the leaves of *cuc3* mutants [50–52]. *CUC1* is not expressed in developing leaves and is not involved in leaf margin patterning [51].

Members of the arabidopsis *DESIGUAL* (*DEAL*) family encode proteins with a Domain of Unknown Function 1218 (DUF1218). The VASCULATURE COMPLEXITY AND CONNECTIVITY (VCC, also called DEAL1) protein [53–55] seems to be required for bilateral symmetry at very early stages of leaf organogenesis, regulating auxin maxima formation. In the leaf primordia of *vcc* mutant plants, the domains of auxin and CUC2 accumulation are randomly clustered or separated and, therefore, *vcc* mutant leaves have mispositioned serrations. In addition, the left and right margins, and in turn the whole leaf lamina, become bilaterally asymmetric in the *vcc* mutants [54].

The EPIDERMAL PATTERNING FACTOR/EPF-LIKE (EPF/EPFL) family comprises 11 secreted cysteine-rich peptides [56] that regulate the activity of members of the ERECTA (ER) family of plasma membrane-localized leucine-rich repeat (LRR) receptor kinases [ER, ER-LIKE1 (ERL1) and ERL2] [57]. Interactions between these ligands and receptors modulate SAM size and affect organ initiation from the SAM [58,59]. At the protrusions of leaf primordium margins, auxin represses *EPFL2* and promotes *ERL2* expression (Figure 1, #14 and #15). EPFL2 interacts with ER, ERL1, and ERL2 in the cells peripheral to the protrusion, inhibiting the auxin response (Figure 1, #16). This feedback loop also maintains leaf serration growth, restricting the auxin response to the protrusions (Figure 2B) [60]. The *er erl1*, *er erl2*, and *erl1 erl2* double mutants and the *epfl2* single mutant show ectopic auxin responses around the protrusion tips of serrations. These single and double mutants exhibit serrations at the leaf primordium stage, but these serrations are lost later in leaf development, suggesting that the ER family proteins and EPFL2 are required to maintain serrations [60].

In conclusion, alternating maxima of auxin and CUC2 set down the pattern for the serrations of the simple leaves of arabidopsis in leaf primordia, a pattern that seems maintained by VCC/DEAL1, and translated into serration protrusion outgrowth by EPF/EPFL, and into repressed cell expansion in serration sinuses by CUC3. In the subsequent sections, we will explore how cytokinins fit within the well-established model for auxin function in simple leaf margin patterning.

Where cytokinins and auxin act and interact

Cytokinins act in many aspects of plant growth, development, and physiology; they are involved in root and shoot growth [61–64], vasculature differentiation [62,63], photomorphogenesis [62], chloroplast [61,62] and stomata development [64], leaf development and senescence [65], plasmodesmatal transport in leaves [66], seed development, and fertility [61–63]. They also mediate plant responses to environmental signals, and to biotic and abiotic stresses [67], such as cold [68,69], osmotic, and water deficit [70,71] stress. Cytokinins can stimulate cell division [72,73], but can also promote cell differentiation [74,75], which indicates that their functions depend on the developmental context.

There is a wealth of published information on cytokinin metabolism and signaling [63,76]. The interplay between auxin and cytokinins has been demonstrated for several processes in plant

development [77–82], and these hormones each regulate the biosynthesis of the other [83,84] (Boxes 2 and 3).

Root development

Cytokinins act antagonistically with auxin in establishing the root stem cell niche [109]. Cytokinin activity appears first during embryonic development in the founder cell of this niche and remains in the apical daughter cell of the founder cell, which will generate the root quiescent center. Cytokinin activity is abolished in the basal cell lineage by auxin, which activates the transcription of *ARABIDOPSIS RESPONSE REGULATOR7 (ARR7)* and *ARR15*, encoding type-A ARRs. This antagonistic interaction has been disrupted by an ethanol-inducible RNA interference construct against *ARR7* (*ARR7-i*) in *arr15* embryos; in the presence of ethanol, these embryos showed defects in the root stem cell system, with ectopic cytokinin activity in the basal cell linage and altered cell shape, number, and identity [109].

The interaction between auxin and cytokinins controls root meristem size. In this developmental context, auxin promotes cell division in the root stem cell niche and proximal root meristem, while cytokinins favor cell differentiation in the root elongation zone [74]. SHORT HYPOCOTYL2 (SHY2, also named Aux/IAA3) inhibits the auxin response by binding to the AUXIN RESPONSE FACTOR (ARF) transcriptional regulators [110], while auxin stimulates the ubiquitin-mediated proteolysis of SHY2 [111,112] (Figure 1, #18). At the root meristem, the cytokinin-dependent transcription factor ARR1 activates *SHY2* expression [74] (Figure 1, #19). In the transition zone between dividing and expanding cells, SHY2 regulates the reciprocal inhibition of auxin and cytokinins: SHY2 inhibits auxin transport by negatively regulating the expression of *PIN1*, *PIN3*, and *PIN7*, and inhibits cytokinin biosynthesis by repressing *ISOPENTENYLTRANSFERASE 5 (IPT5)* [74] (Figure 1, #20 and #21). In addition, ARR1 [113] controls auxin degradation in external root tissues by inducing the expression of *GRETCHEN HAGEN 3.17* (*GH3.17*), which encodes an enzyme that mediates auxin conjugation with glutamate [114]. In this way, cytokinins restrict auxin activity and, hence, root meristem size.

Local accumulation of auxin precedes the development of lateral organs, such as secondary roots, leaves, and flowers [2,115], and cytokinins restrict the area of auxin maxima in these incipient organs, hence regulating the separation of auxin maxima [77,79]. The depletion of cytokinins caused by the constitutive expression of *35S:CKX1* (*CYTOKININ DEHYDROGENASE/OXIDASE 1*)

Box 2. Cytokinin biosynthesis and degradation

Cytokinins are a group of adenine-derived plant hormones that have an isoprenoid or aromatic side chain bound to the N^6 of the purine ring. The most abundant active cytokinin forms are of the isoprenoid type: isopenteniladenine (iP), *trans*-zeatin (tZ), and dihydrozeatin (DHZ) [85].

The first step of cytokinin biosynthesis is mediated by ISOPENTENYLTRANSFERASE1 (IPT1) and IPT3–IPT8. These seven enzymes differ in their spatiotemporal accumulation [86,87], and catalyze the transfer of a prenyl group from dimethylallyl diphosphate to the N^6 position of ADP or ATP, yielding isopentenyl ribotides [88] (Figure 1, #17). The second step of the pathway is the hydroxylation of the isoprenoid side chain of isopentenyl ribotides, catalyzed by the cytochrome P450 enzymes CYP735A1 and CYP735A2, which generate *trans*-zeatin ribotides [89]. The release of free bases (the active forms of cytokinins) from cytokinin ribotides is then catalyzed by nine cytokinin nucleoside 5′-monophosphate phosphoribohydrolases of the LONELY GUY (LOG) family [90], which were first discovered in rice (*Oryza sativa*) [91].

Cytokinins undergo different chemical modifications, like glycosylation by uridine diphosphate glycosyltransferases (UGTs), which inactivate these hormones; the inactivated cytokinins can be reactivated if their sugar moiety (usually glucose) is bound to oxygen, or not if it is bound to nitrogen [92]. The CYTOKININ DEHYDROGENASE/OXIDASE 1 (CKX1) to CKX7 enzymes, which have distinct patterns of gene expression and subcellular localization, cleave cytokinins and thus irreversibly inactivate them [93–95].

Box 3. Cytokinin signaling

Cytokinin signaling relies on a His-Asp phosphorylation signaling cascade [96] initiated by the cytokinin receptors ARABIDOPSIS HISTIDINE KINASE2 (AHK2), AHK3, and AHK4 (also named CYTOKININ RESPONSE1 [CRE1] and WOODEN LEG [WOL]). Interaction between cytokinins and an AHK triggers phosphorylation of a His residue of the AHK; the phosphoryl group is transferred to an Asp residue of AHK [97], and then to a His of an ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN (AHP), which moves into the nucleus after being phosphorylated. Five AHPs (AHP1–AHP5) exist and are phosphorylated in this way [98]. A sixth member of this family, AHP6, does not contain the conserved His residue that is required for phosphorylation, but competes with the remaining AHPs for access to the receptors, thereby negatively regulating cytokinin signaling [99].

In the nucleus, the phosphoryl group carried by AHP1–AHP5 is transferred to an ARABIDOPSIS RESPONSE REGULATOR (ARR) [100]. Two ARR classes have been distinguished based on their sequences and domain structures: arabidopsis has 11 type-A [101] and ten type-B [102] ARRs. The phosphoryl-activated type-B ARRs contain a Myb-like DNA-binding domain and bind to the promoters of cytokinin-responsive genes [103–105]. Type-B ARRs increase chromatin accessibility at the promoters of cytokinin-responsive genes, allowing changes in the transcriptional regulation of these genes that may explain the pleiotropic and context-dependent effects of cytokinins [106]. Type-A ARRs lack the Myb-like DNA-binding domain for transcriptional regulation but contain the phosphoryl receiver domain. Therefore, type-A ARRs negatively regulate cytokinin signaling, probably by competing with type-B ARRs for phosphoryl and by phospho-dependent interactions with target proteins [107].

CYTOKININ RESPONSE FACTORS (CRFs) are a sub-group of six transcriptional regulators of the APETALA2/ETHYLENE RESPONSE FACTOR (AP2/ERF) family that are expressed and rapidly accumulate in the nucleus in response to cytokinins. CRFs act in parallel to the type-B ARRs, with which they share many target genes [108]. Type-A ARRs and CKXs are encoded by early cytokinin-responsive genes, establishing a negative regulatory feedback loop to buffer the cytokinin response [104].

increases lateral root density, because auxin maxima, whose formation precedes lateral root emergence, are abnormally clustered in this transgenic line [116]. Cytokinin biosynthesis occurs near existing lateral root primordia, at the lateral root founder cells, preventing the formation of new lateral roots there by inhibiting *PIN* expression and, therefore, auxin maxima formation [116–118]. Cytokinins also reduce PIN1 levels at the plasma membrane in a nontranscriptional manner, by promoting selective PIN1 endocytosis and lytic degradation in vacuoles at specific polar domains. This mechanism modifies cellular PIN1 polarities, thus changing auxin flow directionality and auxin localization in lateral root organogenesis [119,120]. The increased lateral root density phenotype is also shown by *aza-guanine resistant 2* (*azg2*) loss-of-function mutants; *AZG2* encodes a putative cytokinin diffusion facilitator [121,122]. By contrast, in *amiRPUP14*, a line producing an artificial microRNA (amiRNA) that post-transcriptionally inhibits the expression of the *PURINE PERMEASE 14* (*PUP14*) gene, encoding a transmembrane cytokinin translocator, cytokinin activity is increased, which results in delayed seedling root growth and suppression of lateral roots [123].

Organ emergence from the SAM

In the SAM, tissue domains with cytokinin activity also separate auxin maxima, allowing the sequential and properly spaced emergence of leaf primordia [79]. Moreover, inflorescence phyllotaxis is altered when cytokinin activity is de-repressed in the SAM. In the arabidopsis Col-0 wild type, the successive angles and distances between consecutive siliques, generated as a consequence of the spatiotemporal initiation of the corresponding flower primordia at the SAM, follow a pattern that shows mild variation [124]. However, when cytokinin activity is increased, siliques become abnormally clustered or dispersed and oriented incorrectly. This observation has been made in the transgenic *amiRPUP14* line [123], the *ahp6* single mutants [124], and the *ckx3 ckx5* double mutant [125]. Spatial coordination of auxin and cytokinin responses is also required for controlling flower sepal initiation and size determination [81]. Moreover, cytokinins and auxin are antagonistic in the axillary meristems (the stem cell niches that form at the axils of leaves and generate lateral branches), whose initiation is inhibited by auxin and promoted by cytokinins [126].

Ovule formation

Models for simple leaf serration development and ovule formation have been proposed that involve auxin, but a role for cytokinins has been proposed only for ovule formation [45,60,127,128] (Figure 2A,C). The decreased cytokinin perception in the *cre1-12 ahk2-2 ahk3-3* triple mutant causes a reduction in ovule number, which also happens in *cuc2-1 pSTK::CUC1_RNAi* (*cuc2-1* mutant plants expressing an RNA interference construct targeting *CUC1* under the control of the ovule-specific *SEEDSTICK* promoter) and *pin1-5* [129,130]. Conversely, ovule number is increased as a consequence of the increment in cytokinin content in the *ckx3 ckx5* double mutant, in which cytokinin inactivation is reduced [125], or by treatment with exogenous cytokinins [129,130]. Exogenous application of cytokinin also rescues the number of ovules in *cuc2-1 pSTK::CUC1_RNAi* plants but not in *pin1-5* plants. Since cytokinins increase *PIN1* expression even in *cuc2-1 pSTK::CUC1_RNAi* plants, these hormones have been proposed to modulate ovule development by acting as intermediates between CUC1 or CUC2 and PIN1 [130].

The participation of cytokinins in gynoecium development and ovule formation is also supported by the functional analysis of the *CRF* genes (Box 3): the *crf2 crf3 crf6* triple mutant exhibits reduced ovule number, pistil length, and *PIN1* expression [131]. An enhanced cytokinin response was observed in *Nicotiana tabacum* BY-2 protoplasts transiently transformed with the *35S::CUC1* or *35S::CUC2* constructs, which overexpress *CUC1* or *CUC2*, respectively, and a reduction in the content of cytokinin active forms was detected in *cuc2-1 pSTK::CUC1_RNAi* inflorescences prior to fertilization [132]. In pistils, CUC1 and CUC2 repress the *UGT73C1* and *UGT85A3* genes, which encode enzymes that reversibly inactivate cytokinins by O-glucosylation [132,133] (Box 2 and Figure 1, #22).

In this section, we provide examples of the crosstalk between auxin and cytokinins in very different developmental events, showing that in several cases where auxin maxima set a pattern for organogenesis, cytokinins function in antagonizing auxin – such as separating auxin maxima – or act in ways that have yet to be deciphered. In addition, cytokinins seem to mediate different, developmental context-dependent, effects on PIN1 in different tissues; for example, in roots, cytokinins seem to deplete PIN1 at specific polar domains, resulting in repatterning of PIN1 polarities at the tissue level, which in turn modifies auxin flow directionality [120]. In ovules, however, cytokinins promote *PIN1* expression [130].

Known roles of cytokinins in compound leaf development

In the development of tomato (*S. lycopersicum*) compound leaves, cytokinin contents and responses modulate the extent of their compoundness. Cytokinin signaling can be visualized using the two-component signaling sensor (TCS), a synthetic promoter containing concatemerized binding motifs for type-B ARRs [109]; TCSn and TCSv2 are improved versions of TCS [134]. The cytokinin response was visualized using *TCSv2:3XVENUS* in the SAM and at the margins of the developing tomato leaves [135]. Moreover, super-compound leaves are formed when cytokinin activity is genetically increased, as in the *FIL$_{pro}$>>AtIPT7* transgenic line (*FIL$_{pro}$*, the *FILAMENTOUS FLOWER* promoter, acts specifically in lateral organs) [136], *FIL$_{pro}$>>miR319* [137], or the loss-of-function *clausa* (*clau*) mutant [135]. CLAU is a Myb transcription factor that promotes the end of the morphogenetic phase in developing tomato leaves. CLAU attenuates cytokinin signaling, as shown by the increased *TCSv2:3XVENUS* activity and reduced expression of the *type-A TRRs TRR3* and *TRR5* in the *clau* mutant background [135]. CLAU also promotes gibberellin (GA) responses partially by inducing the expression of the *SlGA20oxidase-1* gibberellin biosynthesis gene [137]. miR319 represses *LANCEOLATE* (*LA*), the tomato ortholog of arabidopsis *TCP4*; LA promotes gibberellin responses and differentiation [138], and reduces sensitivity to cytokinins [137] (Figure 1, #23 and 24).

As expected, when cytokinin activity decreases, as in the miR319-resistant *La-2* mutant [137] and the *FIL$_{pro}$>>AtCKX3* [136], *pFIL>>CLAU* and *pCLAU>>CLAU* [135] transgenic lines, tomato leaves are simpler than those of the wild type. Furthermore, cytokinin depletion caused by *CKX3* overexpression suppresses the super-compound and lobed leaf phenotype caused by *KNOX1* overexpression in tomato and arabidopsis, respectively. Conversely, increasing cytokinin biosynthesis by *FIL$_{pro}$>>AtIPT7* compensates for downregulation of *KNOX1*, revealing that KNOX1 proteins act through cytokinins for compound leaf development [136] as they do in the SAM (Figure 1, #1).

In *C. hirsuta*, the homeodomain leucine zipper (HD-Zip) class I transcription factor REDUCED COMPLEXITY (RCO) is required for compound leaf development by repressing growth at the flanks of leaflets [13,139]. *RCO* is part of a tandem gene triplication, and the arabidopsis genome has only one of these three genes, *LATE MERISTEM IDENTITY1* (*LMI1*) [139,140]. In *C. hirsuta*, *RCO* is expressed at the basal sinuses of leaflets [13,139,140], and *LMI1* is expressed in the rest of the compound leaf margin; the expression pattern of *LMI1* is similar in arabidopsis and *C. hirsuta* [139,140]. RCO directly regulates genes controlling cytokinin homeostasis: it activates the expression of *LONELY GUY7 (LOG7)* and *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN4 (AHP4)*, and inhibits the expression of *CKX2*, *CKX3*, *CKX6*, and *UGT85A1* (Boxes 2 and 3). Consequently, RCO increases endogenous cytokinin levels and the cytokinin response as detected by the *TCSn::TDT* sensor (TDT is a red fluorescent protein). In addition, increasing cytokinin signaling in the *RCO* expression domain (with the *pRCO::ARR1ΔDDK* construct, in which the *RCO* promoter drives the expression of a constitutively active form of the type-B ARR1 of arabidopsis) restores leaf compoundness in the *rco* mutant. These observations indicate that RCO acts, at least partially, through cytokinins [141] (Figure 1, #25).

In summary, evidence from studies of compound leaves in *S. lycopersicum* and *C. hirsuta* thus shows that cytokinins play key roles in promoting leaf compoundness. In addition, the RCO transcription factor activates cytokinins during compound leaf development in *C. hirsuta*.

Evidence for cytokinins participating in simple leaf margin patterning

Mutant or transgenic lines with altered cytokinin homeostasis that exhibit smooth leaf margins

In arabidopsis leaf primordia, depleting cytokinins by increasing their degradation results in developmental defects. The *ANT:CKX3* transgenic line overexpresses *CKX3* under the control of the promoter of *AINTEGUMENTA* (*ANT*), a gene specifically active during leaf primordia development. The consequences of enhanced cytokinin degradation, caused by enhanced CKX3 activity in the *ANT:CKX3* line, include a longer plastochron (the time between successive leaf initiation events) and a reduction in rosette leaf size and vein density [142]. However, little attention has been paid to the leaf margin, which is smoother in the *ANT:CKX3* transgenic line than in the wild type.

IPT enzymes catalyze the first step of cytokinin biosynthesis (Box 2), with IPT3, IPT5, and IPT7 being most specific to the vegetative phase, as other IPTs are not expressed in rosette leaves or are expressed in all tissues [87]. Similar to the *ANT:CKX3* transgenic line, the *ipt3 ipt5 ipt7* triple mutant presents a longer plastochron and leaves with smoother margins than the Col-0 wild type (Figure 3A–E) [88]. Type-B ARRs are the final effector targets of the phosphorylation signaling cascade of cytokinins [76] (Box 3). Similar to *ANT:CKX3* and *ipt3 ipt5 ipt7* plants, the *arr1 arr10 arr12* triple mutant, which carries loss-of-function alleles of three members of the major subfamily of type-B ARRs [143], shows smoother leaf margins (Figure 3F,G). Since type-B ARRs are predominantly expressed in actively dividing cells in the SAM and young developing leaves [102], it makes sense that they may influence leaf development, and in particular, leaf margin morphogenesis.

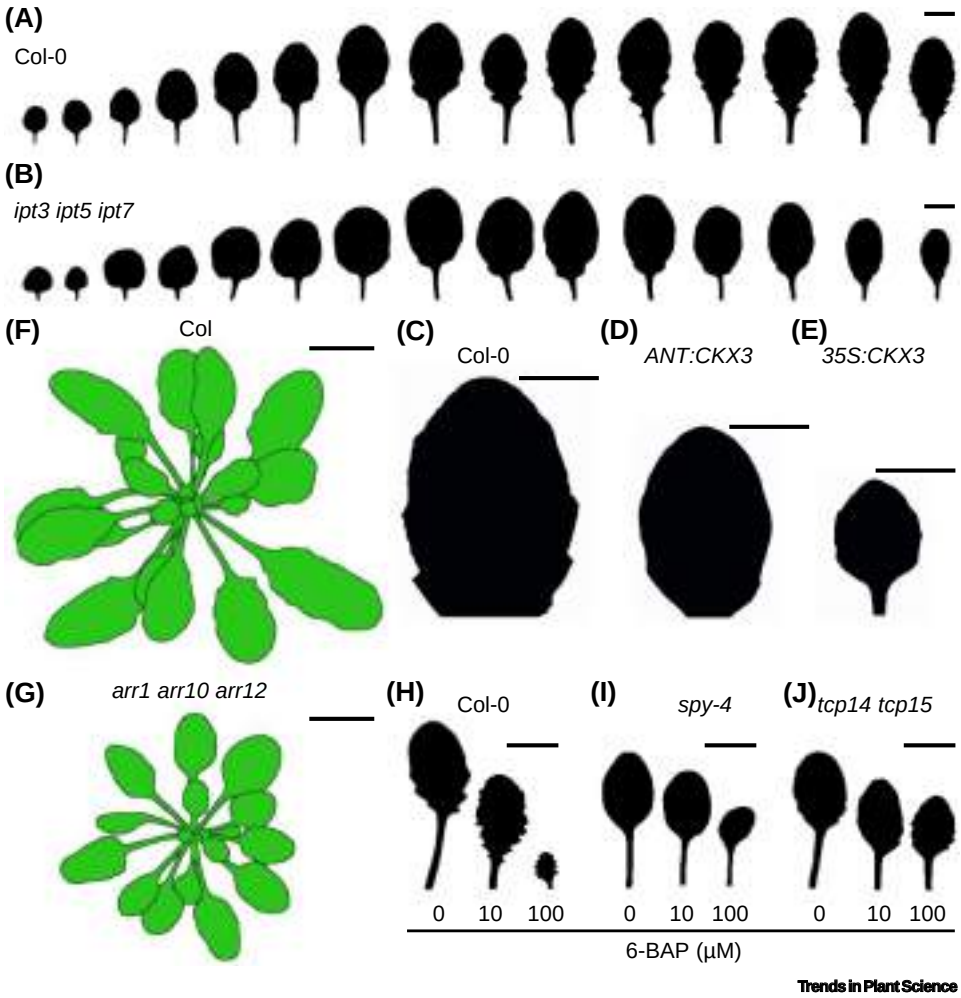


Figure 3. Arabidopsis mutants and transgenic plants affected in cytokinin homeostasis that exhibit smoother leaf margins than their wild types. (A, B) Silhouettes of arabidopsis rosette leaves from successive nodes (1st–15th, from left to right; cotyledons are not shown) of (A) the Col-0 wild type and (B) the *ipt3 ipt5 ipt7* triple mutant, drawn from pictures taken from plants grown in plates and collected 35 days after stratification (das). (C–E) Sixth-node rosette leaves from (C) the Col-0 wild type, and (D) *ANT:CKX3* and (E) *35S:CKX3* transgenic plants. (F, G) Rosettes of (F) Col (named in this way by the authors of [143]) wild type and (G) *arr1 arr10 arr12* plants grown under short days (8-h daylength) photographed 60 das. (H-J) Fifth-node rosette leaves of (H) the Col-0 wild type and (I) the *spy-4* and (J) *tcp14 tcp15* mutants treated with different concentrations of 6-BAP for 3 weeks. Scale bars: (A, B, and F–J) 1 cm, and (C–E) 0.5 cm. Reproduced with permission of the journals and the authors from (C–E) [142], (F, G) [143], and (H–J) [148]. Although the *ipt3 ipt5 ipt7* triple mutant was previously studied in [88], the images shown in (A, B) were obtained in our laboratory. Abbreviations: 6-BAP, 6-benzylaminopurine; *ANT*, *AINTEGUMENTA*; *arr*, *arabidopsis response regulator*; *CKX*, *CYTOKININ DEHYDROGENASE/OXIDASE*; *ipt*, *isopentenyltransferase*; *spy, spindly; tcp, teosinte branched1-cycloidea-proliferating cell factor*.

Cytokinins mediate multiple cellular effects at different stages of leaf development, as shown by dexamethasone-inducible transgenes that increase or decrease cytokinin content by inducing cytokinin biosynthesis or degradation, respectively [75]. At the proliferation phase, cytokinins were found to contribute to maintaining cell proliferation, hence delaying the transition to the cell expansion phase. Both the increase and decrease of cytokinin content at the proliferation phase generated smaller leaves, because of reduced cell expansion or a reduced number of cells, respectively. After the proliferation phase, however, cytokinins stimulated cell expansion

and differentiation. Therefore, cytokinin biosynthesis activation at this stage largely increased leaf size and biomass, while cytokinin degradation had the opposite effect [75]. These observations show some developmental context-dependent aspects of cytokinin activity, and may help us interpret the smooth leaf margin phenotypes caused by enhanced cytokinin degradation in *ANT:CKX3* transgenic plants, as well as by reduced cytokinin biosynthesis in *ipt3 ipt5 ipt7* and reduced cytokinin perception in *arr1 arr10 arr12* multiple mutant plants described above. Indeed, these phenotypes can be explained by either a reduced number or density of serrations, or a reduction in sinus depth; in an analogy with compound leaf morphology, the former corresponds to a reduction in the number of leaflets and the latter to a reduction in leaflet size. Consequently, the cytokinin defective lines discussed in this section may be suffering defects in auxin patterning itself or, alternatively, their altered proliferation/expansion arising from low cytokinin levels could reduce the growth differences between protrusions and sinuses, thus flattening the serrations.

Cytokinins affect proximo-distality in maize leaves

Maize (*Zea mays*) leaves consist of a proximal sheath and a distal blade, with the ligule and auricle structures at the boundary between sheath and blade. The *Hairy Sheath Frayed1* (*Hsf1*) mutant of maize harbors a missense, gain-of-function mutation in the *Histidine Kinase1* (*ZmHK1*) gene and this mutation increases affinity of the ZmHK1 protein for cytokinins, hence increasing cytokinin response. Cytokinin hypersignaling in incipient leaf primordia of the *Hsf1* mutant causes the morphological phenotype of its expanded leaves: ectopic outgrowths in the distal blade, which seem to correspond to sheath, auricle, and ligule proximal structures. Treatment with exogenous cytokinins increases the severity of the *Hsf1* leaf phenotype and phenocopies *Hsf1* in wild-type plants [144]. These results demonstrate that cytokinins are involved in leaf proximo-distal patterning of maize. Although the leaf phenotype of the *Hsf1* mutant had only been explained from the point of view of proximo-distality, we speculate that a plausible complementary explanation is that its outgrowths evidence the effects of cytokinins in leaf margin morphogenesis.

Antagonistic roles of class I and class II (CIN) TCPs in leaf development

The class I TCP genes *TCP14* and *TCP15* are expressed in leaf primordia, where they regulate cell division [145]. Arabidopsis *SPINDLY* (*SPY*) encodes an *O*-linked *N*-acetylglucosamine transferase that catalyzes the addition of *N*-acetylglucosamine to a hydroxyl group of a serine or threonine, or to a thiol group of a cysteine of the target protein [146,147]. SPY negatively regulates gibberellin responses and stabilizes TCP14 and TCP15 to facilitate cytokinin responses in leaves (Figure 1, #26–28). Col-0 plants treated with the synthetic cytokinin 6-benzylaminopurine (6-BAP) produced highly serrated leaves. However, this effect was not shown in *spy* or *tcp14 tcp15* plants, which exhibit smoother leaf margins than those of Col-0 (Figure 3H–J) [148,149]. Furthermore, the KISS ME DEADLY (KMD) family of four F-box proteins targets type-B ARRs [150] and TCP14 [151] for degradation, inhibiting cytokinin responses (Figure 1, #29 and 30). These observations show a functional relationship between class I TCPs and cytokinins.

CIN-TCPs inhibit *CUC* gene expression and the formation of leaf serrations [152]. Particularly, TCP3 directly activates the expression of miR164, *AS1*, and *SHY2*, all of which suppress *CUC* expression [39] (Figure 1, #31–34). TCP4 also interacts with CUC2 and CUC3, inhibiting their transcriptional regulatory activity and thus the formation of serrations in arabidopsis and leaflets in *C. hirsuta* [153] (Figure 1, #35). *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE9* (*SPL9*) is post-transcriptionally repressed by miR156 [154,155], and encodes a protein that binds TCP4, thereby hindering its dimerization with CUC2 and CUC3 (Figure 1, #36 and #37). miR156 levels decline in successive leaf nodes, de-repressing *SPL9* and releasing CUC factors from TCP4, hence allowing a type of heteroblasty in which the number of serrations or leaflets gradually increases in successive leaves [153]. Intriguingly, TCP4 inhibits cytokinin activity by

inducing the expression of *ARR16* [156] (Figure 1, #38), which encodes a type-A ARR that inhibits cytokinin responses [101]. This observation suggests a conserved role for arabidopsis TCP4 and *S. lycopersicum* LA [137]. Taken together, these findings implicate the TCP family, with antagonistic activities of its class I and II proteins, in regulating developmental responses to cytokinins.

The *YUCCA* (*YUC*) gene family has 11 members in arabidopsis and encodes flavin monooxygenases that catalyze the hydroxylation of the amino group of tryptamine, yielding *N*-hydroxyl-tryptamine, a rate-limiting step in tryptophan-dependent auxin biosynthesis [157] (Figure 1, #39). TCP15 represses the transcription of *YUC1* and *YUC4* [158], and TCP4 activates that of *YUC5* [40,159] (Figure 1, #40 and #41), providing another example of the antagonistic activities of class I and class II TCPs in auxin biosynthesis. Moreover, some *YUC* genes may be required for leaf margin development. On the one hand, the *yuc1 yuc2 yuc4 yuc6* quadruple mutant shows narrow leaves that lack hydathodes [160]. On the other hand, the WUS-RELATED HOMEOBOX1 (WOX1), WOX3 [also named PRESSED FLOWER (PRS)] and WOX5 transcription factors redundantly promote auxin biosynthesis along the leaf margin and are required for lateral leaf growth [161]. In a study of the mechanism of pluripotency acquisition during callus formation on callus-inducing medium, callus tissue structure was similar to that of root apical meristem. In the callus cell layer able to regenerate organs, WOX5 promotes auxin production and simultaneously enhances cytokinin sensitivity via repressing type-A ARRs, which favors pluripotency at this cell layer [162]. These results indicate that several *YUC* genes redundantly act on leaf margin shaping and opens the possibility that WOX5 regulates auxin production and cytokinin signaling in this process.

Role of cytokinins in the juvenile-to-adult phase transition

Juvenile and adult rosette leaves are easily distinguishable in arabidopsis [163]: juvenile leaves are small, relatively rounded and smooth or slightly serrated, while adult leaves are bigger, elliptical, more serrated and have abaxial trichomes [164–166]. As already mentioned, miR156, SPL9, TCP4, CUC2, and CUC3 participate in this heteroblastic process [153]. In addition, SPL2, SPL9, SPL11, SPL13A/B, and SPL15 induce expression of the *MIR172* gene [155,167]; miR172 represses genes encoding transcriptional regulators that repress the juvenile-to-adult phase transition and the transition from vegetative to reproductive development, such as *APETALA2* (*AP2*)-like *TARGET OF EAT1* (*TOE1*) and *TOE2* [168].

Cytokinins favor the juvenile-to-adult phase transition during vegetative growth [169,170]. As expected from plants with impaired cytokinin signaling or reduced cytokinin levels, more leaves show juvenile features in *AtML1:ARR1-SRDX*, *AtML1:CKX1*, *ahk2 ahk3*, *arr1 arr10 arr12*, and *CKX1ox* (overexpressing *CKX1*) plants than in Col-0 [169]. *AtML1* is the arabidopsis *MERISTEM LAYER 1* gene, whose promoter is epidermis-specific, and the SRDX motif converts transcriptional activators into dominant repressors. Conversely, as expected from plants with enhanced cytokinin signaling or increased cytokinin levels, the onset of adult features was observed in earlier leaves in *AtML1:LOG4*, *repressor of cytokinin deficiency 2* (*rock2*, a gain-of-function mutation of *AHK2* [171]) and *ckx3 ckx4 ckx5 ckx6* plants. Moreover, SPL (Figure 1, #42), miR172, TOE1, and TOE2 seem to be required for cytokinin activity in phase transitions [169].

In conclusion, genotypes with reduced cytokinin status show a prolonged juvenile phase, and a higher cytokinin status undergo an earlier transition to the adult phase. These results provide additional support for the hypothesis that cytokinins affect leaf margin morphogenesis, and strongly suggest that these hormones also act on most if not all other developmental features that distinguish juvenile from adult rosette leaves.

Gene expression patterns that coincide at the basal region of leaf primordia

As mentioned above, the *TCSn::GFP* sensor is useful to monitor transcriptional activation in response to a cytokinin stimulus in arabidopsis [134]. This reporter is expressed in arabidopsis Col-0 (Figure 4A) in a pattern similar to that of Pro_{CUC2}*:GUS* [46] (Figure 4B), at the basal region of leaf primordia, and excluded from the protrusions of serrations. As also mentioned above, a role for cytokinins in leaf compoundness has been demonstrated in tomato [135,136]. In developing tomato leaves, the expression of the *CUC* homolog *GOBLET* (*GOB*) is similar to that of *CUC2* in arabidopsis leaf primordia [172]. In addition, the cytokinin response, as visualized by *TCSv2:3XVENUS* [135], is similar in tomato and arabidopsis leaves (Figure 4C,D). These observations further support the hypothesis of a conserved role for cytokinins, regulating the extent of tomato leaf compoundness and that of margin serration in arabidopsis leaves.

Since the *TCSn* promoter contains binding motifs for type-B ARRs [63], the *TCSn::GFP* expression pattern matches that of cytokinin-responsive genes [104], such as *CKX5* [94] (Figure 4E). The expression of the cytokinin biosynthesis gene *LOG2* shown by $LOG2_{pro}$*:GUS* is also observed at the basal region of arabidopsis leaf primordia [90]. As mentioned above, the *RCO* promoter of *C. hirsuta* is specifically active at the basal sinuses of leaflets [139], where RCO activates cytokinins [141]. The *RCO* promoter drives reporter gene expression in proximal and internal regions of arabidopsis leaf primordia (Figure 4F). Interestingly, arabidopsis plants carrying the *pRCO::ARR1ΔDDK* or *pRCO::RCOg-VENUS* transgenes show complex, highly lobed, leaves. This morphological phenotype is due to reduced cell size and cell differentiation at the *RCO* expressing region, compared to the wild-type Col-0. Therefore, regulation of leaf margin shape by RCO is at least partially mediated by cytokinins, which reduce cell expansion [141], an effect that has also been observed as a result of the increase of cytokinin content at the proliferative phase of leaf development [75]. As mentioned above, some leaf primordia cells acquire meristematic activity, creating a proliferative region at the junction between the lamina and the petiole [7,8] (Figure 3G). The expression of *TCSn::GFP*, *CUC2*, *type-B ARRs*, *CKX5*, *LOG2*, and *RCO* occurs at the proliferative region of leaf primordia margins (Figure 4G).

Other genes related to cytokinins, such as *CYCD3;2* and *CYCD3;3* encoding D-type cyclins [173], and *TCP14* [145], are expressed mainly in the basal region of developing leaves. Expression of *CYCD3;1*, *CYCD3;2*, and *CYCD3;3* [173] (Figure 4H–J), and of *TCP14*, and *TCP15* [145] (Figure 4K–N) has been visualized by a β-glucuronidase (GUS) reporter in leaf primordia. Cytokinins activate *CYCD3;1* expression [174], and the *cycd3;1-3* triple mutant is impaired in aspects of cytokinin responses such as meristem maintenance, branching, and shoot formation [173].

The precise spatial relationships of *CUC* expression and cytokinin perception, visualized based on *TCS*-based sensors, is not known. Although both patterns seem to overlap at the sinuses of serrations, they may be spatially separated at a cellular level, a possibility that should be clarified in the future. This research will require reporters that are more sensitive than those based on the *TCS* promoters.

Concluding remarks

Cytokinins are known regulators of auxin in different contexts but also have some roles apparently not related to auxin. An important underexplored question is if – and if this were the case how – cytokinins influence simple leaf margin development, a process where the role of auxin has been studied in depth (see Outstanding questions). Cytokinins favor leaf compoundness in tomato

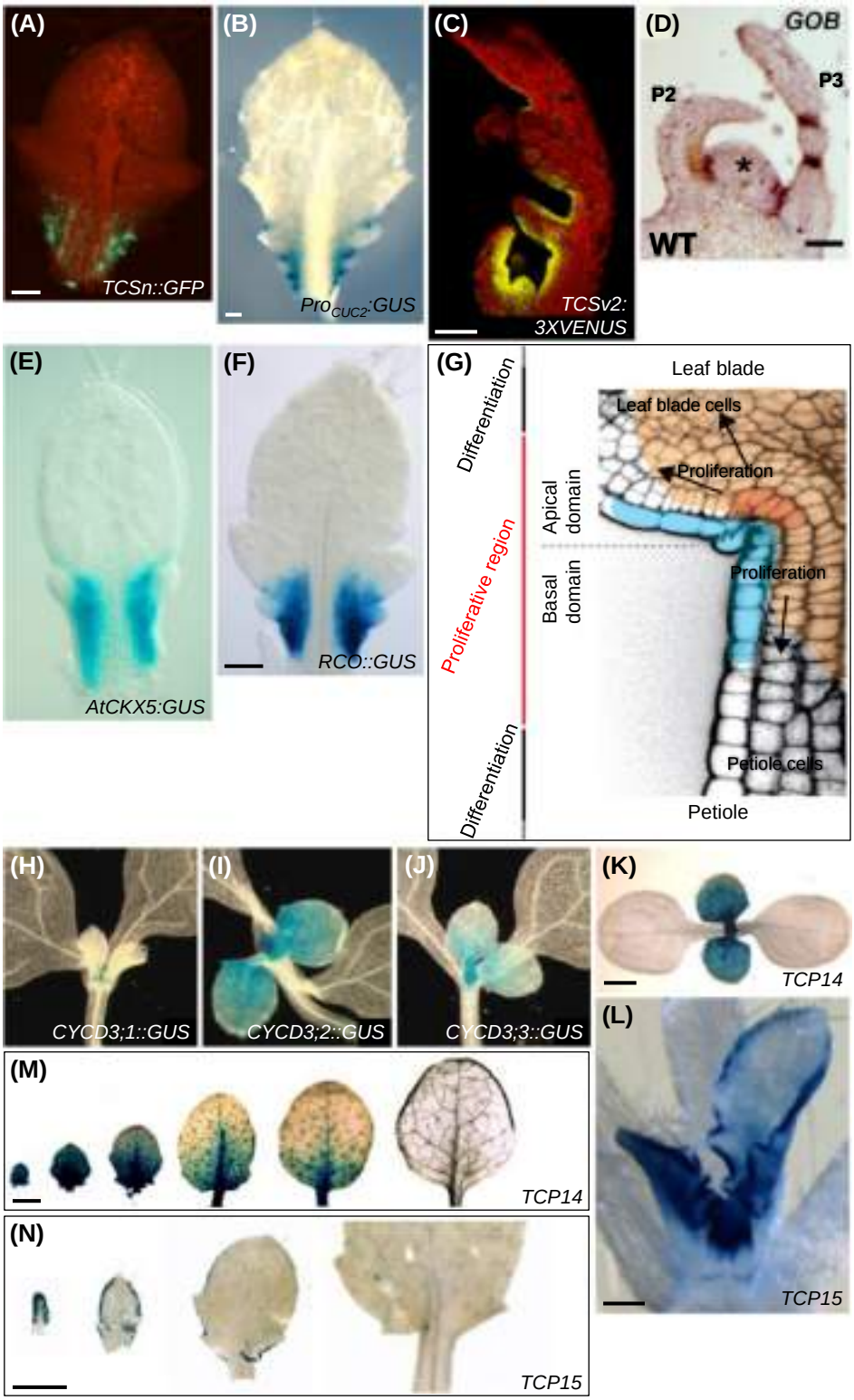
Outstanding questions

Do cytokinins contribute to leaf margin shaping in simple leaves, as they do in compound leaf development?

Cytokinins contribute to the separation of secondary roots, and of aerial organs emerging from the SAM; do they similarly contribute to the separation of simple leaf serrations?

Do CUC factors regulate the activity of cytokinins in simple leaf primordia margins, as they do in ovule boundaries?

What is the extent of the contribution of cytokinins to the juvenile-to-adult phase transition in arabidopsis rosette leaves?



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[136] and *C. hirsuta* [141], but their role in margin patterning in the simple leaves of arabidopsis remains unclear; rather, auxin was thought to provide all the positional information required for producing leaf serrations in arabidopsis [45,60]. Here, we reviewed observations suggesting that cytokinins also participate in forming the serrations in arabidopsis leaf margins. The genetic and pharmacological evidence discussed here points to a clear involvement: mutant (*ipt3 ipt5 ipt7*, *arr1 arr10 arr12*, *spy-4*, and *tcp14 tcp15*) or transgenic (*ANT:CKX3*) plants with reduced cytokinin activity show smoother leaf margins than those of Col-0 (Figure 3), and treatment with the synthetic cytokinin 6-BAP increases serration in Col-0 leaves [148].

In addition, current models for ovule formation and leaf serration development share some intriguing commonalities [45,60,128,130] (Figure 2C). Cytokinin activity may be regulated by CUC factors for leaf margin morphogenesis, as in ovule formation [133]. This hypothesis, which should be tested by cell-level studies, is supported by the similar expression patterns of *GOBLET* in tomato, *CUC2* in arabidopsis, and the *TCS* sensors in both species (Figure 4), and by the fact that TCP factors regulate both *CUC2* and cytokinin activities; while class II CIN-TCPs inhibit CUC2 and cytokinin activities, class I TCPs facilitate cytokinin responses (Figure 1).

In the SAM, CUC [175–177] and EPFL [59] factors and cytokinins [79] participate in lateral organ spacing. In the roots, cytokinins separate lateral primordia by inhibiting the formation of new auxin maxima [116–118,123]. As in these two types of developmental events and ovules, cytokinins may separate auxin maxima in leaves, and hence contribute to creating or maintaining lobes or serrations.

Since cytokinins promote leaf compoundness in plants with compound leaves, studying their role in margin morphogenesis of dicotyledonous simple leaves will provide additional insight into leaf development. Special attention should be paid to the relationship of cytokinins with auxin and the CUC and SPL proteins in three developmental processes: the margin patterning that determines the number and position of auxin maxima at leaf primordia, the control of margin growth during leaf expansion, and the progression of the juvenile-to-adult phase transition in leaves.

Figure 4. Morphogenetic role of the junction between the petiole and the lamina of the leaf primordium. (A) Expression of the *TCSn::GFP* cytokinin response reporter (green signal) and (B) Pro_{CUC2}*:GUS* (blue staining) in leaf primordia, restricted to the basal sinuses. (C) Expression of the *TCSv2:3XVENUS* reporter (yellow signal) at the SAM and the margin of a developing leaf in tomato. (D) *In situ* hybridization of a longitudinal SAM section with a *GOB* probe in tomato. *GOB* is expressed at the boundaries between leaf primordia and the SAM, and in narrow stripes at the leaf margins, flanking the places where the leaflet primordia will emerge. An asterisk marks the SAM. P indicates the plastochron number. (E) *AtCKX5:GUS* expression (blue staining) localized at the base of the youngest emerging leaves, marking the developing leaf petiole. (F) *RCO::GUS* expression in an arabidopsis leaf surrounding the emerging serrations. (G) Leaf primordia proliferative region model. The proliferative region is maintained at the junction region between the lamina (blade) and the petiole (between apical and basal domains) and supplies both lamina and petiole cells. Colors represent the expression domains of *AN3* (pale orange), *CYCD4;2* (intense orange), and *SPT* (blue). A discussion of the role of these genes is beyond the scope of this review. (H–J) Expression of the *CYCD3;1*, *CYCD3;2*, and *CYCD3;3* genes shown by a GUS reporter. Copyright (2007) National Academy of Sciences, U.S.A. (K–N) Expression pattern of *TCP14* (*pTCP14:TCP14:GUS*) and *TCP15* (*pTCP15:TCP15:GUS*) (K, L) in young seedlings, 7 days after germination, and (M, N) in leaves, 20 days after germination, shown according to their sequence of initiation (left to right). Scale bars: (A, B, D, and F) 100 μm, (C) 200 μm, (K) 1.5 mm, (L) 30 μm, and (M, N) 1 mm. Reproduced with minor modifications with permission of the journals and the authors from (B) [46], (C) [135], (D) [172], (E) [94], (F) [139], (G) [7], (H–J) [173], and (K–N) [145]. (A) Although the *TCSn::GFP* line was previously studied in [134], the image shown in (A) was obtained in our laboratory. Abbreviations: *AN3, ANGUSTIFOLIA3; CKX, CYTOKININ DEHYDROGENASE/OXIDASE; CUC2; CUP-SHAPED COTYLEDON2; CYCD, CYCLIN D; GFP, green fluorescent protein; GOB, GOBLET; GUS, β-glucuronidase; RCO, REDUCED COMPLEXITY; SAM, shoot apical meristem; SPT, SPATULA; TCP, TEOSINTE BRANCHED1-CYCLOIDEA-PROLIFERATING CELL FACTOR; TCS, two-component signaling sensor.*

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Declaration of interests

No interests are declared.

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Cytokinins contribute to Arabidopsis leaf margin morphogenesis

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SUMMARY

The serrated margin of *Arabidopsis thaliana* simple leaves is patterned by the position and size of auxin maxima in leaf primordia margins, which are specified by the auxin efflux carrier PIN1, the transcription factor CUC2, and auxin. Loss-of-function alleles of Arabidopsis *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*) reduce bilateral symmetry in rosette leaves, due to the random position, size, and number of margin protrusions and sinuses.

We discovered that VCC interacts with components of the fatty acid elongase complex, which synthesizes very-long-chain fatty acids that inhibit cytokinin biosynthesis. This finding prompted us to study the effects of cytokinin excess and depletion on leaf development through pharmacological and genetic approaches.

A lower cytokinin content or response reduced the number of auxin maxima in Arabidopsis leaf primordia margins and the extent of serration in expanded leaves. The leaf asymmetry caused by the *vcc-2* loss-of-function allele increased upon treatment with exogenous cytokinins. In addition, cytokinin response was asymmetric between the left and right halves of *vcc-2* leaf primordia.

Here, we demonstrated that cytokinins play a role in shaping the margin of Arabidopsis simple leaves, in crosstalk with auxin. We hypothesize that VCC regulates both auxin and cytokinin responses during Arabidopsis leaf primordia morphogenesis.

Keywords: Leaf margin morphogenesis, simple leaf development, auxin, cytokinins, VCC, VLCFAs, *Arabidopsis thaliana*.

INTRODUCTION

In Arabidopsis (*Arabidopsis thaliana*), leaf primordia appear at the flanks of the shoot apical meristem (SAM) as groups of cells that after an initial phase of proliferation, then switch basipetally to cell expansion and differentiation (Donnelly *et al.*, 1999). In many plant species, including Arabidopsis, local modulation of growth at the margins of leaf primordia produces varied margin shapes (Hagemann & Gleissberg, 1996; Ichihashi *et al.*, 2011; Alvarez *et al.*, 2016; Tsukaya, 2021). Such margin shape diversity is mostly dependent upon the number and relative sizes of serrations or lobes in simple leaves, or leaflets in compound leaves (Hay & Tsiantis, 2006; Runions *et al.*, 2017; Kierzkowski *et al.*, 2019).

Auxin has important functions in specifying serrations and lobes in simple leaves. In the margins of Arabidopsis rosette leaf primordia, auxin localization depends on the auxin efflux carrier PIN-FORMED1 (PIN1), the transcriptional regulator CUP-SHAPED COTYLEDON2 (CUC2), and auxin itself. These three factors regulate each other in feedback loops that in turn create periodic, interspersed domains of auxin and CUC2 activity. The auxin and CUC2 domains specify protrusions and sinuses, respectively, along the developing leaf margin (Heisler *et al.*, 2005; Paciorek *et al.*, 2005; Scarpella *et al.*, 2006; Bilsborough *et al.*, 2011). Formation of auxin maxima and protrusions directly reflects CUC2 levels; indeed, the loss-of-function *cuc2-3* mutant lacks auxin maxima in leaf primordia and its expanded leaves have smooth margins (Hibara *et al.*, 2006; Nikovics *et al.*, 2006; Kawamura *et al.*, 2010). By contrast, plants carrying the *cuc2-1D* gain-of-function allele display increased lobe size and sinus depth (Nikovics *et al.*, 2006; Larue *et al.*, 2009).

The Arabidopsis ERECTA (ER) family includes the ER, ER-LIKE1 (ERL1), and ERL2 receptor kinases, which are involved in regulating auxin response along the leaf primordium margin. At the protrusions, auxin promotes *ERL2* expression and represses that of *EPIDERMAL PATTERNING FACTOR-LIKE2* (*EPFL2*). At the sinuses, the EPFL2 secreted peptide physically interacts with and attenuates the activity of members of the ER family, thereby inhibiting the auxin response. This alternation between auxin and EPFL2 activity represents another feedback loop that restricts the auxin response to protrusions and thus maintains leaf serration growth (Tameshige *et al.*, 2016).

Arabidopsis At2g32280 belongs to a family of 15 members that encode proteins with a Domain of Unknown Function1218 (DUF1218), which is plant specific. At2g32280 was first named *VASCULATURE COMPLEXITY AND CONNECTIVITY* (*VCC*), since its loss-of-function mutant alleles make the cotyledon venation pattern simpler than that of the wild type and cause disconnected veins to appear on cotyledons (Roschzttardtz *et al.*, 2014). In a large-scale screening for SALK T-DNA insertional lines exhibiting leaf phenotypes (Wilson-Sánchez *et al.*, 2014), we identified only one line with bilaterally asymmetric rosette leaves, SALK_047972, which carries a T-DNA in *VCC* and was named *vcc-2* by Roschzttardtz *et al.*

(2014). In agreement with the asymmetric phenotype of *vcc* mutant leaves, we first named *VCC* as *DESIGUAL1* (*DEAL1*). Based on similarity to the DUF1218 domain, we identified three additional members related to *VCC*: At4g21310 (*DEAL2*), At1g11500 (*DEAL3*), and At1g05291 (*DEAL4*). These four genes constitute the DEAL subfamily (Wilson-Sánchez *et al.*, 2018). *VCC* is expressed early in leaf primordia development and appears to be required to produce regular auxin maxima at leaf margins and, thus, for bilateral symmetry at very early stages of leaf organogenesis (Wilson-Sánchez *et al.*, 2018). In *vcc* mutants, auxin and CUC2 domains are unevenly spaced and asymmetrically distributed in both halves of leaf primordia. Therefore, the leaves of *vcc* mutants exhibit bilateral asymmetry because of the mispositioning and mis-sizing of leaf protrusions and sinuses.

Auxin and cytokinin signaling interplay in several plant developmental events (El-Showk *et al.*, 2013). Crosstalk between these phytohormones is required for establishing the root stem cell niche (the group of embryonic root precursor cells) (Müller & Sheen, 2008), regulating root meristem size and root growth (Dello Ioio *et al.*, 2008), and controlling lateral root density, with cytokinins preventing the formation of lateral roots (Chang *et al.*, 2015). In the SAM, tissue domains with cytokinin activity also separate auxin maxima, allowing the sequential and properly spaced emergence of leaf primordia (Ori *et al.*, 2000; Yanai *et al.*, 2005; Su *et al.*, 2011) and flower primordia (Bartrina *et al.*, 2011; Besnard *et al.*, 2014). In ovules, CUC factors positively regulate the cytokinin pathway by repressing the expression of uridine diphosphate glycosyltransferase genes (*UGT*s), whose encoded proteins inactivate cytokinins. In contrast to their effects suppressing lateral root development, cytokinins promote ovule formation and increase ovule number (Cucinotta *et al.*, 2020). Current models describing leaf serration and ovule development share some commonalities. Although cytokinins have been proposed to act downstream of CUC factors in ovule formation, it has not been investigated whether they participate during simple leaf margin development (Bilsborough *et al.*, 2011; Galbiati *et al.*, 2013; Cucinotta *et al.*, 2014).

Cytokinins also contribute to the development of compound leaves in tomato (*Solanum lycopersicum*) and *Cardamine hirsuta*. Class I *KNOTTED1*-like homeobox-containing (*KNOX1*) genes maintain SAM pluripotency by activating cytokinin biosynthesis, and *KNOX1* genes expression is redeployed in the leaf primordia of these plant species (Hay & Tsiantis, 2006; Efroni *et al.*, 2010; Hay & Tsiantis, 2010; Bar & Ori, 2014; Kierzkowski *et al.*, 2019). In addition, an increase in cytokinin content or responses induces the development of super-compound leaves in tomato, while a decrease in cytokinin content or responses has the opposite effect (Shani *et al.*, 2010; Bar *et al.*, 2016; Israeli *et al.*, 2021). Finally, the homeodomain leucine zipper (HD-Zip) class I transcription factor REDUCED COMPLEXITY (RCO) of *C. hirsuta* represses growth at the flanks of leaflets and is required for compound

leaf development (Vlad *et al.*, 2014; Kierzkowski *et al.*, 2019). RCO acts, at least partially, by promoting cytokinin activity (Hajheidari *et al.*, 2019).

Here, we describe the identification of VCC interactors, two of which are components of the fatty acid elongase complex. This complex synthetizes very-long-chain fatty acids (VLCFAs, with an acyl chain of at least 20 carbons), which are known to inhibit cytokinin biosynthesis (Nobusawa *et al.*, 2013). This finding prompted us to revisit the hypothesis that cytokinins play a role in Arabidopsis rosette leaf margin morphogenesis, in cooperation with auxin (Navarro-Cartagena & Micol, 2022). To this end, we obtained multiple combinations of mutations in genes required for cytokinin biosynthesis or signaling and in genes related to auxin homeostasis. We also used cytokinin and auxin response reporters to monitor transcriptional activation in response to these phytohormones and determined that cytokinins modulate the phenotype of the *vcc-2* mutant. We discuss a possible role for VCC in coordinating auxin and cytokinins crosstalk for leaf margin morphogenesis.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of the *Arabidopsis thaliana* (L.) Heynh. wild-type accession Columbia-0 (Col-0) and of *vcc-2* (SALK_047972C) were obtained from the Arabidopsis Biological Resource Center (ABRC). Seeds for the *cuc2-1D*, *arr1 arr10 arr12*, *ckx1* (SALK_204043), *ckx3-1* (SALK_050938), *ckx5-1* (SALK_064309), and *er-3* (SALK_044110) mutants and for the *TCSn::GFP* transgenic line were obtained from the Nottingham Arabidopsis Stock Center (NASC; Nottingham, United Kingdom). Seeds of *cuc2-3*, *CUC2$_{pro}$:CUC2:RFP DR5rev$_{pro}$:VENUS*, *PIN1$_{pro}$:PIN1:GFP*, *ARF11$_{pro}$:GFP* and *DR5rev$_{pro}$:GFP* were provided by P. Laufs, *ipt3 ipt5 ipt7* by T. Kakimoto, *ANT:CKX3* by T. Werner, *rock2-10* and *rock3-1* by T. Schmülling and I. Bartrina, and *pRCO::ARR1ΔDDKK* by M. Tsiantis. Unless otherwise stated, all plants were in the Col-0 genetic background and homozygous for the mutations and transgenes indicated in each case. All transgenic plants were analyzed in the F_3 generation, with the homozygous mutant plants and their corresponding control (in the Col-0 background) isolated from the same cross. The crosses between *ipt3 ipt5 ipt7* and *CUC2$_{pro}$:CUC2:RFP DR5rev$_{pro}$:VENUS* or *PIN1$_{pro}$:PIN1:GFP* were an exception (the same transgenic lines used in the crosses with *ipt3 ipt5 ipt7* were used as controls). All mutations studied in this work are recessive, with the exception of *cuc2-1D*, *rock2-10*, and *rock3-1*. In all experiments, mutant and/or transgenic plants were grown in parallel with their corresponding wild type as a control.

Seed surface sterilization and sowing, as well as plant culture and crosses were performed as previously described (Ponce *et al.*, 1998; Berná *et al.*, 1999; Quesada *et al.*, 2000). All single and higher-order mutants were first genotyped by PCR using the primers listed in Table S1. Plants were grown under sterile conditions on half-strength Murashige and Skoog (MS; Duchefa Biochemie) medium containing either 0.7% (w/v) plant agar (Duchefa Biochemie) or 0.6% Gelrite (Duchefa Biochemie; only in those experiments in which *vcc-2* was involved, with the exception of the 6-benzylaminopurine (6-BAP) treatment, in which Plant Agar was used) supplemented with 1% (w/v) sucrose (Duchefa Biochemie) at 20°C ± 1°C, 60–70% relative humidity, and under continuous fluorescent light of ≈80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Seven (to obtain silhouettes) or 16 (in all other cases) evenly spaced seeds were sown in 14-cm-diameter Petri plates. 6-BAP (Duchefa Biochemie) was dissolved in 1 M NaOH before its addition to the medium. The same amount of NaOH was added to the control medium. For 6-BAP treatments, seeds were sown onto half-strength MS medium containing or not 6-BAP 5 days after stratification (das).

Screen based on the split-ubiquitin yeast two-hybrid membrane-based assay

The split-ubiquitin yeast two-hybrid (Y2H) membrane-based assay (Johnsson & Varshavsky, 1994; Stagljar *et al.*, 1998) was performed by Hybrigenics (Paris, France). The full-length

coding sequence of At2g32280 (1–163 amino acids) was cloned into the pB102 vector, in-frame of the sequence encoding the C-terminal half of ubiquitin (Cub), itself linked to the sequence encoding the artificial transcription factor LexA-VP16 (N-STE2-At2g32280-Cub-LexA-VP16-C). A library of Arabidopsis cDNA (D3ATS_dT) constructed into the pP50 vector (N-NubG-HA-prey-C) was screened to saturation by covering the library complexity about 10 times. Each prey in the library is fused to the N-terminal half of ubiquitin (Nub), which carries a point mutation to reduce its affinity for Cub and, consequently, the number of false positives.

Bait and preys were transferred to a *his3* yeast strain. The *HIS3* gene encodes an enzyme involved in histidine biosynthesis. An interaction between bait and prey reconstitutes ubiquitin from Nub and Cub, which is cleaved by endogenous proteases and releases LexA-VP16, which activates *HIS3* transcription, allowing yeast growth. The re-association of ubiquitin was assayed with diploid cells obtained by mating NMY32-DeltaGal4 [mata] with YHGX13 [matα]. To increase test stringency, 100 mM 3-aminotriazole (3-AT), an inhibitor of histidine biosynthesis, was added to the selection medium. From 54.8 million clones screened, 320 His^+ colonies were selected. The DNA of each positive clone was sequenced and searched against GenBank (NCBI).

A Predicted Biological Score (PBS) was computed to assess interaction reliability. This score is an e-value based on a comparison between the number of independent prey fragments found for a given interactor and the possibility of finding them by chance (background noise). PBS ranges from 0 to 1 and represents the probability of an interaction to be nonspecific; with 1 representing potential false positive interactions (Formstecher *et al.*, 2005). Hybrigenics established several PBS thresholds to define four categories from A (highest confidence) to D. An additional category, PBS E, represents interactions involving prey domains connected to more than six different Arabidopsis bait proteins in the Hybrigenics database.

Phenotypic analyses and morphometry

Leaf morphometry was performed on expanded leaves. Leaf images were taken on a flatbed scanner and rotated until the sagittal plane was vertical using Photoshop CS3 (Adobe). The leaf dissection index (LDI) was calculated as described previously (LDI = perimeter squared/[4π × area]) (Bilsborough *et al.*, 2011). Leaf perimeter and area were measured using NIS-Elements (AR Ver3.22.15) software (Nikon), selecting leaf silhouettes as regions of interest (ROIs). Leaf teeth number was counted using the MorphoLeaf software (Biot *et al.*, 2016), with a neighborhood size of 90 and a maximum negative curvature of 179 for detecting sinuses (teeth are automatically identified between sinuses).

Penetrance of the leaf asymmetry phenotype was calculated as the percentage of plants showing at least one leaf with margin patterning defects. Consensus Col-0 leaf

silhouettes were obtained by overlapping 20 rosette leaves of the 10th node and calculating the median of the stack image. Mutant and Col-0 leaf silhouettes were overlaid for comparisons using Photoshop CS3 (Adobe). To calculate the expressivity (severity) percentage of the asymmetry phenotype, total leaf area and nonoverlapping leaf area between a sample and the Col-0 consensus were measured using ImageJ 1.49v (National Institutes of Health, USA). Expressivity (%) was calculated as (nonoverlapping area/sample area) × 100. At least 10 leaves were analyzed per studied genotype.

Confocal microscopy

Confocal laser scanning microscopy images were obtained with a D-Eclipse C1 confocal microscope equipped with a DS-Ri1 digital camera and EZ-C1 software (Nikon). Visualization of fluorescent proteins was performed on leaf primordia mounted in deionized water on glass slides. GFP and RFP or chlorophyll autofluorescence were excited at 488 nm with an argon ion laser and at 543 nm with a helium-neon laser, respectively. Their emissions were detected with a 515/30-nm and 605/75-nm barrier filter, respectively. Optical sections encompassing 10 µm from the adaxial to the abaxial epidermis were photographed and overlapped with the "Volume Render" command from the "Data" menu of EZ-C1. In all figures involving successive nodes, primordia from the same genotype belonged to a unique plant.

Quantification of GFP fluorescence was performed from ROIs automatically detected with NIS-Elements establishing the following parameters within the Binary/Define threshold/RGB menu: green pixel intensity ≥ 15 and size ≥ 30. Fluorescence intensity (green pixel mean) of the ROIs was extracted from the "Histogram under binary" option (Fig. S1). Samples without any region above the threshold yielded a fluorescence intensity of zero.

Transcriptome deep sequencing (RNA-seq) analysis

Total RNA was isolated from 100 mg of Col-0, *vcc-2*, *cuc2-3*, and *cuc2-1D* tissue collected 14 das at the same hour of the day using TRIzol reagent (Thermo Fisher Scientific, Invitrogen). Roots, shoots, and leaves longer than 5 mm were removed to obtain plant material enriched in leaf primordia. Samples were collected on ice and immediately frozen in liquid nitrogen. Total RNA concentration and quality assessments, RNA-seq library construction and sequencing, mapping of reads to the Arabidopsis genome (TAIR10), and identification of differentially expressed genes between Col-0 and *vcc-2*, *cuc2-3*, or *cuc2-1D* were performed as previously described (Navarro-Quiles *et al.*, 2022). Genes with a *p*-value < 0.05 adjusted with the false discovery rate (FDR) estimation method of Benjamini and Hochberg were considered differentially expressed.

Gene Ontology (GO; http://www.geneontology.org/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) pathway enrichment analyses were

performed by Novogene. GO enrichment analysis of differentially expressed genes was implemented in the GOseq R package. GO terms with a corrected *p*-value < 0.05 were considered significantly enriched. KEGG pathway enrichment analysis was tested using KOBAS (Wu *et al.*, 2006).

RESULTS

VCC physically interacts with components of the fatty acid elongase complex

VCC is predicted to have four transmembrane domains and two soluble domains (Roschzttardtz *et al.*, 2014). To identify VCC interactors, we conducted a split-ubiquitin yeast two-hybrid membrane-based screen, which can identify protein–protein interactions involving transmembrane proteins. Accordingly, we fused VCC to the C-terminal half of ubiquitin as bait and fused all preys to the N-terminal half of ubiquitin (Johnsson & Varshavsky, 1994; Stagljar *et al.*, 1998). We obtained 320 interactions, 274 of which could be classified by a Predicted Biological Score (PBS; see Materials and Methods). These 274 clones represented 58 non-redundant genes (Tables 1 and S1), nine of which were related to fatty acid metabolism and five to endomembrane system protein trafficking.

The interactor with the highest number of hits and PBS was PASTICCINO2 (PAS2), a 3-hydroxy acyl-CoA dehydratase (Bach *et al.*, 2008). PAS2 and another interactor, ECERIFERUM10 (CER10), an enoyl-CoA reductase (Zheng *et al.*, 2005), are members of the fatty acid elongase complex. This complex is composed of four endoplasmic reticulum-bound enzymes and catalyzes the elongation of fatty acids with 16–18 carbon atoms to 20 or more, which are known as VLCFAs (Tehlivets *et al.*, 2007; Bach *et al.*, 2008; Roudier *et al.*, 2010). VLCFAs are synthetized in epidermal cells and regulate the expression of cytokinin biosynthesis genes in vascular tissues (Nobusawa *et al.*, 2013). The interaction of VCC with PAS2 and CER10 prompted us to examine a possible relationship between VCC and cytokinins in shaping simple leaf margins.

Genetic and pharmacological evidence of a role for cytokinins in Arabidopsis rosette leaf margin patterning

ISOPENTENYL TRANSFERASE (*IPT*) paralogs encode enzymes that catalyze the first step in cytokinin biosynthesis. Among the nine known *IPT* genes, *IPT3*, *IPT5*, and *IPT7* are the most highly expressed in the vegetative phase of Arabidopsis life cycle (Miyawaki *et al.*, 2004; Miyawaki *et al.*, 2006). To assess the potential role of cytokinins in leaf margin patterning, we first compared rosette leaf margin shape in Col-0, *cuc2-3*, *cuc2-1D*, and *ipt3 ipt5 ipt7* plants, all of which were already available (see Materials and Methods), and in the *cuc2-1D ipt3 ipt5 ipt7* quadruple mutant, which we obtained in this study (Fig. 1a-j). Under our growth conditions, the rosette leaves of the *ipt3 ipt5 ipt7* triple mutant exhibited smoother margins than those of Col-0, with fewer and smaller protrusions and sinuses. The observed phenotypes of the *cuc2-3* and *cuc2-1D* mutants were as previously described and that of *cuc2-1D ipt3 ipt5 ipt7* was similar to that of *ipt3 ipt5 ipt7*.

To quantify the observed differences in margin shape, we used the leaf dissection index (LDI) (Bilsborough *et al.*, 2011). LDI measures the complexity of leaf shape; a circle yields an

LDI value of 1, and more dissected forms yield higher values. We obtained LDIs of 2.32 ± 0.08 (Col-0), 2.21 ± 0.09 (*cuc2-3*), 3.52 ± 0.21 (*cuc2-1D*), 1.70 ± 0.03 (*ipt3 ipt5 ipt7*), and 1.77 ± 0.04 (*cuc2-1D ipt3 ipt5 ipt7*) (n = 10; Fig. 1k). The lower LDI value of *cuc2-3* leaves suggests that the mild serration of wild-type leaves requires CUC2, while the gain-of-function allele *cuc2-1D* produces lobed leaves more dissected than Col-0 (+1.21 in the LDI), in agreement with previous results (Nikovics *et al.*, 2006; Larue *et al.*, 2009; Bilsborough *et al.*, 2011). The smooth margins of the *ipt3 ipt5 ipt7* leaves indicated that cytokinins are also necessary for the serrations of wild-type leaves. Notably, the loss of cytokinin biosynthesis suppressed the phenotype of *cuc2-1D* in the *cuc2-1D ipt3 ipt5 ipt7* quadruple mutant background. The suppression of the *cuc2-1D* phenotype was also evident in other organs: *cuc2-1D* plants had short siliques that curled at their apices (Larue *et al.*, 2009), as well as an abnormal shoot phyllotaxis (Peaucelle *et al.*, 2007), while these traits were partially restored in the *cuc2-1D ipt3 ipt5 ipt7* quadruple mutant (Fig. 2). The suppression of lobe development in *cuc2-1D ipt3 ipt5 ipt7*, which accumulates high levels of CUC2, indicates that the function of CUC2 on serration formation is contingent on the presence of cytokinins.

The *arr1 arr10 arr12* triple null mutant lacks 3 of the 10 type-B ARABIDOPSIS RESPONSE REGULATORs (ARRs), which act downstream of cytokinin signaling by positively regulating the expression of cytokinin-responsive genes by directly binding to their promoters (Mason *et al.*, 2005; Argyros *et al.*, 2008). The *ANT:CKX3* transgenic line overexpresses *CYTOKININ DEHYDROGENASE/OXIDASE 3*, which encodes an enzyme that catalyzes the irreversible cleavage of cytokinins, under the control of the promoter from *AINTEGUMENTA* (*ANT*), a gene specifically active during leaf primordia development (Holst *et al.*, 2011). We determined that both the *arr1 arr10 arr12* triple mutant and *ANT:CKX3* transgenic plants show smoother leaf margins than Col-0 (Fig. S2). The effects of reduced cytokinin signaling in *arr1 arr10 arr12* and of increased cytokinin degradation in *ANT:CKX3* plants on leaf margin shape suggest that proper cytokinin activity is required for Arabidopsis leaf margin patterning.

We also analyzed the leaf phenotypes of other mutants with higher endogenous cytokinin contents or responses. We thus characterized the *ckx1 ckx3-1 ckx5-1* triple mutant, whose leaves contained more serrations (10.00 ± 2.00) than those of Col-0 (7.55 ± 1.64; n = 29 leaves from the 9^{th}–11^{th} nodes; $p = 0.0001$ in a Student's *t*-test) (Fig. S3). The *repressor of cytokinin deficiency 2-10* (*rock2-10*) and *rock3-1* gain-of-function alleles in *ARABIDOPSIS HISTIDINE KINASE 2* (*AHK2*) and *AHK3*, respectively, encode constitutively active cytokinin receptors (not dependent on cytokinin binding) (Bartrina *et al.*, 2017). We observed that the *rock2-10* and *rock3-1* mutants also have sharper teeth than Col-0 (Fig. S4).

Since treatment with the synthetic cytokinin 6-BAP increases leaf serration in Col-0 plants (Steiner *et al.*, 2012; Efroni *et al.*, 2013), we tested its effects on *ipt3 ipt5 ipt7* plants (Fig. S5a-e). Treatment with 15 nM 6-BAP partially restored serrations in *ipt3 ipt5 ipt7* leaves,

mainly from those of the 10th and subsequent rosette nodes (Fig. S5f-j), and shortened the leaf plastochron, which was longer in *ipt3 ipt5 ipt7* than in Col-0 (Fig. S5k-w). These results confirm that cytokinin deficiency causes the observed smooth leaf margin phenotype in *ipt3 ipt5 ipt7*.

To further test the effect of an increased response to cytokinins on leaf margin patterning, we used the *pRCO::ARR1ΔDDK* construct. *ARR1ΔDDK* encodes a constitutively active form of Arabidopsis ARR1 under the control of the *RCO* leaf margin–specific promoter from *C. hirsuta* and yields extra-lobed leaves in Arabidopsis (Hajheidari *et al.*, 2019). The heterologous expression pattern of *RCO* in Arabidopsis (Vlad *et al.*, 2014) is similar to that of *CUC2* (Nikovics *et al.*, 2006), as they are expressed at the sinuses of the base of leaf primordia (Navarro-Cartagena & Micol, 2022). We compared Arabidopsis *pRCO::ARR1ΔDDK* transgenic plants and *cuc2-1D* mutant plants (Fig. S6). We noticed two types of phenotypically mutant *pRCO::ARR1ΔDDK* plants (Fig. S6c, d), the less lobed type being similar to *cuc2-1D* (Fig. S6e-g). This observation also indicated that cytokinin activity in specific regions of the leaf primordium margin can modify leaf margin shape in a manner similar to that shown by the gain of CUC2 function in the *cuc2-1D* mutant. Taken together, these results reinforce the hypothesis that cytokinins contribute to the formation of leaf lobes or serrations.

Cytokinins regulate the number of auxin maxima in leaf primordia margins

CUC2 and PIN1 are known to play key roles in shaping Arabidopsis simple leaf margins (Bilsborough *et al.*, 2011). To assess whether the spatial distribution of these factors may be compromised in *ipt3 ipt5 ipt7* plants, we crossed this triple mutant to the $CUC2_{pro}$*:CUC2:RFP* $DR5rev_{pro}$*:VENUS* double transgenic line, as well as to $PIN1_{pro}$*:PIN1:GFP* (Xu *et al.*, 2006) plants. The synthetic *DR5rev* promoter is auxin responsive (Friml *et al.*, 2003). In $CUC2_{pro}$*:CUC2:RFP* $DR5rev_{pro}$*:VENUS* leaf primordia, we detected the red fluorescent protein (RFP) signal in margin sinuses and the VENUS signal in developing veins and margin protrusions, where auxin maxima form (Fig. 3a-d). We observed no ectopic expression of $CUC2_{pro}$*:CUC2:RFP* or $DR5rev_{pro}$*:VENUS* in *ipt3 ipt5 ipt7* (Fig. 3g-j). In leaf primordia collected from the 8th to 11th rosette nodes, we noticed fewer auxin maxima (as shown by the VENUS signal at protrusions) in *ipt3 ipt5 ipt7* leaf primordia (4.3 ±1.64; n = 23) when compared to Col-0 (8.31 ± 1.82; n = 36; $p < 0.0001$ in a Student's *t*-test). PIN1 appeared to properly localize in the cell membranes of *ipt3 ipt5 ipt7* leaf primordia protrusions (Fig. 3e, f, k, l), suggesting that a perturbation of the polarity of auxin transport is not causal for the smooth leaf margin of this triple mutant.

Cytokinin response in leaf primordia depends on *CUC2* and *ER*

To monitor the cytokinin response, we used the *TCSn::GFP* reporter, consisting of the *TCSn* promoter, which harbors 24 concatemerized binding motifs for activated type-B ARRs in an

optimized configuration, driving green fluorescent protein (*GFP*) expression (Zürcher *et al.*, 2013). In Col-0 leaf primordia, the *TCSn::GFP* reporter was expressed in developing veins and at the basal region of the lamina, being more intense at the flanks of the primary vein and excluded from the margin protrusions, based on GFP fluorescence intensity (Fig. 4a-d). The *TCSn::GFP* expression pattern was similar to that described for *CUC2* (Nikovics *et al.*, 2006) and for genes known to be activated by type-B ARRs (Zürcher & Müller, 2016), like *CKX5* (Werner *et al.*, 2003). Cytokinin signaling at the base of the lamina and in the developing veins decreased and increased, respectively, with the developmental stage of leaf primordia.

To determine if the cytokinin response is altered in mutants affected in genes involved in margin morphogenesis and in the generation of auxin maxima, we obtained genetic combinations of *TCSn::GFP* with the *cuc2-3* and *cuc2-1D* mutations, as well as with *er-3* (Durbak & Tax, 2011) and *ipt3 ipt5 ipt7*. The *ipt3 ipt5 ipt7* line was introduced as a control to monitor the response of *TCSn::GFP* in a cytokinin-depleted background. Indeed, we detected no GFP signal in the *ipt3 ipt5 ipt7 TCSn::GFP* line (Fig. 4e-h). Although the expression pattern of *TCSn::GFP* was maintained in all other transgenic lines under study, we determined that cytokinin perception is low in *cuc2-3* (Fig. 4i-l), high at the basal flanks in *cuc2-1D* (Fig. 4m-p), and very low in *er-3* (Fig. 4q-t). We confirmed these observations by measuring GFP fluorescence intensity at the basal region of leaf primordia in all five transgenic lines (Fig. S1). These observations suggest that cytokinin perception during leaf margin patterning is dependent upon the presence of both CUC2 and ERECTA.

Cytokinin and VCC interplay during leaf margin morphogenesis

As mentioned above, our split-ubiquitin Y2H membrane-based assay suggested that VCC interacts with components of the fatty acid elongase complex. Since VLCFAs inhibit cytokinin biosynthesis (Nobusawa *et al.*, 2013), we considered that the morphological phenotype of *vcc-2* leaves might be related to an altered cytokinin response. To investigate a potential relationship between VCC and cytokinins, we grew Col-0 and *vcc-2* plants in the presence of 10 nM 6-BAP, a synthetic cytokinin. This pharmacological treatment had no effect on Col-0 plants, while it increased the penetrance of the *vcc-2* leaf margin phenotype from 47%, in agreement with Wilson-Sánchez *et al.* (2018), to 81% (Fig. 5a), and its expressivity from 17% to 30% (Fig. 5b-d).

To facilitate the study of the *vcc-2* mutant, we searched for an environmental condition that caused full penetrance and kept a constant expressivity of the *vcc-2* phenotype. The use of Gelrite gelling agent instead of plant agar met this criterion. Hence, we used Gelrite for all plant cultures involving *vcc-2*, with the exception of the previously described 6-BAP treatment, in which we used plant agar.

To test the effect of cytokinin deficiency on the *vcc-2* leaf phenotype, we obtained the *vcc-2 ipt3 ipt5 ipt7* quadruple mutant, which was indistinguishable from the *ipt3 ipt5 ipt7* triple mutant and extremely different from the *vcc-2* single mutant (Fig. S7a-d). In addition, we observed a frequently abnormal *TCSn::GFP* expression pattern in *vcc-2* leaf primordia: the GFP signal was asymmetrically distributed between the left and right sides of the lamina, in terms of both position and intensity, and with ectopic expression in lobes (Fig. 6). We concluded that there is a functional relationship between VCC and cytokinins in leaf margin development, based on the observations that cytokinin excess (by 6-BAP treatment) or deficit (in the *ipt3 ipt5 ipt7* background) enhances or suppresses, respectively, the *vcc-2* mutant phenotype, which also exhibited an altered cytokinin response based on *TCSn::GFP*.

ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) negatively regulates cytokinin signaling. In the SAM, AHP6 creates cytokinin signaling inhibitory fields that are required for proper fruit phyllotaxis (Besnard *et al.*, 2014). Interestingly, we observed that the *vcc-2* mutant shows visible defects in fruit distribution along the stem, with abnormally clustered siliques that sometimes emerged from the same node. We obtained the *vcc-2 ahp6-1* double mutant and examined its fruit phyllotaxis (Fig. S8a-d). While most divergence angles between consecutive siliques were close to 150° in Col-0, other angles (from 0° to 30°, and from 270° to 360°) appeared in the *ahp6-1* and *vcc-2* single mutants and were more frequent in the *vcc-2 ahp6-1* double mutant (Fig. S8f-h). Clustered siliques also appeared in these mutants (Fig. S8i-l), but it was unclear if the double mutant phenotype was synergistic or merely additive.

Expanded leaf margin aberrations and auxin signaling perturbations during leaf primordia development are spatially correlated in the *vcc-2* mutant

The regular pattern of auxin response and CUC2 interspersed domains along the leaf margin seen in the wild type (Bilsborough *et al.*, 2011) fails to properly form in *vcc-2* primordia (Wilson-Sánchez *et al.*, 2018). Auxin promotes Aux/IAA (AUXIN/INDOLEACETIC ACID-INDUCED PROTEIN) degradation, which frees members of the AUXIN RESPONSE FACTOR (ARF) family to bind to auxin-responsive elements (AREs) at the promoters of primary auxin-responsive genes (Teale *et al.*, 2006). *ARF11$_{pro}$:GFP* is expressed at leaf protrusion tips (Fig. 7a-d), as is the classic auxin response reporter *DR5rev$_{pro}$:GFP* (Fig. 7m-p; the *DR5* promoter is composed of concatemerized AREs) (Friml *et al.*, 2003). In *vcc-2* leaf primordia, the *ARF11$_{pro}$:GFP* signal was bilaterally asymmetric, and its expression in the abnormally large lobes of the mutant was extended (Fig. 7e-l). We had previously detected *DR5rev$_{pro}$:GFP* signal in *vcc-2* leaf margins in an irregular spatial arrangement compared to Col-0 (Wilson-Sánchez *et al.*, 2018). Since these first observations were based on plants grown on culture medium prepared with plant agar, we repeated these observations using Gelrite as an

alternative gelling agent. Under this new culture condition, auxin signaling was not only asymmetrically distributed in *vcc-2* leaf primordia, but also spread across large margin regions that, in some cases, included sinuses (Fig. 7q-x). These results highlight the role of VCC in the auxin signaling pathway and underscore the correlation between the increased severity of the morphological phenotype of *vcc-2* expanded leaves and increased auxin response perturbation in leaf primordia. Finally, the *vcc-2 cuc2-3* double mutant, which showed a similar leaf margin shape to that of *cuc2-3* plants when grown on plant agar (Wilson-Sánchez *et al.*, 2018), showed a phenotype intermediate between that of *vcc-2* and *cuc2-3* single mutants, with serrated leaves, when grown on Gelrite (Fig. S9), suggesting that *VCC* and *CUC2* do not genetically interact.

Transcriptome profiles of the *cuc2-3*, *cuc2-1D*, and *vcc-2* mutants

To ascertain whether CUC2 is molecularly related to cytokinin homeostasis, we performed a transcriptome analysis of leaf primordia, collected 14 das, from Col-0, *cuc2-3* and *cuc2-1D* seedlings. In the *cuc2-3* mutant, we identified 791 upregulated genes and 2,251 downregulated genes, relative to Col-0 (Fig. S10a; Supplemental Data Set 1). *CUC2* was among the downregulated genes, in agreement with Maugarny-Calès *et al.* (2019). In the *cuc2-1D* mutant, 448 genes were upregulated, including *CUC2*, and 598 were downregulated (Fig. S10b; Supplemental Data Set 2).

We conducted a classification of differentially expressed genes in *cuc2* mutants according to GO and KEGG terms. In *cuc2-3*, the significantly enriched GO categories included many terms related to phosphate (kinase [GO:0016301], phosphatase [GO:0016791], ATPase [GO:0016887], and GTPase [GO:0003924] activities) and signal transduction (GO:0007165) (Fig. S10c; Supplemental Data Set 3A). In the *cuc2-1D* mutant, we detected no significantly enriched GO categories (Fig. S10d; Supplemental Data Set 4A). In the case of the significantly enriched KEGG terms, *cuc2-3* only included the plant–pathogen interaction (ath04626) category (Fig. S10e; Supplemental Data Set 3B), while *cuc2-1D* was enriched in the terms circadian rhythm (ath04712) and the regulation of different metabolites (Fig. S10f; Supplemental Data Set 4B).

Notably, most, if not all known, cytokinin-related genes were downregulated in *cuc2-3* (Table 2), including genes for cytokinin biosynthesis, signaling, and response. This result was in agreement with the low response of the *TCSn::GFP* reporter in *cuc2-3* leaf primordia (Fig. 4i-l). However, none of these genes were deregulated in *cuc2-1D*. This gain-of-function mutant showed high-intensity fluorescence from the *TCSn::GFP* reporter, but the differences with Col-0 were less remarkable than with *cuc2-3* (Fig. 4m-o). We hypothesize that cytokinin activity may be modulated by CUC2 in leaves but in an asymmetrically CUC2 dose-dependent

manner, since the gain of CUC2 function did not exhibit the opposite effect of the CUC2 loss of function.

We only identified 66 upregulated and 55 downregulated genes in the *vcc-2* mutant, including *VCC* (Fig. S11a; Supplemental Data Set 5). Unexpectedly, we did not find differentially expressed genes related to VLCFAs or any plant hormone (Fig. S11b-c; Supplemental Data Set 6). This finding would suggest that VCC acts downstream of VLCFAs, auxin, and cytokinins. Another possibility is that transcript levels from a subset of genes only deregulated in specific cells are masked when analyzing a pool of mRNAs from a homogenized tissue during RNA extraction. Hence, cell-level gene expression studies may be required to characterize the regions with ectopic cytokinin response in *vcc-2* leaf primordia.

DISCUSSION

A role for cytokinins in simple leaf margin development

The serration of the margin of simple leaves such as those of Arabidopsis has been satisfactorily explained by the distribution of auxin along the leaf primordium margin, which is regulated by CUC2 and EPFL2. These factors form mutually exclusive domains with auxin: the phytohormone accumulates in growing protrusions, which are flanked by sinuses where CUC2 (Bilsborough *et al.*, 2011) and EPFL2 (Tameshige *et al.*, 2016) act.

We previously postulated a role for cytokinins in Arabidopsis leaf margin patterning, in crosstalk with auxin (Navarro-Cartagena & Micol, 2022). Here, we provide evidence for the participation of cytokinins in Arabidopsis simple leaf margin patterning. Cytokinin depletion reduced leaf margin serration and suppressed the leaf and flower mutant phenotypes of the *cuc2-1D* mutant (Figs. 1 and 2). The slightly increased leaf complexity of *cuc2-1D ipt3 ipt5 ipt7* compared to *ipt3 ipt5 ipt7* may be explained by the role of CUC2 in the formation of auxin maxima during leaf morphogenesis, which are also necessary for the development of leaf protrusions (Maugarny-Calès *et al.*, 2019), or by the residual activity encoded by other *IPT* genes, which are less specific of the vegetative phase than *IPT3*, *IPT5*, and *IPT7* (Miyawaki *et al.*, 2004).

The addition of the synthetic cytokinin 6-BAP to the culture medium partially restored serration in *ipt3 ipt5 ipt7* leaves (Fig. S2). We did not see increased serration in Col-0 plants treated with 6-BAP nor a reduction in leaf size, unlike that reported by Steiner *et al.* (2012) and Efroni *et al.* (2013). These different results may be due to the differences in culture conditions. Indeed, these authors grew their plants in pots under photoperiod conditions and sprayed a solution of 6-BAP onto the plants. By contrast, we grew all our plants on plates in continuous light, and we added 6-BAP to the culture medium directly.

The reduction in cytokinin response in the *arr1 arr10 arr12* triple null mutant and the increase in cytokinin degradation in the *ANT:CKX3* transgenic plants produced smoother leaf margins than those of Col-0 (Fig. S3). Conversely, lines with increased cytokinin activity showed more dissected leaves compared to the wild type. This was the case for the *pRCO::ARR1ΔDDKK* transgenic plants (Fig. S6) and the *ckx1 ckx3 ckx5* triple mutant (Fig. S3). These observations reinforce the hypothesis of a role for cytokinins in leaf serration patterning.

We established that the cytokinin response reporter *TCSn::GFP* was expressed at the flanks of the basal region of leaf primordia laminae of otherwise wild-type plants, where it was excluded from lobes. We also observed its weak expression in petioles (Fig. 4a-d). This expression pattern substantially overlapped with that of *CUC2* (Fig. 3a-d) (Nikovics *et al.*, 2006). *TCSn::GFP* signal was absent from *ipt3 ipt5 ipt7* plants, was lower in the *cuc2-3* and *er-3* backgrounds, and slightly higher in *cuc2-1D* relative to Col-0 (Fig. 4). These observations

suggest that cytokinin activity may be regulated by CUC factors in leaf margin patterning, as is known to take place in ovule formation (Bartrina *et al.*, 2011; Bencivenga *et al.*, 2012; Galbiati *et al.*, 2013; Cucinotta *et al.*, 2018; Cucinotta *et al.*, 2020). The poor *TCSn::GFP* signal in *er-3* plants also suggests a connection between ER activity and cytokinins. Such connection may also occur in the SAM and ovules, whose development depends upon interactions between members of the EPFL and ER families (Aida *et al.*, 1999; Tsukaya, 2021; Navarro-Quiles *et al.*, 2022) as well as on cytokinin activity (Jasinski *et al.*, 2005; Cucinotta *et al.*, 2020).

Leaf primordia displayed fewer auxin maxima in *ipt3 ipt5 ipt7* (Fig. 3), which may partially explain the smoother margin of expanded leaves in this mutant. Cytokinins maintain cell proliferation during the proliferation phase of developing leaves and later stimulate cell expansion and differentiation (Skalák *et al.*, 2019). Hence, cytokinin depletion will reduce the number of auxin maxima and may perturb the proliferation/expansion balance, thereby flattening serrations.

Finally, cytokinins may have a conserved morphogenetic role, favoring complexity in the formation of compound leaves in tomato and *C. hirsuta* (Shani *et al.*, 2010; Bar *et al.*, 2016; Hajheidari *et al.*, 2019), and also in the serration patterning of simple leaves in Arabidopsis. Additional support for this hypothesis is provided by the increased cytokinin activity caused by the *pRCO::ARR1ΔDDK* transgene, which reduces cell expansion and differentiation at Arabidopsis margin sinuses (Hajheidari *et al.*, 2019), as *RCO* does in *C. hirsuta* (Vlad *et al.*, 2014; Kierzkowski *et al.*, 2019).

A role for VCC in the crosstalk between cytokinins and auxin in leaf margin primordia

VCC (DEAL1) appears to be involved in the regulation of where auxin maxima localize along the primordia margin of both leaf halves and, therefore, in the establishment of leaf bilateral symmetry (Wilson-Sánchez *et al.*, 2018). Here, we showed that VCC also contributes to control cytokinin activity during leaf development. Indeed, the *TCSn::GFP* reporter in *vcc-2* primordia exhibited an asymmetrical distribution of GFP signal and ectopic expression in protrusion tips, regions where this reporter is normally almost absent in Col-0 primordia (Fig. 6). This observation suggested that VCC was required for a symmetrical cytokinin response between the two halves of the leaf primordia laminae and for its repression at protrusion tips. In addition, the leaf asymmetry phenotype of *vcc-2* was enhanced or suppressed in conditions of cytokinin excess (by 6-BAP treatment) or depletion (in the *ipt3 ipt5 ipt7* background) (Figs. 5 and S8). Our results suggest a functional relationship between VCC and cytokinins during leaf development, which also occurs during cotyledon vein patterning (Yanagisawa *et al.*, 2021).

VCC was localized to the endoplasmic reticulum membrane (Wilson-Sánchez *et al.*, 2018), where it may interact with components of the VLCFA elongase complex (Table 1). VLCFAs regulate cytokinin biosynthesis (Nobusawa *et al.*, 2013) and polar auxin transport

(Roudier *et al.*, 2010). It is worth noting here that cytokinin receptors are mainly located on the endoplasmic reticulum membrane (Caesar *et al.*, 2011; Wulfetange *et al.*, 2011; Lomin *et al.*, 2017; Romanov *et al.*, 2018) and that their gain of function affects leaf development (Bartrina *et al.*, 2017). Moreover, proteins involved in auxin homeostasis, metabolism, and signaling (Friml & Jones, 2010) as well as ethylene receptors (Grefen *et al.*, 2008) also localize to the endoplasmic reticulum. For these reasons, the endoplasmic reticulum has been proposed to be an important organelle for phytohormonal crosstalk (Caesar *et al.*, 2011). We hypothesize that VCC plays a pivotal role in the crosstalk between auxin and cytokinins, because it links auxin, VLCFAs, and cytokinins.

Cytokinins contribute to the maintenance of the undifferentiated state of the SAM (Su *et al.*, 2011), prevent the proliferative arrest at the reproductive meristem (Merelo *et al.*, 2022) in Arabidopsis, and lengthen the proliferation phase of leaf development, favoring compound leaves in tomato and *C. hirsuta* (Shani *et al.*, 2010; Israeli *et al.*, 2021). In Arabidopsis leaf primordia, changes in cytokinin response in terms of intensity (in *cuc2-3* and *er-3* mutants) or location (in *vcc-2*) may influence meristematic activity in developing leaves, extending or shortening the duration of the cell proliferation phase and therefore promoting or reducing leaf complexity. In *vcc-2* plants, the random cytokinin response along the primordia margin, and hence of meristematic capacity, may contribute to the growth differences between the left and right halves of leaf primordia and consequently to leaf bilateral asymmetry.

The RCO transcription factor is required for compound leaves in *C. hirsuta* and acts, at least partially, by promoting cytokinin biosynthesis and signaling (Hajheidari *et al.*, 2019). Ectopic expression of *RCO* in *C. hirsuta* leaves appears to cause ectopic activity of cytokinins and has morphological consequences that are reminiscent of those seen in *vcc* mutants in Arabidopsis. In *C. hirsuta rco pmutRCO::RCOcds-VENUS* plants expressing *RCO* from a promoter variant lacking all *RCO*-binding sites and thus unable to feed back to repress its own expression, leaflets are less rounded and show irregular shapes compared to the wild type; the margin of the simple leaves of Arabidopsis *vcc* mutants is irregular, which causes bilateral asymmetry (Fig. S12). *C. hirsuta* and Arabidopsis are closely related species; they belong to the same lineage within the Brassicaceae family that also includes *Arabidopsis lyrata* (Hu *et al.*, 2011) and *Capsella rubella* (Slotte *et al.*, 2013), all of which have fully sequenced genomes (Gan *et al.*, 2016). Therefore, it is reasonable to speculate that cytokinins may modulate leaf composition in *C. hirsuta* and leaf serration in Arabidopsis. However, we determined here that *VCC* and *CUC2* did not genetically interact. Nevertheless, the presence of serrations in the *vcc-2 cuc2-3* double mutant may indicate that the ectopic cytokinin activity shown by *TCSn::GFP* reporter caused by *vcc-2* mutation partially bypasses the requirement for *CUC2* in the development of serrations in Arabidopsis, as occurs with *pRCO::ARR1ΔDDK* for *RCO* in *C. hirsuta* complex leaf formation (Hajheidari *et al.*, 2019).

Different experimental evidence demonstrates that cytokinins and auxin crosstalk in different plant developmental contexts. From our work, we propose that these hormones interact during simple leaf margin morphogenesis. Although the contribution of auxin in this process has previously been intensely studied, the role of cytokinins has not been deeply investigated, despite its known participation in the morphogenesis of compound leaves (Navarro-Cartagena & Micol, 2022). In this article, we demonstrate that cytokinins modulate the margin shape of simple leaves and that the role of VCC in leaf bilateral symmetry may be related not only to auxin, but also to cytokinins. Further research will be needed to understand the crosstalk between these plant hormones in simple leaf margin morphogenesis.

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AUTHOR CONTRIBUTIONS

J.L.M. conceived, designed, and supervised the research, provided resources, and obtained funding. Several experiments were codesigned by S.N.-C., D.W.-S., and J.L.M. S.N.-C. performed most of the experiments. D.W.-S. performed the split-ubiquitin yeast two-hybrid membrane-based assay. S.N.-C. and J.L.M. wrote the manuscript. All authors revised and approved the manuscript.

TABLES

Table 1. Selected VCC interactors identified in a split-ubiquitin yeast two-hybrid membrane-based screen

PBS*	Number of hits	AGI code	Protein name	Description
A	33	At5g10480	PASTICCINO2 (PAS2)	3-Hydroxyacyl-CoA dehydratase, member of the very-long-chain fatty acid (VLCFA) elongase complex. Tyrosine phosphatase-like protein involved in cell division and differentiation
D	3	At3g55360	ECERIFERUM10 (CER10)	Enoyl-CoA reductase, member of the VLCFA elongase complex, involved in all VLCFA elongation reactions that are required for cuticular wax biosynthesis and storage lipid and sphingolipid metabolism
B	14	At4g34100	ECERIFERUM9 (CER9)	Involved in cuticular wax biosynthesis
D	9	At2g03140		Alpha/beta-hydrolase superfamily protein
D	1	At4g20870	FATTY ACID HYDROXYLASE2 (FAH2)	Fatty acid hydroxylase
C	7	At3g15820	REDUCED OLEATE DESATURATION1 (ROD1)	Functions as a phosphatidylcholine:diacylglycerol cholinephosphotransferase Catalyzes a major reaction for the transfer of 18:1 into phosphatidylcholine for desaturation and for the reverse transfer of 18:2 and 18:3 into the triacylglycerol biosynthesis pathway
D	7	At5g01460		Limb development membrane protein 1 (LMBR1)-like integral membrane family protein
D	9	At4g14965	MEMBRANE-ASSOCIATED PROGESTERONE BINDING PROTEIN4 (MAPR4)	
D	3	At3g51730		Saposin B domain-containing protein
C	4	At2g23310	RER1C1	Golgi membrane protein involved in returning the molecules that are exported from the endoplasmic reticulum (ER) to the Golgi apparatus back to the endoplasmic reticulum
D	1	At4g09580	T25P22.20	SNARE-associated Golgi protein family

Table 1 (continued). Selected VCC interactors identified in a split-ubiquitin yeast two-hybrid membrane-based screen

PBS*	Number of hits	AGI code	Protein name	Description
D	1	At2g02370		SNARE-associated Golgi protein family
D	1	At4g38790	T9A14.70	Endoplasmic reticulum lumen protein retaining receptor family
D	1	At3g54300	VESICLE-ASSOCIATED MEMBRANE PROTEIN727 (VAMP727)	Member of Synaptobrevin-like protein family; required for trafficking of storage proteins to protein storage vacuoles
D	1	At1g44350	IAA-LEUCINE RESISTANT (ILR)-LIKE GENE6 (ILL6)	Protein similar to IAA amino acid conjugate hydrolase
D	1	At1g26210	SOFL1 (SOB FIVE-LIKE1)	Acts redundantly with SOFL2 as a positive regulator of cytokinin levels

*Predicted Biological Score. A, B, C, and D: Very high, high, good, and moderate confidence in the interaction, respectively. E: Warning of nonspecific interaction.

Table 2. Cytokinin-related differentially expressed genes in an RNA-seq analysis of *cuc2-3* plants

AGI code	Gene name	Gene full name and description	Fold-change	*p*-value	FDR
At1g68460	*IPT1*	*ISOPENTENYLTRANSFERASE 1*	0.16	4.35E–06	1.08E–04
At2g28305	*LOG1*	*LONELY GUY 1*. Cytokinin riboside 5′-monophosphate phosphoribohydrolase	0.52	4.97E–09	2.47E–07
At5g03270	*LOG6*	*LONELY GUY 6*. Cytokinin riboside 5′-monophosphate phosphoribohydrolase	0.53	5.39E–03	4.11E–02
At5g06300	*LOG7*	*LONELY GUY 7*. Cytokinin riboside 5′-monophosphate phosphoribohydrolase	0.38	1.87E–06	5.13E–05
At5g35750	*AHK2*	*ARABIDOPSIS HISTIDINE KINASE 2*	0.59	1.28E–05	2.82E–04
At3g16857	*ARR1*	*ARABIDOPSIS RESPONSE REGULATOR 1*	0.62	1.22E–04	1.95E–03
At4g16110	*ARR2*	*ARABIDOPSIS RESPONSE REGULATOR 2*	0.69	6.92E–03	4.96E–02
At3g48100	*ARR5*	*ARABIDOPSIS RESPONSE REGULATOR 5*	0.56	2.47E–05	5.03E–04
At1g19050	*ARR7*	*ARABIDOPSIS RESPONSE REGULATOR 7*	0.51	2.85E–08	1.19E–06
At3g57040	*ARR9*	*ARABIDOPSIS RESPONSE REGULATOR 9*	0.62	7.10E–04	8.32E–03

FIGURE LEGENDS

Figure 1. Suppression of the *cuc2-1D* leaf phenotype in the *ipt3 ipt5 ipt7* background. (a-e) Representative rosettes of (a) the wild-type Col-0, (b) the loss-of-function *cuc2-3* and (c) gain-of-function *cuc2-1D* single mutants, (d) the loss-of-function *ipt3 ipt5 ipt7* triple mutant, and (e) the *cuc2-1D ipt3 ipt5 ipt7* quadruple mutant. (f-j) Silhouettes of rosette leaves from successive nodes (from left to right, 1^{st}–15^{th}) of (f) Col-0, (g) *cuc2-3*, (h) *cuc2-1D*, (i) *ipt3 ipt5 ipt7*, and (j) *cuc2-1D ipt3 ipt5 ipt7* plants. (k) Extent of margin serration, quantified using the leaf dissection index of leaves from the 9^{th}–12^{th} nodes. Data are means ± standard deviation (SD). Asterisks indicate values significantly different from Col-0 (except in the comparison between *ipt3 ipt5 ipt7* and *cuc2-1D ipt3 ipt5 ipt7* plants) in a Student's *t*-test (**p* < 0.05, ***p* < 0.005, and ****p* < 0.0001; n = 10). Pictures were taken (a-e) 21 and (f-k) 35 days after stratification (das). Scale bars, 1 cm.

Figure 2. Suppression of the flower and silique morphological phenotypes of *cuc2-1D* in the *ipt3 ipt5 ipt7* background. (a-e) Flowers, (f-j) siliques, and (k-o) silique spacing along the stem of (a, f, k) Col-0, (b, g, l) *cuc2-3*, (c, h, m) *cuc2-1D*, (d, i, n) *ipt3 ipt5 ipt7*, and (e, j, o) *cuc2-1D ipt3 ipt5 ipt7* plants. Pictures were taken 49 das. Red arrowheads indicate abnormally clustered siliques. Scale bars, (a-j) 1 mm, and (k-o) 1 cm.

Figure 3. Expression patterns of the $CUC2_{pro}$*:CUC2:RFP*, $DR5rev_{pro}$*:VENUS* and $PIN1_{pro}$*:PIN1:GFP* reporters in *ipt3 ipt5 ipt7* leaf primordia. (a-d, g-k) $CUC2_{pro}$*:CUC2:RFP* $DR5rev_{pro}$*:VENUS* (red and greenish yellow, respectively) primordia from successive nodes (9^{th}–12^{th} for Col-0 and 8^{th}–11^{th} for *ipt3 ipt5 ipt7*). (e, k) Visualization of $PIN1_{pro}$*:PIN1:GFP* (green) expression in leaf primordia corresponding to an undetermined (from 9^{th}–11^{th}) node of (e) Col-0 or (k) *ipt3 ipt5 ipt7*. (f, l) PIN1:GFP cell membrane localization in the margin of a leaf primordium of (f) Col-0 and (l) *ipt3 ipt5 ipt7* plants. Due to their different plastochrons, Col-0 primordia (a-f) were collected 14–15 das, and those of *ipt3 ipt5 ipt7* (g-l) were collected 17–18 das. In this and all other figures, all primordia shown for a given genotype were collected from the same single plant. Scale bars, 0.1 mm.

Figure 4. Cytokinin response in *ipt3 ipt5 ipt7*, *cuc2-3*, *cuc2-1D*, and *er-3* leaf primordia. *TCSn::GFP* expression pattern was visualized in (a-d) Col-0, (e-h) *ipt3 ipt5 ipt7*, (i-l) *cuc2-3*, (m-p) *cuc2-1D*, and (q-t) *er-3* leaf primordia from successive nodes (8^{th}–11^{th}). Chlorophyll autofluorescence is shown in red. Primordia were collected 14–15 das, exceptions being those of *ipt3 ipt5 ipt7* plants, which were collected 17–18 das due to their longer plastochron. Scale bars, 0.1 mm.

Figure 5. Effects of 6-BAP treatment on leaf asymmetry in *vcc-2*. (a) Effect of 6-BAP on the penetrance of the leaf asymmetry phenotype, based on 15 replicates of nine Col-0 or *vcc-2* plants. (b) Effect of 6-BAP on the expressivity (severity) of the leaf asymmetry phenotype, based on 10 Col-0 and 25 *vcc-2* leaves from the 9^{th}–11^{th} nodes. The Col-0 values are not equal to 0 because no individual leaf equals the Col-0 consensus. Data are means ± SD. Asterisks indicate values significantly different from untreated plants in a Student's *t*-test (*$p < 0.0001$). (c) Method employed for the calculation of the expressivity of the mutant phenotype (see Materials and Methods). (d) Four representative leaf silhouettes of each genotype and treatment. Pictures were taken 35 das. Scale bars, 1 cm.

Figure 6. Cytokinin response in *vcc-2* leaf primordia. The *TCSn::GFP* expression pattern was visualized in (a-d) Col-0 and (e-p) *vcc-2* leaf primordia from successive nodes (7^{th}–10^{th}) collected 14 das. Chlorophyll autofluorescence is shown in red. Scale bars, 0.1 mm.

Figure 7. Auxin response in *vcc-2* leaf primordia. (a-l) *$ARF11_{pro}$:GFP* and (m-x) *$DR5rev_{pro}$:GFP* expression visualized in (a-d, m-p) Col-0 and (e-l, q-x) *vcc-2* primordia from successive nodes (7^{th}–10^{th}) collected 14 das. Chlorophyll autofluorescence is shown in red. Scale bars, 0.1 mm.

SUPPLEMENTAL INFORMATION FIGURES

Figure S1. Quantification of GFP fluorescence in leaf primordia.

Figure S2. Reduced leaf margin serration in the *arr1 arr10 arr12* triple mutant and the *ANT:CKX3* transgenic line.

Figure S3. Increased leaf margin serration in the *ckx1 ckx3 ckx5* triple mutant.

Figure S4. Increased teeth sharpness from *rock2-10* and *rock3-1* leaf margins.

Figure S5. Effects of 6-BAP on the margin shape of leaf primordia and expanded leaves in *ipt3 ipt5 ipt7* plants.

Figure S6. Comparison of the leaf phenotypes of the *cuc2-1D* gain-of-function mutant and the *pRCO::ARR1ΔDDK* transgenic line.

Figure S7. Genetic interaction between *vcc-2* and *ipt3 ipt5 ipt7*.

Figure S8. Genetic interaction between *vcc-2* and *ahp6-1*.

Figure S9. Genetic interaction between *vcc-2* and *cuc2-3*.

Figure S10. Differentially expressed genes identified from RNA-seq analysis of the *cuc2-3* and *cu2-1D* mutants relative to Col-0.

Figure S11. Differentially expressed genes identified from an RNA-seq analysis of the *vcc-2* mutant relative to Col-0.

Figure S12. Cytokinin response in young leaves and expanded leaf morphology comparison between *Cardamine hirsuta rco pmutRCO::RCOcds-VENUS* and Arabidopsis *vcc-2* plants.

Table S1. Other VCC protein interactors identified in a split-ubiquitin membrane-based yeast two-hybrid assay.

Table S2. PCR primers used in this work.

Data Set 1. Differentially expressed genes identified from an RNA-seq analysis of *cuc2-3* plants compared to Col-0.

Data Set 2. Differentially expressed genes identified from an RNA-seq analysis of *cuc2-1D* plants compared to Col-0.

Data Set 3. Gene Ontology term enrichment among differentially expressed genes in *cuc2-3* plants compared to Col-0.

Data Set 4. Gene Ontology term enrichment among differentially expressed genes in *cuc2-1D* plants compared to Col-0.

Data Set 5. Differentially expressed genes identified from an RNA-seq analysis of *vcc-2* plants compared to Col-0.

Data Set 6. Gene Ontology term enrichment among differentially expressed genes in *vcc-2* plants compared to Col-0.

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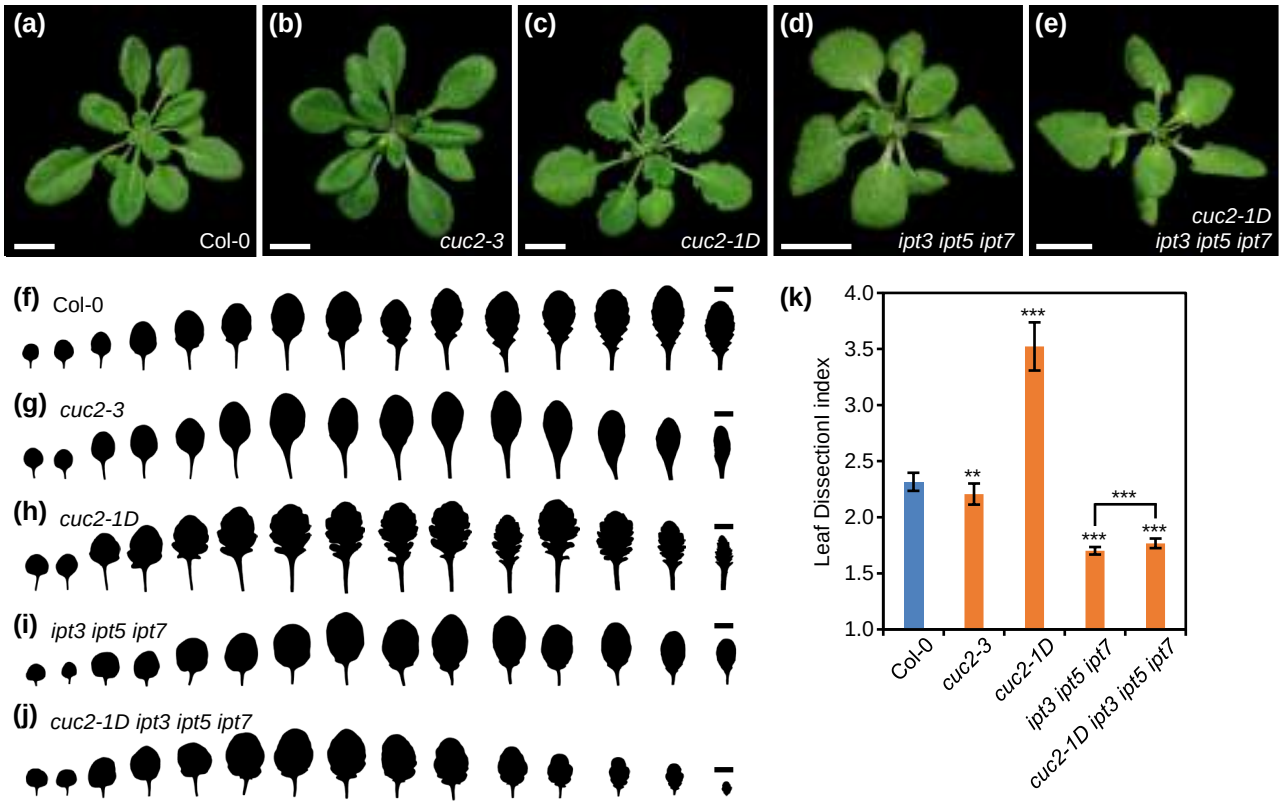
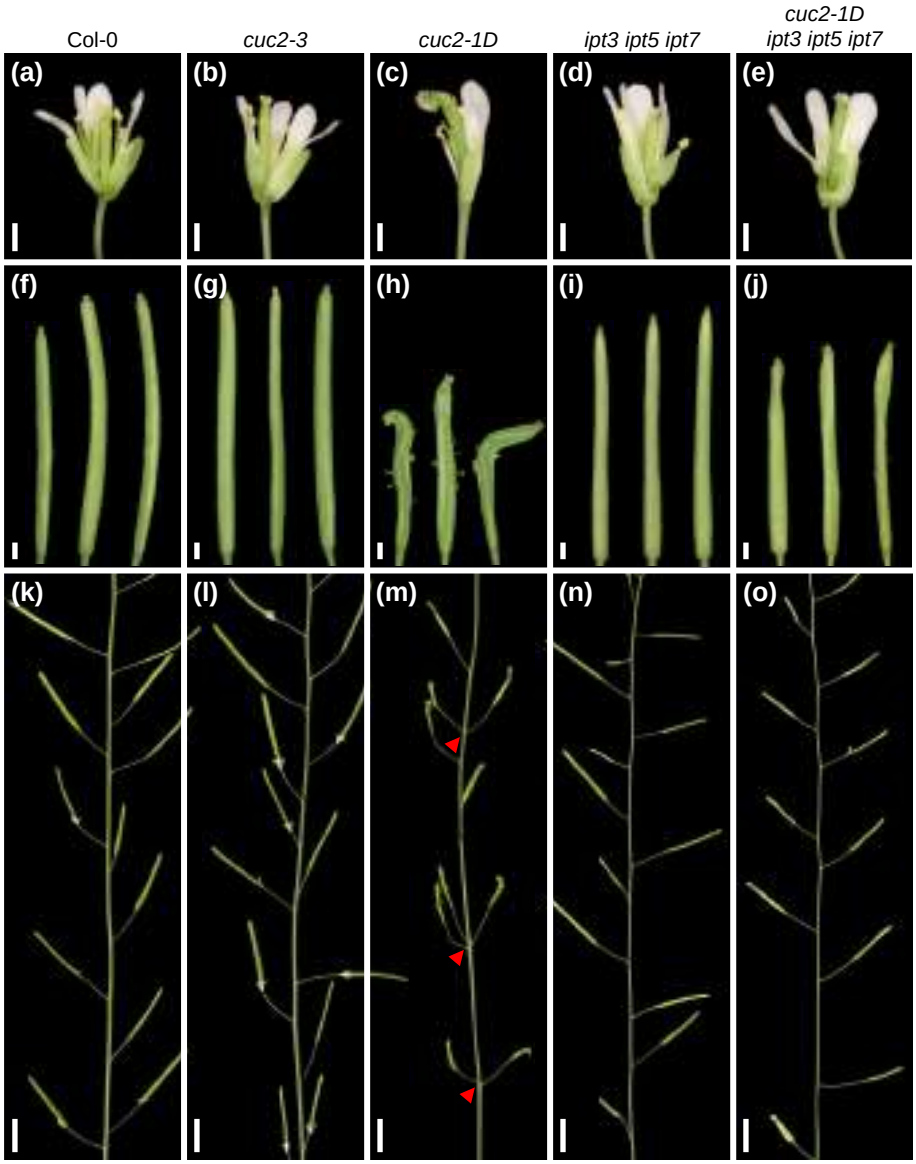
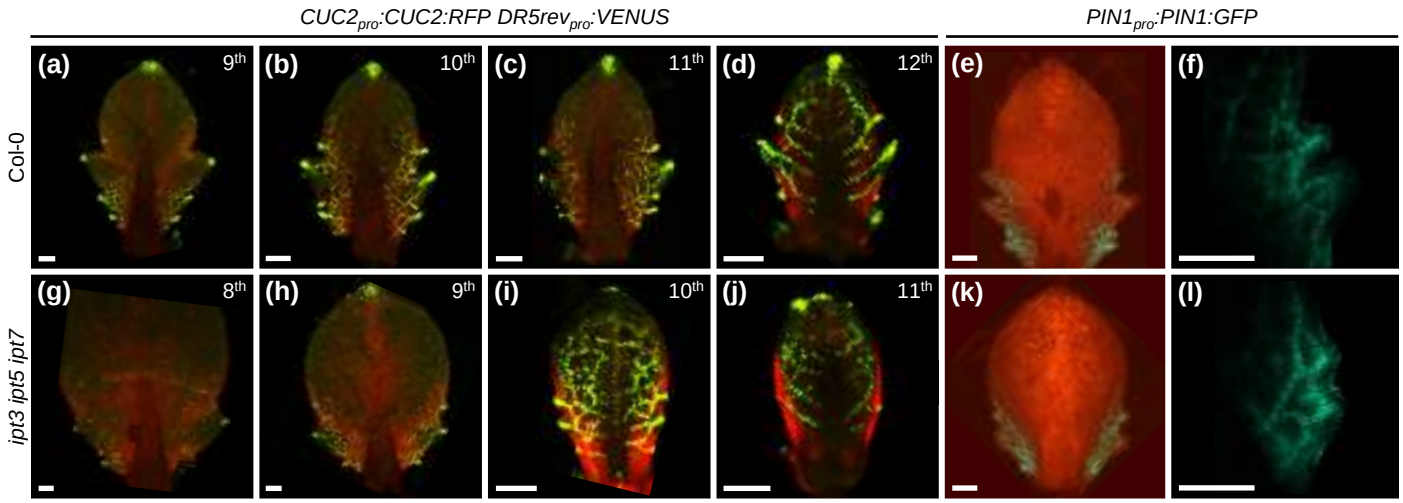
Navarro-Cartagena *et al.*, Figure 1



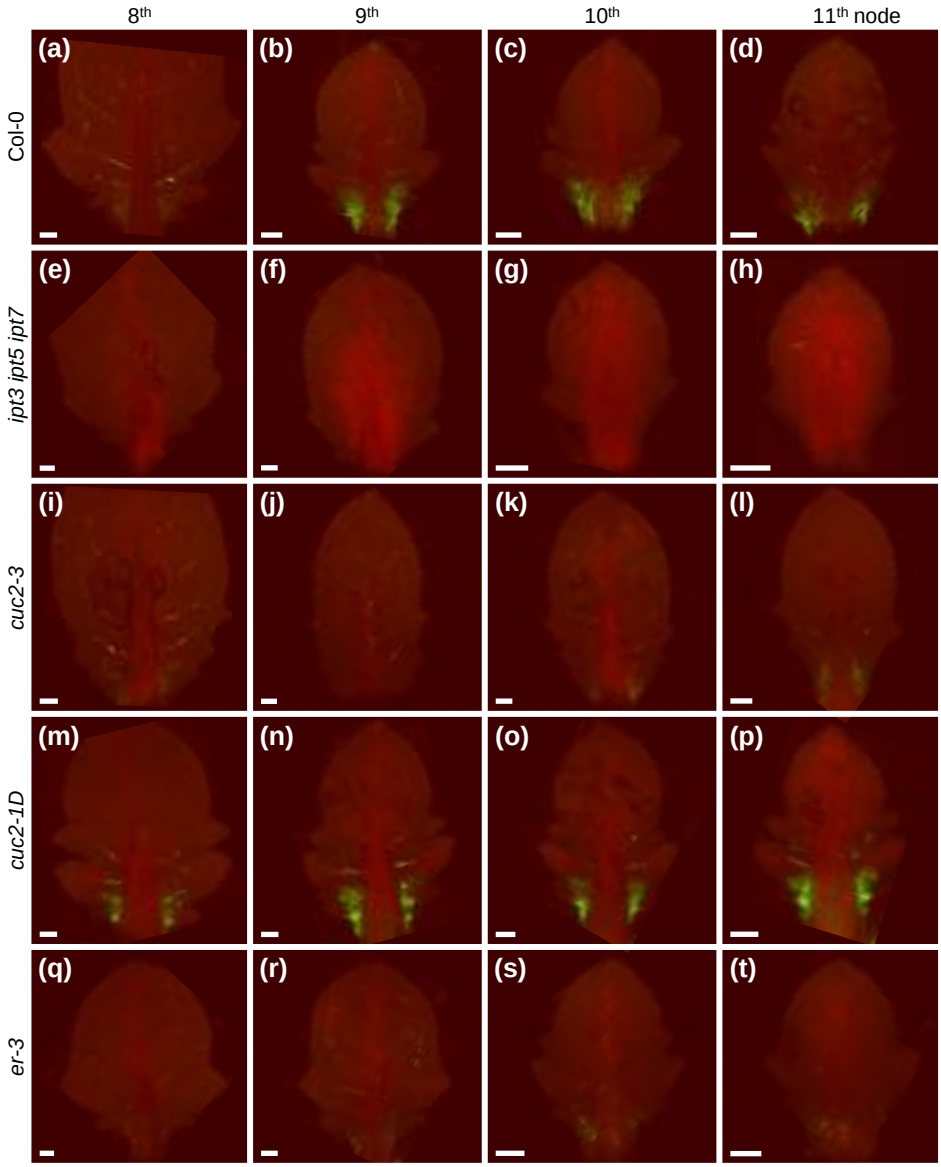
Navarro-Cartagena *et al*., Figure 2



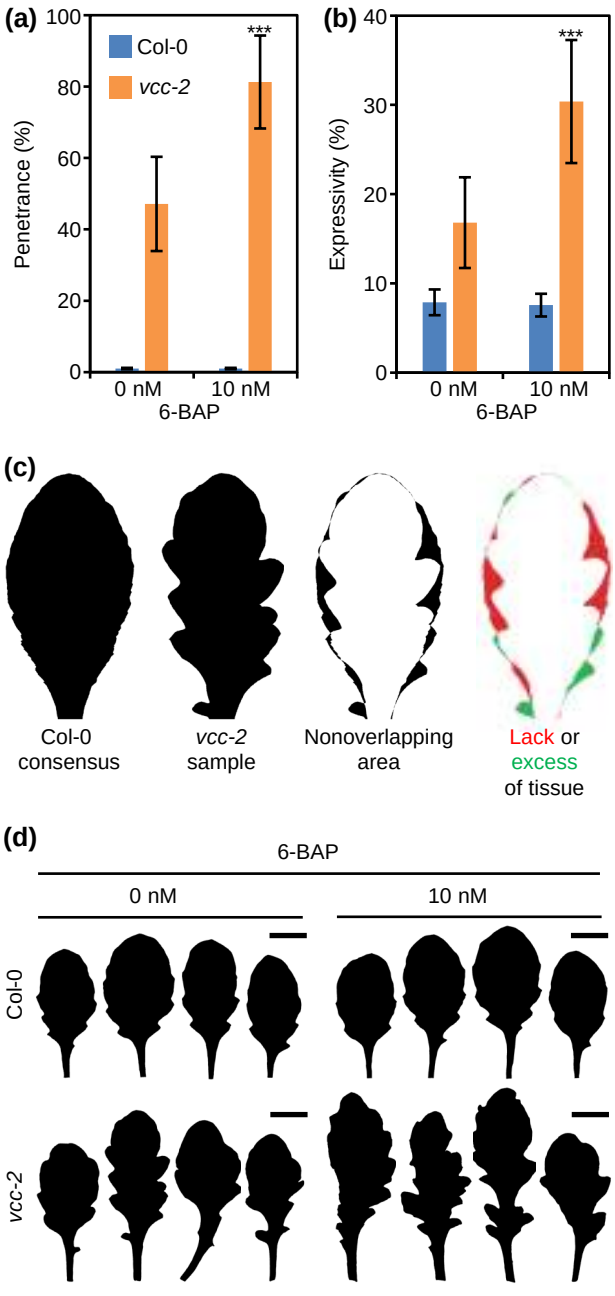
Navarro-Cartagena *et al*., Figure 3



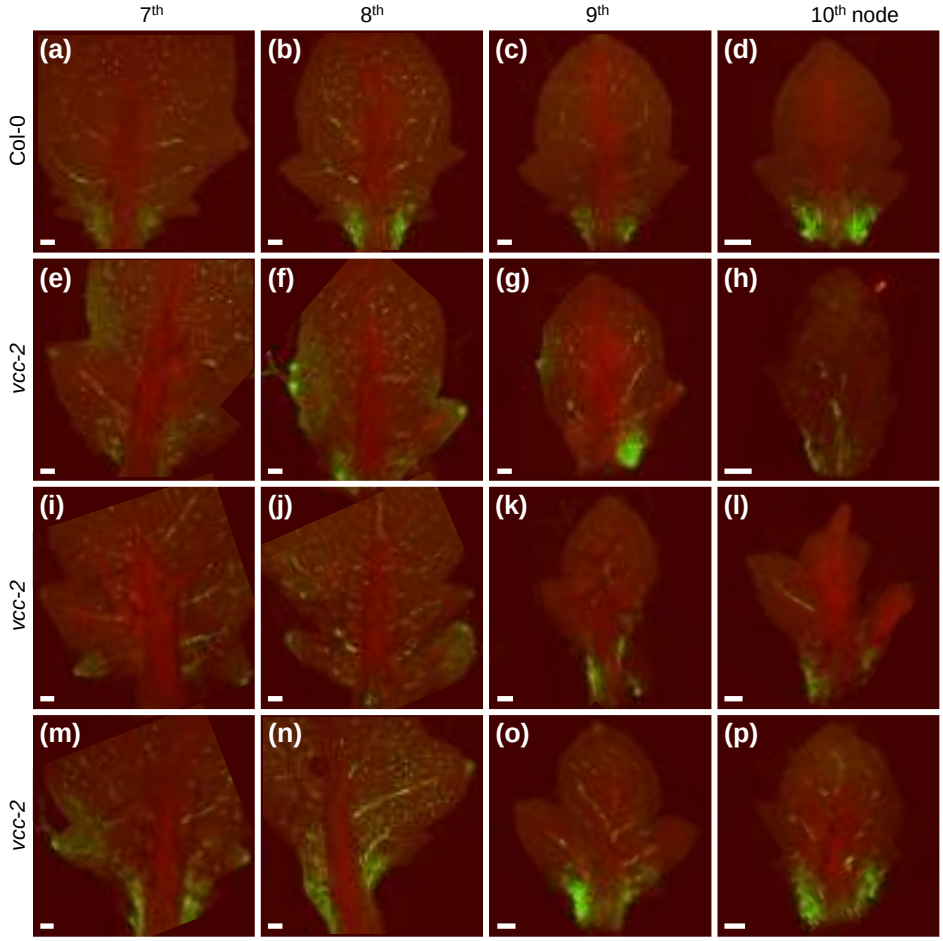
Navarro-Cartagena *et al*., Figure 4



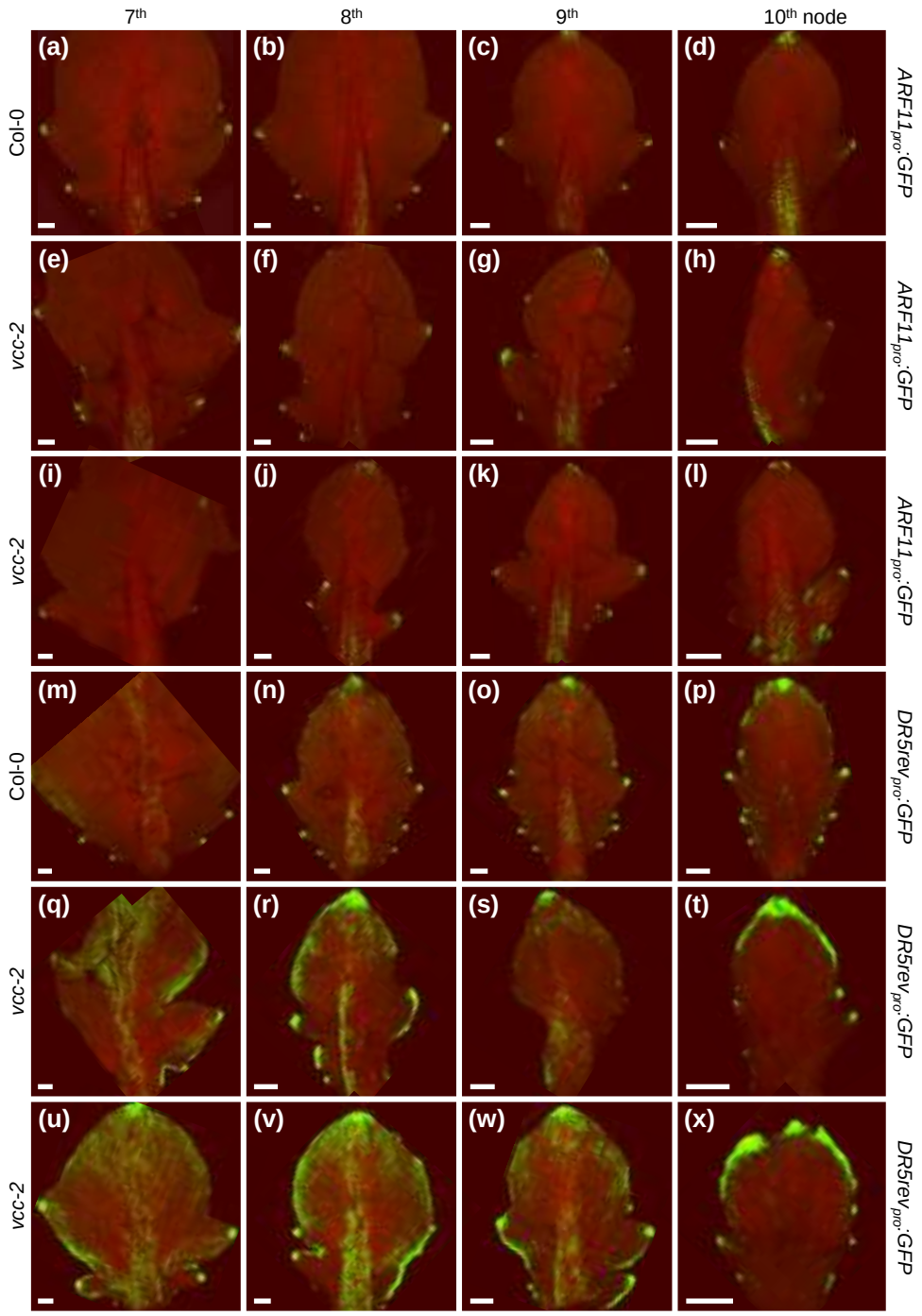
Navarro-Cartagena *et al.*, Figure 5



Navarro-Cartagena *et al*., Figure 6



Navarro-Cartagena *et al*., Figure 7



Cytokinins contribute to Arabidopsis leaf margin morphogenesis

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Supplementary Figures and Tables

Supplementary Data Sets not included in this file

- Navarro-Cartagena et al_Data Set 1.xlsx
- Navarro-Cartagena et al_Data Set 2.xlsx
- Navarro-Cartagena et al_Data Set 3.xlsx
- Navarro-Cartagena et al_Data Set 4.xlsx
- Navarro-Cartagena et al_Data Set 5.xlsx
- Navarro-Cartagena et al_Data Set 6.xlsx

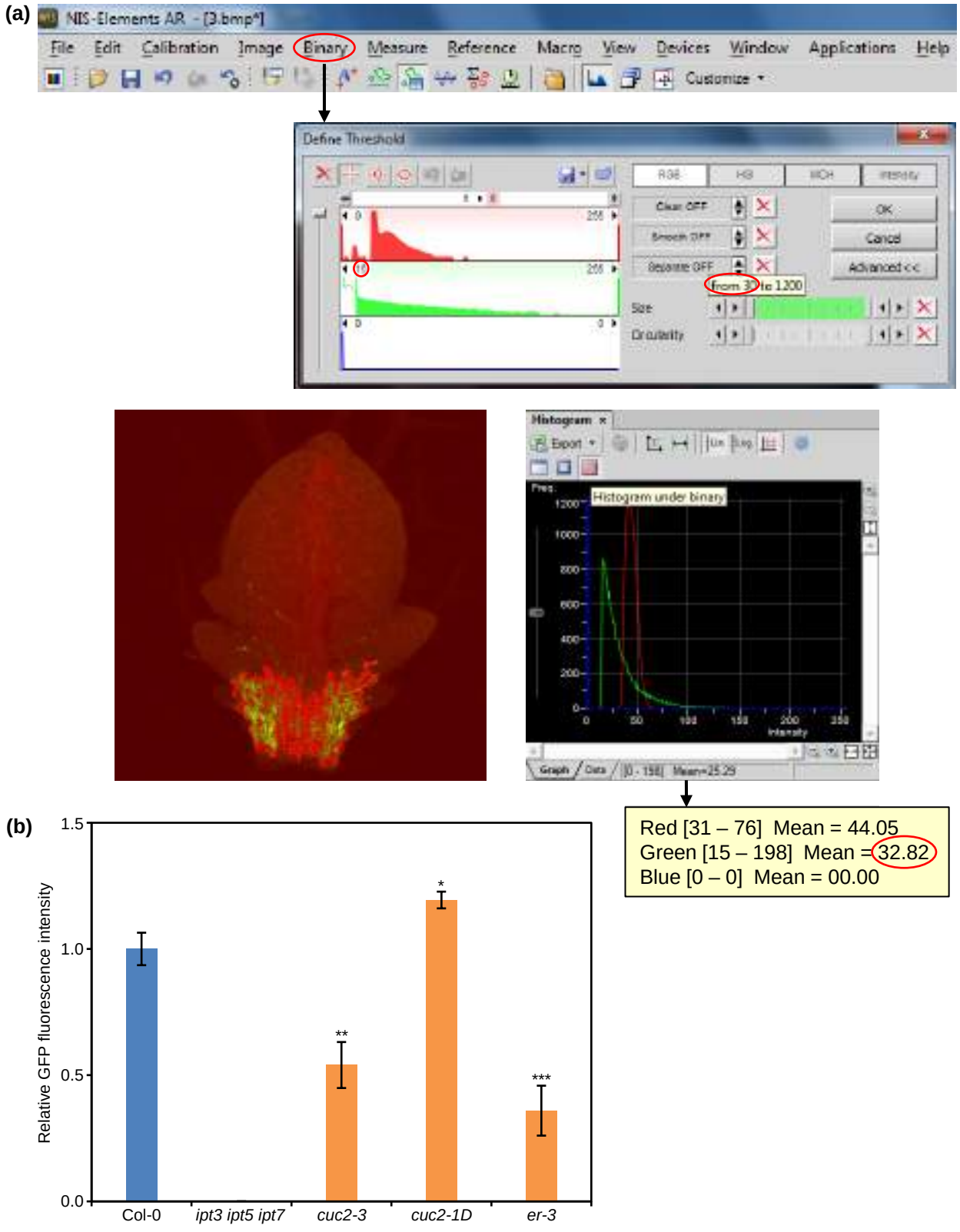

Figure S1. Quantification of GFP fluorescence in leaf primordia. (a) Nikon NIS-Elements (AR Ver3.22.15) software was used, with the following options: binary → define threshold → RGB → green range: 15–255; size range: from 30 to 1200 pixels. Green pixel mean (fluorescence intensity) of the region of interest (ROI; surrounded by the red outline) was extracted from the histogram under binary. Samples without any region above the threshold yielded a fluorescence intensity value of zero. (b) Relative GFP fluorescence intensity in leaf primordia from successive rosette nodes (9^{th}-11^{th}) collected 14-15 das, exceptions being those of *ipt3 ipt5 ipt7* plants, which were collected 17-18 das due to their longer plastochron. Values were normalized to those of Col-0. Data are means ± SD. Asterisks indicate values significantly different from Col-0 in a Student's *t*-test (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$; n = 26, 21, 31, 26, and 25 in Col-0, *ipt3 ipt5 ipt7*, *cuc2-3*, *cuc2-1D*, and *er-3*, respectively).

Figure S2. Reduced leaf margin serration in the *arr1 arr10 arr12* triple mutant and the *ANT:CKX3* transgenic line. (a, b) Silhouettes of leaves from successive rosette nodes (1st–12th) of (a) Col-0 and (b) *arr1 arr10 arr12* plants. Leaves were collected (a) 25 and (b) 35 das from plants transferred from plates to pots at 5 das; *arr1 arr10 arr12* growth in plates was arrested at the very early seedling stage, producing only a few leaves. (c, d) Silhouettes of leaves from successive rosette nodes (1st–15th) of (c) Col-0 or (d) *ANT:CKX3* plants grown in plates for 35 days. Scale bars, 1 cm.

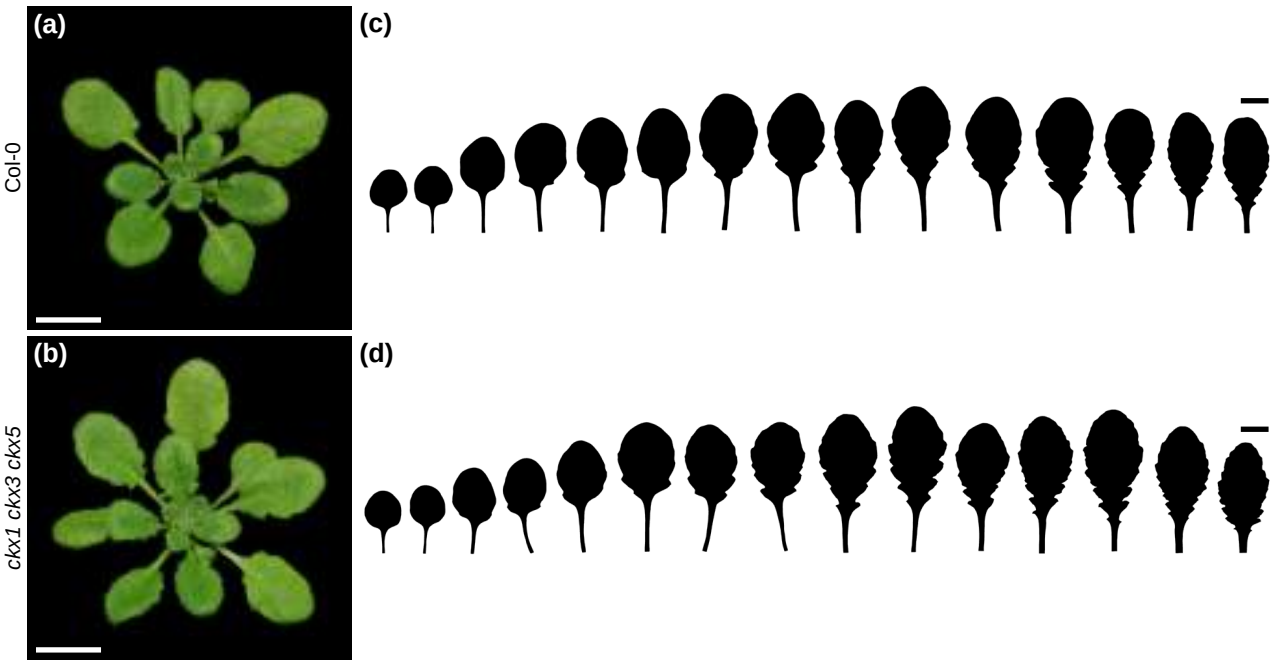

Figure S3. Increased leaf margin serration in the *ckx1 ckx3 ckx5* triple mutant. (a, b) Rosettes and (c, d) silhouettes of leaves from successive rosette nodes (1^{st}–15^{th}) of (a, c) Col-0 and (b, d) *ckx1 ckx3 ckx5* plants. Pictures were taken at (a, b) 21 and (c, d) 35 das. Scale bars, 1 cm.

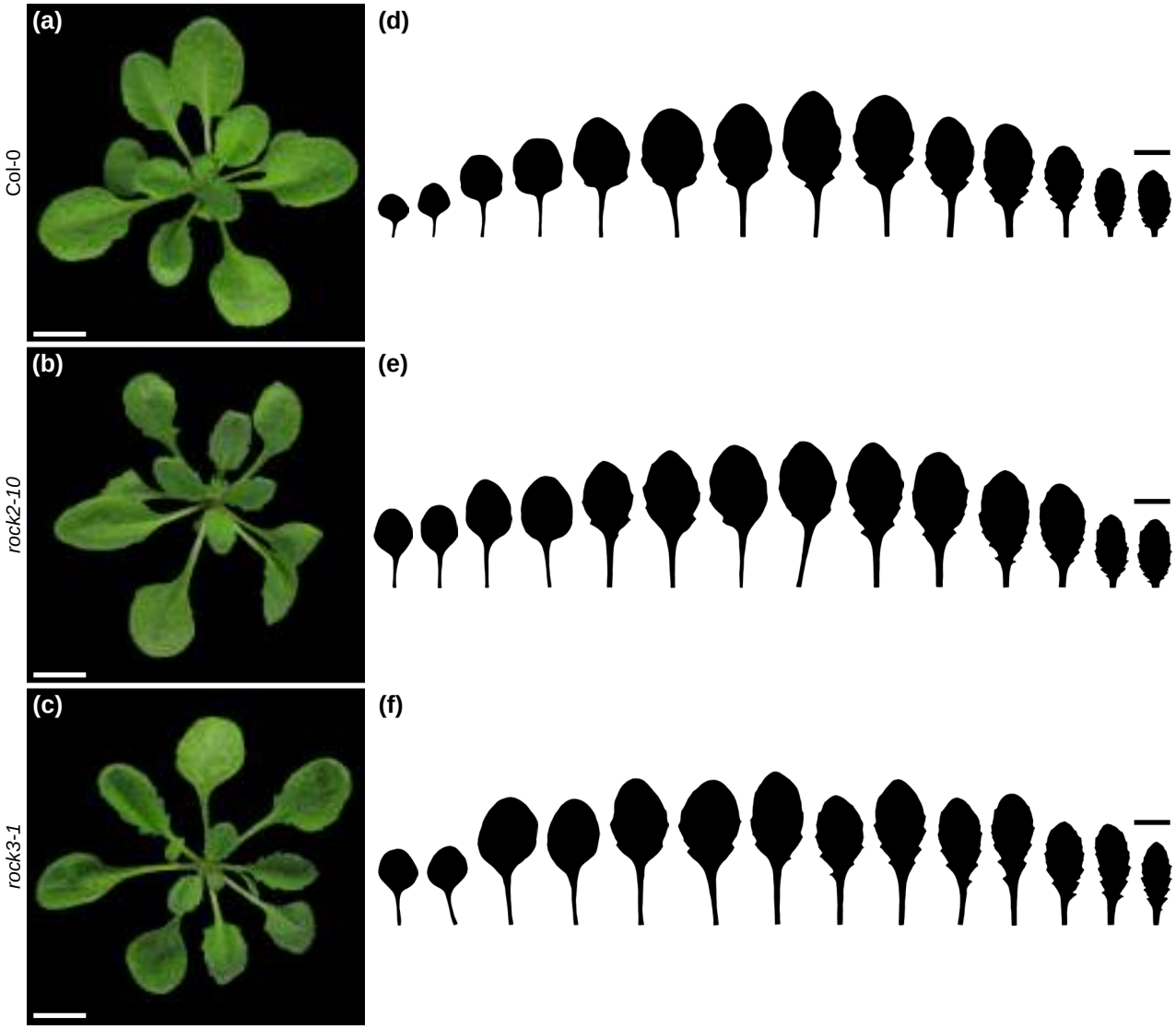

Figure S4. Increased teeth sharpness from *rock2-10* and *rock3-1* leaf margins. (a-c) Rosettes and (d-f) silhouettes of leaves from successive rosette nodes (1^{st}-14^{th}) of (a, d) Col-0, (b, e) *rock2-10*, and (c, f) *rock3-1* plants. Pictures were taken at (a-c) 21 and (d-f) 28 das. Scale bars, 1 cm.

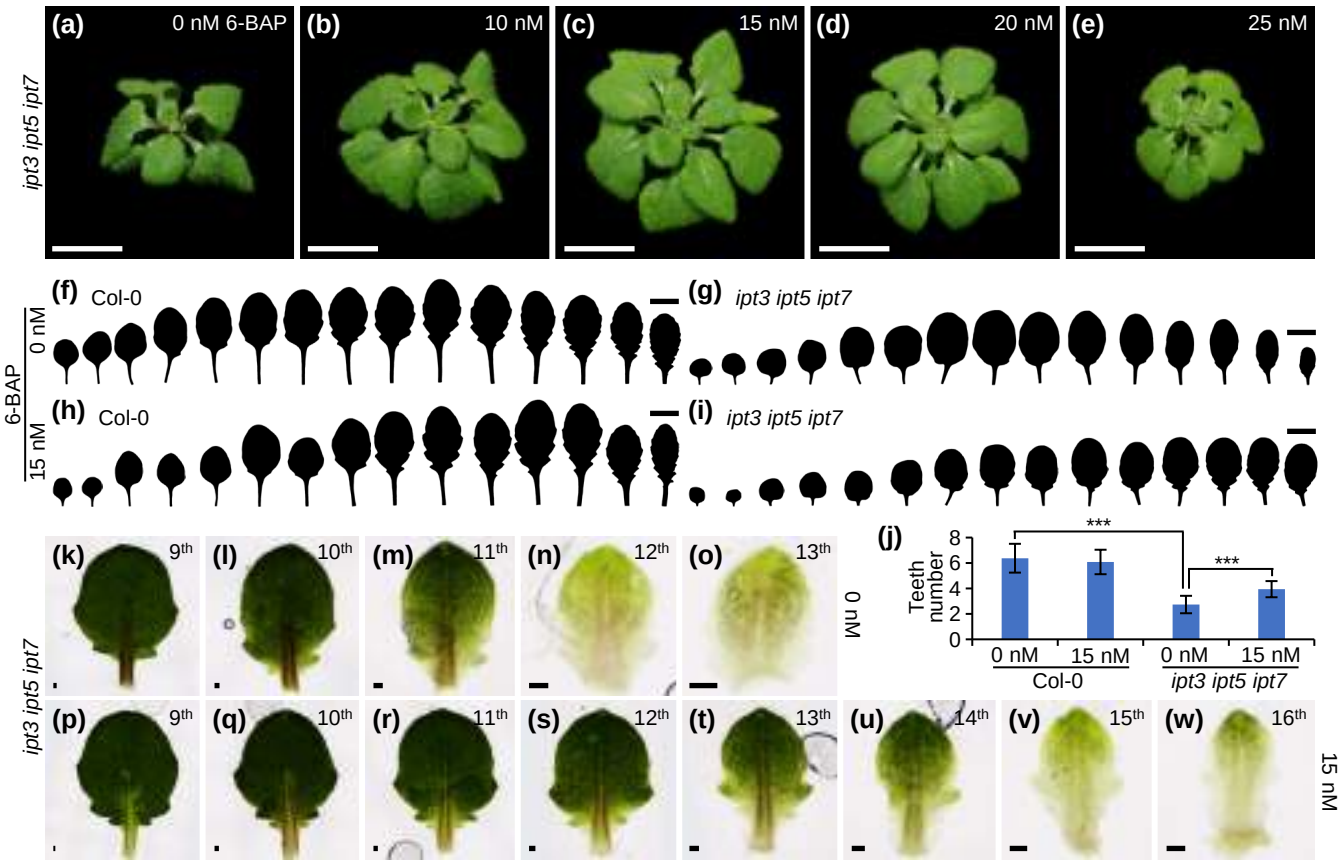

Figure S5. Effects of 6-BAP on the margin shape of leaf primordia and expanded leaves of *ipt3 ipt5 ipt7* plants. (a-e) *ipt3 ipt5 ipt7* rosettes grown on culture medium containing the indicated 6-BAP concentrations. Since the addition of 20 and 25 nM 6-BAP visibly reduced plant growth, 15 nM 6-BAP was used for further treatments. (f-i) Silhouettes of leaves from successive rosette nodes (1^{st}–15^{th}) of (f, h) Col-0 or (g, i) *ipt3 ipt5 ipt7* plants grown on culture medium (h, i) containing 15 nM 6-BAP or (f, g) no added phytohormone. (j) Number of margin teeth in leaves from successive rosette nodes (9^{th}–11^{th}). Data are means ± SD. Asterisks indicate values significantly different from Col-0 in a Student's *t*-test (***$p < 0.0001$; $n \geq 18$). (k-w) Bright-field micrographs of leaf primordia from successive rosette nodes (9^{th}–13^{th} or 9^{th}–16^{th}) collected from *ipt3 ipt5 ipt7* plants grown on culture medium (p-w) with or (k-o) without the addition of 15 nM 6-BAP. *ipt3 ipt5 ipt7* plants developed three extra leaves (u-w) in the presence of 6-BAP. Pictures were taken at (a-e) 25, (f-i) 35, and (k-t) 21 das. Scale bars, (a-i) 1 cm and (k-w) 0.1 mm. All concentrations shown in this figure refer to 6-BAP.

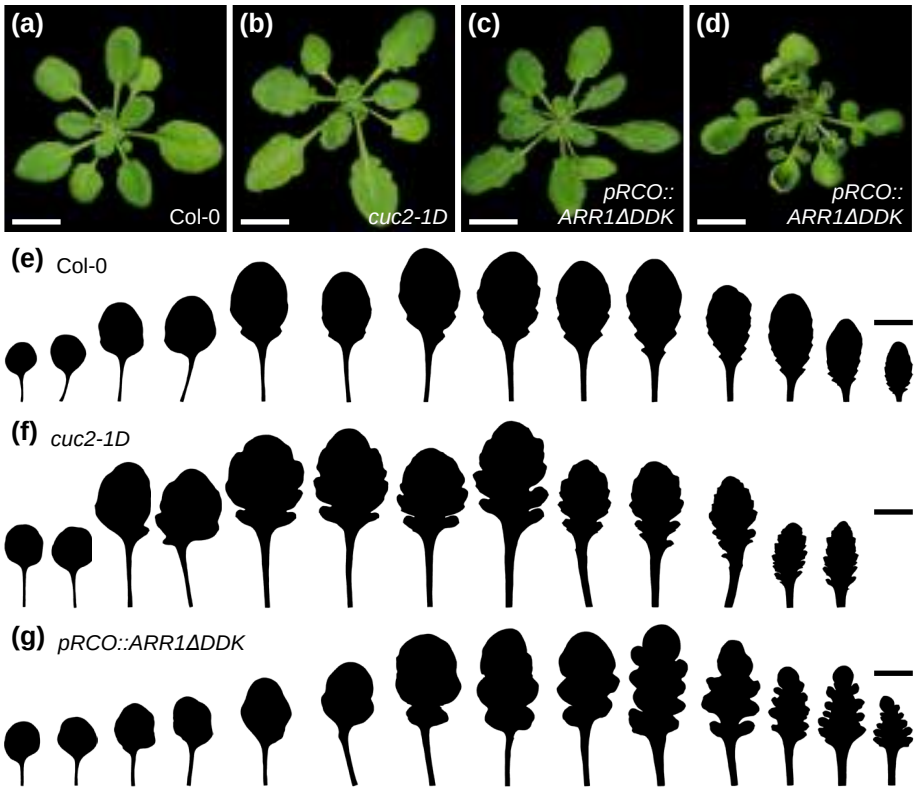

Figure S6. Comparison of the leaf phenotypes of the *cuc2-1D* gain-of-function mutant and the *pRCO::ARR1ΔDDK* transgenic line. (a-d) Rosettes and (e-g) silhouettes of leaves from successive rosette nodes (1^{st}–14^{th}) of (a, e) Col-0, (b, f) *cuc2-1D*, and (c, d, g) *pRCO::ARR1ΔDDK* plants. The silhouettes shown in (g) correspond to a *pRCO::ARR1ΔDDK* plant showing a phenotype like that shown in (c). Pictures were taken at (a-d) 21 and (e-f) 28 das. Scale bars, 1 cm.

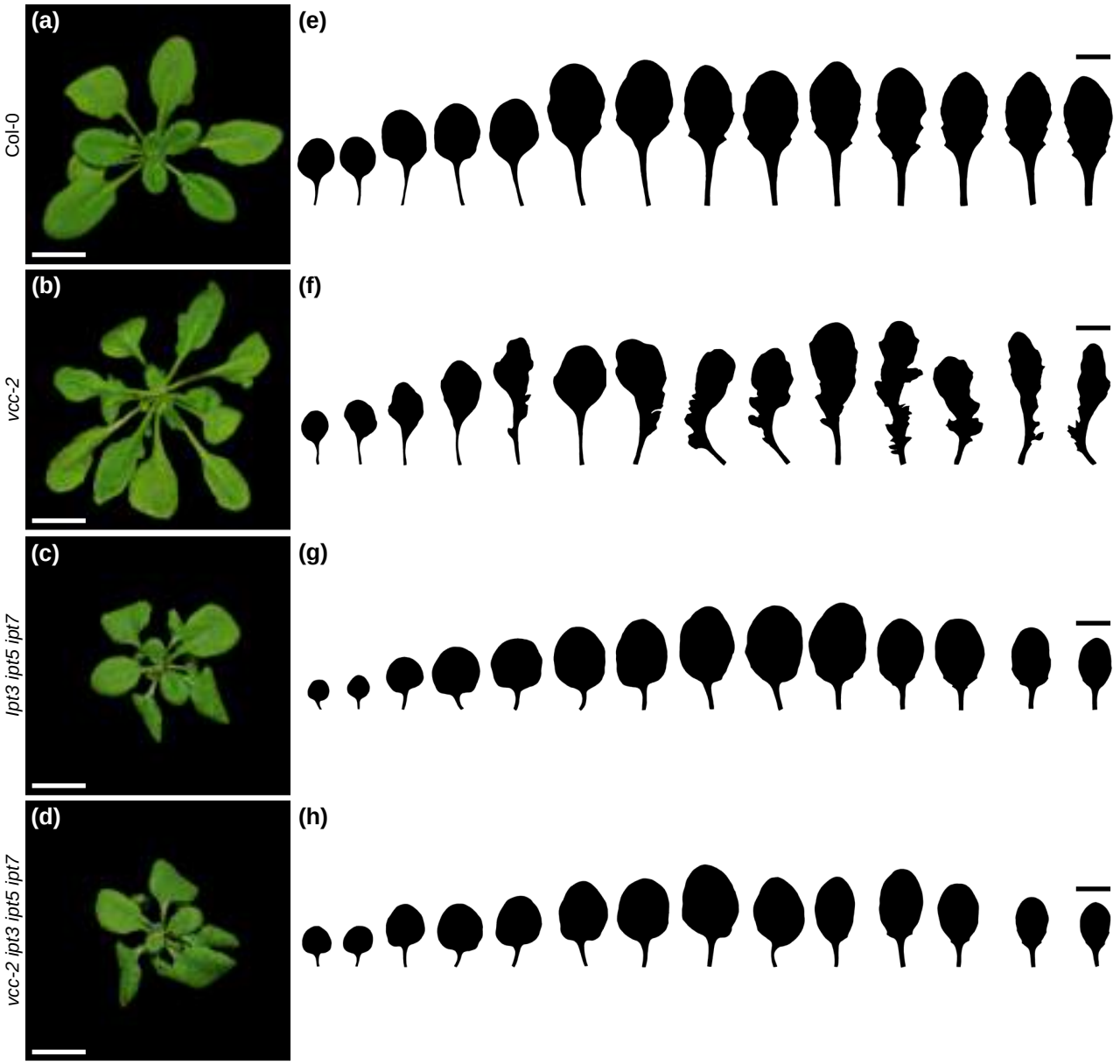

Figure S7. Genetic interaction between *vcc-2* and *ipt3 ipt5 ipt7*. (a-d) Rosettes and (e-h) silhouettes of leaves from successive rosette nodes (1st–14th) of (a, e) Col-0, (b, f) *vcc-2*, (c, g) *ipt3 ipt5 ipt7*, and (d, h) *vcc-2 ipt3 ipt5 ipt7* plants. Pictures were taken at (a-d) 21 and (e-h) 28 das. Scale bars, 1 cm.

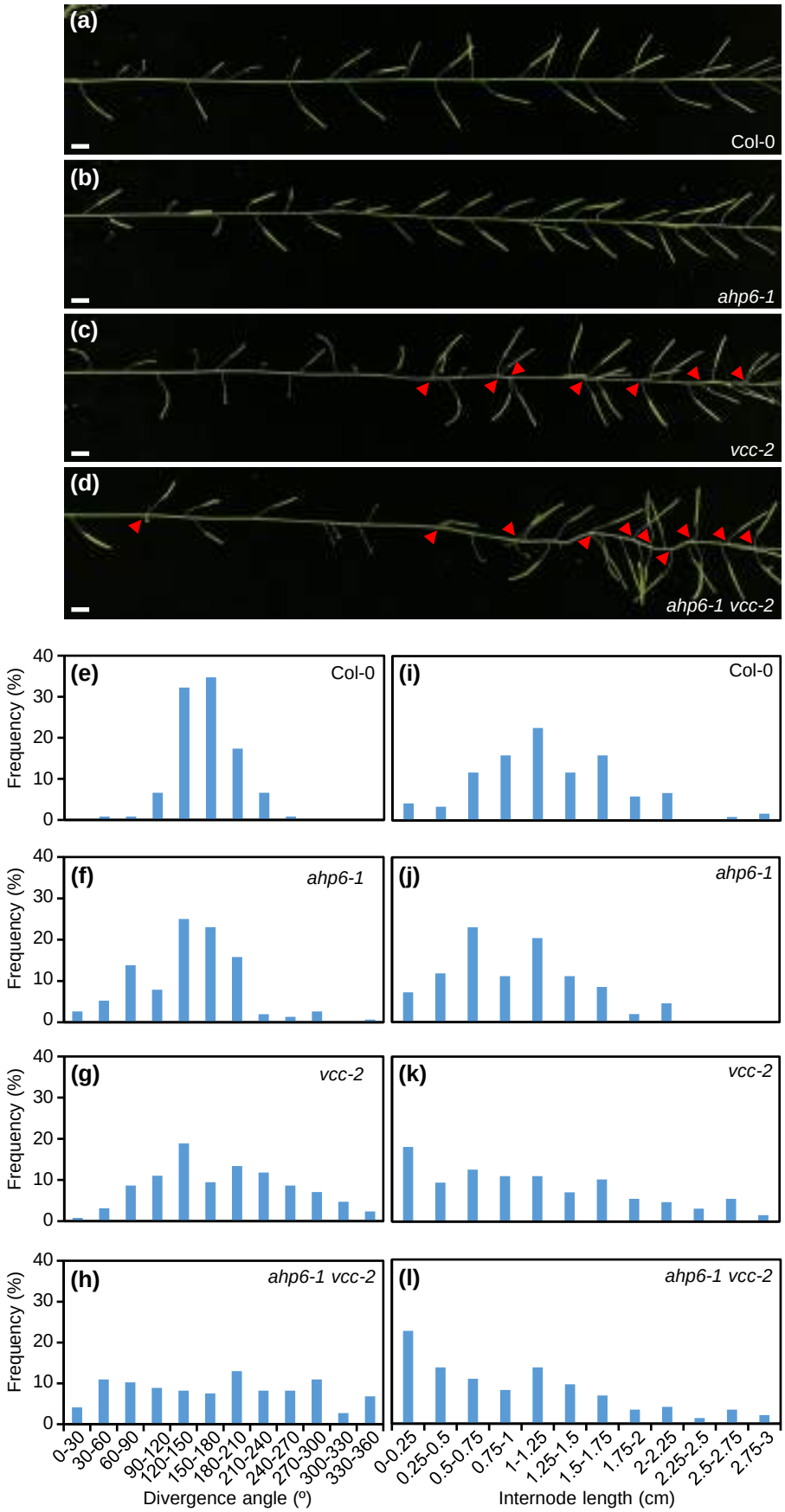

Figure S8. Genetic interaction between *vcc-2* and *ahp6-1*. (a-d) Silique spacing along main stem segments from (a) Col-0, (b) *ahp6-1*, (c) *vcc-2*, and (d) *ahp6-1 vcc-2* plants. Red arrowheads indicate abnormally clustered siliques. Scale bars, 1 cm. (e-h) Frequencies of divergence angles and (i-l) internode lengths between successive siliques along the stem in (e, i) Col-0, (f, j) *ahp6-1*, (g, k) *vcc-2*, and (h, l) *ahp6-1 vcc-2* main stems, expressed as the percentage of the measurements made (n ≥ 120).

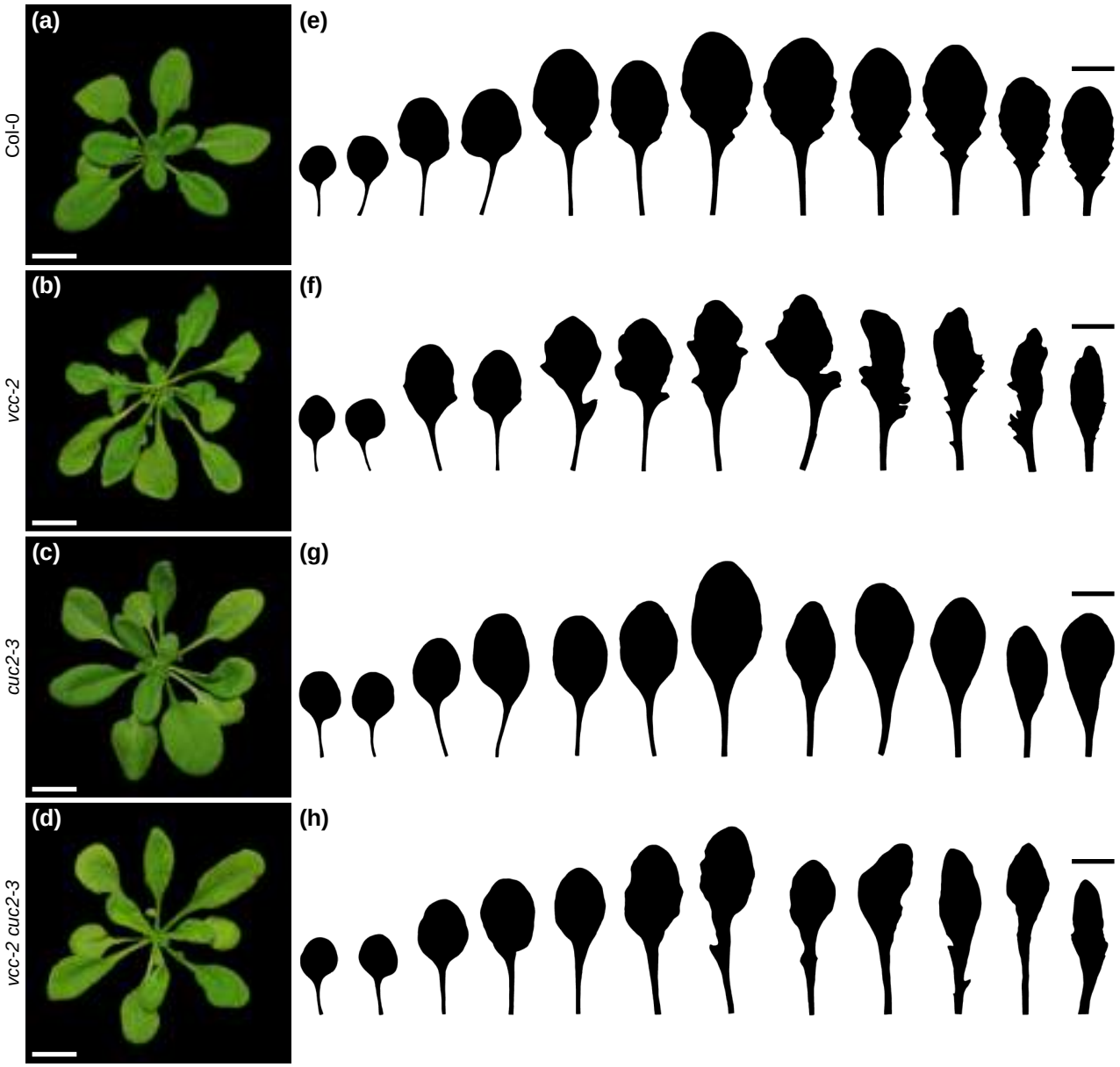

Figure S9. Genetic interaction between *vcc-2* and *cuc2-3*. (a-d) Rosettes and (e-h) silhouettes of leaves from successive rosette nodes (1^{st}–12^{th}) of (a, e) Col-0, (b, f) *vcc-2*, (c, g) *cuc2-3*, and (d, h) *vcc-2 cuc2-3* plants. Pictures were taken at (a-d) 21 and (e-h) 28 das. Scale bars, 1 cm. Pictures in (a) and (b) are the same as those in Figs. S7a and S7b, respectively.

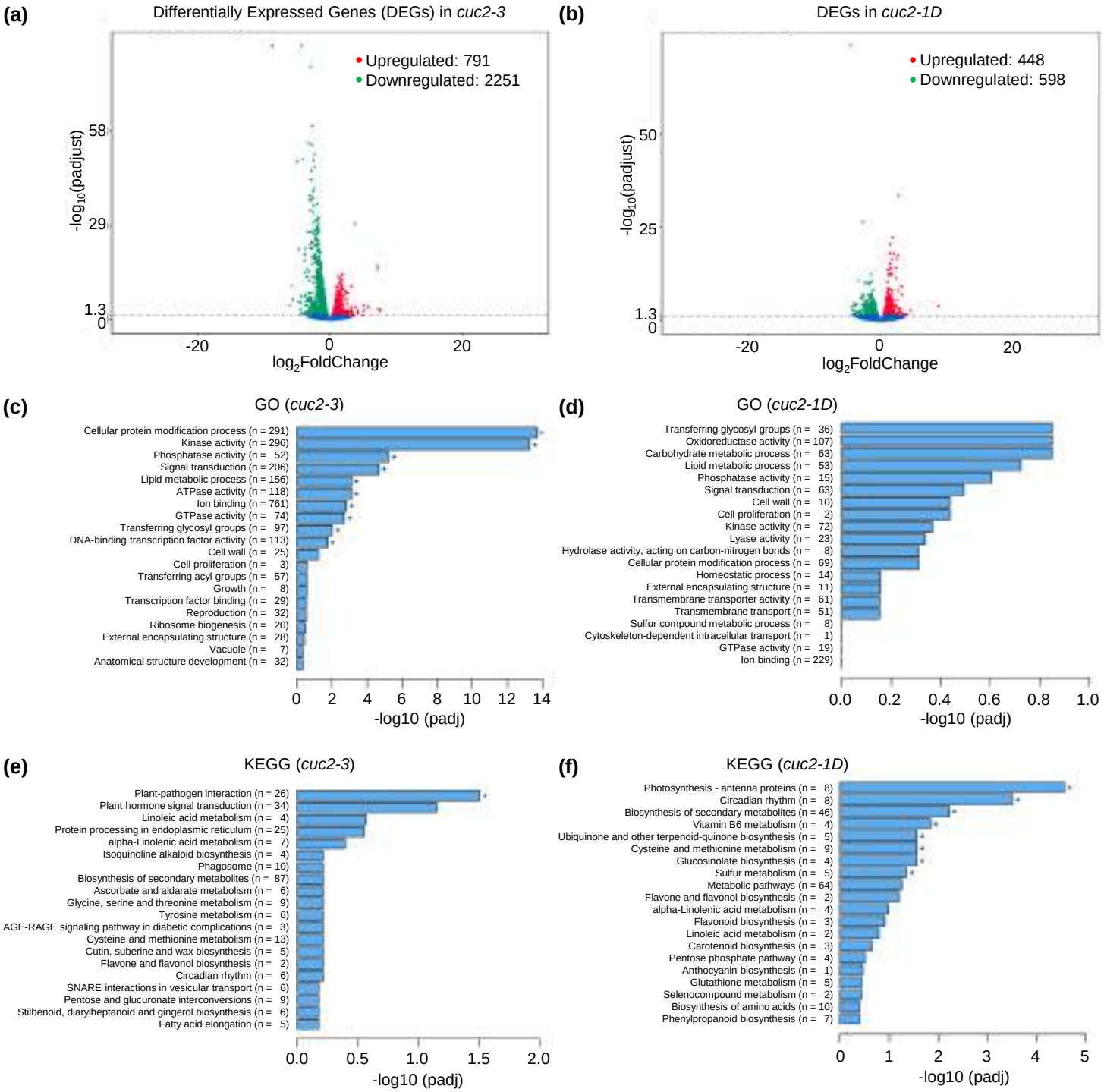

Figure S10. Differentially expressed genes identified from RNA-seq analysis of the *cuc2-3* and *cu2-1D* mutants relative to Col-0. (a-b) Volcano plots used to infer the overall distribution of DEGs. (c, d) Gene Ontology (GO) and (e, f) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses. Three biological replicates were assayed. Threshold was set as *p*-adj < 0.05. n: number of differentially expressed genes concerning a given GO or KEGG term.

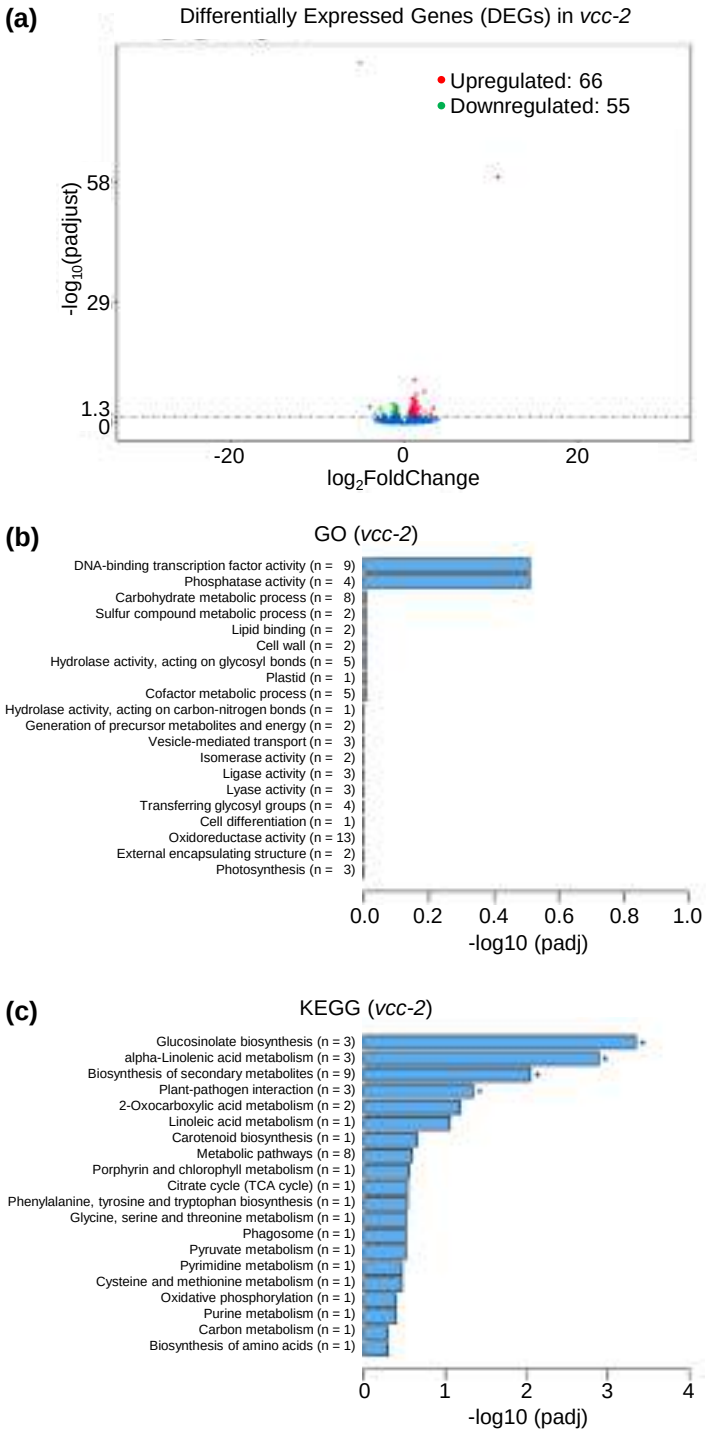

Figure S11. Differentially expressed genes identified from an RNA-seq analysis of the *vcc-2* mutant relative to Col-0. See Fig. S10 legend for details.

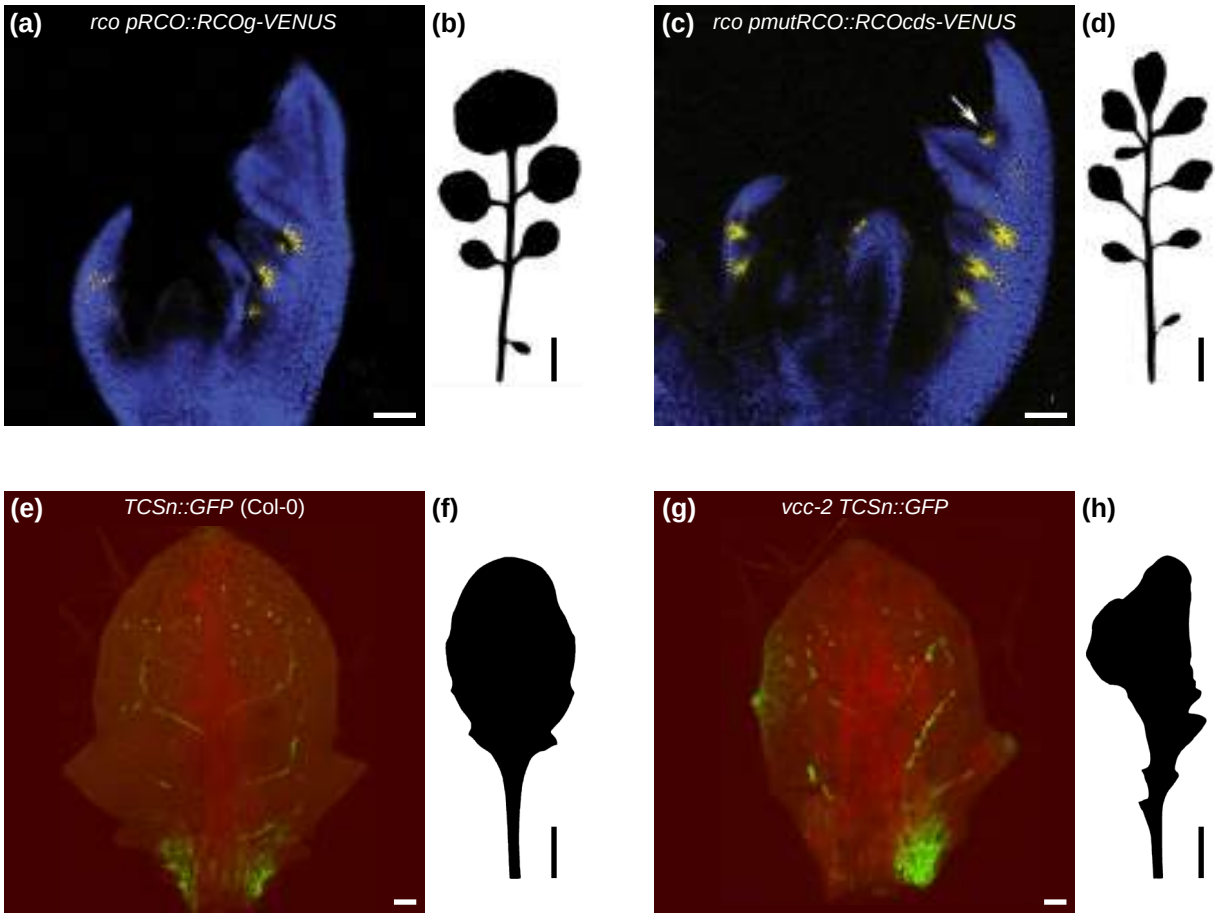

Figure S12. Cytokinin response in young leaves and expanded leaf morphology comparison between *Cardamine hirsuta rco pmutRCO::RCOcds-VENUS* and Arabidopsis *vcc-2* plants. In *C. hirsuta*, RCO directly represses transcription of the *RCO* gene, establishing a negative autoregulatory feedback loop that defines the *RCO* expression pattern and promotes cytokinin activity. The mutated *RCO* promoter *pmutRCO* lacks RCO-binding sites, thereby lacking autoregulation. (a, b) In *C. hirsuta rco pRCO::RCOg-VENUS* plants, (a) the RCO-VENUS signal is restricted to the base of leaflets in young leaves, and (b) expanded leaves are phenotypically wild type. (c, d) In *rco pmutRCO::RCOcds-VENUS* plants, (c) the RCO-VENUS signal intensity increases and ectopically accumulates at the boundaries of terminal leaflets (arrow), (d) and expanded leaves show lateral leaflets with increased complexity and an additional subdivision of the terminal leaflet. (e, f) In the Arabidopsis wild-type Col-0, (e) expression of the cytokinin response reporter *TCSn::GFP* is excluded from lobes at leaf primordia, and (f) expanded leaves are phenotypically wild type. (g, h) In the *vcc-2* mutant, (g) ectopic and asymmetric cytokinin response visualized by *TCSn::GFP* is observed in leaf primordia, and (h) expanded leaves show an increased margin complexity and bilateral asymmetry. Scale bars, (a, c) 50 µm, (b, d, f, h) 1 cm, and (e, g) 0.1 mm. Images (a)-(d) were taken from Hajheidari *et al.* (2019) with permission from the authors.

Table S1. Other VCC interactors identified in a screen based in the split-ubiquitin yeast two-hybrid membrane-based assay

PBS*	Number of hits	Gene code	Protein	Description
D	1	At4g25570	ACYB-2	Cytochrome b561/ferric reductase transmembrane family protein
D	1	At1g14730		Cytochrome b561/ferric reductase transmembrane family protein
D	2	At2g18960	ARABIDOPSIS H^+ ATPASE1 (AHA1)	Plasma membrane proton ATPase
D	2	At5g57350	H^+-ATPASE3 (HA3)	Member of plasma membrane H^+-ATPase family
D	2	At4g36530	AP22.96	Alpha/beta hydrolase family
D	2	At5g38660	ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT1 (APE1)	
D	2	At5g46110	ACCLIMATION OF PHOTOSYNTHESIS TO ENVIRONMENT2 (APE2)	Chloroplast triose-phosphate/3-phosphoglycerate translocator
D	3	At1g04340		HR-like lesion-inducing protein-like
E	24	At1g45688	COMPANION OF CELLULOSE SYNTHASE1 (CC1)	Interacts with the cellulose synthase complex and microtubules
D	1	At1g57680	CANDIDATE G-PROTEIN COUPLED RECEPTOR1 (CAND1)	Plasminogen activator inhibitor
D	4	At1g72120	NPF5.14	Major facilitator superfamily protein. Putative peptide/nitrate transporter
D	1	At5g13400		Major facilitator superfamily protein. Putative peptide/nitrate transporter
D	5	At2g21120	ANTIVIRAL RNAI-DEFECTIVE2 (AVI2), ENHANCER OF RDR6 3 (ENOR3)	Putative magnesium transporter
D	4	At3g26670		Putative magnesium transporter
D	1	At3g06180		Ribosomal protein L34e superfamily protein
D	1	At4g33380		Dimethylallyl, adenosine tRNA methylthiotransferase
D	3	At5g66190	FERREDOXIN-$NADP^+$-OXIDOREDUCTASE1 (FNR1)	Leaf-type ferredoxin:NADPH dehydrogenase/oxidoreductase

Table S1 (continued.) Other VCC interactors identified in a screen based in the split-ubiquitin yeast two-hybrid membrane-based assay

PBS*	Number of hits	Gene code	Protein	Description
C	3	At1g25230	F4F7.38	Calcineurin-like metallo-phosphoesterase superfamily protein
D	9	At5g35220	ETHYLENE-DEPENDENT GRAVITROPISM-DEFICIENT AND YELLOW-GREEN1 (EGY1)	Membrane-associated and ATP-independent metalloprotease; carries out beta-casein degradation and is involved in chloroplast and plastid biogenesis
D	1	At2g20230	F11A3.22	Tetraspanin family protein
D	1	At2g04780	FASCICLIN-LIKE ARABINOOGALACTAN7 (FLA7)	Fasciclin-like arabinogalactan protein
D	1	At5g44920	TIR-KASH PROTEIN (TIK)	Toll-Interleukin-Resistance domain-containing protein. KASH domain protein that localizes to the nuclear envelope and affects nuclear morphology
D	7	At5g45420	MEMBRANE ANCHORED MYB (MAMYB)	R2R3-MYB family transcription factor. Duplicated SANT DNA-binding domain-containing protein
D	1	At5g16480	PLANT AND FUNGI ATYPICAL DUAL-SPECIFICITY PHOSPHATASE5 (PFA-DSP5)	Atypical dual-specificity phosphatase
B	9	At5g06320	NDR1/HIN1-LIKE3 (NHL3)	Similar to tobacco hairpin-induced gene (HIN1) and Arabidopsis non-race specific disease resistance gene (NDR1)
B	5	At1g44575	NONPHOTOCHEMICAL QUENCHING4 (NPQ4)	PHOTOSYSTEM II SUBUNIT S [PSII-S (CP22)], a ubiquitous pigment (chlorophyll/xanthophyll)-binding protein involved in nonphotochemical quenching
D	2	At3g61430	PLASMA MEMBRANE INTRINSIC PROTEIN1A (PIP1A)	Aquaporin
E	16	At4g35100	PLASMA MEMBRANE INTRINSIC PROTEIN3 (PIP3)	Aquaporin

Table S1 (continued). Other VCC interactors identified in a screen based in the split-ubiquitin yeast two-hybrid membrane-based assay

PBS*	Number of hits	Gene code	Protein	Description
D	1	At2g37180	PLASMA MEMBRANE INTRINSIC PROTEIN2C (PIP2C), RESPONSIVE TO DESICCATION28 (RD28)	Functions as aquaporin and is involved in desiccation
D	2	At1g11260	SUGAR TRANSPORTER1 (STP1)	H^+/hexose cotransporter
D	1	At2g42220	T24P15.13	Rhodanese-like domain-containing protein/Cell cycle control phosphatase superfamily protein
D	3	At5g44020		Acid phosphatase class B family protein (HAD superfamily, subfamily IIIB)
D	1	At2g45470	FASCICLIN-LIKE ARABINOGALACTAN PROTEIN8 (FLA8)	Fasciclin-like arabinogalactan protein. Possibly involved in embryogenesis and seed development
D	2	At2g36460	FRUCTOSE-BISPHOSPHATE ALDOLASE6 (FBA6)	Aldolase superfamily protein
D	2	At3g13410		2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
D	1	At3g16180	NITRATE TRANSPORTER 1.12 (NRT1.12)	Low affinity nitrate transporter expressed in the plasma membrane and found in the phloem of the major veins of leaves. It is responsible for nitrate redistribution to young leaves
D	1	At5g62880	RHO-RELATED PROTEIN FROM PLANTS11 (ROP11)	GTPase involved in cell wall patterning. Locally activated to form plasma membrane domains, which direct formation of cell wall pits in metaxylem vessel cells through interaction with cortical microtubules
B	25	At1g22750		Transmembrane protein
B	22	At1g44920		Transmembrane protein
D	1	At5g52420	PSI-INTERACTING ROOT-CELL ENRICHED3 (PRCE3)	Transmembrane protein
D	1	At5g17610	K10A8.90	Unknown protein
D	1	At3g61870		Unknown protein

*Predicted Biological Score. A, B, C and D: Very high, high, good and moderate confidence in the interaction, respectively. E: Warning of non-specific interaction.

Table S2. PCR primers used for the genotyping of single and multiple mutants in this work

Oligonucleotide names	Gene	Oligonucleotide sequences (5′ → 3′)	
		Forward primer (F or LP)	Reverse primer (R or RP)
SALK_047972_LP/RP	*VCC*	GATGTAGCTGCTGCAATCCTC	TTTCAAGTTTCCCGTGACAAG
KG21969 _LP/RP	*IPT3*	TGGAGAGATTCGCCATGTGACAG	CCAACTTGTCGTATATCATTCGTACAGTG
SALK_133407_LP/RP	*IPT5*	CCTTTCCTCAACATATGCTCG	AGAAGTCTCTCCGTCGTCTCC
SALK_001940_LP/RP	*IPT7*	CTCTCGGGGTAAATGTCACAC	TTGACAACTCACGACTCGTTG
SALK_044110_LP/RP	*ER*	CCGGGTAATGAAGAGACATTG	GCAACGTTGCTGGAGATTAAG
SAIL_605_C09_LP/RP	*CUC2*	CAAGATTTGAAACAGCCTTATCG	ATCCCAACAAGTGCACAAGTC
CUC2_F/R	*CUC2*	CAACTGTGAGCGTAAGCAG	GGTCAAAGTCAAACCCTAGC
AHP6_F/R	*AHP6*	AAGCAAGACCTGTTCACATTAG	GTTGCGGAGTAACTTCTCG
KG		CAACACGTGGGTTAATTAAGAATTCAGTAC	
LBb1.3		ATTTTGCCGATTTCGGAAC	
LB1		GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	

Oligonucleotide sequences were taken from http://signal.salk.edu/tdnaprimers.2.html, except CUC2_F/R.

X.- AGRADECIMIENTOS

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