Seedling developmental defects upon blocking CINNAMATE-4-HYDROXYLASE are caused by perturbations in auxin transport

Ilias El Houari^{1,2} , Caroline Van Beirs^{1,2} , Helena E. Arents^{1,2} , Huibin Han³ , Alexandra Chanoca^{1,2} , Davy Opdenacker^{1,2} , Jacob Pollier^{1,2,4} , Véronique Storme^{1,2} , Ward Steenackers^{1,2} , Mussa Quareshy⁵ , Richard Napier⁵ , Tom Beeckman^{1,2} , Jiří Friml³ , Bert De Rybel^{1,2} , Wout Boerjan^{1,2*} and Bartel Vanholme^{1,2*}

¹Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 71, Ghent B-9052, Belgium; ²VIB Center for Plant Systems Biology, Technologiepark 71, Ghent B-9052, Belgium; ³Institute of Science and Technology (IST) Austria, Klosterneuburg 3400, Austria; ⁴VIB Metabolomics Core, Ghent 9052, Belgium; ⁵School of Life Sciences, University of Warwick, Coventry, CV4 7AL, UK

Summary

- The phenylpropanoid pathway serves a central role in plant metabolism, providing numerous compounds involved in diverse physiological processes. Most carbon entering the pathway is incorporated into lignin. Although several phenylpropanoid pathway mutants show seedling growth arrest, the role for lignin in seedling growth and development is unexplored.
- We use complementary pharmacological and genetic approaches to block CINNAMATE-4-HYDROXYLASE (C4H) functionality in *Arabidopsis* seedlings and a set of molecular and biochemical techniques to investigate the underlying phenotypes.
- Blocking C4H resulted in reduced lateral rooting and increased adventitious rooting apically in the hypocotyl. These phenotypes coincided with an inhibition in AUX transport. The upstream accumulation in *cis*-cinnamic acid was found to be likely to cause polar AUX transport inhibition. Conversely, a downstream depletion in lignin perturbed phloem-mediated AUX transport. Restoring lignin deposition effectively reestablished phloem transport and, accordingly, AUX homeostasis.
- Our results show that the accumulation of bioactive intermediates and depletion in lignin jointly cause the aberrant phenotypes upon blocking C4H, and demonstrate that proper deposition of lignin is essential for the establishment of AUX distribution in seedlings. Our data position the phenylpropanoid pathway and lignin in a new physiological framework, consolidating their importance in plant growth and development.

Authors for correspondence:

Bartel Vanholme

Email: Bartel.Vanholme@psb.vib-ugent.be

Wout Boerjan

Email: Wout.Boerjan@psb.vib-ugent.be

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Introduction

The phenylpropanoid pathway (PPP) is a plant-specific metabolic pathway converting the aromatic amino acids phenylalanine or tyrosine into a broad range of secondary metabolites, including coumarins, phenolic acids, stilbenes and flavonoids (Vogt, 2010). These molecules are involved in a diverse set of biological and physiological processes in plants, ranging from pigmentation to plant defence responses (Saslowky *et al.*, 2000; Noel *et al.*, 2005; Chong *et al.*, 2009; Schmid *et al.*, 2014; Brunetti *et al.*, 2018; R. Vanholme *et al.*, 2019b). In quantitative terms, the majority of the carbon entering the PPP is allocated to the biosynthesis of monolignols such as coniferyl and sinapyl alcohol (Boerjan *et al.*, 2003; R. Vanholme *et al.*, 2019a). The monolignols are the main building blocks of lignin (Boerjan *et al.*, 2003; Ralph *et al.*, 2019; R. Vanholme *et al.*, 2019a), a

biopolymer deposited in the plant cell wall to safeguard the plant's structural integrity as well as long-distance water transport.

Mutations in the PPP often come with dwarfism, and three different models have been proposed that explain these phenotypes (Muro-Villanueva *et al.*, 2019). Two models suggest a change in lignin content and composition to be the causal factor. According to the first model, lignin depletion in xylem vessels causes a weakening in the strength of these cells. The tension forces generated by transpiration then cause the collapse of weakened xylem vessels, thus disrupting xylem functionality (Franke *et al.*, 2002; Coleman *et al.*, 2008; De Meester *et al.*, 2018; Muro-Villanueva *et al.*, 2019). As such, the upward transport of water in the plant is disrupted. The second model proposes a cell wall integrity system to induce dwarfism. The depletion in lignin content or alteration of lignin composition in the cell wall can lead to the induction of a defence response in the plant, which would then negatively impact growth (Gallego-Giraldo *et al.*,

*These authors share last authorship.

2018). The mediator complex was found to play a central role in the growth inhibition underlying disruption of cell wall integrity (Bonawitz *et al.*, 2014; Dolan *et al.*, 2017). In contrast with the previous two, the third model explaining dwarfism in PPP mutants proposes a differential accumulation of bioactive intermediates to be at the basis of the growth phenotypes. Several intermediates or end-products of the PPP have been described to have bioactive properties (B. Vanholme *et al.*, 2019). Some of the best-described include flavonoids (Peer & Murphy, 2007), salicylic acid (Zhao *et al.*, 2015; Tan *et al.*, 2020) and *cis*-cinnamic acid (Steenackers *et al.*, 2017, 2019), but preliminary evidence exists for many more. For some PPP mutants the phenotypes previously have been linked to accumulating flavonoids and salicylic acid (Besseau *et al.*, 2007; Gallego-Giraldo *et al.*, 2011), although for flavonoids this has been disputed (Li *et al.*, 2010).

The phenotypically most severe *Arabidopsis* PPP mutants are defective in single copy genes, such as *HYDROXYCINNA MOYL-COA SHIKIMATE/QUINATE HYDROXYCINNA MOYL TRANSFERASE (HCT)*, *CINNAMATE-4-HYDROXYLASE (C4H)* and *COUMARATE-3-HYDROXYLASE (C3H)* (Hoffmann *et al.*, 2004; Abdulrazzak *et al.*, 2006; Besseau *et al.*, 2007; Schilmiller *et al.*, 2009; Gallego-Giraldo *et al.*, 2011). A previously described *c4h* T-DNA mutant showed seedling lethality and early arrest of leaf expansion (Schilmiller *et al.*, 2009). By contrast, whereas knockdown mutants showing leaky expression of *C4H* also show a strongly decreased lignin content (*ref3-1*, *ref3-2* and *ref3-3*), they were able to develop into adult plants (Schilmiller *et al.*, 2009). This indicates that a complete block of the pathway is necessary to bring about seedling growth arrest. However, the underlying cause for the seedling-stage growth phenotypes blocking lignin production was never investigated. This makes the role for lignin in such an early stage of plant development still enigmatic, leaving open several possible unexplored functionalities.

Here, we investigated the early developmental defects caused by loss-of-function of *C4H*, which is the second enzyme active in the PPP, converting *trans*-cinnamic acid (*t*-CA) to *p*-coumaric acid (*p*CA) (Fig. 1a). Both *c4h* mutant seedlings (*c4h-4*) and seedlings treated with the *C4H* inhibitor piperonylic acid (PA) (Schalk *et al.*, 1998) showed a perturbed lateral rooting and an accumulation of adventitious roots specifically in the apical region of etiolated hypocotyls. We showed that these phenotypes can be attributed to a perturbation in AUX transport, likely caused by both the accumulation of the AUX transport inhibitor *cis*-cinnamic acid (*c*-CA) and a depletion of lignin. Our results thus show that two proposed models explaining growth perturbation in PPP mutants can underlie the same phenotype within one mutant. In addition, the depletion in lignin caused a perturbation in phloem-mediated AUX transport, presumably due to a disrupted xylem functionality. The restoration of lignin deposition in the *c4h-4* mutant and in PA-treated seedlings effectively restored both transport streams, resulting in a restoration of AUX distribution over the seedling. Our findings thus propose lignin deposition to be not only essential in providing mechanical strength to support large plant structures, but also necessary for the correct organization of seedling growth and architecture.

Materials and Methods

Plant material, transgenic lines, chemicals and growth conditions

Arabidopsis thaliana of the Col-0 ecotype was used for all assays unless stated otherwise. The following transgenic lines in the Col-0 ecotype were used: *coi1-21* (Kim *et al.*, 2013), *etr1-3* (Zipfel *et al.*, 2004), *tir1afb2afb3* (Dharmasiri *et al.*, 2005), *pDR5::LUC* (Moreno-Risueno *et al.*, 2010), *tt4* (Brown *et al.*, 2001). The CINNAMATE-4-HYDROXYLASE (*C4H*) *c4h-4* mutant (GK-753B06; (Kleinboelting *et al.*, 2012)) was obtained from the NASC institute. Seeds were vapour-phase sterilized and grown on ½ Murashige & Skoog (MS) medium (pH 5.7) containing 2.15 g MS basal salt mixture powder (Duchefa), 10 g sucrose, 0.5 g MES monohydrate, 8 g plant tissue culture agar per litre. The medium was supplemented with one of the following compounds: piperonylic acid (PA; Sigma Aldrich), naphthylphthalamic acid (NPA; Sigma Aldrich), coniferaldehyde (ConAld; Sigma Aldrich), SA (Sigma Aldrich), *p*-coumaric acid (*p*CA; Sigma Aldrich) and quercetin (Sigma Aldrich). These compounds were prepared as a stock solution in dimethyl sulfoxide (DMSO) and were added to the autoclaved medium before pouring the plates. Seeds were stratified via a 2-d cold treatment. For primary root analysis, the plates were incubated for a 14-d period in a vertical orientation in the tissue culture (TC) room under a 16 h : 8 h, light : dark photoperiod at 21°C. For the adventitious root (AR) assays, seeds were given a light pulse and then transferred for 7 d to darkness at 21°C. Subsequently the plates were transferred to the TC room for 7 d. For assessment of the involvement of *cis*-cinnamic acid, plants were grown in both the TC room and in a climate-controlled box containing LED lighting.

Phenotyping

Primary root characteristics were assessed by counting the lateral roots along the primary root using a stereomicroscope (CETI Binocular Zoom Stereo; Thermo Fisher Scientific, Waltham, MA, USA). The plates were scanned using an Expression 11000XL (Epson, Nagano, Japan) and root length was measured using IMAGEJ software. Lateral root density (LRD) was calculated by dividing the number of lateral roots for each plant by the respective primary root length. For assessment of adventitious root characteristics, the number of adventitious roots was counted separately for the top third and lower two-thirds along the hypocotyl using a stereomicroscope. The plates were scanned using an Epson Expression 11000XL, and hypocotyl length was measured using IMAGEJ. Statistics were performed using SAS ENTERPRISE GUIDE 8 and SAS 9.4 software (Supporting Information Methods S1).

pDR5::LUC assays

Plates containing ½MS medium were sprayed with 1 mM D-luciferin solution (Duchefa Biochemie, Haarlem, the Netherlands)

Fig. 1 Inhibition of C4H in *Arabidopsis thaliana* results in strong seedling phenotypes. (a) The first two steps of the general phenylpropanoid pathway, with the conversion of phenylalanine to cinnamic acid by PAL and subsequent conversion to *p*-coumaric acid by C4H. Alternatively, *trans*-cinnamic acid can isomerize to its *cis*-isomer under influence of UV. The known C4H-inhibitor PA is indicated on the figure in red. PAL, PHENYLALANINE AMMONIA LYASE; C4H, CINNAMATE-4-HYDROXYLASE; PA, piperonylic acid. (b) Phenotype of 4-wk-old Col-0 wild-type (WT), heterozygous *c4h*-mutant and homozygous *c4h*-mutant. An inset is made focusing on the homozygous *c4h*-mutant. Bar, 0.1 cm. (c, d) Average primary root length (c) and lateral root density (LRD) (d) of WT and *c4h-4* seedlings ($n > 20$). (e) Average number of adventitious roots (ARs) of WT and *c4h-4* etiolated seedlings ($n > 50$). (f) Phenotype of etiolated WT and *c4h-4* seedlings grown on 1/2 Murashige & Skoog (MS) medium. Bar, 0.5 cm. Yellow arrow, ARs located in the top third; blue arrow, ARs located at the bottom two-thirds. (g, h) PA dose-response curves for primary root length (g) and LRD (h) ($n > 20$). (i) PA dose-response graph for average number of ARs of etiolated seedlings ($n > 40$). (j) Phenotype of etiolated seedlings grown on 1/2 MS medium supplemented with or without 50 μM PA. Bar, 1 cm. Yellow arrow, ARs located in the top third; blue arrow, ARs located at the bottom two-thirds. An inset was made focusing on the adventitious roots formed upon PA treatment. Bar, 0.1 cm. Yellow coloration, top third of the hypocotyl; blue coloration, lower two-thirds. Error bars represent 95% confidence intervals. Asterisks indicate significant differences compared to the corresponding mock-treatment (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; (c) Student's *t*-test; (d, e, h, i) GEE model; (g) ANOVA, Dunnett's test).

and seedlings subsequently were transferred to these plates. The *pDR5::LUC* images were taken by a Lumazone machine carrying a CCD camera (Princeton Instruments, Trenton, NJ, USA). The CCD camera was controlled by WINVIEW/32 software imaging the luciferase (LUC) expression automatically every 10 min (exposure time, 10 min) for 12 h. The picture series were saved as TIFF format for further analysis and a Kymograph (http://www.embl.de/camnet/html/body_kymograph.html) was generated with IMAGEJ. For quantification of the LUC signal, pixel intensity was measured using IMAGEJ over the hypocotyl after 1 h of imaging. For each seedling, the signal intensity was normalized for the length of the hypocotyl.

Auxin transport assays

Col-0 seeds were sown *in vitro* on $\frac{1}{2}$ MS medium plates contain 50 μ M PA or the same amount of DMSO for 4 d in the darkness, upon which the etiolated seedlings were transferred to the respective light conditions for another 2 d. A positive control was performed using 10 μ M NPA. A droplet of 5 μ l ^{3} H-IAA (20 μ l ^{3} H-IAA added into 10 ml of $\frac{1}{2}$ MS medium with 1.25% agar) was applied to the apical part of the hypocotyls. After an incubation in darkness for 6 h, the roots were removed and hypocotyls were collected. Samples were homogenized in liquid nitrogen and incubated with 1 ml of Opti-Fluor scintillation cocktail (PerkinElmer, Waltham, MA, USA) overnight. The amount of transported ^{3} H-IAA then was measured in a scintillation counter (300SL; Hidex, Turku, Finland) for 300 s with three technical repetitions.

Vascular conductivity assays

Seedlings were given a 7-d dark treatment and then a 5-d light treatment. For phloem transport assays, one of the cotyledons of the etiolated seedlings was slightly damaged. The seedling was then placed on a plate containing $\frac{1}{2}$ MS medium with the top part of the hypocotyl being placed on parafilm, and a 1- μ l droplet of a 2 mM solution of 5(6)-carboxyfluorescein diacetate (CFDA; Sigma-Aldrich) was applied to the cotyledon. The seedlings then were scored for the presence of signal in the root-hypocotyl junction over a period of 90 min with a 10-min time interval. For xylem transport assays the hypocotyls of etiolated seedlings were excised via a cut slightly above the root-hypocotyl junction. The hypocotyl was placed on a plate containing $\frac{1}{2}$ MS medium with the bottom part of the hypocotyl being placed on parafilm. A 2- μ l droplet of a 2 mM solution of CFDA was applied to the bottom part of the hypocotyl. The seedlings then were scored for the presence of signal in the shoot apical meristem (SAM) over a period of 30 min with a 5-min time interval.

Wiesner staining

Whole etiolated seedlings were placed in cold acetone for 30 min. Seedlings then were placed in Wiesner reagent (3% phloroglucinol, two volumes 100% EtOH, one volume 37% HCl) for 5 min and mounted on slides containing chloral hydrate. Whole-

seedling imaging was performed using a VHX-5000 microscope (Keyence, Osaka, Japan). Detailed images of the vascular tissue were taken using an Olympus BX53 microscope.

Vascular anatomy

Plant material was fixated in 4% (m/v) paraformaldehyde, 1% (v/v) glutaraldehyde and 0.02 M sodium phosphate buffer (pH 7.2). The material was washed and dehydrated by subsequent incubation steps of 2 h in ethanol (EtOH) solutions with increasing concentrations (30%, 50%, 70%, 85%, 95%). Infiltration was performed according to Technovit 7100 (Kulzer, Hanau, Germany) manufacturer instructions. The two-step embedding was performed as described previously (De Smet *et al.*, 2004). Sections of 10 μ m were cut using a microtome (Reichert-Jung Supercut 2050; Leica Biosystems, Wetzlar, Germany) and placed on Superfrost® slides (Menzel Gläser; Thermo Fisher Scientific). The staining was done with 0.1% Toluidine Blue O (Sigma-Aldrich) for 10 min, and counterstaining with ruthenium red (Sigma), with washing steps between and after staining. DePeX Mounting Medium (VWR International, Radnor, PA, USA) treatment was performed and pictures were taken using a DIC light microscope (Leica BX51) with an industrial digital camera using TOUPVIEW 3.7 software.

Results

Blocking the phenylpropanoid pathway affects seedling development

In order to study the early growth phenotypes of *c4h* knock-out plants, we investigated the previously described GABIKAT T-DNA line (GK-753B06; *c4h-4*; Schilmiller *et al.*, 2009). In accordance with previous results the mutant showed seedling growth arrest (Fig. 1b), with a strongly perturbed leaf development. Several growth parameters were quantified by growing *c4h-4* seedlings *in vitro* on $\frac{1}{2}$ MS medium at 14 d after stratification (DAS). Compared to the wild-type (WT), mutant seedlings showed a reduction in primary root length and lateral root density (LRD; Fig. 1c,d), accompanied by an outgrowth of adventitious roots (ARs) at the root-hypocotyl junction.

In order to study the induction of the ARs in more detail, an AR-induction assay was implemented. This assay allows the analysis of the position of ARs along the length of the hypocotyl. Stratified WT and *c4h-4* seeds were sown on $\frac{1}{2}$ MS medium and given a light pulse before being stored in total darkness for 7 d. The light pulse triggers germination and storing plants in darkness allows for etiolation of the hypocotyl. The plates with etiolated seedlings subsequently were transferred to long-day growth conditions, which allows for AR outgrowth. After 7 d under long-day growth conditions adventitious rooting was assessed. *c4h-4* seedlings showed a significant increase in AR number (Fig. 1e,f) and, notably, the outgrowth of AR in the *c4h-4* seedlings was restricted almost primarily to the top third of the hypocotyl (Fig. 1e,f).

As a result of its early growth arrest, the analysis of the *c4h-4* required the use of a segregating line, making its implementation in the experiments impractical. Therefore, for follow-up studies we chose to use PA, which is a well-described C4H-inhibitor (Schalk *et al.*, 1998; Naseer *et al.*, 2012; Van de Wouwer *et al.*, 2016). Col-0 seeds were germinated on medium containing PA over a concentration range of 0–50 μ M. At 14 DAS a dose-dependent reduction of both primary root length and LRD (Fig. 1g,h) as well as an induction of ARs at the root–shoot junction were observed. These results are in line with the data obtained for the *c4h-4* mutant. Stratified Arabidopsis seeds then were etiolated on $\frac{1}{2}$ MS medium containing a concentration range of PA (0–100 μ M). Although to a lesser extent than the *c4h-4* mutant, seedlings treated with PA also showed a significant increase in AR number (Fig. 1i,j) in the apical region of the hypocotyl. Compared to the untreated control, the average number of ARs in the top third of the hypocotyl was significantly higher for all PA concentrations tested. By contrast, a slight increase of ARs in the lower section of the hypocotyl was observed only for seedlings treated with 25 μ M PA.

Overall, the data demonstrate that perturbation of C4H severely affects seedling development. This highlights the importance of a functional PPP during the early stages of plant growth, and validates its function in establishing early plant architecture.

Inhibition of C4H severely perturbs the PPP

C4H is active as the second enzyme in the PPP, and the entire flux through the pathway is shuttled via this step. Perturbing the function of C4H therefore is expected to have major consequences on the levels of PPP intermediates and end-products. To assess the effect of the inhibition of C4H on the metabolome of the plant, an LC-MS metabolite profiling of etiolated WT, *c4h-4* and PA-treated seedlings was performed (Table 1; Methods S2). Both *c4h-4* and PA-treated seedlings showed an accumulation of compounds upstream of C4H (i.e. phenylalanine and products of cinnamic acid catabolism) and a depletion of products downstream of C4H (e.g. flavonoids and products leading to lignin building blocks). The results underline the pivotal role that C4H plays in the PPP, as exemplified by the severe perturbations in metabolite levels upon C4H inhibition, and confirm C4H functionality to be strongly disrupted in both the *c4h-4* mutant and upon PA treatment. In addition, the shared phenotypes of the *c4h-4* mutant and PA-treated seedlings are reflected in the PCA plot of the metabolic profiles, with the *c4h-4* mutant and PA-treated seedlings clustering together (Fig. 2). The slight disparity in clustering of the *c4h-4* mutant and PA could be explained by the differences in degree of pathway perturbation and the presence of PA and its catabolism products (Table 1).

Together, these results confirm the pivotal role of C4H in secondary plant metabolism and prove the adequacy of PA-treatment as a substitute for the *c4h-4* mutation. The observed depletion in lignin building blocks is in line with previous observations in weaker *c4h* mutants (Schilmiller *et al.*, 2009). The depletion in lignin, however, is difficult to reconcile with the observed developmental defects, including the specific apical

induction of ARs. The latter has been observed only rarely, but was previously linked to phytohormonal interplay (Rasmussen *et al.*, 2017). Therefore, we further investigated the role of phytohormones in AR-induction upon inhibition of C4H.

Inhibition of C4H perturbs AUX transport

The induction and outgrowth of ARs are complex processes regulated by phytohormones, among which are JA, ETH and AUX (Gutierrez *et al.*, 2012; Verstraeten *et al.*, 2014; Steffens & Rasmussen, 2016; Lakehal & Bellini, 2019; Lakehal *et al.*, 2019; Alallaq *et al.*, 2020; Lakehal *et al.*, 2020). The potential involvement of these phytohormones in AR spacing of *c4h* mutants was investigated using corresponding signalling mutants, namely *coi1-21* (JA), *etr1-3* (ETH) and *tir1afb2afb3* (AUX). As PA-treated WT plants were shown to be an adequate substitute for the *c4h-4* mutant, AR growth was assessed for all signalling mutant lines upon treatment with and without 50 μ M PA. Of the different lines tested, only the *tir1afb2afb3* mutant showed no increase in ARs upon PA treatment (Fig. 3a). Additionally, the distribution of ARs along the hypocotyl upon PA treatment was unaffected in this mutant. These data show that PA-mediated AR-proliferation depends on the canonical AUX signalling pathway.

In order to visualize the effect of PA on AUX distribution in the hypocotyl, seeds of the AUX reporter line *pDR5::LUC* were etiolated on medium supplemented with 50 μ M PA. The LUC activity then was followed in the hypocotyl by imaging the plants over a 12-h time period, upon which a kymograph was generated (Fig. 3b; Xuan *et al.*, 2015). The kymograph revealed a strong apical accumulation of signal upon PA-treatment. Additionally, the signal intensity was lower in the basal parts of the hypocotyl compared to mock-treated hypocotyls. To obtain quantitative evidence, the *pDR5::LUC* signal was measured at the 1-h mark over the hypocotyl for 50 hypocotyls treated with/without 50 μ M PA. Upon PA treatment, a strong apical accumulation of signal was observed in the apical third of the hypocotyl that dropped to near zero for the basal two-thirds (Fig. 3c). This signal distribution contrasted with the mock-treated plants, which showed a steady decline in signal from the top to the bottom of the hypocotyl. These results indicate that inhibition of C4H perturbs AUX homeostasis, most likely by affecting AUX transport.

It could be hypothesized that PA itself activates the AUX signalling cascade in the reporter lines by binding to an AUX receptor. After all, PA and IAA are similar in size (166 and 175 Da, respectively) and share a planar aromatic skeleton decorated with a carboxylic acid characteristic for AUXs (Veldstra, 1953; Fig. 3d). However, despite the similarities, the carbon skeletons differ, with PA being a benzodioxane and IAA an indole, and the length of the side chains is different. In line with these structural dissimilarities, molecular docking of PA in the binding pocket of the AUX receptor TIR1 showed it to adopt a pose distinctly out of alignment with the pose of IAA (Fig. 3e; Methods S3), making it unlikely that PA itself can activate AUX signalling. Evidence confirming this finding was obtained via Surface Plasmon Resonance (SPR), where interaction kinetics of PA with either TIR1

Table 1 Inhibition of CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis thaliana* perturbs the phenylpropanoid pathway (PPP).

No.	RT	<i>m/z</i>	Name	Wild-type (WT)	<i>c4h-4</i> mutant	Fold change <i>c4h-4</i> - WT	PA	Fold Change PA - WT
Phenylalanine and cinnamate derivatives								
1	2.19	164.0715	Phenylalanine	20.25 ± 4.25	117.39 ± 28.03	5.80	48.97 ± 11.24	2.42
2	9.69	355.1028	Cinnamoyl hexose (formic acid adduct)	0.61 ± 0.72	2215.55 ± 598.94	>100	1850.64 ± 604.39	>100
3	8.62	355.1028	Cinnamoyl hexose (formic acid adduct)	0.49 ± 0.36	1125.18 ± 370.58	>100	1179.51 ± 441.18	>100
4	12.46	263.0550	Cinnamoyl malate	0.00 ± 0.00	40.44 ± 12.27	>100	26.32 ± 7.96	>100
Flavonoids								
5	6.93	623.1592	Isorhamnetin glucoside rhamnoside 1	60.07 ± 19.24	0.00 ± 0.00	>100	1.45 ± 1.03	41.48
6	8.50	623.1603	Isorhamnetin glucoside rhamnoside 2	1822.91 ± 532.53	0.44 ± 0.49	>100	128.30 ± 97.33	14.21
7	5.49	609.1447	Kaempferol dihexoside	613.37 ± 139.24	0.00 ± 0.00	>100	16.63 ± 10.47	36.88
8	5.54	755.1994	Kaempferol dihexoside rhamnoside 1	299.57 ± 81.86	0.03 ± 0.10	>100	26.81 ± 25.24	11.17
9	6.99	755.2016	Kaempferol dihexoside rhamnoside 2	875.62 ± 217.82	0.00 ± 0.00	>100	9.07 ± 6.27	96.54
10	6.23	593.1481	Kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-D-glucopyranoside	55.62 ± 10.28	0.00 ± 0.00	>100	2.91 ± 3.93	19.11
11	8.08	739.2069	kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-D-glucopyranoside-7-O-a-L-rhamnopyranoside 1	1436.33 ± 380.07	0.00 ± 0.00	>100	18.44 ± 23.38	77.90
12	6.22	739.2070	Kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-D-glucopyranoside-7-O-a-L-rhamnopyranoside 2	5768.48 ± 1279.59	8.53 ± 7.94	>100	486.21 ± 379.86	11.86
13	8.26	593.1498	Kaempferol-rutinoside (hex + rha)	1389.46 ± 369.87	2.42 ± 2.68	>100	616.29 ± 626.08	2.25
Ferulic acid derivatives								
14	7.24	551.1752	4/7/9-O-hexoside G(8-O-4) ferulic acid 1	130.59 ± 31.32	0.00 ± 0.00	>100	10.08 ± 3.35	12.95
15	7.58	551.1756	4/7/9-O-hexoside G(8-O-4) ferulic acid 2	161.06 ± 24.72	0.00 ± 0.00	>100	9.71 ± 3.54	16.59
16	5.94	551.1753	4/7/9-O-hexoside G(8-O-4) ferulic acid 3	210.85 ± 48.69	0.02 ± 0.05	>100	7.40 ± 2.52	28.49
17	6.98	551.1753	4/7/9-O-hexoside G(8-O-4) ferulic acid 4	390.72 ± 84.75	0.09 ± 0.11	>100	38.14 ± 11.76	10.24
18	6.70	551.1757	4/7/9-O-hexoside G(8-O-4) ferulic acid 5	1053.87 ± 177.17	0.00 ± 0.00	>100	83.44 ± 26.10	12.63
19	6.43	551.1759	4/7/9-O-hexoside G(8-O-4) ferulic acid 6	4063.76 ± 746.22	1.60 ± 1.90	>100	303.70 ± 82.14	13.38
24	4.07	371.0971	5-hydroxyferulic acid + hexose 1	70.56 ± 15.11	0.01 ± 0.02	>100	5.83 ± 2.28	12.10
25	3.60	371.0976	5-hydroxyferulic acid + hexose 2	375.78 ± 124.87	0.01 ± 0.04	>100	15.27 ± 7.95	24.61
26	5.96	339.1107	Coniferaldehyde 4-O-hexoside	68.02 ± 20.82	3.07 ± 2.25	22.12	12.47 ± 7.74	5.45
27	3.84	387.1289	Coniferin (formic acid adduct)	1932.65 ± 718.88	4.31 ± 2.75	>100	136.05 ± 68.97	14.21
28	3.20	357.1172	Dihydroferulic acid + hexose	43.24 ± 12.28	0.01 ± 0.01	>100	0.02 ± 0.07	>100
29	11.34	535.1445	Dihydroferuloyl-beta-keto acid + hexose + 136 Da	109.93 ± 31.85	0.00 ± 0.00	>100	26.80 ± 7.77	4.10
30	8.44	753.2220	Disinapoyl hexose + hexose	134.27 ± 37.61	0.02 ± 0.05	>100	66.57 ± 59.17	2.02
31	3.72	355.1025	Ferulic acid 4-O-hexoside 1	323.49 ± 55.67	0.12 ± 0.20	>100	20.92 ± 7.90	15.46
32	5.35	355.1029	Ferulic acid 4-O-hexoside 2	1539.51 ± 379.00	1.07 ± 1.05	>100	48.18 ± 21.33	31.95
33	5.44	355.1029	Ferulic acid 4-O-hexoside 3	2490.13 ± 485.84	19.86 ± 5.71	>100	253.94 ± 77.65	9.81
34	5.86	355.1027	Feruloyl hexose	646.73 ± 146.47	0.08 ± 0.10	>100	10.75 ± 6.05	60.16
35	6.21	581.1845	Feruloyl hexose + 226 Da 1	118.84 ± 32.12	0.25 ± 0.30	>100	12.92 ± 4.92	9.20

Table 1 (Continued)

No.	RT	<i>m/z</i>	Name	Wild-type (WT)	<i>c4h-4</i> mutant	Fold change <i>c4h-4</i> - WT	PA	Fold Change PA - WT
36	8.24	581.1856	Feruloyl hexose + 226 Da 2	382.95 ± 93.27	0.00 ± 0.00	>100	21.58 ± 7.87	17.75
37	2.61	563.1600	Feruloyl hexose 4-O-hexoside (formic acid adduct)	108.31 ± 30.85	0.19 ± 0.24	>100	13.17 ± 5.97	8.23
38	4.53	325.0919	Feruloyl pentose 1	264.37 ± 112.58	45.61 ± 16.79	5.80	32.40 ± 12.72	8.16
39	5.10	325.0921	Feruloyl pentose 2	456.51 ± 163.43	3.59 ± 1.77	>100	18.61 ± 8.00	24.54
40	5.92	583.2011	G 4-O-hexoside(8-O-4)G (formic acid adduct)	248.86 ± 94.48	0.00 ± 0.00	>100	0.08 ± 0.12	>100
41	10.13	533.1648	G(8-5)feruloyl hexose	264.64 ± 30.42	0.43 ± 0.44	>100	11.12 ± 4.03	23.80
42	9.20	519.1856	G(8-5)G hexoside	199.61 ± 62.44	0.00 ± 0.00	>100	0.40 ± 0.47	>100
43	8.19	469.0800	G(8-O-4)ferulic acid sulfate	841.14 ± 152.38	1.28 ± 2.49	>100	165.15 ± 91.89	5.09
44	10.32	389.1229	G(8-O-4)ferulic acid	105.31 ± 23.63	0.01 ± 0.02	>100	17.53 ± 4.92	6.01
45	10.25	505.1336	G(8-O-4)ferulic acid + malate	95.31 ± 22.14	0.24 ± 0.27	>100	15.27 ± 4.58	6.24
46	8.24	521.2011	G(red8-5)G + hexose 1	163.90 ± 28.32	0.00 ± 0.00	>100	0.60 ± 0.71	>100
47	9.08	521.2011	G(red8-5)G + hexose 2	715.41 ± 68.49	0.01 ± 0.03	>100	12.73 ± 7.75	56.21
49	5.54	385.1136	Sinapic acid 4-O-hexoside 1	18148.45 ± 3185.04	276.41 ± 117.33	65.66	4779.87 ± 2163.07	3.80
50	6.10	385.1134	Sinapic acid 4-O-hexoside 2	6030.09 ± 1234.35	74.89 ± 33.67	80.52	1623.39 ± 629.03	3.71
51	5.00	385.1129	Sinapic acid 4-O-hexoside 3	161.43 ± 37.22	0.02 ± 0.05	>100	63.81 ± 23.86	2.53
52	4.39	385.1132	Sinapic acid 4-O-hexoside 4	762.84 ± 139.35	5.45 ± 4.03	>100	304.76 ± 99.96	2.50
53	6.67	352.1029	Sinapoyl glutamate 1	175.36 ± 48.08	1.79 ± 1.15	98.19	70.07 ± 26.78	2.50
54	6.03	352.1029	Sinapoyl glutamate 2	248.59 ± 69.12	3.78 ± 2.11	65.72	101.05 ± 35.24	2.46
55	9.31	339.0715	Sinapoyl malate 1	2921.54 ± 735.01	48.09 ± 25.75	60.76	1930.41 ± 595.57	1.51
56	9.01	339.0714	Sinapoyl malate 2 PA derivatives	3618.53 ± 554.83	67.86 ± 36.76	53.33	2349.53 ± 649.93	1.54
57	9.52	165.0190	Piperonylic acid	0.00 ± 0.00	0.04 ± 0.10	0.00	298.39 ± 82.70	>100
58	5.80	373.0769	Piperonylic acid hexose (formic acid adduct)	0.90 ± 1.06	8.14 ± 19.46	9.05	5954.44 ± 2250.56	>100

List of compounds were characterized upon LC-MS profiling of etiolated Col-0 WT, *c4h-4* and Col-0 seedlings treated with 50 μ M PA ($n > 7$). Each datum represents eight biological replicates. For each metabolite, a unique number (No.), mass-to-charge ratio (*m/z*), retention time (RT), peak area $\pm$ SD and fold-change compared to the WT are given.

or the related AUX receptor AFB5 were followed. Hereby, no evidence was found that PA could bind either the TIR1 or AFB5 receptor, showing that it is neither an agonist nor an anti-AUX (Fig. 3f).

Fig. 2 Piperonylic acid (PA)-treatment serves as an adequate substitute for the *c4h-4* mutant in *Arabidopsis thaliana* (C4H, CINNAMATE-4-HYDROXYLASE). Principal component analysis score plots for the metabolic profiles obtained by LC-MS of etiolated Col-0 wild-type (WT), *c4h-4* and 50 μ M PA-treated WT seedlings ($n > 7$). Each datapoint represents eight biological replicates.

Next we quantified AUX transport in etiolated seedlings grown on medium supplemented with/without 50 μ M PA. Treatment with the AUX transport inhibitor NPA was included as a positive control. This experiment revealed that the AUX transport capacity measured upon treatment with both PA and NPA was significantly reduced when compared to that of mock-treated plants (Fig. 3g). Together, these results indicate that inhibition of C4H affects AUX homeostasis in the hypocotyl via the inhibition of AUX transport.

Auxin transport inhibition upon blocking C4H is caused partially by *c*-CA accumulation

The perturbation of AUX transport upon inhibition of C4H could find its origin in the accumulation of bioactive molecules derived from compounds upstream of C4H. Interestingly, two such molecules have been described as having AUX transport regulatory properties: *cis*-cinnamic acid (*c*-CA), an AUX transport inhibitor Steenackers *et al.*, 2019), and SA, an AUX transport modulator (Tan *et al.*, 2020).

c-CA is the UV-mediated isomerization product of the C4H substrate *trans*-cinnamic acid (*t*-CA) (Wong *et al.*, 2005; Steenackers *et al.*, 2019). Our metabolomic profiling upon PA treatment showed the accumulation of conjugation products of cinnamic acid (Table 1; Methods S4), suggesting that cinnamic

Fig. 3 Inhibition of CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis thaliana* results in AUX transport inhibition. (a) Average number of adventitious roots (ARs) of etiolated Col-0 wild-type (WT), the jasmonate signalling mutant *coi1-21*, the ethylene signalling mutant *etr1-3* and the AUX signalling mutant *tir1afb2afb3* grown on $\frac{1}{2}$ MS medium supplemented with (+) or without (–) 50 μ M PA ($n > 20$). Yellow coloration, top third of the hypocotyl; blue coloration, lower two-thirds. (b) Kymographs of *pDR5::LUC* intensity along the hypocotyl of etiolated seedlings during a 12 h period (LUC, luciferase). Seedlings were etiolated and grown on plates with/without 50 μ M PA ($n > 50$). (c) Graph showing normalized intensity of the *pDR5::LUC* signal over the hypocotyl of etiolated *Arabidopsis* seedlings grown on plates with or without 50 μ M PA ($n > 50$). SAM, shoot apical meristem. (d) Chemical structures of indole-3-acetic acid (IAA) and piperonylic acid (PA). (e) Docking results in the best possible position for IAA and PA in the lower region of the TIR1 pocket. The bottom shows the superimposed structures of PA and IAA from two perspectives (f) Surface Plasmon Resonance analysis of AUX-dependent interaction of TIR1 and AFB5 with Aux/IAA DII. Each sensorgram shows the binding with 50 μ M IAA (blue), an AUX-free injection (red) and the data for 50 μ M PA (green). Lower panels show the sensorgrams from the anti-AUX assay with 5 μ M IAA (blue dashed line) or 5 μ M IAA plus 50 μ M PA (green). (g) Auxin transport capacity of mock, PA and naphthylphthalamic acid (NPA)-treated seedlings ($n = 9$). DPM, disintegrations per min. Error bars represent 95% confidence intervals. Asterisks indicate significant differences compared to the corresponding mock-treatment (***, $P < 0.0001$; (a) GEE model, (g) ANOVA, Dunnett's test).

acid itself accumulates upon inhibition of C4H. Moreover, because all experiments had been conducted under growth conditions incorporating UV radiation, the accumulation of ϵ -CA might explain the observed phenotypes. According to this hypothesis, growing PA-treated plants under UV-free light should reduce the ϵ -CA content and attenuate the growth phenotypes. Despite the assumed requirement of UV to form ϵ -CA, basal concentrations of both CA isomers were detected in mock-treated seedlings grown under both light with (UV-light) and without UV. Treatment with PA significantly increased ϵ -CA concentrations for both light conditions (Fig. 4a), but ϵ -CA concentrations were increased only under UV-light (Fig. 4b). Thus,

besides the indication for an as of yet unknown UV-independent mechanism for plants to produce ϵ -CA, the data revealed that UV increases ϵ -CA concentrations in PA-treated seedlings.

If ϵ -CA is indeed the causal agent for the PA-triggered perturbation of AUX transport, avoiding ϵ -CA accumulation by growing PA-treated seedlings under light without UV should restore AUX transport. To investigate this, AUX transport was quantified for plants grown under light with and without UV (Fig. 4c). In mock-treated plants AUX transport capacity was not significantly different for both light conditions. PA, on the other hand, significantly decreased AUX transport capacity for both light treatments, although not to the same extent. Auxin transport was

significantly more inhibited for PA-treated plants grown under UV-light as compared to plants grown under light without UV. The dependency of PA on UV-light to inhibit AUX transport is in line with the proposed model indicating the involvement of *c*-CA as an AUX transport inhibitor. However, the mild but significant inhibition of AUX transport upon PA-treatment under UV-free conditions was unexpected as *c*-CA concentrations were not increased in these plants. These data suggest that, in addition to *c*-CA, other players are involved in the AUX transport inhibition upon blocking of C4H.

In order to determine whether the light-dependent variations in AUX transport translate to phenotypic changes, AR growth was assessed under both light conditions (Fig. 4d). Changing light conditions did not affect the total number of ARs formed upon PA treatment over the entire hypocotyl. However, the distribution of AR over the hypocotyl was altered, as UV-grown plants showed a significantly higher increase in ARs in the top part of the hypocotyl upon PA treatment. This indicates that *c*-CA has a partial involvement in the outgrowth of ARs in the top part of the hypocotyl upon PA-treatment. These results are in line with the AUX transport measurements, showing an effect of PA that is more pronounced in plants grown under UV-light. However, the lack of a full complementation of the PA-phenotype upon preventing *c*-CA accumulation further supports the hypothesis that besides *c*-CA other players are involved in the PA-triggered AR phenotypes.

Salicylic acid, which also is produced upstream of C4H, also has been described as an AUX transport modulator (Zhao *et al.*, 2015; Tan *et al.*, 2020). To test the potential involvement of SA in the PA-mediated perturbation of AUX homeostasis, we assessed AR growth for the *NahG-OE* Arabidopsis line (Gaffney *et al.*, 1993) upon treatment with and without 50 μ M PA. The *NahG-OE* line encodes a salicylate hydroxylase that catabolizes SA, thus inactivating it. Upon PA-treatment, *NahG-OE* plants showed an accumulation of AR in the top third similar to that of the WT (Fig. 4e), refuting the involvement of SA in affecting AUX homeostasis upon PA-treatment.

c-CA accumulation and a depletion in monolignols: a dual mechanism at play

Besides accumulation of upstream compounds, the inhibition of C4H causes a depletion of products downstream of C4H (Table 1), which also could explain part of the phenotypes. To identify whether the depletion of a compound downstream of C4H is responsible for the observed inhibition of AUX transport in PA-treated seedlings, a cotreatment was performed with 50 μ M PA and 200 μ M *p*CA, which is the product of C4H. The cotreatment did not lead to a decrease in total AR formation (Fig. 4f). Interestingly however, there was a significant drop in ARs in the apical third of the hypocotyl compared to plants treated with PA only (Fig. 4f), suggesting that a player downstream of C4H is involved.

One group of molecules downstream of C4H known to modulate AUX transport are the flavonoids, among which quercetin is one of the most well-studied (Peer & Murphy, 2007). Our

previous metabolomic profiling of PA-treated etiolated seedlings showed a strong depletion in flavonoid content. To assess whether a depletion of quercetin could account for the AR phenotype a cotreatment of 50 μ M PA with 100 μ M quercetin was performed. Cotreatment with quercetin did not result in complementation of AR growth or distribution (Fig. 4g), suggesting that it is not involved. As quercetin is not the only flavonoid known to modulate AUX transport, a *tt4* mutant deficient in the production of flavonoids (Brown *et al.*, 2001) was analyzed alongside a WT control. The *tt4* mutant did not show a significant increase in ARs in the top third of the hypocotyl (Fig. 4h), suggesting that flavonoids are not the downstream factor involved in the growth defects upon inhibition of C4H.

As the majority of the carbon entering the phenylpropanoid pathway is incorporated into lignin, blocking the pathway has a strong negative effect on the lignin content (Van de Wouwer *et al.*, 2016). To examine whether a depletion of lignin is causal to the phenotypes, we repeated the complementation assays with 100 μ M ConAld, which was chosen for this purpose based on its stability under light and it being a precursor to both the S- and G-units that constitute the majority of the lignin polymer (Boerjan *et al.*, 2003). In accordance to the cotreatment with *p*CA, cotreatment with ConAld partially restored the number and distribution of ARs in PA-treated etiolated seedlings (Fig. 4i). These results strongly indicate that a depletion of monolignols is involved in the phenotypes induced upon inhibition of C4H.

Both cotreatment with PA and ConAld as well as growing PA-treated seedlings under UV-free light partially complemented the adventitious rooting phenotype. It therefore was assessed whether the treatments act additively. For this purpose, seedlings were etiolated on medium with or without 50 μ M PA and 100 μ M ConAld and grown under light with or without UV (Fig. 4j). Upon cotreatment with ConAld under UV-free light conditions, PA did not significantly change the number nor the distribution of ARs. Together these results show that a dual mechanism is at play for the establishment of the AR phenotypes upon inhibition of C4H, with both an accumulation of *c*-CA and a depletion in monolignols involved.

Lignin depletion perturbs long-distance AUX transport

The depletion of monolignols was shown to explain part of the developmental defects upon C4H inhibition, suggesting that lignin is crucial for proper seedling development. We therefore assessed whether the phenotypic restoration upon cotreatment with ConAld corresponded to a restoration in AUX distribution. For this purpose, seeds of the AUX reporter line *pDR5::LUC* were etiolated on medium supplemented with or without 50 μ M PA and 100 μ M ConAld. The intensity of the *pDR5::LUC* signal then was measured over the hypocotyl. Mock-treated plants showed a steady decline in signal from the top to the bottom of the hypocotyl, and upon PA treatment a strong apical accumulation of signal again was observed in the apical third of the hypocotyl (Fig. 5a). Cotreatment of PA with ConAld significantly decreased the apical accumulation of signal, hereby partially complementing the perturbation in AUX distribution. These results thus are in line

with the phenotypic data, and signify that lignin deposition is vital for the correct distribution of AUX over the plant.

In order to further assess the involvement of lignin in the observed phenotypes, Arabidopsis seedlings were etiolated on medium with or without 50 μM PA and 100 μM ConAld.

Lignin distribution was visualized by Wiesner staining. Although seedlings treated with PA showed a strongly reduced staining in the vascular tissue, staining in this region was partially complemented for upon cotreatment with ConAld (Fig. 5b). Hypocotyl cross-sections were stained with toluidine blue to visualize lignin

Fig. 4 Auxin transport inhibition upon blocking CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis thaliana* is caused by both an accumulation of *cis*-cinnamic acid (*c*-CA) and a depletion in monolignols. (a, b) Profiling of *trans*-cinnamic acid (*t*-CA) (a) and *c*-CA (b) concentrations of etiolated seedlings grown on ½ MS medium supplemented with or without 50 µM piperonylic acid (PA) and UV ($n = 5$). Values on the y-axis indicate the absolute amount of *t*-CA (a) or *c*-CA (b) per mg DW. (c) Auxin transport capacity of etiolated seedlings treated with or without 50 µM PA and UV ($n = 9$). (d–j) Average number of ARs of etiolated (d) Col-0 grown on ½ MS medium supplemented with or without 50 µM PA and UV ($n > 60$). (e) Col-0 and *NahG*-OE, deficient in SA, supplemented with or without 50 µM PA ($n > 100$). (f, g) Col-0 grown on ½ MS medium supplemented with 50 µM PA and either 200 µM *p*-coumaric acid (*p*CA) (f) or 100 µM quercetin (g) ($n > 40$). (h) Col-0 and the flavonoid mutant *tt4* ($n > 100$). (i) Col-0 grown on ½ MS medium supplemented with or without 50 µM PA and 100 µM coniferaldehyde (ConAld) ($n > 50$). (j) Col-0 grown on ½ MS medium supplemented with or without 50 µM PA, 100 µM ConAld and UV ($n > 50$). Yellow coloration, top third of the hypocotyl; blue coloration, lower two-thirds. Error bars represent 95% confidence intervals. Letters a–d are given to distinguish statistically significant results ($P < 0.01$; (a–c) ANOVA, Tukey's honestly significant difference test; (d–j) GEE model).

and counterstained with ruthenium red to visualize the plant cell walls. This revealed a reduced blue staining in the xylem vessels upon PA treatment, which pointed towards a reduced lignin content in these cells (Fig. 5c). In line with the Wiesner staining, the reduced lignin content was partially complemented upon cotreatment with ConAld.

In order to correlate these observations to xylem functionality, we assessed xylem transport capacity in the hypocotyl using CFDA (Melnyk *et al.*, 2015). *Arabidopsis* seedlings were etiolated on medium with or without 50 µM PA and 100 µM ConAld. The hypocotyl was cut above the root–hypocotyl junction and a droplet of CFDA was applied at the base of the hypocotyl. The presence of the fluorescent signal was then monitored over time in the SAM. Although fluorescence observed in the SAM rapidly increased for mock-treated hypocotyls (Fig. 5d), this occurred at a much later time point for the PA-treated seedlings. Additionally, only a fraction of the PA-treated hypocotyls showed fluorescence in the SAM after 30 min. Xylem transport capacity was fully restored upon cotreatment of PA with ConAld, showing that the partial complementation of lignin in the xylem observed previously is sufficient for a restoration of xylem functionality.

The perturbed xylem functionality could explain the AUX transport inhibition via the Münch model (Munch, 1930; Knoblauch *et al.*, 2016). Long-distance AUX transport is known to occur via the phloem, and the Münch model proposes phloem transport to be driven by a pressure gradient generated at the top of the plant. This gradient is established by the upward transport of water via the xylem, meaning that a defect in xylem transport could lead to a perturbed phloem and therefore AUX transport. To test this hypothesis, phloem transport capacity was assessed in etiolated seedlings using CFDA. *Arabidopsis* seedlings were etiolated on medium with or without 50 µM PA and 100 µM ConAld. A droplet of CFDA was applied to the cotyledons, upon which the fluorescent signal was monitored over time in the root–hypocotyl junction (Fig. 5e). In contrast with the mock-treated hypocotyls, only 1% of the PA-treated seedlings showed fluorescence in the root–shoot junction at the final time point. Analogous to the xylem transport, cotreatment of PA with ConAld complemented the phloem transport capacity, showing that the adequate deposition of lignin is required for proper phloem transport.

Confirmation of results in the *c4h-4* mutant

In order to obtain conclusive evidence that the inhibition of C4H leads to the inhibition of long-distance AUX transport, key

experiments were repeated using the heterozygous *c4h-4* mutant. AR growth was assessed for WT and the segregating *c4h-4* mutant seeds upon treatment with or without 100 µM ConAld. As expected, treatment of the segregating *c4h-4* mutant population with 100 µM ConAld resulted in substantial phenotypic complementation, which encumbered identification of the homozygous *c4h-4* seedlings. The seedlings therefore were genotyped to identify the homozygous mutants. Analogous to PA-treated seedlings, etiolated homozygous *c4h-4* mutant plants treated with ConAld showed a significant complementation of AR-growth in the top third of the hypocotyl (Fig. 6a). These results thus confirm the inhibition of C4H and subsequent depletion in monolignols to be causal to the increased AR induction and their specific apical spacing, hereby again showing the requirement of lignin deposition in the establishment of AUX distribution in seedlings.

In order to confirm the involvement of the vascular tissue and, thus, long-distance AUX transport in the establishment of these phenotypes, we repeated the xylem and phloem transport assays using the *c4h-4* mutant. Seeds from the Col-0 WT and a segregating seedstock of the *c4h-4* mutant were etiolated on medium supplemented with or without 100 µM ConAld. Xylem transport was assessed as described above using the fluorescent tracker CFDA. Homozygous *c4h-4* mutants were identified afterwards by genotyping. Similar to PA treatment, the *c4h-4* mutant showed a severely perturbed xylem functionality (Fig. 6b). Treatment of the *c4h-4* mutant with ConAld significantly restored xylem transport, although transport velocity appeared slower than for the Col-0 WT. Phloem transport was assessed as described above for etiolated Col-0 WT and the segregating *c4h-4* mutant treated with or without 100 µM ConAld. Homozygous *c4h-4* mutants were afterwards identified by genotyping. Again, results were analogous to those obtained for PA-treated seedlings, with phloem transport being almost entirely perturbed in the *c4h-4* mutant (Fig. 6c) and treatment with ConAld completely restoring phloem transport in the mutant.

Together, these results indicate that a two-fold mechanism comprising both a depletion in lignin and an accumulation of *c*-CA lie at the basis of AUX transport inhibition upon blocking C4H (Fig. 7). As such, the requirement of a functional PPP and lignin deposition in the establishment of AUX transport and homeostasis in plants is illustrated. Hereby, we demonstrate the role of the PPP in the determination of plant development and architecture.

Fig. 5 Inhibition of CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis thaliana* perturbs long-distance AUX transport through the phloem due to a depletion in lignin. (a) Graph showing normalized intensity of the *pDR5::LUC* signal of etiolated *Arabidopsis* seedlings grown on plates with or without 50 μM piperonyl acid (PA) and 100 μM coniferaldehyde (ConAld) ($n > 30$). These were then transferred to $\frac{1}{2}$ MS medium and treated for 1 h with luciferin, upon which the signal intensity was measured and normalized for the length of the hypocotyl. (b) Wiesner staining of etiolated seedlings grown on $\frac{1}{2}$ MS medium supplemented with or without 50 μM PA and 100 μM ConAld. For each treatment, the entire hypocotyl is shown on the left (bar, 1 mm) and a magnification on the right (bar, 10 μm). (c) Transverse sections stained with toluidine blue and counterstained with ruthenium red of etiolated seedlings grown on $\frac{1}{2}$ MS medium supplemented with or without 50 μM PA and 100 μM ConAld. Pictures are representative of > 25 hypocotyls. Bar, 10 μm. (d) Xylem transport assays using the fluorescent probe 5(6)-carboxyfluorescein diacetate (CFDA). Etiolated seedlings were grown on $\frac{1}{2}$ MS medium supplemented with or without 50 μM PA and 100 μM ConAld, and the hypocotyl was excised directly above the hypocotyl–root junction. CFDA was administered to the bottom of the hypocotyl and fluorescence was assessed over time in the shoot apical meristem (SAM) ($n > 100$). (e) Phloem transport assays using the fluorescent probe CFDA. Etiolated seedlings were grown on $\frac{1}{2}$ MS medium supplemented with or without 50 μM PA and 100 μM ConAld before CFDA was administered to the cotyledons. Fluorescence then was assessed over time in the hypocotyl–root junction ($n > 100$). Error bars represent 95% confidence intervals.

Fig. 6 Confirmation of results in the *Arabidopsis thaliana* CINNAMATE-4-HYDROXYLASE (C4H) *c4h-4* mutant. (a) Average number of adventitious roots (ARs) of etiolated Col-0 wild-type (WT) and *c4h-4* seedlings grown on $\frac{1}{2}$ MS medium supplemented with or without 100 μ M coniferaldehyde (ConAld) ($n > 50$). Yellow coloration, top third of the hypocotyl; blue coloration, lower two-thirds. Error bars represent 95% confidence intervals. Letters a–d are given to distinguish statistically significant results for top third and a'–d' for bottom two thirds ($P < 0.01$; GEE model). (b) Xylem transport assays using the fluorescent probe 5(6)-carboxyfluorescein diacetate (CFDA). Etiolated Col-0 WT and *c4h-4* seedlings were grown on $\frac{1}{2}$ MS medium supplemented with or without 100 μ M ConAld and the hypocotyl was excised directly above the hypocotyl–root junction. CFDA was administered to the bottom of the hypocotyl and fluorescence was assessed over time in the shoot apical meristem (SAM) ($n > 20$). (c) Phloem transport assays using the fluorescent probe CFDA. Etiolated Col-0 WT and *c4h-4* seedlings were grown on $\frac{1}{2}$ MS medium supplemented with or without 100 μ M ConAld before CFDA was administered to the cotyledons. Fluorescence then was assessed over time in the hypocotyl–root junction ($n > 30$). Error bars represent 95% confidence intervals.

Discussion

Inhibiting the function of CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis* seedlings either in the *c4h-4* mutant or upon piperonylic acid (PA) treatment resulted in severe and distinct phenotypes, with an impeded lateral root development and a strong accumulation of adventitious roots specifically in the apical part of the hypocotyl. These phenotypes were found to be caused by a perturbation in AUX transport, resulting in the accumulation of AUX apically in the hypocotyl. The underlying cause of the perturbation in AUX homeostasis was found to be two-fold: a downstream depletion in lignin and, presumably, an

upstream accumulation of the endogenous AUX transport inhibitor *cis*-cinnamic acid (*c*-CA).

The *c*-CA previously was presumed to be produced via isomerization of *trans*-cinnamic acid (*t*-CA) under exposure to UV light (Wong *et al.*, 2005; Steenackers *et al.*, 2019). However, herein, we found *c*-CA also to be present in plants grown under UV-free conditions. Additionally, the concentrations of *c*-CA present in the mock-treated plant were similar for plants grown in light with or without UV radiation. This indicates a UV-independent biosynthesis route toward *c*-CA, possibly via a dedicated enzymatic biosynthesis. Upon inhibition of C4H there is a strong accumulation of *t*-CA. However, this accumulation of *t*-CA is

Fig. 7 Proposed model explaining the phenotypic effects upon inhibition of CINNAMATE-4-HYDROXYLASE (C4H) in *Arabidopsis thaliana*. Graphical model displaying the physiological effects upon blocking C4H in the hypocotyl. (a) Given a functional phenylpropanoid pathway, baseline levels of *cis*-cinnamic acid (*c*-CA) are produced and normal lignin deposition takes place. Functional polar AUX transport and phloem transport result in normal AUX distribution pattern. (b) Upon blocking C4H, *c*-CA concentrations are elevated due to increased UV-mediated conversion from *trans*-cinnamic acid (*t*-CA), which would lead to reduced cell-to-cell AUX transport. In addition, lignin deposition is reduced, leading to a perturbed long-distance AUX transport through the phloem. As a consequence, AUX distribution is affected, resulting in an apical accumulation of AUX. Dashed arrow from phenylalanine to *c*-CA, putative biosynthetic route towards *c*-CA; dashed black upwards arrow, increase in *t*-CA concentrations; red cross, loss of C4H functionality; dashed red arrow, increase in *c*-CA concentrations leading to the inhibition of polar AUX transport; dashed blue arrow, depletion in lignin concentrations leading to the perturbation of long-distance AUX transport (greater size of blue arrow represents greater contribution of lignin depletion in the observed phenotypes). PAL, PHENYLALANINE AMMONIA LYASE; *p*CA, *para*-coumaric acid.

only accompanied by an increase in *c*-CA in UV-grown plants. When C4H is inhibited in plants grown in UV-free light there is a strong accumulation in concentrations of *t*-CA only. As without UV the excess in *t*-CA does not result in an increase in *c*-CA concentrations, it appears that the biosynthesis of *c*-CA is independent of *t*-CA. A possible biosynthesis route towards *c*-CA could be directly from phenylalanine. Together, the presented results help us in further understanding the role of *c*-CA as an endogenous plant growth regulator.

By further dissecting the underlying molecular causes of the phenotypes, the involvement of other pathway intermediates and end products that are known AUX transport modulators (SA, flavonoids) was refuted (Peer & Murphy, 2007; Tan *et al.*, 2020). Our findings indicated lignin deposition as an indirect requirement for phloem-mediated AUX transport. The drop in lignin content when blocking the phenylpropanoid pathway (PPP) alters AUX homeostasis resulting in the developmental defects. Lignin deposition, however, is known to occur in the xylem but not the phloem vessels, pointing towards the perturbation in xylem functionality as a primary cause of effect. Although we cannot exclude alternative hypotheses, the adverse effect of xylem transport perturbation on phloem transport capacity could be explained by the Münch model (Munch, 1930; Knoblauch *et al.*, 2016), which states that phloem transport is driven by a pressure gradient generated at the apical part of the plant. This gradient is established by the transport of water via the xylem. Thus, impaired lignification would disrupt xylem functionality and, indirectly, also phloem transport. As an altered lignin content of the xylem has been described for numerous PPP mutants (R. Vanholme *et al.*, 2019a), it is likely that phloem transport also is affected in these mutants. An impairment in AUX transport and phloem transport could explain some of the phenotypes observed in PPP mutants. For example, many PPP mutants show dwarfism and a lack of apical dominance (Hoffmann *et al.*, 2004; Schilmiller *et al.*, 2009; Bonawitz & Chapple, 2013; De Meester *et al.*, 2018). Whereas the reduction in plant height is considered a direct consequence of a perturbed water transport, the loss of apical dominance could be a consequence of an impaired AUX transport capacity. In addition, the transport of solutes and sugars that typically occurs via the phloem will be affected. Disturbing transport of these solutes would effectively disrupt the efficient distribution of energy throughout the plant, further contributing to the stunted growth of some of these mutants.

Auxin transport inhibition previously has been proposed to cause the growth defects of an *HYDROXYCINNAMOYL-COA SHIKIMATE/QUINATE HYDROXYCINNAMOYL TRANSFERASE (HCT)-RNAi* line (Besseau *et al.*, 2007). The growth defects in this *hct* mutant were linked to the upstream accumulation of flavonoids, which were presumed to cause an observed inhibition in AUX transport. Reducing flavonoid content in the *hct* mutant by silencing *CHALCONE SYNTHASE (CHS)*, which is involved in flavonoid production, indeed was accompanied with a restoration of both growth and AUX transport. However, in a later study, the growth defects observed in the *hct* mutant were found to be independent of flavonoids (Li *et al.*, 2010). Here, the authors pointed towards a slight restoration in

lignification in the *chs/hct* double mutant compared to the *hct* line to allow for the growth restoration. Indeed, low levels of lignin deposition seemed to allow for a large portion of plant growth considering the much stronger growth perturbation of the *c4h-4* knock-out mutant compared to partial *c4h* loss-of-function mutants (*ref3-1*, *ref3-2* and *ref3-3*; (Schilmiller *et al.*, 2009)). However, a possible link between the increase in lignin content and the restoration in AUX transport in the *chs/hct* mutant has so far not been investigated. If AUX transport inhibition in the *hct* mutant also was caused by a defective lignification, the observed restoration of AUX transport in the *chs/hct* mutant would thus also be caused by the small restoration of lignification and not the reduction in flavonoid content.

As lignin hinders the efficient conversion of plant biomass to fermentable sugars, reducing lignin content in plants is part of ongoing strategies to optimize plant biomass for a more efficient processing (Vanholme *et al.*, 2013). Understanding the phenotypes associated with lowering lignin content in plants is therefore a prerequisite in the light of these valorization purposes. Previously, phenotypes of PPP mutants were hypothesized to be caused by either an accumulation of products upstream or a depletion of products downstream in the pathway (Muro-Villanueva *et al.*, 2019). Here we show that in the case of C4H both models contribute, as polar AUX transport inhibition is caused by the accumulation of *c*-CA, and long-distance AUX transport is inhibited by a depletion of lignin. Remarkably, both models contribute to the same phenotype – namely, the apical accumulation of ARs. Nevertheless, as phenylpropanoids are involved in a wide range of physiological processes, the phenotypes induced by accumulating compounds are expected to be different for each PPP mutant. By thus charting a role for both the accumulation and depletion of PPP intermediates in the establishment of the phenotypes upon inhibition of C4H, this research improves our understanding of the underlying factors contributing to the growth phenotypes of PPP mutants. In particular, by establishing a role for lignin already at the early seedling stage, we demonstrate a fundamental position for lignin apart from providing plant rigidity linked to upright growth. These observations thus have clear implications pertaining the ongoing strategies to engineer the PPP towards a more efficient bio-based economy.

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Author contributions

IEH, AC, RN, TB, JF, BDR, WB and BV designed the experiments; IEH, CVB, HEA, HH, JP, WS and MQ performed the experiments; VS, DO and JP contributed new tools; and IEH and BV wrote the manuscript. WB and BV share last authorship.

ORCID

Helena E. Arents <https://orcid.org/0000-0003-4758-5758>
Tom Beeckman <https://orcid.org/0000-0001-8656-2060>
Caroline Van Beirs <https://orcid.org/0000-0002-6376-2307>
Wout Boerjan <https://orcid.org/0000-0003-1495-510X>
Alexandra Chanoca <https://orcid.org/0000-0003-4554-9526>
Jiří Friml <https://orcid.org/0000-0002-8302-7596>
Huibin Han <https://orcid.org/0000-0002-8758-4752>
Ilias El Houari <https://orcid.org/0000-0003-3985-4196>
Richard Napier <https://orcid.org/0000-0002-0605-518X>
Davy Opdenacker <https://orcid.org/0000-0003-0633-6368>
Jacob Pollier <https://orcid.org/0000-0002-1134-9238>
Mussa Quareshy <https://orcid.org/0000-0001-8115-9803>
Bert De Rybel <https://orcid.org/0000-0002-9551-042X>
Véronique Storme <https://orcid.org/0000-0003-4762-6580>
Bartel Vanholme <https://orcid.org/0000-0002-7214-7170>

References

- Abdulrazzak N, Pollet B, Ehling J, Larsen K, Asnaghi C, Ronseau S, Proux C, Erhardt M, Seltzer V, Renou J-P. 2006. A coumaroyl-ester-3-hydroxylase insertion mutant reveals the existence of nonredundant meta-hydroxylation pathways and essential roles for phenolic precursors in cell expansion and plant growth. *Plant Physiology* 140: 30–48.
- Alallaq S, Ranjan A, Brunoni F, Novák O, Lakehal A, Bellini C. 2020. Light controls *de novo* adventitious root regeneration by modulating jasmonate and cytokinin homeostasis in Norway spruce hypocotyls. *bioRxiv*, 2020.03.11.985838
- Besseau S, Hoffmann L, Geoffroy P, Lapierre C, Pollet B, Legrand M. 2007. Flavonoid accumulation in Arabidopsis repressed in lignin synthesis affects auxin transport and plant growth. *Plant Cell* 19: 148–162.
- Boerjan W, Ralph J, Baucher M. 2003. Lignin biosynthesis. *Annual Review of Plant Biology* 54: 519–546.
- Bonawitz ND, Chapple C. 2013. Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Current Opinion in Biotechnology* 24: 336–343.
- Bonawitz ND, Im Kim J, Tobimatsu Y, Ciesielski PN, Anderson NA, Ximenes E, Maeda J, Ralph J, Donohoe BS, Ladisch M. 2014. Disruption of Mediator rescues the stunted growth of a lignin-deficient Arabidopsis mutant. *Nature* 509: 376–380.
- Brown DE, Rashotte AM, Murphy AS, Normanly J, Tague BW, Peer WA, Taiz L, Muday GK. 2001. Flavonoids act as negative regulators of auxin transport *in vivo* in Arabidopsis. *Plant Physiology* 126: 524–535.
- Brunetti C, Fini A, Sebastiani F, Gori A, Tattini M. 2018. Modulation of phytohormone signaling: a primary function of flavonoids in plant–environment interactions. *Frontiers in Plant Science* 9: 1042.
- Chong J, Poutaraud A, Hugueney P. 2009. Metabolism and roles of stilbenes in plants. *Plant Science* 177: 143–155.
- Coleman HD, Samuels AL, Guy RD, Mansfield SD. 2008. Perturbed lignification impacts tree growth in hybrid poplar—a function of sink strength, vascular integrity, and photosynthetic assimilation. *Plant Physiology* 148: 1229–1237.
- De Meester B, de Vries L, Özparpucu M, Gierlinger N, Corneillie S, Pallidis A, Goeminne G, Morreel K, De Bruyne M, De Rycke R. 2018. Vessel-specific reintroduction of cinnamoyl-CoA reductase1 (CCR1) in dwarfed *ccr1* mutants restores vessel and xylary fiber integrity and increases biomass. *Plant Physiology* 176: 611–633.
- De Smet I, Chaerle P, Vanneste S, De Rycke R, Inzé D, Beeckman T. 2004. An easy and versatile embedding method for transverse sections. *Journal of Microscopy* 213: 76–80.
- Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M. 2005. Plant development is regulated by a family of auxin receptor F box proteins. *Developmental Cell* 9: 109–119.
- Dolan WL, Dilkes BP, Stout JM, Bonawitz ND, Chapple C. 2017. Mediator complex subunits *MED2*, *MED5*, *MED16*, and *MED23* genetically interact in the regulation of phenylpropanoid biosynthesis. *Plant Cell* 29: 3269–3285.
- Franke R, Hemm MR, Denault JW, Ruegger MO, Humphreys JM, Chapple C. 2002. Changes in secondary metabolism and deposition of an unusual lignin in the *ref8* mutant of Arabidopsis. *The Plant Journal* 30: 47–59.
- Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science* 261: 754–756.
- Gallego-Giraldo L, Escamilla-Trevino L, Jackson LA, Dixon RA. 2011. Salicylic acid mediates the reduced growth of lignin down-regulated plants. *Proceedings of the National Academy of Sciences, USA* 108: 20814–20819.
- Gallego-Giraldo L, Pose S, Pattathil S, Peralta AG, Hahn MG, Ayre BG, Sunuwar J, Hernandez J, Patel M, Shah J *et al.* 2018. Elicitors and defense gene induction in plants with altered lignin compositions. *New Phytologist* 219: 1235–1251.
- Gutierrez L, Mongelard G, Floková K, Păcurar DI, Novák O, Staswick P, Kowalczyk M, Păcurar M, Demailly H, Geiss G. 2012. Auxin controls Arabidopsis adventitious root initiation by regulating jasmonic acid homeostasis. *Plant Cell* 24: 2515–2527.
- Hoffmann L, Besseau S, Geoffroy P, Ritzenhaler C, Meyer D, Lapierre C, Pollet B, Legrand M. 2004. Silencing of *hydroxycinnamoyl-coenzyme A shikimate/quininate hydroxycinnamoyltransferase* affects phenylpropanoid biosynthesis. *Plant Cell* 16: 1446–1465.
- Kim J, Dotson B, Rey C, Lindsey J, Bleeker AB, Binder BM, Patterson SE. 2013. New clothes for the jasmonic acid receptor COI1: delayed abscission, meristem arrest and apical dominance. *PLoS ONE* 8: e60505.
- Kleinboelting N, Hup G, Kloetgen A, Viehoever P, Weisshaar B. 2012. GABI-Kat SimpleSearch: new features of the *Arabidopsis thaliana* T-DNA mutant database. *Nucleic Acids Research* 40: D1211–1215.
- Knoblauch M, Knoblauch J, Mullendore DL, Savage JA, Babst BA, Beecher SD, Dodgen AC, Jensen KH, Holbrook NM. 2016. Testing the Münch hypothesis of long distance phloem transport in plants. *eLife* 5: e15341.
- Lakehal A, Bellini C. 2019. Control of adventitious root formation: insights into synergistic and antagonistic hormonal interactions. *Physiologia Plantarum* 165: 90–100.
- Lakehal A, Chaabouni S, Cavel E, Le Hir R, Ranjan A, Raneshan Z, Novák O, Păcurar DI, Perrone I, Jobert F. 2019. A molecular framework for the control of adventitious rooting by TIR1/AFB2-Aux/IAA-dependent auxin signaling in Arabidopsis. *Molecular Plant* 12: 1499–1514.
- Lakehal A, Dob A, Rahnesan Z, Novák O, Escamez S, Alallaq S, Strnad M, Tuominen H, Bellini C. 2020. *ETHYLENE RESPONSE FACTOR 115* integrates jasmonate and cytokinin signaling machineries to repress adventitious rooting in Arabidopsis. *bioRxiv*. 2019.2012. 2027.886796.
- Li X, Bonawitz ND, Weng J-K, Chapple C. 2010. The growth reduction associated with repressed lignin biosynthesis in *Arabidopsis thaliana* is independent of flavonoids. *Plant Cell* 22: 1620–1632.
- Melnik CW, Schuster C, Leyser O, Meyerowitz EM. 2015. A developmental framework for graft formation and vascular reconnection in *Arabidopsis thaliana*. *Current Biology* 25: 1306–1318.
- Moreno-Risueno MA, Van Norman JM, Moreno A, Zhang J, Ahnert SE, Benfey PN. 2010. Oscillating gene expression determines competence for periodic Arabidopsis root branching. *Science* 329: 1306–1311.
- Munch E. 1930. *Stoffbewegungen in der Pflanze*. Jena, Germany: Gustav Fischer.
- Muro-Villanueva F, Mao X, Chapple C. 2019. Linking phenylpropanoid metabolism, lignin deposition, and plant growth inhibition. *Current Opinion in Biotechnology* 56: 202–208.

- Naseer S, Lee Y, Lapierre C, Franke R, Nawrath C, Geldner N. 2012. Casparian strip diffusion barrier in Arabidopsis is made of a lignin polymer without suberin. *Proceedings of the National Academy of Sciences, USA* 109: 10101–10106.
- Noel JP, Austin MB, Bomati EK. 2005. Structure–function relationships in plant phenylpropanoid biosynthesis. *Current Opinion in Plant Biology* 8: 249–253.
- Peer WA, Murphy AS. 2007. Flavonoids and auxin transport: modulators or regulators? *Trends in Plant Science* 12: 556–563.
- Ralph J, Lapierre C, Boerjan W. 2019. Lignin structure and its engineering. *Current Opinion in Biotechnology* 56: 240–249.
- Rasmussen A, Hu Y, Depaepe T, Vandenbussche F, Boyer F-D, Van Der Straeten D, Geelen D. 2017. Ethylene controls adventitious root initiation sites in Arabidopsis hypocotyls independently of strigolactones. *Journal of Plant Growth Regulation* 36: 897–911.
- Saslowsky DE, Dana CD, Winkel-Shirley B. 2000. An allelic series for the *chalcone synthase* locus in Arabidopsis. *Gene* 255: 127–138.
- Schalk M, Cabello-Hurtado F, Pierrel M-A, Atanassova R, Saindrenan P, Werck-Reichhart D. 1998. Piperonylic acid, a selective, mechanism-based inactivator of the *trans*-cinnamate 4-hydroxylase: a new tool to control the flux of metabolites in the phenylpropanoid pathway. *Plant Physiology* 118: 209–218.
- Schilmiller AL, Stout J, Weng JK, Humphreys J, Ruegger MO, Chapple C. 2009. Mutations in the *cinnamate 4-hydroxylase* gene impact metabolism, growth and development in Arabidopsis. *The Plant Journal* 60: 771–782.
- Schmid NB, Giehl RF, Döll S, Mock H-P, Strehmel N, Scheel D, Kong X, Hider RC, von Wirén N. 2014. Feruloyl-CoA 6'-hydroxylase1-dependent coumarins mediate iron acquisition from alkaline substrates in Arabidopsis. *Plant Physiology* 164: 160–172.
- Steenackers W, El Houari I, Baekelandt A, Witvrouw K, Dhondt S, Leroux O, Gonzalez N, Corneillie S, Cesarino I, Inzé D. 2019. *cis*-Cinnamic acid is a natural plant growth-promoting compound. *Journal of Experimental Botany* 70: 6293–6304.
- Steenackers W, Klíma P, Quareshy M, Cesarino I, Kumpf RP, Corneillie S, Araújo P, Viaene T, Goeminne G, Nowack MK. 2017. *cis*-Cinnamic acid is a novel, natural auxin efflux inhibitor that promotes lateral root formation. *Plant Physiology* 173: 552–565.
- Steffens B, Rasmussen A. 2016. The physiology of adventitious roots. *Plant Physiology* 170: 603–617.
- Tan S, Abas M, Verstraeten I, Glanc M, Molnár G, Hajný J, Lasák P, Petřík I, Russinova E, Petrášek J. 2020. Salicylic acid targets protein phosphatase 2A to attenuate growth in plants. *Current Biology* 30: 381–395.e8.
- Van de Wouwer D, Vanholme R, Decou R, Goeminne G, Audenaert D, Nguyen L, Höfer R, Pesquet E, Vanholme B, Boerjan W. 2016. Chemical genetics uncovers novel inhibitors of lignification, including p-iodobenzoic acid targeting CINNAMATE-4-HYDROXYLASE. *Plant Physiology* 172: 198–220.
- Vanholme B, Desmet T, Ronsse F, Rabaey K, Van Breusegem F, De Mey M, Soetaert W, Boerjan W. 2013. Towards a carbon-negative sustainable bio-based economy. *Frontiers in Plant Science* 4: 174.
- Vanholme B, El Houari I, Boerjan W. 2019. Bioactivity: phenylpropanoids' best kept secret. *Current Opinion in Biotechnology* 56: 156–162.
- Vanholme R, De Meester B, Ralph J, Boerjan W. 2019a. Lignin biosynthesis and its integration into metabolism. *Current Opinion in Biotechnology* 56: 230–239.
- Vanholme R, Sundin L, Seetso KC, Kim H, Liu X, Li J, De Meester B, Hoengenaert L, Goeminne G, Morreel K. 2019b. COSY catalyses *trans*–*cis* isomerization and lactonization in the biosynthesis of coumarins. *Nature Plants* 5: 1066–1075.
- Veldstra H. 1953. The relation of chemical structure to bio-logical activity in growth substances. *Annual Review of Plant Physiology* 4: 151–198.
- Verstraeten I, Schotte S, Geelen D. 2014. Hypocotyl adventitious root organogenesis differs from lateral root development. *Frontiers in Plant Science* 5: 495.
- Vogt T. 2010. Phenylpropanoid biosynthesis. *Molecular Plant* 3: 2–20.
- Wong WS, Guo D, Wang XL, Yin ZQ, Xia B, Li N. 2005. Study of *cis*-cinnamic acid in *Arabidopsis thaliana*. *Plant Physiology and Biochemistry* 43: 929–937.
- Xuan W, Audenaert D, Parizot B, Möller BK, Njo MF, De Rybel B, De Rop G, Van Isterdael G, Mähönen AP, Vanneste S. 2015. Root cap-derived auxin pre-patterns the longitudinal axis of the Arabidopsis root. *Current Biology* 25: 1381–1388.
- Zhao X, Wang J, Yuan J, Wang XL, Zhao QP, Kong PT, Zhang X. 2015. NITRIC OXIDE-ASSOCIATED PROTEIN1 (*AtNOA1*) is essential for salicylic acid-induced root waving in *Arabidopsis thaliana*. *New Phytologist* 207: 211–224.
- Zipfel C, Robatzek S, Navarro L, Oakeley EJ, Jones JD, Felix G, Boller T. 2004. Bacterial disease resistance in Arabidopsis through flagellin perception. *Nature* 428: 764–767.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Methods S1 Statistical analysis.

Methods S2 Metabolite profiling.

Methods S3 Auxin binding and docking experiments.

Methods S4 GC-MS analysis.

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