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

Ilias El Houari1,2, Caroline Van Beirs1,2, Helena E. Arents1,2, Huibin Han3, Alexandra Chanoca1,2, Davy Opdenacker1,2, Jacob Pollar1,2,4, Véronique Storme1,2, Ward Steenackers1,2, Mussa Qureshy5, Richard Napier5, Tom Beeckman1,2, Jiří Friml3, Bert De Rybel1,2, Wout Boerjan1,2* and Bartel Vanholme1,2*

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

Authors for correspondence:
Bartel Vanholme
Email: Bartel.Vanholme@psb.vib-ugent.be
Wout Boerjan
Email: Wout.Boerjan@psb.vib-ugent.be

Received: 15 January 2021
Accepted: 6 March 2021

doi: 10.1111/nph.17349

Key words: Arabidopsis, auxin, cis-cinnamic acid, phenylpropanoids, piperonylic acid, lignin, metabolomics, roots.

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 (Saslowsky 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., 2003; Ralph et al., 2019; Vanholme et al., 2019a).

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.
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 mediate to be at the basis of the growth phenotypes. Several mutants proposes a differential accumulation of bioactive intermediates 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* (Dhartmasiri 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 vapoour-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), naphtylphthalamic 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 Epson 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 root length 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 ½ 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 ½ MS medium supplemented with or without 50 µM PA. Bar, 1 cm. Yellow coloration, top third of the hypocotyl; blue coloration, lower 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/eamnet/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 1/2MS medium plates contain 50 μM 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 μM NPA. A droplet of 5 μl 3H-IAA (20 μl 3H-IAA added into 10 ml of 1/2MS 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 3H-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 1/2MS medium with the top part of the hypocotyl being placed on parafilm, and a 1-μl 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 after 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 1/2MS medium with the bottom part of the hypocotyl being placed on parafilm. A 2-μl 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 aceton 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 μm 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. DePcX Mounting Medium (VWR International, Radnor, PA, USA) treatment was performed and pictures were taking 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 GABI-KAT 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 1/2MS 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 seedlings were sown on 1/2MS 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 mutant was 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 μM. 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 ½MS medium containing a concentration range of PA (0–100 μM). 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 μM 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 (Schulmiller 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; Allallaq 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 mutants lines upon treatment with and without 50 μM 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 μM 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 μM 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).

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>m/z</th>
<th>Name</th>
<th>Wild-type (WT)</th>
<th>c4h-4 mutant</th>
<th>Fold change c4h-4 - WT</th>
<th>PA</th>
<th>Fold Change PA - WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.19</td>
<td>164.0715</td>
<td>Phenylalanine and cinnamate derivatives</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>9.69</td>
<td>355.1028</td>
<td>Cinnamoyl hexose (formic acid adduct)</td>
<td>0.61 ± 0.72</td>
<td>2215.55 ± 598.94</td>
<td>&gt;100</td>
<td>1850.64</td>
<td>604.39</td>
</tr>
<tr>
<td>3</td>
<td>8.62</td>
<td>355.1028</td>
<td>Cinnamoyl hexose (formic acid adduct)</td>
<td>0.49 ± 0.36</td>
<td>1125.18 ± 370.58</td>
<td>&gt;100</td>
<td>1179.51</td>
<td>441.18</td>
</tr>
<tr>
<td>4</td>
<td>12.46</td>
<td>263.0550</td>
<td>Cinnamyl malate</td>
<td>0.00 ± 0.00</td>
<td>40.44 ± 12.27</td>
<td>&gt;100</td>
<td>26.32</td>
<td>7.96</td>
</tr>
<tr>
<td>5</td>
<td>6.93</td>
<td>623.1592</td>
<td>Isorhamnetin glucoside rhamnose 1</td>
<td>60.07 ± 19.24</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>1.45</td>
<td>1.03</td>
</tr>
<tr>
<td>6</td>
<td>8.50</td>
<td>623.1603</td>
<td>Isorhamnetin glucoside rhamnose 2</td>
<td>1822.91 ± 532.53</td>
<td>0.44 ± 0.49</td>
<td>&gt;100</td>
<td>128.30</td>
<td>97.33</td>
</tr>
<tr>
<td>7</td>
<td>5.49</td>
<td>609.1447</td>
<td>Kaempferol dihexoside</td>
<td>613.37 ± 139.24</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>16.63</td>
<td>10.47</td>
</tr>
<tr>
<td>8</td>
<td>5.54</td>
<td>755.1994</td>
<td>Kaempferol dihexoside</td>
<td>299.57 ± 81.86</td>
<td>0.03 ± 0.10</td>
<td>&gt;100</td>
<td>26.81</td>
<td>25.24</td>
</tr>
<tr>
<td>9</td>
<td>6.99</td>
<td>755.2016</td>
<td>Kaempferol dihexoside</td>
<td>875.62 ± 217.82</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>9.07</td>
<td>6.27</td>
</tr>
<tr>
<td>10</td>
<td>6.23</td>
<td>593.1481</td>
<td>Kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-o-glucopyranoside</td>
<td>55.62 ± 10.28</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>2.91</td>
<td>3.93</td>
</tr>
<tr>
<td>11</td>
<td>8.08</td>
<td>739.2069</td>
<td>Kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-o-glucopyranoside</td>
<td>1436.33 ± 380.07</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>18.44</td>
<td>23.38</td>
</tr>
<tr>
<td>12</td>
<td>6.22</td>
<td>739.2070</td>
<td>Kaempferol-3-O-a-L-rhamnopyranosyl(1,2)-b-o-glucopyranoside</td>
<td>5768.48 ± 1279.59</td>
<td>8.53 ± 7.94</td>
<td>&gt;100</td>
<td>486.21</td>
<td>379.86</td>
</tr>
<tr>
<td>13</td>
<td>8.26</td>
<td>593.1498</td>
<td>Kaempferol-rutinoside (hex + rha)</td>
<td>1389.46 ± 369.87</td>
<td>2.42 ± 2.68</td>
<td>&gt;100</td>
<td>616.29</td>
<td>626.08</td>
</tr>
<tr>
<td>14</td>
<td>7.24</td>
<td>551.1752</td>
<td>Ferulic acid derivatives</td>
<td>130.59 ± 31.32</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>10.08</td>
<td>3.35</td>
</tr>
<tr>
<td>15</td>
<td>7.58</td>
<td>551.1756</td>
<td>4/7/9-O-hexoside G(8-O-4)</td>
<td>161.06 ± 24.72</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>9.71</td>
<td>3.54</td>
</tr>
<tr>
<td>16</td>
<td>5.94</td>
<td>551.1753</td>
<td>4/7/9-O-hexoside G(8-O-4)</td>
<td>210.85 ± 48.69</td>
<td>0.02 ± 0.05</td>
<td>&gt;100</td>
<td>7.40</td>
<td>2.52</td>
</tr>
<tr>
<td>17</td>
<td>6.98</td>
<td>551.1753</td>
<td>4/7/9-O-hexoside G(8-O-4)</td>
<td>390.72 ± 84.75</td>
<td>0.09 ± 0.11</td>
<td>&gt;100</td>
<td>38.14</td>
<td>11.76</td>
</tr>
<tr>
<td>18</td>
<td>6.70</td>
<td>551.1757</td>
<td>4/7/9-O-hexoside G(8-O-4)</td>
<td>1053.87 ± 177.17</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>83.44</td>
<td>26.10</td>
</tr>
<tr>
<td>19</td>
<td>6.43</td>
<td>551.1759</td>
<td>4/7/9-O-hexoside G(8-O-4)</td>
<td>4063.76 ± 746.22</td>
<td>1.60 ± 1.90</td>
<td>&gt;100</td>
<td>303.70</td>
<td>82.14</td>
</tr>
<tr>
<td>24</td>
<td>4.07</td>
<td>371.0971</td>
<td>5-hydroxyferulic acid + hexose 1</td>
<td>70.56 ± 15.11</td>
<td>0.01 ± 0.02</td>
<td>&gt;100</td>
<td>5.83</td>
<td>2.28</td>
</tr>
<tr>
<td>25</td>
<td>3.60</td>
<td>371.0976</td>
<td>5-hydroxyferulic acid + hexose 2</td>
<td>375.78 ± 124.87</td>
<td>0.01 ± 0.04</td>
<td>&gt;100</td>
<td>15.27</td>
<td>7.95</td>
</tr>
<tr>
<td>26</td>
<td>5.96</td>
<td>339.1107</td>
<td>Coniferaldehyde 4-O-hexoside</td>
<td>68.02 ± 20.82</td>
<td>3.07 ± 2.25</td>
<td>22.12</td>
<td>12.47</td>
<td>7.74</td>
</tr>
<tr>
<td>27</td>
<td>3.84</td>
<td>387.1289</td>
<td>Coniferal (formic acid adduct)</td>
<td>1932.65 ± 718.88</td>
<td>4.31 ± 2.75</td>
<td>&gt;100</td>
<td>136.05</td>
<td>68.97</td>
</tr>
<tr>
<td>28</td>
<td>3.20</td>
<td>357.1172</td>
<td>Dihydroferulic acid + hexose</td>
<td>43.24 ± 12.28</td>
<td>0.01 ± 0.01</td>
<td>&gt;100</td>
<td>0.02</td>
<td>0.07</td>
</tr>
<tr>
<td>29</td>
<td>11.34</td>
<td>535.1445</td>
<td>Dihydroferuloyl-beta-keto acid + hexose + 136 Da</td>
<td>109.93 ± 31.85</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>26.80</td>
<td>7.77</td>
</tr>
<tr>
<td>30</td>
<td>8.44</td>
<td>753.2220</td>
<td>Disinapoyl hexose + hexose</td>
<td>134.27 ± 37.61</td>
<td>0.02 ± 0.05</td>
<td>&gt;100</td>
<td>66.57</td>
<td>59.17</td>
</tr>
<tr>
<td>31</td>
<td>3.72</td>
<td>355.1025</td>
<td>Ferulic acid 4-O-hexoside 1</td>
<td>323.49 ± 55.67</td>
<td>0.12 ± 0.20</td>
<td>&gt;100</td>
<td>20.92</td>
<td>7.90</td>
</tr>
<tr>
<td>32</td>
<td>5.35</td>
<td>355.1029</td>
<td>Ferulic acid 4-O-hexoside 2</td>
<td>1539.51 ± 379.00</td>
<td>1.07 ± 1.05</td>
<td>&gt;100</td>
<td>48.18</td>
<td>21.33</td>
</tr>
<tr>
<td>33</td>
<td>5.44</td>
<td>355.1029</td>
<td>Ferulic acid 4-O-hexoside 3</td>
<td>2490.13 ± 485.84</td>
<td>19.86 ± 5.71</td>
<td>&gt;100</td>
<td>253.94</td>
<td>77.65</td>
</tr>
<tr>
<td>34</td>
<td>5.86</td>
<td>355.1027</td>
<td>Feruloyl hexose</td>
<td>646.73 ± 146.47</td>
<td>0.08 ± 0.10</td>
<td>&gt;100</td>
<td>10.75</td>
<td>6.05</td>
</tr>
<tr>
<td>35</td>
<td>6.21</td>
<td>581.1845</td>
<td>Feruloyl hexose + 226 Da</td>
<td>118.84 ± 32.12</td>
<td>0.25 ± 0.30</td>
<td>&gt;100</td>
<td>12.92</td>
<td>4.92</td>
</tr>
</tbody>
</table>
Next we quantified AUX transport in etiolated seedlings grown on medium supplemented with/without 50 µM 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.

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 µM PA-treated WT seedlings (n > 7). Each datapoint represents eight biological replicates.

Table 1 (Continued)

<table>
<thead>
<tr>
<th>No.</th>
<th>RT</th>
<th>m/z</th>
<th>Name</th>
<th>Wild-type (WT)</th>
<th>c4h-4 mutant</th>
<th>Fold change c4h-4 - WT</th>
<th>PA</th>
<th>Fold Change PA - WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>36</td>
<td>8.24</td>
<td>581.1856</td>
<td>Feruloyl hexose + 226 Da 2</td>
<td>382.95 ± 93.27</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>21.58 ± 7.87</td>
<td>17.75</td>
</tr>
<tr>
<td>37</td>
<td>2.61</td>
<td>563.1600</td>
<td>Feruloyl hexose 4-O-hexoside (formic acid adduct)</td>
<td>108.31 ± 30.85</td>
<td>0.19 ± 0.24</td>
<td>&gt;100</td>
<td>13.17 ± 5.97</td>
<td>8.23</td>
</tr>
<tr>
<td>38</td>
<td>4.53</td>
<td>325.0919</td>
<td>Feruloyl pentose 1</td>
<td>264.37 ± 112.58</td>
<td>45.61 ± 16.79</td>
<td>5.80</td>
<td>32.40 ± 12.72</td>
<td>8.16</td>
</tr>
<tr>
<td>39</td>
<td>5.10</td>
<td>325.0921</td>
<td>Feruloyl pentose 2</td>
<td>456.51 ± 163.43</td>
<td>3.59 ± 1.77</td>
<td>&gt;100</td>
<td>18.61 ± 8.00</td>
<td>24.54</td>
</tr>
<tr>
<td>40</td>
<td>5.92</td>
<td>583.2011</td>
<td>G 4-O-hexoside(8-O-4)G (formic acid adduct)</td>
<td>248.86 ± 94.48</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>0.08 ± 0.12</td>
<td>&gt;100</td>
</tr>
<tr>
<td>41</td>
<td>10.13</td>
<td>533.1648</td>
<td>G(8-5)feruloyl hexose</td>
<td>264.64 ± 30.42</td>
<td>0.43 ± 0.44</td>
<td>&gt;100</td>
<td>11.12 ± 4.03</td>
<td>23.80</td>
</tr>
<tr>
<td>42</td>
<td>9.20</td>
<td>519.1856</td>
<td>G(8-5)G hexoside</td>
<td>199.61 ± 62.44</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>0.40 ± 0.47</td>
<td>&gt;100</td>
</tr>
<tr>
<td>43</td>
<td>8.19</td>
<td>469.0800</td>
<td>G(8-O-4)ferulic acid sulfate</td>
<td>841.14 ± 152.38</td>
<td>1.28 ± 2.49</td>
<td>&gt;100</td>
<td>165.15 ± 91.89</td>
<td>5.09</td>
</tr>
<tr>
<td>44</td>
<td>10.32</td>
<td>389.1229</td>
<td>G(8-O-4)ferulic acid</td>
<td>105.31 ± 23.63</td>
<td>0.01 ± 0.02</td>
<td>&gt;100</td>
<td>17.53 ± 4.92</td>
<td>6.01</td>
</tr>
<tr>
<td>45</td>
<td>10.25</td>
<td>505.1336</td>
<td>G(8-O-4)ferulic acid + malate</td>
<td>95.31 ± 22.14</td>
<td>0.24 ± 0.27</td>
<td>&gt;100</td>
<td>15.27 ± 4.58</td>
<td>6.24</td>
</tr>
<tr>
<td>46</td>
<td>8.24</td>
<td>521.2011</td>
<td>G(red8-5)G + hexose 1</td>
<td>163.90 ± 28.32</td>
<td>0.00 ± 0.00</td>
<td>&gt;100</td>
<td>0.60 ± 0.71</td>
<td>&gt;100</td>
</tr>
<tr>
<td>47</td>
<td>9.08</td>
<td>521.2011</td>
<td>G(red8-5)G + hexose 2</td>
<td>715.41 ± 68.49</td>
<td>0.01 ± 0.03</td>
<td>&gt;100</td>
<td>12.73 ± 7.76</td>
<td>56.21</td>
</tr>
<tr>
<td>49</td>
<td>9.54</td>
<td>385.1136</td>
<td>Sinapic acid 4-O-hexoside 1</td>
<td>18148.45 ± 3185.04</td>
<td>276.41 ± 117.33</td>
<td>65.66</td>
<td>4779.87 ± 2163.07</td>
<td>3.80</td>
</tr>
<tr>
<td>50</td>
<td>6.10</td>
<td>385.1134</td>
<td>Sinapic acid 4-O-hexoside 2</td>
<td>6030.09 ± 1234.35</td>
<td>74.89 ± 33.67</td>
<td>80.52</td>
<td>1623.39 ± 629.03</td>
<td>3.71</td>
</tr>
<tr>
<td>51</td>
<td>5.00</td>
<td>385.1129</td>
<td>Sinapic acid 4-O-hexoside 3</td>
<td>161.43 ± 37.22</td>
<td>0.02 ± 0.05</td>
<td>&gt;100</td>
<td>63.81 ± 23.86</td>
<td>2.53</td>
</tr>
<tr>
<td>52</td>
<td>4.39</td>
<td>385.1132</td>
<td>Sinapic acid 4-O-hexoside 4</td>
<td>762.84 ± 139.35</td>
<td>5.45 ± 4.03</td>
<td>&gt;100</td>
<td>304.76 ± 99.96</td>
<td>2.50</td>
</tr>
<tr>
<td>53</td>
<td>6.67</td>
<td>352.1029</td>
<td>Sinapoyl glutamate 1</td>
<td>175.36 ± 48.08</td>
<td>1.79 ± 1.15</td>
<td>98.19</td>
<td>70.07 ± 26.78</td>
<td>2.50</td>
</tr>
<tr>
<td>54</td>
<td>6.03</td>
<td>352.1029</td>
<td>Sinapoyl glutamate 2</td>
<td>248.59 ± 69.12</td>
<td>3.78 ± 2.11</td>
<td>65.72</td>
<td>101.05 ± 35.24</td>
<td>2.46</td>
</tr>
<tr>
<td>55</td>
<td>9.31</td>
<td>339.0715</td>
<td>Sinapoyl malate 1</td>
<td>2921.54 ± 735.01</td>
<td>48.09 ± 25.75</td>
<td>60.76</td>
<td>1930.41 ± 595.57</td>
<td>1.51</td>
</tr>
<tr>
<td>56</td>
<td>9.01</td>
<td>339.0714</td>
<td>Sinapoyl malate 2</td>
<td>3618.53 ± 554.83</td>
<td>67.86 ± 36.76</td>
<td>53.33</td>
<td>2349.53 ± 649.93</td>
<td>1.54</td>
</tr>
<tr>
<td>57</td>
<td>9.52</td>
<td>165.0190</td>
<td>Piperonylic acid</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.10</td>
<td>0.00</td>
<td>298.39 ± 82.70</td>
<td>&gt;100</td>
</tr>
<tr>
<td>58</td>
<td>5.80</td>
<td>373.0769</td>
<td>Piperonylic acid hexose (formic acid adduct)</td>
<td>0.90 ± 1.06</td>
<td>8.14 ± 19.46</td>
<td>9.05</td>
<td>5954.44 ± 2250.56</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

List of compounds were characterized upon LC-MS profiling of etiolated Col-0 WT, c4h-4 and Col-0 seedlings treated with 50 µM 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 ± 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).

© 2021 The Authors
New Phytologist © 2021 New Phytologist Foundation
www.newphytologist.com
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 t-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 µM 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 µM PA and 200 µM pCA, 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 µM PA with 100 µM 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 trol mutant deficient in the production of flavonoids (Brown et al., 2001) was analyzed alongside a WT control. The trol 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 µM 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 pCA, 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 µM PA and 100 µM 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 µM PA and 100 µM 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...
etiolated seedlings using CFDA. Arabidopsis seedlings were etiolated to test this hypothesis, phloem transport capacity was assessed in the hypocotyl above 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 seedlings, etiolated hormone mutants treated 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 piperonylic acid (PA) and 100 µM coniferaldehyde (ConAld) (n > 30). These were then transferred to ½ 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 ½ 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 ½ 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 ½ 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 ½ 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.
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 black arrow, increase in t-CA concentrations; 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; pCA, 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 & Chappell, 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.

### Acknowledgements

This work was supported by the Fonds voor Wetenschappelijk Onderzoek – Vlaanderen (FWO) through project number G008116N and personal grants to IEH (1504020N) and CVB (11D8220N). RN acknowledges the support of BBSRC award BB/L009366/1. Part of the work was performed by IEH at the Sainsbury Laboratory, University of Cambridge funded by EMBO (STF-8658). We would like to thank Ottoline Leyser for hosting performance of these experiments, and Martin van Rongen, Sally Ward and Zoe Nahas for technical advice. We would like to thank the VIB Metabolomics Core Facility and Geert Goeminne for processing of the LC-MS samples. We would like to thank Luuk Scholten for technical support.
Author contributions

IEH, AC, RN, TB, JF, BDR, WB and BV designed the experiments; IEH, CKB, 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

Helen A. Arends https://orcid.org/0000-0003-4758-5758
Tom Beckman https://orcid.org/0000-0001-8566-2060
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 Frišl https://orcid.org/0000-0002-8302-7596
Huibin Han https://orcid.org/0000-0002-8758-4752
Ilías 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


© 2021 The Authors
*New Phytologist* © 2021 New Phytologist Foundation

---

**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.

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.