Arogenate Dehydratase Isoenzymes Profoundly and Differentially Modulate Carbon Flux into Lignins*

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*Running Title: Arogenate dehydratases and lignification

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Keywords: Arogenate; lignin; phenylalanine; guaiacyl:syringyl ratios; carbon allocation; arogenate dehydratase; plastid; metabolic networks; laser microscope dissection; vascular bundles; interfascicular fibers; pyrolysis GC/MS

Background hypothesis: That the plastid localized arogenate dehydratase (ADT) gene family differentially controls carbon flux for lignin deposition, with the latter substance being the main contributor to lignocellulosic recalcitrance.

Results: Single and multiple ADT knockouts resulted in differential control over lignin content/composition.

Conclusion: First evidence for Phe-upstream metabolism differentially controlling carbon flux into distinct secondary cell wall types.

Significance: Upstream metabolic networks regulate secondary cell-wall formation.

SUMMARY

How carbon flux differentially occurs in vascular plants following photosynthesis for protein formation, phenylpropanoid metabolism (i.e. lignins), and other metabolic processes is not well understood. Having previously discovered/deduced that a six-membered arogenate dehydratase (ADT 1-6) gene family encodes the final step in Phe biosynthesis in Arabidopsis thaliana, this raised the fascinating question whether individual ADT isoenzymes (or combinations thereof) differentially modulated carbon flux to lignins, proteins, etc. If so, unlike all other lignin pathway manipulations which target cell wall/cytosolic processes, this would be the first example of a plastid (chloroplast) associated metabolic process influencing cell-wall formation. Homozygous T-DNA insertion lines were thus obtained for 5 of the 6 ADTs, these being used to generate double, triple and quadruple knockouts (KOs) in different combinations. The various mutants so obtained gave phenotypes with profound but distinct reductions in lignin amounts, encompassing a range spanning from near to wild type levels to reductions of up to ~68%. In the various KOs, there were also marked changes in guaiacyl:syringyl (G:S) ratios ranging from ~3:1 to 1:1, respectively, these being rationalized due to differential carbon flux into vascular bundles (vb) versus that of fiber cells. Laser-microscope dissection/pyrolysis GC/MS, histochemical staining/lignin analyses, and pADT::GUS-localization, suggested that ADT5 preferentially affects carbon flux into the vb, whereas the adt3456 knockout additionally greatly reduced carbon flux into fiber cells. This plastid-localized metabolic step can thus profoundly differentially affect carbon flux into lignins in distinct anatomical regions, and provides incisive new insight into different factors affecting G:S ratios and lignin primary structure.

The final step of Phe biosynthesis, catalyzed by arogenate dehydratase (ADT) in planta (1-3), is potentially a major regulatory point due to both its pivotal position at the branch-point of Tyr and Phe biosynthesis (see Fig. 1), and as a linkage point between plastid/chloroplast localized shikimate-
chorismate and cytosolic/membrane/cell wall associated phenylpropanoid metabolic networks. Together, these pathways comprise some of the most metabolically intensive networks in vascular plants. Indeed, depending upon the species, up to 50% of captured photosynthetic carbon can be in the form of Phe-derived phenylpropanoids (4,5). Furthermore, downstream phenylpropanoid-derived products can have important but distinct physiological functions in planta, including fragrances/flavors, defense molecules, UV protectants, pigments and cell wall structural biopolymers, e.g. allyl/prophenyl phenols, lignans (6), flavonoids (7), (proantho)cyaniids, stilbenes (8), phytoalexins (e.g. isoflavones) (9), lignins (10) and suberins (11,12). The broad physiological functions of phenylpropanoid-derived metabolites thus translate into a diverse and ever-changing demand for the pathway intermediate Phe in different tissues and organs, i.e. in addition to Phe utilization for protein synthesis and other metabolic pathways.

Curiously, the question of pivotal regulatory metabolic networks upstream of Phe, and profoundly altering carbon flux/allocation into phenylpropanoid/lignin metabolism versus protein synthesis etc., had essentially not been addressed before. Instead, previous biotechnological manipulations targeted the presumed entry point to the phenylpropanoid pathway, phenylalanine ammonia lyase, as well as various downstream monolignol pathway steps (see Anterola and Lewis (13) and Davin et al. (10) for a discussion). Most of these approaches, however, did not take into consideration of the potentially seamless integration of related upstream, but differentially localized, metabolic networks associated with carbon flux into phenylpropanoids, and transcriptional regulation thereof. This was relevant since, in previous metabolic flux studies leading to monolignols in loblolly pine (Pinus taeda), it was established that factors apparently affecting Phe availability helped control/modulate carbon flux into phenylpropanoid metabolism (14,15), i.e. rather than phenylalanine ammonia lyase having a central rate-limiting role as had often been reported due to its entry point position to phenylpropanoids.

The ADT family was thus considered a potentially promising candidate for involvement in regulating the previously documented changes in Phe availability in plants, due to its branch-point position in the shikimate-chorismate pathway, and its sensitivity to feedback inhibition by Phe. Indeed, we had previously characterized all six ADT isoenzymes from Arabidopsis thaliana, and provided molecular and biochemical evidence supporting the arogenate route as the major mode of Phe biosynthesis (Fig. 1) (3). Specifically, three isoenzymes, ADT3, ADT4 and ADT5 demonstrated exclusive substrate preference for arogenate, while isoenzymes ADT1, ADT2 and ADT6 displayed instead a strong substrate preference for arogenate, but also had limited ability to utilize prephenate (3). Further confirmatory observations of a strong substrate preference for arogenate were subsequently made for one rice ADT isoenzyme (16) and three petunia ADT isoenzymes (17). Feedback inhibition of ADTs was also demonstrated to be an important factor influencing Phe biosynthesis, accumulation, and turnover as feedback insensitive ADTs in both rice and Arabidopsis were found to accumulate circa 55 and 160 times more Phe, respectively, compared to wild type (WT) (16). Thus, given the important role of ADT, and considering the vast range of uses for Phe in planta, it was instructive to determine if different isoenzymes potentially participated in distinct metabolic networks involving Phe.

In order to begin to delineate the potential individual physiological contributions of specific ADT isoenzymes, Arabidopsis lines containing knockouts (KOs) of single and multiple ADT genes were generated, with these then being initially analyzed for potential differential effects on phenylpropanoid metabolism (specifically lignification). It was thus established that lines with a combination of ADT4 and ADT5 KOs had profoundly altered lignin contents, including the various triple and quadruple KOs involving those isoenzymes, which gave even more pronounced effects. This is, therefore, the first demonstration that modulation of a network pathway step (ADT) upstream of phenylpropanoid metabolism, localized in plastids/chloroplasts, can differentially alter carbon allocation/flux into lignification (phenylpropanoid metabolism), versus formation of Phe for either protein synthesis or some other metabolic pathway.
EXPERIMENTAL PROCEDURES

All commercial kits were used according to the manufacturer’s instructions, with any minor deviations noted.

Generation and Confirmation of Single, Double, Triple and Quadruple ADT Knockout Lines—T-DNA insertion lines for all six ADT genes in Arabidopsis (supplemental Table S1), were obtained from either SIGnAL (18) or INRA (19). For each T-DNA insertion line, DNA was extracted from leaves of individual plants using the RedExtract kit (Sigma) with these samples then individually used as a template for two PCR reactions with different primer sets. For SALK lines, gene-specific left and right primers LP + RP, respectively, were used to amplify WT-specific PCR products, and left-border primer site “c1” (Lbc1) + RP were used to amplify T-DNA-specific PCR products (supplemental Table S1: Lbc1: 5′-CACAATCCCACTATCCTTCGC-3′). For the INRA line, the T-DNA-specific primer FLAG-LB (5′-GACGTAACATAAGGGACTGACC-3′) was substituted for Lbc1. Homozygous T-DNA insertion lines were identified as those having T-DNA-specific PCR products only, these being sequenced to confirm the presence and the specific site of each T-DNA insertion. ADT KO lines were confirmed using RT-PCR with primers designed to the 3′-end of each ADT mRNA transcript. Confirmed single KO lines were then crossed together to generate double heterozygous ADT KOs in all combinations, with double homozygous lines being identified in the subsequent generation using the same PCR screening approach described above. The same strategy was used to create triple and quadruple KO lines, using double and triple KO parental lines, respectively. Each double, triple and quadruple KO line was independently confirmed using the PCR strategy described above.

Complementation of adt5 line—Complementation was carried out by expressing ADT5 under control of its native promoter in the adt5 KO line. First, a 1,888 bp fragment upstream of ADT5 was amplified using promoter-specific primers (see supplemental Table S2). The ADT5 coding gene was then cloned into the pENTR™/D-TOPO® vector to generate entry constructs. After full sequence verification, the entry vector construct was subcloned into a pK2GW7 binary vector (20). The CaMV 35S promoter of the vector construct was next swapped for the adt5 promoter following established procedures (21). The confirmed construct was transformed into Agrobacterium and used to transform the adt5 KO line using standard protocols (22).

Arabidopsis Growth and Harvest Conditions—All confirmed homozygous KO, complementation and WT lines were grown in soil with four plants per pot in Washington State University greenhouses (16 h days, 27–28°C; 8 h nights, 24–26°C; 200 ppm nitrogen-based fertilizer added 5 days a week). For lignin analyses, the main stems of at least 48 plants were harvested weekly from after initial stem emergence up to maturity (~3.5 to 10 weeks). The weights and lengths of 20 inflorescence stems from each line were measured, with these then subsequently cut into 0.5 to 1 cm long pieces, lyophilized, and stored at room temperature prior to lignin analyses. For histochemical staining, two main stems for each ADT KO, and WT line were harvested at 7 weeks.

Real Time RT-PCR Analysis of ADT KO Lines—Stem tissue for WT and selected ADT KO lines were harvested 5 weeks after planting, flash frozen in liquid N2 and stored at -80°C until use. Frozen tissue was ground using a mortar and pestle, and ~90 to 110 mg was transferred to a 1.5 ml microcentrifuge tube. Total RNA was extracted using the Spectrum™ Plant Total RNA Extraction Kit (Sigma-Aldrich). RNA quantity and quality was assessed using a Nanodrop 2000c spectrometer (Thermo Fisher Scientific Inc.), and mRNA (1 μg) was reverse transcribed to cDNA using Superscript III (Invitrogen).

Gene-specific primers for each ADT isoform and housekeeping gene, TIP41-like (AT4G34270; (23)) were designed using Primer Premier 6.10 software (Premier Biosoft International) (see supplemental Table S2). The SYBR Green Real Time RT-PCR kit (Invitrogen) was used for real time RT-PCR reactions, with 0.05 μg cDNA and 62.5 pmol primers for each reaction. Triplicate reactions were run on a Mx 3505P Real Time Thermocycler (Stratagene), and data was analyzed with Mx Pro QPCR software (Stratagene).

Arogenate Dehydratase Assays—The following method from Jung et al. (1), modified by Maeda et al. (17), was applied for assaying ADT activity in
Arabidopsis stems. Approximately 20 g of stem tissue was harvested, immediately ground in liquid N$_2$, and extracted with 30 ml lysis buffer (20 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 35 mg leupeptin, and 35 mL of plant cell and tissue extract protease inhibitor cocktail [Sigma-Aldrich]). The crude lysate was then subjected to an ammonium sulfate precipitation, with both the 20–40% and 40–80% fractions collected. Each fraction was desalted with a PD-10 column (GE Healthcare) then concentrated to approximately 50 μl using an Amicon Ultra-4 Centrifugal Filter (Millipore). An aliquot (5 μl) of each protein extract (containing 30 μg and 370 μg for the 20 to 40% and 40 to 80% fractions, respectively) was added to the total volume of the 12 μl reaction mixture containing 250 μM arogenate and 20 mM Tris, pH 8.0. After incubation at 37°C for 15 min, the reaction was stopped by addition of 10 μl of MeOH with 2 μl of 10 mM alanine added as internal standard. The assay mixtures were vortexed and centrifuged, with half of the sample derivatized with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacacetamide and analyzed by GC-MS, and the other half being derivatized using the Pico-tag system (Waters), analyzed by HPLC as previously described (24).

Free Amino Acid Analysis—Total free amino acid pools were extracted from 5 week old WT and ADT KO stems, using methanol:chloroform: water (12:5:3), as previously described (24). Amino acids were derivatized with the AccQ•Tag Ultra Derivatization Kit (Waters) and analyzed by Ultra-Performance Liquid Chromatography (Waters). Phe and Tyr levels in WT were ~18 and ~12 pmol/mg dry weight, respectively, while those of the ADT KO lines ranged from 12–21 and 5–11 pmol/mg dry weight, respectively, suggesting no massive changes occurred in Phe/Tyr levels between WT and ADT KO lines.

Histochemical Staining—Histochemical staining and imaging of fresh hand-cut sections taken near the base of Stage 3 (25) (7 weeks old) stems of both mutants and WT lines were carried out as previously described by Patten et al. (26). For detection of presumed guaiacyl (G)-lignin, stem cross-sections were placed in a phloroglucinol-HCl (0.1%, w/v) solution for 30 min (27,28), transferred to a glass slide and observed under the differential interference contrast setting, using an Olympus System Microscope, Model BHT (Olympus Optical Co., Ltd, Tokyo, Japan). The Māule reaction was used for presumed syringyl (S)-lignin component detection; hand cut cross-sections of stems were treated for 10 min with KMnO$_4$ (0.5%, w/v, filtered through a 0.45 μm MillexHV filter) and rinsed with distilled H$_2$O (29). Samples were then treated with HCl (0.1%, v/v) for 5 min, rinsed, mounted in concentrated NH$_4$OH, with observation as described above.

Estimations of Lignin Contents and Compositions—Extractive-free stem cell wall residues (CWR) were obtained through extraction with EtOH:toluene (1:1, v/v), EtOH and distilled H$_2$O, as described previously (26,30-32). Estimations of lignin contents were made using the AcBr method (33), as described in Blee et al. (34) and adjusted for G:S monomeric ratios, using estimated AcBr extinction coefficients (280 nm) of 15.31, 18.61 and 14.61 g$^{-1}$ cm$^{-1}$ for p-hydroxyphenyl (H), G and S units, respectively (32). Cleavable monomeric (G/S) compositions and contents were also estimated by thioacidolysis using the general procedures of Lapierre et al. (35) and Rolando et al. (36), as described by Blee et al. (34), and quantified by comparison using standard curves with authentic standards. Pyrolysis GC/MS was carried out as previously described (26) using a pyrolyzer PYR-4A:Shimadzu interfaced with a
cloning into the primers, respectively, facilitated directional PciI restriction sites to forward and reverse each were amplified using the primer sets shown. Regions upstream of ADT4 and ADT5 (~1800bp each) were amplified using the primer sets shown in supplemental Table S2. Addition of PstI and PciI restriction sites to forward and reverse primers, respectively, facilitated directional cloning into the pCAMBIA 1305.2 vector. Confirmed pADT::GUS-pCAMBIA constructs were transformed into Agrobacterium for stable transformation of WT Arabidopsis (22). Second-generation transformed (T2) plants were treated with X-gluc for visualization of GUS expression as previously described (21,39). Stems were hand-sectioned and stained with 0.1mM X-gluc solution for 45 min. Images were taken using a high resolution light microscope (Leitz).

RESULTS

Generation and Gross Phenotype Comparisons of ADT Knockout Lines—T-DNA insertion lines for each ADT isoenzyme used in this study (supplemental Table S1) were obtained from either the SALK Institute Genomic Analysis Laboratory (SIGnAL) (18), or the Institut National de la Recherche Agronomique (INRA) (19). Following screening to obtain the various homozygous lines, it was determined that ADT1 and ADT3-6 had their corresponding mRNA transcripts apparently abolished (supplemental Fig. S2); these lines were thus considered as confirmed single KO lines. The exception was ADT2 (the sole line obtained from INRA) in which ADT2 mRNA was still present in the homozygous T-DNA insertion line, and this was not utilized further. The five confirmed single KO lines: adt1, adt3, adt4, adt5 and adt6 were then crossed together to generate double ADT KO lines in all combinations: adt1/3, adt1/4, adt1/5, adt1/6, adt3/4, adt3/5, adt3/6, adt4/5, adt4/6, and adt5/6, respectively. Similarly, confirmed homozygous double KOs were crossed together to create triple KOs, with adt1/3/4, adt1/3/5, adt1/4/5, adt3/4/5 and adt4/5/6 also being obtained. Additionally, an adt3/4/5/6 quadruple knockout was generated by crossing together adt3/4/5 and adt4/5/6.

The Arabidopsis growth/development time-frame involves three distinct stages (25), as previously described in the comprehensive study of the lignin pathway altered ref8, fah 1–2 and C4H::F5H Arabidopsis lines (26). During this time-frame, phenotypic assessment of the above ADT mutant lines at each weekly harvesting point was carried out, in order to identify single or double KO lines with potential reductions in lignin contents, alterations in stem lengths and weights, and/or presence of prostrate phenotypes. Of the single KOs, only adt5 had a slight decrease in stem weights and lengths (to ~90% of WT levels, Fig. 2, A and B), and had a partially prostrate (Fig. 3E) phenotype that was phenotypically distinct from WT (Fig. 3A). The remaining single KO lines, adt1, adt3 and adt4 each had relatively similar stem lengths and weights to WT over the course of growth and development (Fig. 2, A and B), and also did not appear visually to be phenotypically different from WT (Fig. 3, B–D). Additionally, one double KO, adt4/5, initially stood out as having reduced stem weights and lengths to ~82% of WT levels (Fig. 2, A and B), and a rather prostrate phenotype (Fig. 3F) relative to WT (Fig. 3A). Based on these observations, additional crosses were made to obtain two triple KOs that shared the same combination of adt4 and adt5; i.e. adt1/4/5 and adt3/4/5. Both triple KOs
also had obvious prostrate phenotypes (Fig. 3, G and H), and adt1/4/5 had similar stem weights and lengths to adt4/5, corresponding to ~85% of WT levels whereas adt3/4/5 displayed further reductions in both stem weight and length, corresponding to ~71% of WT levels (Fig. 2, A and B). Interestingly, the ADT isoenzymes ADT3, ADT4 and ADT5, which are in the same phylogenetic cluster, ‘subgroup III’ (3), appeared to cause the greatest phenotypic effect, and therefore it was instructive to test if the fourth ADT in that subgroup (ADT6) caused any further changes. Thus, we also obtained a quadruple KO, adt3/4/5/6, in which all members of subgroup III (3) were disrupted. This line had further reductions in stem weights and lengths (Fig. 2, A and B) corresponding to ~67% of WT levels, as well as displaying a prostrate phenotype (Fig. 3I).

Based on these phenotype analyses, the single KOs adt1, adt3, adt4 and adt5, double KO adt4/5, triple KOs adt1/4/5 and adt3/4/5, and quadruple KO adt3/4/5/6 were subjected to both histochemical and lignin analyses in order to assess the individual and/or combinatorial contribution of these genes to lignification. Lignin analyses (estimated lignin contents and monomeric compositions) were carried out weekly (see below).

**ADT Expression Levels in Selected ADT Knockout lines**—Real-time RT-PCR was used to further verify that ADT transcripts were either not detectable or greatly reduced in each corresponding ADT KO line and, additionally, to identify any potential increased expression in non-targeted ADTs (supplemental Fig. S3). The relative mRNA expression levels of all six ADTs were also measured in WT and each of the eight selected KO lines described above. For each of these lines, ADT transcripts for each corresponding knocked out gene were absent, except for ADT1 and ADT6, which were reduced to <25% WT levels (supplemental Fig. S3). It is possible that the latter two genes are able to produce a small amount of transcript, despite being homozygous for T-DNA insertions, since the insertions in ADT1 and ADT6 are present in the 1st intron and the 5′-UTR, respectively, and may allow for a small amount of correctly processed mRNA to be transcribed from these genes. Similar findings have been described elsewhere for T-DNA insertion mutants (40).

Consistent with the findings by Rippert et al. (41), ADT4 and ADT5 had the highest expression levels in WT stems, with the remaining ADTs also being expressed in stems, but at lower levels (supplemental Fig. S3). Expression of these two genes was increased slightly, but perhaps significantly, compared to WT in certain lines: ADT4 expression was increased in the adt3 and adt5 KO lines, while ADT5 was increased in the adt3 KO, while no change in expression was observed in the adt1 and adt4 KO lines. Small increases in expression were also noted for ADT1, ADT2 and ADT3, for certain ADT KO lines (supplemental Fig. S3), while there were no apparent increases observed for ADT6 in any lines.

As a decrease in expression levels does not (necessarily) translate directly to a decrease in overall ADT activity, we also attempted to measure ADT activity in Arabidopsis stems. While ADT activity has been detected in certain species (1,2) it has never been described in Arabidopsis. Nevertheless, crude enzyme extracts were performed using WT stem tissue, using the modified protocol described by Maeda et al. (17). Both arogenate and prephenate were tested as substrates; however, in both cases no ADT or PDT activity could be observed (data not shown). Since no activity could be detected in WT stems, the KO lines were not assayed since they were expected to have even lower levels of ADT activity. A small decrease in the levels of free Phe was observed, however, in the stems of each KO line as compared to WT, with the exception of adt1 and adt3. These changes were observable in 5 week old stem tissue, but by 7 weeks (when lignification was complete) they had returned to WT levels (data not shown, Corea et al., unpublished).

**Histochemical Analyses of Single and Multiple ADT Knockouts**—Qualitative histochemical analyses of 7 week old basal stem sections (Fig. 4 and Supplemental Fig. S4), for both staining of guaiacyl (G) and syringyl (S) lignin containing phenotypes, were also carried out using phloroglucinol-HCl (for G) (26-28) and Mäule (for S) (26,29) reagents, respectively. G-lignin component staining, used frequently for detection of coniferoyl-alcohol derived moieties (see Fig. 5 for structures), was near identical throughout the vb and if regions of WT, adt1, adt3 and adt4 lines (supplemental Fig. S4, A–D) lines, whereas
presumed G moieties were apparently less readily detectable in if regions of adt5, adt4/5, adt1/4/5, adt3/4/5 and adt3/4/5/6 (Fig. 4, B–F), as gauged by the decreased levels of red-pink staining in these cross-sections. Also apparent in the phloroglucinol-HCl treated sections were irregularly-shaped and partially collapsed cell walls in the metaxylem (mx) within the vb of adt1/4/5 and adt3/4/5 lines (Fig. 4, H and I), in contrast to the WT line where this deformation was not evident (Fig. 4G). There was little, if any, visible difference in presumed S (sinapyl alcohol derived) moieties for any of the KO lines using the Mäule reagent (Fig. 4, K–O and supplemental Fig. S4, F–H), as compared to WT (Fig. 4J and supplemental Fig. S4 E).

**Estimated Lignin Contents/Compositions of Single and Multiple ADT Knockouts**—The potential effects on lignification in the various ADT KO lines were next assessed, relative to the WT line, with stems from each plant line harvested weekly. Extractive-free stem cell wall residues (CWR) were thus subjected to “acetyl bromide (AcBr) lignin” (33,34) and thioacidolysis (34-36) analyses, in order to estimate gross lignin contents/compositions. Note though that “AcBr lignin” methods can over-estimate lignin contents of ~24.2% CWR. The other single KOs adt1, adt3 and adt5 were, however, slightly reduced compared to WT, with ~257, 244 and 250 μmol/g CWR, respectively) and quadruple KO, adt3/4/5/6 (~95 μmol/g CWR). Their amounts ranged from ~27 – 16% by weight of the estimated AcBr lignin content. The other single KOs adt1, adt3 and adt5 were, however, slightly reduced compared to WT, with ~257, 244 and 250 μmol/g CWR, respectively. Larger reductions were also observed for the double KO, adt4/5 (~147 μmol/g CWR), triple KOs, adt1/4/5 and adt3/4/5 (~116 and ~88 μmol/g CWR, respectively) and quadruple KO, adt3/4/5/6 (~95 μmol/g CWR). Their amounts ranged from ~27 – 16% by weight of the putative lignin present, with such reductions in releasable monomers being frequently observed when overall lignin contents are reduced.

The adt5 line was, however, significantly decreased in G cleavable monomers (Fig. 5C), but apparently had slightly higher levels of S
monomers compared to WT (Fig. 5D). Similarly, the double adt4/5 KO had even more pronounced reductions in G monomer levels released, with little effect on the S amounts compared to WT. However, in the corresponding triple and quadruple KOs, adt1/4/5, adt3/4/5 and adt3/4/5/6, there were further reductions in both G and S releasable monomers, compared to the adt4/5 KO. Again, the adt5 line complemented with the native ADT5 gene resulted in restoration of G+S levels (~310μmol/g CWR), and G:S ratios to that of WT levels.

Pyrolysis GC/MS Analyses—It was next instructive to compare the lignin-derived pyrolysis products released from S-enriched if and G-enriched vb tissues using both WT and adt1/4/5 lines, in a manner as previously successfully carried out on ref8, fah 1-2 and C4H::F5H lines (26). First, WT and adt1/4/5 stem CWR samples were individually subjected to pyrolysis GC/MS and analyzed. Products were identified by either retention time, co-elution with authentic standards, and mass spectroscopic fragmentation data. Simple inspection of releasable lignin-derived pyrolysis products in these two GC/MS chromatograms indicated substantial reductions in G and S components (relative to H-derived moieties) in the adt1/4/5 line (Fig. 6B) on comparison to WT (Fig. 6A), with the largest reduction being with G-derived components. That is, in the WT line, the pyrolysis products identified were of H (peaks 1–3, 5 and 8), G (peaks 4, 6, 7, 10, 11, 14, 15, 17, 19, 24 and 29) and S (peaks 13, 20 and 23), respectively, with the G-components most abundant (Fig. 6A, Table 1 and supplemental Fig. S1). By contrast, the pyrolysis products obtained for the adt1/4/5 CWR detected the presence of H- (peaks 1–3, and 5), G- (peaks 4, 6, 7, 10, 11, 14, 15 and 19) and S- (peaks 13, 20, 23 and 26) with significant reduction in both G/S derived products relative to H-moieties (Fig. 6B, Table 1 and supplemental Fig. S1). These data thus provisionally agreed with thioacidolysis results that also indicated a greater reduction in G-lignin-derived monomers as compared to S-monomeric moieties in the adt1/4/5 line.

Next, laser micro-dissection was employed to excise circa 5000 individual vb and if sections from the WT and adt1/4/5 lines, with these also being subjected to pyrolysis GC/MS. Analysis of the vb regions of adt1/4/5 (Fig. 6D, Table 1 and supplemental Fig. S1) resulted in identification of H- (peaks 1–3 and 5), G- (peaks 4, 6, 10, 11, 15, 17 and 19) and S- (peaks 13, 20 and 23) pyrolysis fragments, with both G and S moieties being significantly reduced relative to the H-derived components (with the S-moieties being barely detectable). By contrast, the analysis of the if regions gave a chromatogram with H- (peaks 1–3 and 5), G- (peaks 4, 11 and 17) and S- (peaks 13, 20 and 21) pyrolysis fragments (Fig. 6C, Table 1 and supplemental Fig. S1). In this case, however, the relative amounts of S-components still remained high, with the most notable effect being on G-component reductions. These results are thus consistent with histochemical staining using phloroglucinol, which indicated that the reduction was greatest in the G-lignin constituents of the if.

GUS-expression patterns in stems—Expression of ADT4 and ADT5 was visualized using the GUS expression system. Putative promoter regions for ADT4 and ADT5 were fused in-frame with the GUS gene and stably transformed into WT Arabidopsis (see Experimental Procedures). Staining patterns were observed in 4-week stem cross sections. ADT4 and ADT5 were shown to have overlapping expression patterns, which were localized to the vascular cambium regions in the vb (Fig. 7A and B, respectively). Staining was not observed in the interfascicular cambium or other regions of the stem.

DISCUSSION

ADT Manipulations and Phenotypic Effects—The possible differential contribution of distinct ADTs in impacting upon carbon flux into the lignin pathway was investigated given its branch point position between shikimate-chorismate and phenylpropanoid metabolism. It was confirmed by real-time RT-PCR that ADT mRNA transcripts were below or near below detection from each corresponding ADT KO line examined with the exception of ADT2 (supplemental Fig. S3). The latter was not successfully knocked out (see Experimental Procedures). Vastly reduced levels of ADT1 transcript were observed in adt1 and adt1/4/5, while a small amount of ADT6 transcript was found in adt3/4/5/6; in each case, their levels were significantly reduced though compared to WT.
While slight increases in non-targeted ADTs were observed in certain ADT KO lines, there was no apparent trend for increased ‘compensation’ in the double, triple and quadruple KOs, relative to the single KOs. This was clearly demonstrated in the adt3/4/5/6 KO line, for which ADT3, ADT4, ADT5 and ADT6 transcript levels were decreased/abolished, but the remaining ADT1 and ADT2 transcript levels were apparently identical to those of WT. These results suggest the observed phenotypes are a direct result of diminished ADT gene expression levels in the knocked out ADTs. While we were unable to confirm that the decrease in ADT expression led to a reduction of ADT activity in planta, the decrease in ADT expression is fully consistent with the phenotypes observed for these plants.

Phe pool sizes were also slightly affected in adt4, adt5, adt4/5, adt1/4/5, adt3/4/5, adt3/4/5/6, but only during the time of growth and development where lignification was occurring; later in development, when lignification slowed, there was no detectable difference compared to WT (Corea et al., manuscript in preparation).

Depending upon the plant line generated, there were relatively significant, but distinct, reductions in stem lengths (to a range of circa 70-83% of WT, see Fig. 2B), when adt5 was ‘knocked out’ in combination with adt4, adt1/4, adt3/4, and adt3/4/6, respectively. This was also noted in dry weight stem tissue determinations which showed reductions to circa 86 and 76% of WT levels for adt1/4/5 and adt3/4/5, relative to WT. Such relatively small effects on biomass production, however, contrast with numerous other studies on monolignol pathway step modulations. The latter frequently result in, for example, extremely dwarfed plant lines (with greatly reduced biomass) and significantly compromised vasculature (for a discussion see 10).

Comparison of Histochemical and Pyrolysis GC/MS Analyses of Vascular Bundles and Interfascicular Fibers—Guaiacyl and syringyl entities are often qualitatively detected using either the histochemical staining reagent, phloroglucinol - HCl, for G-components in lignified tissues, or the Mäule reagent for S-derived lignin moieties. Histochemical staining of the various lines generated herein (adt1, adt3, adt4, adt5, adt4/5, adt1/4/5, adt3/4/5 and adt3/4/5/6) on comparison to WT, thus provided some useful insights into the limitations of these staining protocols. Specifically, the double, triple and quadruple mutants containing the adt5 knockout gave essentially no indication that G-moieties were present in if regions, whereas the vb moieties were positively even though there was considerable distortion/weakening of the metaxylem (mx) cell walls (Fig. 4, B–F). By contrast, for each of the lines examined using the Mäule reagent, S-staining was notably detected in if cell walls, as well as in fiber-containing cell walls within the vb (Fig. 4, K–O). These qualitative data thus suggested the absence of G-moieties in the if regions.

Pyrolysis GC/MS of stem cross-sections (CWRs) of both WT and adt1/4/5 were also carried out, and these resulted in facile detection of various H, G, and S-derived monomers (Fig. 6, A and B; Table 1). By comparison to the H-derived constituents, however, the levels of both G- and S-derived moieties were much reduced overall in the adt1/4/5 line, with the highest reduction being in G-derived moieties (Fig. 6B). Laser-microscope dissected if and vb sections of both WT and adt1/4/5 lines, when subjected to pyrolysis GC/MS, next provided considerable insight into the type of lignins present in these distinct anatomical regions. For the if region of adt1/4/5, it was evident (relative to H-derived monomers) that the amounts of G-derived pyrolysis products were substantially diminished relative to S-derived moieties, with the latter being comparatively more readily detectable (Fig. 6C). Thus, the if regions still contained a S-enriched lignin even though overall lignin amounts were substantially reduced. Failure to detect G-moieties in if regions of some of these mutants by histochemical staining thus demonstrates a serious limitation in this qualitative staining protocol, when G-levels are low (relative to S- and H-derived moieties).

Laser microscope dissection/pyrolysis GC/MS of WT if regions resulted in facile detection of H- (peaks 1 and 3), G- (peaks 4, 6, 7, 10, 11, 14, 15, 17–19 and 24) and S- (13, 20–23, 27 and 28) lignin derived moieties, with the G- and S-moieties predominating relative to the H-derived component (data not shown, see Patten et al. (26)). By contrast, analysis of the if regions of the adt1/4/5 indicated substantial reductions in G-component release, with both S and H component
matured and ultimately senesced (Fig. 8A) stages (sampled weekly) until the plant stems of the different lines generated at different growth thioacidolysis released G + S monomer levels in (adt1/4/5) (10,26). At maturation, however, the estimated lignin contents, as previously observed examined essentially gave linear increases overall variation in samples tested, all of the lines Although there was considerable experimental compositions and contents were also systematically studied over a period of 3-10 weeks, reflecting the three phases of Arabidopsis growth/development until maturation and senescence (25,26). As indicated above, massive, yet differential, reductions in lignin contents and compositions were observed, via manipulation of this plastid-localized enzyme family.

We next plotted out correlations between estimated “AcBr lignin” contents versus thioacidolysis released G + S monomer levels in the different lines generated at different growth stages (sampled weekly) until the plant stems matured and ultimately senesced (Fig. 8A). Although there was considerable experimental variation in samples tested, all of the lines examined essentially gave linear increases overall in releasable G + S monomer amounts relative to estimated lignin contents, as previously observed (10,26). At maturation, however, the adt4/5 (▲), adt1/4/5 ( ●), adt3/4/5 ( ●) and adt3/4/5/6 ( ●) lines had G + S monomer release/“AcBr lignin” levels that did not surpass the 4 week old levels for the WT line. As documented elsewhere (26), there was also an initial ~5% “AcBr lignin” deposition, where essentially no G + S moieties are released; this early stage “AcBr lignin”, presumed H-derived (from p-coumaryl alcohol), can also include other non-lignin components, as discussed in Jourdes et al. (32) and Davin et al. (10). Most importantly though, the essentially linear correlations of G + S monomer release versus estimated lignin contents are considered indicative of a biochemical process in place leading to (near) conservation of the 8–O–4’ inter-unit linkage frequency in these various lignins. This again reflects control exercised over lignin macromolecular assembly (10).

It was instructive to also compare G and S monomer release from the different lines generated. As discussed earlier above (Results), the adt5 KO ( ●) had the largest (of any single adt mutant) reductions in lignin contents and thioacidolytic monomer release (Fig. 8, B and D), relative to WT. However, the effect on reductions in monomer release essentially only impacted G-monomer levels (circa 74% of WT, Fig. 8B) but not that of the S-constituents, which (if anything) were slightly increased (Fig. 8D). These data thus provisionally suggested that, at a minimum, essentially the same amount of cleavable S-lignin-derived monomers was being generated in the adt5 line as for WT. The overall amounts of G and S moieties released from adt1 ( ●), adt3 ( ●), and adt4 ( ●) lines were also similar to that of WT at maturity, albeit with a slight increase in levels of G- moieties for adt4 (Fig. 8, B and D). In the latter case, there also appeared to be a small increase in total ‘AcBr’ lignin contents (circa 10% more relative to WT). Given the large G-monomer reduction levels in adt5, but not on S-levels, these data provisionally suggested that the primary effect was on the lignin forming biochemical machinery in the G-enriched vb rather than in the various regions containing fiber cells.

Examination of the double, triple and quadruple Kos (Fig. 8, C and E), all of which contained the adt5 KO, was also very informative, as this added and extended to the observations made above. As indicated earlier, the first of these, adt4/5 ( ▲), displayed a quite pronounced prostrate phenotype (Fig. 3D), and also at maturity had ‘AcBr’ lignin contents estimated to be ~61% of WT (Fig. 5A). However, at maturity, there was again essentially no difference in the amount of releasable S-monomers, relative to WT (Fig. 8E), whereas by contrast the G-monomers released were reduced to circa 35% of WT levels (Fig. 8C). These data were thus again consistent with the primary target for lignin and G-monomer reduction being within the vb region.

For the triple (adt1/4/5 and adt3/4/5) and quadruple (adt3/4/5/6) knockouts, the releasable G-monomer levels were reduced further down to
order to maintain physiological integrity, and that relatively modest lignin reductions can occur in levels. These data thus again suggest that only due to their decreased lignin levels, relative to WT resulting phenotypes were, however, substantially pathway was simply significantly reduced. The manipulations was that carbon allocation to the lignin biosynthesis (42,43).

Such differential effects on lignin deposition are striking, however, given the different subcellular localizations of ADTs in chloroplasts/plastids for Phe formation versus monolignol pathway enzymes being cytosol/membrane localized. This is the first time though that a plastid/chloroplast localized upstream step involving the shikimate-chorismate pathway has been shown to have specific isoenzymes dedicated to profoundly and differentially directing carbon flux into Phe for lignin biosynthesis (42,43).

That is, importantly, the effect of these manipulations was that carbon allocation to the pathway was simply significantly reduced. The resulting phenotypes were, however, substantially weakened from a structural vasculature viewpoint due to their decreased lignin levels, relative to WT levels. These data thus again suggest that only relatively modest lignin reductions can occur in order to maintain physiological integrity, and that loss of physiological function occurs when lignin levels are significantly decreased. This, in turn, demonstrates the limitations in plasticity of physiological responses/structural properties when lignins are not fully deposited.

Towards Identifying Physiological Roles of ADT Isoenzymes and ADT Phylogeny—Our earlier phylogenetic tree comparisons (3) also indicated that ADT3 and ADT6 are in the same cluster (subgroup III) as ADT4 and ADT5, whereas ADT1 and ADT2 are in subgroups 1 and 2, respectively. All six Arabidopsis ADT genes were though found to be expressed in stems, leaves, roots, flowers, siliques and seeds (3,41). Of these, ADT2 was the most highly expressed in leaves and seeds, and it was also suggested earlier to have a “housekeeping” role in Phe biosynthesis (41). By contrast, ADT4 and ADT5 were more highly expressed in stems and roots, whereas the remaining isoenzymes had generally much lower levels of expression (supplementary Fig. S3) (41). Co-expression of ADTs with other Arabidopsis genes was also explored using the Botany Array Resource Expression Angler (44); each of the six ADTs was used as bait and this showed evidence for differential transcriptional regulation of the ADTs. ADT3, ADT4, ADT5 and ADT6 were provisionally co-regulated with numerous shikimate, phenylpropanoid, and aromatic amino acid biosynthesis genes (supplemental Table S4A). Of particular significance, in light of the findings herein, ADT4 and ADT5 are provisionally co-expressed with at least one gene of each step in the monolignol pathway between Phe and the three monolignols, p-coumaryl, coniferyl and sinapyl alcohol, with the exception of F5H (supplemental Table S4A). By contrast, ADT1 and ADT2 were mainly involved in basic cellular functions, such as transcription, translation, cell division, and nucleic or amino acid biosynthesis (supplemental Table S4B).

Together, transcriptional expression patterns and co-expression data strongly support the finding that ADT4 and ADT5 function primarily in Phe biosynthesis as a precursor for lignin. When these two genes were knocked out in combination, a differential reduction in G- and S- monomers was now also reduced to circa 72, 58 and 67% of WT amounts, respectively (Fig. 8E). Thus, with the triple and quadruple knockouts there was now clearly also an effect on S-monomer amounts and hence on (S) lignin deposition in the fiber cells. Overall, the lignin contents in these lines were also significantly reduced down to ~52, 50 and 32% of WT levels, respectively, this representing some of the largest reductions in lignin levels ever reported in genetic manipulations.

Taken together, these data again point to different modulation of ADT-regulated carbon flux into distinct anatomical regions (vb versus fiber cells). As a result, it was not unexpected that the G:S ratios in the lignins so obtained also changed markedly with different KO lines from ~3:1 (WT) to ~1:1 (adt3/4/5/6). This was because these cell types still contained the biochemical machinery to predominantly generate G- and S-derived monomers, respectively, i.e. thereby generating different amounts of G- and S-lignins.

In this respect, the GUS expression patterns of both ADT5 and ADT4 indicated that they were localized to the vascular cambium adjacent to the vb (Fig. 7), whereas the others were localized as relatively faint bands throughout the cambial regions (data not shown). Thus, this expression data is also consistent with the analyses of the vb, and the substantial decrease in G-levels in same.

Such differential effects on lignin deposition are striking, however, given the different subcellular localizations of ADTs in chloroplasts/plastids for Phe formation versus monolignol pathway enzymes being cytosol/membrane localized. This is the first time though that a plastid/chloroplast localized upstream step involving the shikimate-chorismate pathway has been shown to have specific isoenzymes dedicated to profoundly and differentially directing carbon flux into Phe for lignin biosynthesis (42,43).

That is, importantly, the effect of these manipulations was that carbon allocation to the pathway was simply significantly reduced. The resulting phenotypes were, however, substantially weakened from a structural vasculature viewpoint due to their decreased lignin levels, relative to WT levels. These data thus again suggest that only relatively modest lignin reductions can occur in order to maintain physiological integrity, and that
both ADT4 and ADT5 (Fig. 7). In the latter case, both genes were specifically localized to the vascular cambium, which is adjacent to the G-rich vb. Together, these results indicated that a difference in the ratio of these lignin monomers is due to the distinctive localization pattern of these genes. Yet while knocking out all four genes in subgroup III resulted in the greatest reductions in lignin levels, the adt3/4/5/6 quadruple KO did not significantly reduce further the remaining estimated ~29% lignin (present in the adt3/4/5 KO) as gauged by G + S monomer release. That is, there was no complete depletion of releasable G- and S- monomers in the various knockout lines examined, this, in turn, raising interesting questions possibly about the role of the remaining isoenzymes, including ADT2. While we currently envisage a primary role for it in protein formation, it is also possible that the H-components in lignin are largely derived from ADT2 generated Phe, as well as being responsible for some of the carbon in the “residual” G + S lignins being formed.

It thus also now remains to be comprehensively and systematically established as to which specific ADT genes are differentially expressed within specific cell types of Arabidopsis tissues and organs, what their physiological roles are more precisely, and how these metabolic networks are organized and regulated.

CONCLUSIONS

The roles of specific plastid/chloroplast localized ADTs in differentially modulating carbon flux into lignin biogenesis into distinct anatomical regions of the vasculature now provides an exciting opportunity to further unravel the complexities of lignin biosynthesis at the cellular and tissue levels. This is of particular interest given the plastid/chloroplast localization of ADTs, versus the phenylpropanoid enzyme localizations in the cytosol (including membrane associated processes), and cell-wall lignification itself. Indeed, the selective “conscription” of specific ADTs for lignin formation in different cell types may also represent an important evolutionary point during the transition of aquatic plants to a land-based environment. Accordingly, our studies provide a wonderful opportunity to establish in future: Specific roles of each ADT in various aspects of phenylpropanoid and lignin forming metabolic processes, including detailed metabolomic analyses, and yet a further means to probe lignin primary structure (45), as well as the physiological consequences of lignin deposition reduction. Furthermore, these findings provide a means to study to what extent lignin levels can be reduced without affecting overall physiological functions. These studies also provide new insight into additional factors affecting G:S ratios given that differential carbon flux into distinct anatomical regions (vb versus if/fiber cells) resulted in alteration in G:S ratios from 3:1 (WT) to 1:1 for adt3/4/5/6. Once again, however, the reduction of carbon flux into the lignin pathway results in a very unplastic physiological response, in terms of, for example, impaired stem integrity resulting from lignin reduction.

Acknowledgments—The authors express gratitude to: Julianna Gothard for growing Arabidopsis wild type and mutant plants; Ericka Duncan, Victor Barona, Andres Lozano Garcia and Luis Oleas Chavez for laser microscope dissection of vascular bundles and interfascicular fibers; and to Diana Bedgar for arogenate dehydratase assays. Thanks are also extended to Dr. Michael Knoblauch and Dr. Valerie Lynch-Holm of the Franceschi Microscopy and Imaging Center for use of their microscopy facility.

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FOOTNOTES

*This work was mainly supported by a grant from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences (DE-FG-0397ER20259) which involved all of the lignin, laser microscope dissection, and pyrolysis GC/MS work. Support from the BioEnergy Science Center, the U.S. Department of Energy Bioenergy Research Center supported by the Office of Biological and Environmental Research in the DOE Office of Science, and the United States Department of Agriculture (#683A757612), as well as by the G. Thomas and Anita Hargrove Center for Plant Genomic Research, is greatly appreciated. This support enabled some of the mutant lines to be screened/selected for subsequent analyses. Complementation studies were supported by the USDA-NIFA supported Northwest Advanced Renewable Alliance (#2011-68005-30416).

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3The abbreviations used are: ADT, arogenate dehydratase; H, p-hydroxyphenyl; G, guaiacyl; KO, knockout; S, syringyl; WT, wild type.
FIGURE LEGENDS

FIGURE 1. Proposed biosynthetic pathways to Phe, Tyr and Trp in plants, where the body of evidence supports the aromate pathways to Phe/Tyr.

FIGURE 2. Growth and development parameters of Arabidopsis WT and ADT KO lines. A, stem fresh/dry weights and B, lengths measured weekly from 3.5 to 10 weeks with an average of >20 stems. Trend lines were estimated using a polynomial line of best fit. Selected examples of tissue dry weights are also provided (Fig. 2A).

FIGURE 3. Plant phenotypes at 5 weeks of growth/development. WT (A), adt1 (B), adt3 (C) and adt4 (D) lines displayed similar upright phenotypes, whereas adt5 (E), adt4/5 (F), adt1/4/5 (G), adt3/4/5 (H) and adt3/4/5/6 (I) stems were weakened, and unable to fully support their weight. Scale bars = 10 cm.

FIGURE 4. Histochemical staining of stems from 7 week old WT and ADT KO lines. WT (A, G, J), adt5 (B, K), adt4/5 (C, L), adt1/4/5 (D, H, M), adt3/4/5 (E, I, N) and adt3/4/5/6 (F, O) were treated with phloroglucinol-HCl (A–I) and Mäule (J–O) reagents. Positions of the interfascular fiber (if) regions, and vascular bundles (vb) are indicated in WT (A, J). Increased magnification of the vb in A, D and E are shown in G, H and I, respectively, with metaxylem (mx), protoxylem (px) and xylem fibers (xf) labeled in G. Fainter staining of G-moieties was detected using phloroglucinol-HCl in the if regions of adt5, adt4/5, adt1/4/5, adt3/4/5, and adt3/4/5/6 (C–F, respectively), whereas there was no apparent decrease in the vb in any KO lines. However, increased magnification (G–I) indicated that mx cell wall integrity is affected in triple KO lines (H and I), with numerous irregularly shaped/partially collapsed vessels (denoted by *) present in these lines. Scale bars: 50 μm.

FIGURE 5. Estimated lignin content/compositional analyses of Arabidopsis WT and ADT KO lines. Estimation of lignin contents using “AcBr” (A), and levels of thioacidolysis G + S- (B), G- (C) and S- (D) derived lignin monomeric cleavage products released as a function of time (growth and development), with structures of monolignols, p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) and their corresponding derivatives following thioacidolysis (E).

FIGURE 6. Pyrolysis GC/MS chromatograms of WT and the adt1/4/5 KO line, showing H-, G and S-derived pyrolysis products (indicated in red, blue and green, respectively). Cell wall residues from WT (A) and adt1/4/5 (B) stem tissues, together with laser-microdissected interfascicular regions (C) and vascular bundles (D) from adt1/4/5. [For peak numbers, see Table 1, and for structures see supplemental Fig. S1.]

FIGURE 7. GUS expression patterns for ADT4 and ADT5 in 4-week old Arabidopsis stem cross-sections. A, pADT4::GUS expression pattern. B, pADT5::GUS expression pattern. Native promoters from both genes drove expression of GUS specifically in the vascular cambium (vc) region adjacent to the vascular bundle (vb), but not in the cambium adjacent to the interfascicular fiber (if) region. Other distinct anatomical regions of the stem are indicated as follows: cortex (co), epidermis (ep), interfascicular cambium (ic) region, pith (pi), phloem (ph), and phloem cap (pc). Scale bars: 50 μm.

FIGURE 8. Comparison of thioacidolysis-determined G- and/or S- lignin-derived monomer contents versus total “AcBr lignin” contents, for single KO lines, adt1, adt3, adt4, and adt5, as well as multiple KO lines, adt4/5, adt1/4/5, adt3/4/5 and adt3/4/5/6. Total G+S-derived thioacidolysis monomeric degradation products compared to total “AcBr-lignin” for all single and multiple KO lines (A). G-derived thioacidolysis degradation products compared to total “AcBr-lignin” for single (B) and multiple (C) KO lines. S-derived thioacidolysis degradation products compared to total “AcBr-lignin” for single (D) and multiple (E) KO lines.
### TABLE 1

Pyrolysis GC/MS lignin-derived products from *Arabidopsis* WT and *adt1/4/5* cell wall residues (CWR), as well as laser microdissected vascular bundles (vb) and interfascicular fibers (if)

| Compound                        | Unit | Ions                  |
|---------------------------------|------|-----------------------|
| 1 Phenol                        | H    | 94, 66, 65            |
| 2 4-Methylphenol                | H    | 108, 107, 90, 79, 77,51 |
| 3 3-Methylphenol                | H    | 108, 107, 90, 79, 77,51 |
| 4 Guaiacol                      | G    | 124, 109, 81, 53      |
| 5 4-Ethylphenol                 | H    | 122,107, 77, 51       |
| 6 4-Methyguaiacol               | G    | 138, 123, 95, 77, 67  |
| 7 Catechol                      | G    | 110, 92, 81, 64, 63   |
| 8 4-Vinylphenol                 | H    | 120, 119, 91, 65      |
| 9 4-Allylphenol                 | H    | 134,133, 107, 105, 91, 77 |
| 10 4-Ethylguaiacol              | G    | 152, 137, 122         |
| 11 4-Vinylguaiacol              | G    | 150, 135, 107, 77     |
| 12 trans-4-propenylphenol       | H    | 134,133, 105, 77      |
| 13 2,6-Dimethoxyphenol          | S    | 154, 139, 111, 96, 65 |
| 14 Eugenol                      | G    | 164, 149, 137,131, 103, 91, 77 |
| 15 Vanilllin                    | G    | 152, 151, 123, 109, 81 |
| 16 2,6-Dimethoxy-4-methylphenol | S    | 168, 153, 125, 65     |
| 17 trans-isodeugenol            | G    | 164, 149, 131, 103, 91, 77 |
| 18 Acetovanillone               | G    | 166, 151,123          |
| 19 Guaiacylacetone              | G    | 180, 137, 122         |
| 20 2,6-Dimethoxy-4-vinylphenol  | S    | 180, 165, 137, 122    |
| 21 4-Allyl-2,6-dimethoxyphenol  | S    | 194, 179, 147, 131, 119, 91, 77 |
| 22 Syringaldehyde               | S    | 182, 167, 153, 139, 111, 93 |
| 23 trans-2,6-dimethoxy-4-       | S    | 194, 179, 151, 131    |
| propanophenol                   |      |                       |
| 24 trans-coniferol alcohol      | G    | 180, 137, 124         |
| 25 Acetosyringone               | S    | 196, 181, 153         |
| 26 Syringylacetone              | S    | 210, 167, 123, 69     |
| 27 trans-sinapyl aldehyde       | S    | 208, 180, 177, 165, 137 |
| 28 trans-sinapyl alcohol        | S    | 210, 167, 154, 149    |
| 29 Coniferaldehyde              | G    | 178, 161, 147, 135    |

a n.d.: not detected
Figure 1
Figure 3
Figure 5
Figure 6
Figure 7
Figure 8
Arogenate dehydratase isoenzymes profoundly and differentially modulate carbon flux into lignins

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*J. Biol. Chem.* published online February 6, 2012

Access the most updated version of this article at doi: [10.1074/jbc.M111.322164](http://doi.org/10.1074/jbc.M111.322164)

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