A coumaroyl-ester-3-hydroxylase Insertion Mutant Reveals the Existence of Nonredundant meta-Hydroxylation Pathways and Essential Roles for Phenolic Precursors in Cell Expansion and Plant Growth

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Cytochromes P450 monooxygenases from the CYP98 family catalyze the meta-hydroxylation step in the phenylpropanoid biosynthetic pathway. The ref8 Arabidopsis (Arabidopsis thaliana) mutant, with a point mutation in the CYP98A3 gene, was previously described to show developmental defects, changes in lignin composition, and lack of soluble sinapoyl esters. We isolated a T-DNA insertion mutant in CYP98A3 and show that this mutation leads to a more drastic inhibition of plant development and inhibition of cell growth. Similar to the ref8 mutant, the insertion mutant has reduced lignin content, with stem lignin essentially made of p-hydroxyphenyl units and trace amounts of guaiaeryl and syringyl units. However, its roots display an ectopic lignification and a substantial proportion of guaiaeryl and syringyl units, suggesting the occurrence of an alternative CYP98A3-independent meta-hydroxylation mechanism active mainly in the roots. Relative to the control, mutant plants produce very low amounts of sinapoyl esters, but accumulate flavonol glycosides. Reduced cell growth seems correlated with alterations in the abundance of cell wall polysaccharides, in particular decrease in crystalline cellulose, and profound modifications in gene expression and homeostasis reminiscent of a stress response. CYP98A3 thus constitutes a critical bottleneck in the phenylpropanoid pathway and in the synthesis of compounds controlling plant development. CYP98A3 cosuppressed lines show a gradation of developmental defects and changes in lignin content (40% reduction) and structure (prominent frequency of p-hydroxyphenyl units), but content in foliar sinapoyl esters is similar to the control. The purple coloration of their leaves is correlated to the accumulation of sinapoylated anthocyanins.

Plants synthesize a wide variety of natural products based on the phenylpropane skeleton derived from Phe (Petersen et al., 1999). These compounds have an array of important functions, which are not yet fully evaluated or understood and involve a variety of ecological and physiological phenomena (Stafford, 1990; Debeaujon et al., 2000). The derivatives of hydroxycinnamic acids are essential intermediates in lignification and influence the physicochemical properties of the cell walls (Boerjan et al., 2003). Sinapoyl esters function to protect Arabidopsis (Arabidopsis thaliana) from UV irradiation (Landry et al., 1995; Booij-James et al., 2000) and caffeoyl and feruloyl esters play a significant role as antioxidants (Gracia and Logan, 2000; Mathew and Abraham, 2004). Hydroxycinnamic acids are the precursors of a wide range of volatile compounds with most likely allelochemical functions (Dudareva et al., 2004). Other important groups of natural products derived from phenylpropane units are involved in plant response to environmental biotic and abiotic stimuli. For example, stilbenes and isoflavones are important phytoalexins in plants (Nicholson and Hammerschmidt, 1992).
Lignin, which originates from the oxidative polymerization of \(p\)-hydroxycinnamyl alcohols, is a major structural component of secondarily thickened cell walls of tissues with conducting and/or mechanical functions (Lewis and Yamamoto, 1990; Boerjan et al., 2003). Angiosperm lignin is essentially made of guaiacyl (G) and syringyl (S) units, together with weak or trace amount of \(p\)-hydroxyphenyl (H) units. Three hydroxylations are necessary to form these three units, which are catalyzed by three different cytochrome P450 monoxygenases: cinnamate 4-hydroxylase (C4H or CYP73), \(p\)-coumaroyl ester 3'-hydroxylase (C3'H or CYP98), and ferulate 5-hydroxylase (F5H or CYP84; Humphreys and Chapple, 2002). The 3-hydroxylation step was only recently shown to involve the cytochrome P450 CYP98A3 in Arabidopsis (Schoch et al., 2001; Franke et al., 2002a, 2002b; Nair et al., 2002). The \(meta\)-hydroxylation (or 3-hydroxylation) is not catalyzed on the free \(p\)-coumaric acid as anticipated, but on its conjugates with shikimic or quinic acids (Schoch et al., 2001). CYP98A3, together with the associated hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT; Hoffmann et al., 2003, 2004), controls a major branch point dispatching phenolic precursors between the synthesis of lignin monomers and that of flavonoids, stilbenes, and probably coumarins (Fig. 1).

An ethyl methanesulfonate mutant of the CYP98A3 gene has recently been described (Franke et al., 2002a, 2002b). The \(ref8\) mutation was a single missense base change in the last one-third of the coding sequence, which was sufficient to result in a strong impact on plant development and in the inhibition of the formation of sinapoyl esters and of the G and S lignin units. Here we describe isolation of a T-DNA insertion mutant and show that complete inactivation of CYP98A3 has an even greater impact on plant development. Gene inactivation is associated with reduced cell expansion, altered sugar composition of cell walls, and decreased content in crystalline cellulose and pectins.

Changes in plant and cell wall composition are associated with strong perturbations in the expression of genes involved in energetic and stress metabolism, signaling cascades, and cell wall development. In agreement with the results obtained on the \(ref8\) mutant (Franke et al., 2002a), the lignification of the aerial part of this insertion mutant is deeply altered, with a reduced lignin content essentially made of H units and only trace amounts of G and S units. In contrast to stems, roots of the null mutant, however, display an ectopic lignification phenotype and a substantial frequency of G and S lignin units. Similar to the \(ref8\) mutant, only trace amounts of sinapoyl malate (SM) are observed in the null mutant, which not only contains unusual \(p\)-coumaryl derivatives, but also accumulates flavonol glycosides. To get further insight into the role of CYP98A3 and plants less severely affected in their development, we also produced cosuppressed plants. Surprisingly enough, while changes in lignification were similar to those observed in the null mutant, albeit less pronounced, our analyses revealed that the leaves of CYP98A3 cosuppressed plants overaccumulate sinapoylated anthocyanins, and that their levels of SM are similar to the control.

Together, these data indicate (1) an alternative \(meta\)-hydroxylation pathway leading to G and S units in root lignin and, to a lesser extent, in the rachis; (2) cross talk between hydroxycinnamic acid/lignin and flavonoid pathways; and (3) a crucial role of phenolic precursors for cell wall expansion and homeostasis control during plant development.

### RESULTS

**\(cyp98A3\) T-DNA Insertion and Cosuppression Mutants Show Drastic Impairments in Growth and Development**

A \(cyp98A3\) insertional mutant was isolated by PCR screening of a T-DNA-mutagenized population in the...
accession Wassilewskija (Ws) 2 (Krystan et al., 1999) for a T-DNA insertion in the CYP98A3 locus (At2g40890), as described in “Materials and Methods.” A segregation pattern of 3:1 in the progeny of the selfed T1 and T2 mutants, based on kanamycin resistance, and a 1:2:1 segregation (wild-type:heterozygous:homozygous insertion) based on PCR and phenotypic inspection indicated that it contained a single T-DNA insertion locus. Sequencing of the PCR-amplified flanking regions revealed that the T-DNA was inserted in an intron 623 nucleotides downstream of the ATG start codon. Based on PCR amplifications, using a gene-specific primer on either side of the insertion and T-DNA left- and right-border primers, respectively, at least two inverted T-DNAs are present at the insertion site (data not shown). Therefore, a DNA insertion of 12 kb or more in the intron was predicted, which is expected to result in a complete loss of gene function.

The total inactivation of the CYP98A3 gene in homozygous plants was confirmed by RNA-blot hybridization (Fig. 2C) and quantitative reverse-transcription (RT)-PCR (see below). No apparent phenotype was detected for heterozygous cyp98A3 plants. In contrast, visual examination of the progeny immediately indicated that homozygous plants were severely retarded in their growth (Fig. 2, A and B). In 15-d-old seedlings, the cyp98A3 mutation resulted in hypocotyls with shortened length (5 ± 1 mm instead of 31 ± 4 mm for the wild type) and increased diameter (1,033 ± 71 μm instead of 711 ± 55 μm for the wild type). Roots had a swollen aspect (576 ± 31 μm diameter instead of 405 ± 23 μm in the wild type) with increased initiation from the crown and showed reduced growth (13 ± 3 mm length instead of 71 ± 7 mm in the wild type) and gravitropism compared to the wild type (Fig. 2A).

When transferred to soil, homozygous cyp98A3 plants maintained their dwarf phenotype with a rosette never exceeding 1 to 1.5 cm in diameter (Fig. 2B). Plants developed a bushy miniature rosette of round leaves and growth of cyp98A3 plants was arrested latest at the onset of primary stem development, which occurred usually about 2 weeks later than in wild-type plants. Mutants only occasionally initiated bolting stems, which never exceeded 3 cm in height and never developed fertile flowers. Despite this developmental arrest, cyp98A3 plants can survive for months without growing or showing signs of senescence (data not shown). When grown on soil, homozygous mutants also showed a darker leaf coloration compared to wild-type and heterozygous plants.

The reddish-dark bushy appearance and reduced growth of the homozygous null mutants was also shared by plants where CYP98A3 overexpression under the control of a 35S promoter led to gene cosuppression. Approximately 10% of primary transformants (ecotype Columbia [Col-0]) containing a cauliflower mosaic virus (CaMV) 35S::CYP98A3 construct showed a reduced growth phenotype (Fig. 3). These independently transformed plants were arrested at different stages of development. The cosuppression of CYP98A3 in each primary transformant was confirmed by RNA-blot hybridization (data not shown) and quantitative RT-PCR (see below). A gradation of phenotypes was obtained, which appears to be correlated with the onset of the cosuppression (Fig. 3). Some plants arrested earlier in development did not bolt and showed a very limited growth of the rosette (line IV). For others, bolting was delayed and growth of inflorescences stems was retarded (Fig. 3, B and D, lines V to VIII). When plants bolted, inflorescences showed purple stems and cauline leaves. If flowers developed, they were limp, in most cases male sterile, and only occasionally produced viable seeds (Fig. 3, C–E). A few seeds were recovered from primary transformants designated as lines V, VI, and VII in Figure 3, and CYP98A3 expression was monitored throughout the life cycle in T2 plants derived from each line (Fig. 3F). Cosuppression was preceded by a phase of CYP98A3 overexpression for all plants (data not shown), and was fully established only when plants were 5 to 6 weeks old. CYP98A3 transcript levels were then reduced to undetectable levels within the subsequent 3 weeks (Fig. 3F). In fully cosuppressed plants, no CYP98A3 transcripts were detectable in RNA-blot analysis, and no protein was detected based on protein-blot analyses using CYP98A3-directed polyclonal antibodies.
antibodies (data not shown). The timing and degree of the cosuppression observed in each cosuppressed line differed slightly and seems to be correlated with the severity of the phenotype, in particular with the onset of developmental arrest.

**cyp98A3** Mutants Display Modified Lignin in Stems But Ectopic Lignin Deposition in Roots

Given the severe phenotype of the cyp98A3 insertion mutant, plants were compared to wild type both at the same age and at a comparable stature. Lignin yield and composition were determined using all aerial parts from insertion mutant plants (6 weeks old) and wild-type Ws plants (6 weeks old or same size as 6-week-old mutants). Lignin determination by the standard Klason gravimetric method could not be applied to the dwarf null plants because this method is too sample demanding. Instead, the lignin structure was analyzed by thioacidolysis, which specifically gives rise to H, G, and S monomers from H, G, or S lignin arylglycerol lignin units involved in β-O-4 bonds (Lapierre et al., 1995). The total yield in lignin-derived monomers from dry stems was close to 180 μmol g⁻¹ for 6-week-old wild-type and heterozygous cyp98A3 lines (Table I). These monomers were almost exclusively G and S, with a very low amount of H monomers (close to 1% of the \([H+G+S]\) total). In contrast, thioacidolysis of cyp98A3 insertion plants resulted in a much lower yield of total monomers (1.5 μmol g⁻¹). In addition, approximately 95% of the thioacidolysis monomers were representative of H units, while G and S monomers were found only in very low, but detectable, amounts (Table I). For comparison purposes, juvenile wild-type plants with a size similar to that of the 6-week-old mutant were subjected to thioacidolysis. Relative to 6-week-old wild-type plants, these juvenile plants yielded 100 times less lignin-derived monomers, which is comparable to the cyp98A3 insertion mutant. However, the lignin from juvenile wild-type plants was mainly composed of G and S subunits, whereas H units contributed only 3.5% of monomers, compared to 95% in the cyp98A3 mutant (Table I). Taken together, these results mirror the lignin alteration in the ref8 mutant caused by a point mutation in CYP98A3 (Franke et al., 2002a). The drastically low thioacidolysis yield is indicative of very low lignin content. It is also very likely the result of the high frequency of H units in the mutant lignin, since H units have a high tendency to be involved in carbon-carbon or biphenyl-ether interunit bonds that resist thioacidolysis (as also demonstrated by the lignin analyses of the cosuppressed plants). In contrast to the results reported for the ref8 mutant (Franke et al., 2002a), we could detect weak, but quantifiable, amounts of G and S units in the lignin.
of the cyp98A3 null mutant (Table I). This important observation constitutes a first indication that the 3-hydroxylation of phenolic compounds might proceed not exclusively via CYP98A3, which is entirely silenced in the null mutant.

Phloroglucinol staining was performed in an attempt to determine the tissue localization of lignin in the insertion mutant. Cytological examination of the miniature inflorescence stems from cyp98A3 insertion plants was impossible due to the poor mechanical properties of the tissues and crushing of the sections. In contrast, the less severe phenotype of the roots permitted this cytological analysis, and phloroglucinol staining revealed an ectopic lignification phenotype. In addition to xylem cell and fibers, which stain both in wild-type and mutant plant roots, phloroglucinol staining of pith and cortex cells was observed only in the roots of cyp98A3 insertion mutants (Fig. 4, A and B). A similar ectopic lignification phenotype in roots has been previously reported for various mutants affected in cell wall biogenesis and cell expansion (Caño-Delgado et al., 2000) and in plants treated with inhibitors of cellulose synthase (Caño-Delgado et al., 2003). More surprisingly, an ectopic lignification phenotype was also observed in the rachis (stem, cauline leaves, and sepals) of cosuppressed transformants, which developed bolting stems (Fig. 4, C–E). No phloroglucinol-reactive material was, however, detected in the rosette leaves of cosuppressed plants or in the hypocotyls and rosette leaves of the cyp98A3 insertion plants. To clarify the nature of the phloroglucinol-reactive material accumulated, we analyzed the lignin content and structure in stems of cosuppressed plants, as well as the structure of root lignin from 3-week-old cyp98A3 insertion plants. Inflorescence stems of cosuppressed plants were harvested from T2 plants derived from lines V, VI, and VII at an age of 13 weeks, when plant growth was fully arrested and CYP98A3 transcripts were undetectable (Fig. 3F). Due to the limited number of T2 seeds/plants available, material from all three lines was pooled. The extract-free stems of cosuppressed plants were characterized by a 40% reduced Klason lignin content relative to the corresponding Col-0 control (Table II). Upon thioacidolysis, the lignin from cosuppressed plants released H, G, and S monomers in much lower total yield compared to wild type (93% reduction), which is indicative of a high content in resistant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield in (H + G + S)</th>
<th>Molar Frequency of Thioacidolysis Monomers</th>
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<tbody>
<tr>
<td></td>
<td>µmol g⁻¹ dry sample</td>
<td>H</td>
</tr>
<tr>
<td>Dry stems/leaves from</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Six-week-old Ws</td>
<td>162 (8)</td>
<td>0.7 (0.02)</td>
</tr>
<tr>
<td>Six-week-old heterozygous cyp98A3</td>
<td>187 (17)</td>
<td>1.3 (0.1)</td>
</tr>
<tr>
<td>Ws of same size as below</td>
<td>1.2 (0.4)</td>
<td>3.2 (0.02)</td>
</tr>
<tr>
<td>Six-week-old homozygous cyp98A3</td>
<td>1.5 (0.2)</td>
<td>94.6 (0.2)</td>
</tr>
<tr>
<td>Dry roots from</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Three-week-old Ws</td>
<td>63 (0.5)</td>
<td>5.2 (0.1)</td>
</tr>
<tr>
<td>Ws of same size as below</td>
<td>7.5 (0.02)</td>
<td>6.6 (0.7)</td>
</tr>
<tr>
<td>Three-week-old homozygous cyp98A3</td>
<td>1.8 (0.1)</td>
<td>43.1 (9.8)</td>
</tr>
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</table>

Figure 4. Ectopic lignification phenotypes of cyp98A3 insertion and cosuppressed plants. Plant material was stained with phloroglucinol and visualized using whole-mount bright-field microscopy. A, Roots from 3-week-old wild-type (Ws) and cyp98A3 insertion plants (30×). B to E, Different organs from 10-week-old T2 plants derived from the cosuppressed line VII (Fig. 3) were used for phloroglucinol staining. Roots (B, 30×), inflorescence stems (C), cauline leaves (D), and flowers (E, each 10×).
interunit bonds. Similar to cyp98A3 insertion mutants, the cosuppressed plants are characterized by a strong decrease in the relative amounts of S and G units in stems and a dramatic increase in the proportion of H units, which constitute 74% of lignin thioacidolysis monomers compared to less than 1% in stems from wild-type Col-0 plants (Table II). In addition, the dithioketal derivative of p-OH coumaraldehyde was obtained in a low, but quantifiable, amount (about 1.3% of the H main monomers), which indicates that p-OH-coumaraldehyde end groups occur that could contribute to the positive phloroglucinol staining in stems of cosuppressed plants.

The analyses of wild-type Ws roots confirmed that, compared to the corresponding stem lignin, roots contain relatively less S units and correspondingly more G and H units (Sibout et al., 2003), with H units contributing 5% to 7% to the total lignin thioacidolysis monomers, depending on the developmental stage of wild-type plants (Table I). Similar to stems, roots of the cyp98A3 insertion mutants provided a lower total thioacidolysis yield and a much higher proportion of H monomers (43%) compared to the wild-type roots. However, the frequency of G thioacidolysis monomers released by root lignin was not reduced to the same extent as in the case of lignin derived from stems, and the frequency of S monomers was comparable in wild-type and mutant roots (Table I). Indeed, G and S units in the root tissues of the cyp98A3 insertion mutant still constitute more than 56% of the thioacidolysis-derived monomers (wild-type roots contain about 94%; Table I). Together with the persistence of G and S lignin units in the mutant stem, this result further supports the occurrence of an alternative 3-hydroxylation mechanism leading to the ectopic root lignin.

Insertion and Cosuppressed cyp98A3 Lines Accumulate Flavonoids and p-Coumaroyl Derivatives, But Sinapoyl Esters Also Persist

To further characterize the impact of the cyp98A3 mutation on phenolic metabolism, we analyzed soluble phenolics by liquid chromatography (LC)-mass spectrometry (MS) of soluble phenolics. Given the severe phenotype of the insertion mutant, analyses were performed using 15-d-old seedlings when phenotypic differences to wild-type Ws plants are less pronounced. Despite this, the results obtained for cyp98A3 insertion plants displayed a higher variability than those for control Ws plants grown under the same conditions (Table III). The main p-hydroxyccinamic and flavonol derivatives were identified on the basis of their mass and UV spectra (see “Materials and Methods” and supplemental text for details) or, where available, from literature data (Veit and Pauli, 1999; Bloor and Abrahams, 2002; Tohge et al., 2005). In agreement with the qualitative data reported for the leaves of the ref8 mutant (Franke et al., 2002a), the analysis of insertion plantlets confirmed a drastic decrease in SM and the accumulation of p-coumaroyl derivatives. However, it is noteworthy that homozygous insertion mutants are not completely devoid of SM (Fig. 5; Table III). Likewise, E and Z isomers of sinapoyl Glc (SG),

### Table II. Lignin content and structure in extract-free and dry stems of control Col-0 and cosuppressed 13-week-old lines

<table>
<thead>
<tr>
<th>Sample</th>
<th>Klason Lignin Content</th>
<th>Thioacidolysis Yield</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>% by weight</td>
<td>μmol g⁻¹ Klason lignin</td>
</tr>
<tr>
<td>Col-0</td>
<td>18.48 (0.05)</td>
<td>1,600 (90) 0.7 (0.01) 69 (0.3) 30.3 (0.3)</td>
</tr>
<tr>
<td>Cosuppressed</td>
<td>11.65 (0.11)</td>
<td>220 (30) 74 (1) 13 (0.1) 13 (0.1)</td>
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</tbody>
</table>

### Table III. LC-MS determination of soluble phenolics extracted from 15-d-old control (Ws) or null cyp98A3 mutant plants

<table>
<thead>
<tr>
<th>Compound</th>
<th>(M-H)⁻</th>
<th>ng/mg fresh material</th>
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<tbody>
<tr>
<td>Flavonol glycosides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>577</td>
<td>102 (7) 259 (96)</td>
</tr>
<tr>
<td>K2</td>
<td>593</td>
<td>72 (10) 210 (52)</td>
</tr>
<tr>
<td>K3</td>
<td>739</td>
<td>42 (4) 100 (24)</td>
</tr>
<tr>
<td>K4</td>
<td>755</td>
<td>40 (2) 153 (43)</td>
</tr>
<tr>
<td>Q1</td>
<td>609</td>
<td>70 (15) 205 (38)</td>
</tr>
<tr>
<td>Q2</td>
<td>755</td>
<td>11 (2) 35 (5)</td>
</tr>
<tr>
<td>Q3</td>
<td>771</td>
<td>31 (4) 98 (6)</td>
</tr>
<tr>
<td>I1</td>
<td>623</td>
<td>24 (5) 82 (18)</td>
</tr>
<tr>
<td>Total flavonol glycosides</td>
<td>392 (35) 1,142 (275)</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Sinapoyl and coumaroyl derivatives</th>
<th>ng/mg fresh material</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM (E and Z)</td>
<td>339 243 (27) 23 (19)</td>
</tr>
<tr>
<td>SG (E and Z)</td>
<td>385 239 (14) 64 (38)</td>
</tr>
<tr>
<td>CG (E and Z)</td>
<td>325 Tr 225 (53)</td>
</tr>
</tbody>
</table>
putatively identified from their mass and UV spectra, were severely reduced, but not abolished, in insertion mutants compared to Ws control plants. Concomitant to this severe decrease of sinapoyl esters, novel compounds were detected in cyp98A3 insertion plants with mass and UV spectra assignable to p-coumaroyl Glc (CG; E and Z isomers). CG was detected only in trace amounts in wild-type Ws plants (Fig. 5; Table III). In addition, p-coumaroyl malate (CM) was observed as a trace component in this series of cyp98A3 insertion plants, but accumulated to higher amounts in two other sample series (data not shown). No accumulation of p-coumaroyl shikimate or p-coumaroyl quinate could be detected, as confirmed by the analysis of authentic standards.

The characterization of flavonol glycosides by negative electrospray LC-MS revealed that their level was 3-fold higher in the cyp98A3 insertion seedlings relative to the Ws control (Fig. 5; Table III). Comparison of the profiles indicates that the mutation leads to a general enrichment of all flavonol glycosides present in wild-type plants and does not seem to induce the increase of selective or specific flavonol derivatives (Table III).

To analyze the role of CYP98A3 in older tissues, pooled cauline leaves from cosuppressed T2 plants derived from lines V, VI, and VII (Fig. 3), which were arrested at later stages of development, were used. Cauline leaves from wild-type Col-0 were analyzed in parallel by negative electrospray LC-MS. In agreement with literature data (Graham, 1988; Pelletier et al., 1999), the flavonol content of mature leaves from wild-type plants was found to be simpler than that of young plants. Only the three major kaempferol glycosides, K1 to K3, were found in significant amounts (Fig. 5C), whereas quercetin derivatives were only trace components. Similar to results with cyp98A3 insertion plantlets, mature leaves from cosuppressed plants contained 3 times more flavonol glycosides than the corresponding control (data not shown). In addition, quercetin derivatives could be clearly observed on the LC-MS trace of the mutant, while occurring as trace components in the wild type (Fig. 5, C and D). In wild-type plants, these quercetin glycosides are usually observed only at earlier developmental stages (Pelletier et al., 1999) or after UV exposure (Veit and Pauli, 1999). Surprisingly, and in contrast to results obtained with the insertion mutant, the level of SM was not affected in leaves of cyp98A3 cosuppressed plants (Fig. 5D). Also in contrast to results obtained with cyp98A3 insertion mutants, CG and CM were observed only in trace amounts in cosuppressed leaves, comparable to levels in wild-type plants.

The deep purple coloration of leaves from cosuppressed plants prompted us also to examine their anthocyanin content by positive electrospray LC-MS. In addition to the major anthocyanin of Arabidopsis, cyanidin 3-O-[2"O-(6"O-(sinapoyl) xylosyl) 6"O-(p-O-(glucosyl)-p-coumaroyl) glucoside] 5-O-(6"O-malonyl) glucoside (Bloor and Abrahams, 2002),
we observed a series of other, less abundant cyanidin derivatives in cosuppressed plants only (data not shown). These are, on the basis of their mass fragmentation pattern, identical to those recently identified by Tohge et al. (2005), who reported the accumulation of such cyanidin derivatives in the leaves of an Arabidopsis mutant overexpressing a MYB transcription factor. All these acylated cyanidin glycosides, which surprisingly include substantial amounts of sinapoylated derivatives, were below the detection level in leaves of wild-type plants.

In summary, the aerial parts of the cyp98A3 insertion mutant display drastically reduced, although not negligible, levels of sinapoyl esters and S and G lignin units. It is accompanied by a strong increase in p-coumaroyl esters, H lignin units, and flavonoids. This confirms the central role of CYP98A3 as the C3’H in the general phenylpropanoid pathway (Schoch et al., 2001; Franke et al., 2002a, 2002b; Nair et al., 2002). However, the persistence of detectable amounts of SM in cyp98A3 null plants and the presence of G and S lignin units mainly in their roots support the hypothesis of an alternative meta-hydroxylase pathway independent of CYP98A3. The occurrence of large amounts of SM and sinapoylated cyanidin glucosides in completely cosuppressed cyp98A3 plants gives further support to this hypothesis.

Impaired Plant Development of cyp98A3 Mutants Is Accompanied by Reduced Cell Size and Alteration in Cell Wall Polysaccharide Composition

In an attempt to understand the observed inhibition of plant growth, cell size and cell wall modifications were investigated. A statistical analysis of the size of epidermal cells in different leaves and of different leaf areas indicated a significant 6-fold decrease of the cell size in the cyp98A3 insertion mutant (Fig. 6A). This reduced cell size is not accompanied by modification of the cell shape, and an estimation of the total number of cells per leaf based on leaf and cell size (Fig. 6A) revealed no difference to wild-type plants. A substantial, but less dramatic, reduction in cell size was also observed in cosuppressed plants (data not shown). In addition to decreased cell size, an alteration in the cell wall polysaccharide composition was observed.
(Fig. 6B). Polysaccharide analysis of 5-week-old plants revealed a significant increase in Glc released by trifluoroacetic acid (TFA) hydrolysis representing amorphous glucans (mainly amorphous cellulose). A concomitant decrease in the level of TFA-released uronic acids (UA; reflecting a decrease of pectic polysaccharides) compared to wild-type plants of the same age was observed (Fig. 6B). In addition, the amount of Glc released by Seaman hydrolysis, reflecting crystalline cellulose, was 50% lower in the cyp98A3 insertion mutant compared to mature Ws plants. It is interesting to note that the decrease in crystalline cellulose is accompanied by an increase in amorphous cellulose in cyp98A3, suggesting that the overall amount of cellulose is not altered in cell walls of the mutant, but rather its state of crystallinity, and hence possibly the architecture of the wall. However, at 5 weeks, wild-type plants are significantly larger than cyp98A3 homozygous plants. To test whether the differences in the composition of the cell wall polysaccharides did not merely reflect delayed development, wild-type plants were grown for 2 weeks until they reached a size comparable to the size of the 5-week-old null cyp98A3. Certain changes in polysaccharide composition occur during development of wild-type Ws plants. In mature plants, an increase in UAs was observed, accompanied by a slight increase in all neutral sugars analyzed with the exception of TFA-released Glc. In addition, a strong increase of crystalline cellulose was observed (Fig. 6B), which is likely due to the higher proportion of secondary walls present in the older plants. When comparing cyp98A3 insertion mutants to wild-type plants, an increase in amorphous cellulose, and a significant reduction in Ara, reflecting lower levels of arabinogalactan proteins and/or pectin-derived arabinans, was apparent (Fig. 6B).

Cytoskeleton and, in particular, cortical microtubules, are known to be intimately associated with the biogenesis of the cell wall and cellulose microfibril elongation, as well as secondary cell wall depositions and signaling of morphogenetic processes (Baskin, 2001; Wasteneys, 2004). Immunostaining of cortical microtubules in elongating root cells did not reveal any significant perturbation of the transversal orientation of the microtubules. However, the overall microtubule structure was altered and appeared more punctuated (Fig. 6B).

Impaired Development and Reduced Cell Size Is Accompanied by Massive Changes in Gene Expression

The strong alterations in cell wall composition, cell size, and in overall plant development observed in the cyp98A3 insertion mutant go beyond expectations and cannot be explained by modifications in phenylpropanoid metabolism alone. Gene expression-profiling analyses were thus performed to pinpoint the underlying developmental defects. Given the severe phenotype of the mutant, 15-d-old seedlings grown in vitro were used to minimize the impact of differences in plant development on gene expression and to catch a picture of the phenomenon at its onset. Although even at this early stage pleiotropic and other indirect effects cannot be excluded, they were expected to be less pronounced.

In a preliminary experiment, the expression of selected genes upstream in the phenylpropanoid pathway was compared using quantitative real-time RT-PCR analysis. Given that genes encoding phenylpropanoid enzymes display diurnal changes in their expression (Rogers et al., 2005), we examined expression levels throughout the 12-h light cycle in plants grown under a 12-h-light/12-h-dark regime. In wild-type plants, the transcript levels of all phenylpropanoid genes were highest 4 h into the light period. At the end of the day period, they returned to the same levels observed at 0 h (Fig. 7A). This variation in gene expression is in agreement with studies by Rogers et al. (2005), and a comparable cycling of all transcripts analyzed was also observed in cyp98A3 insertion mutants, with the exception of a total inactivation of CYP98A3 (Fig. 7A). While transcript levels of genes encoding Phe ammonia lyase (PAL1; At2g37040) and HCT (At5g48930; Fig. 7A) were comparable in cyp98A3 mutants and wild type at all time points, an increase in C4H (CYP73A5; At2g30490) expression compared to wild type was observed at the 2-, 4-, and 8-h time points in cyp98A3 insertion mutants (Fig. 7A). In 10-week-old cosuppressed T2 plants derived from the primary transformants V, VI, and VII (Fig. 3), which were arrested in growth when analyzed, CYP98A3 gene expression is suppressed to undetectable levels (Figs. 7B and 3F). This suppression was accompanied by a decrease in the expression of PAL1, C4H, and HCT at all time points analyzed (Fig. 7B). However, in all cases, cycling during the daylight period was not altered. Cosuppression of the CYP98A3 gene in adult plants, but not inactivation via a T-DNA insertion in juvenile plantlets, thus appears to result in a repression of the general phenylpropanoid pathway. But the inactivation of CYP98A3 has no impact on the circadian cycle of phenylpropanoid gene expression. The cyp98A3 insertion and wild-type Ws lines grown under the same conditions can thus be compared on a larger scale in juvenile plants.

Microarray analysis of gene expression was thus performed using 15-d-old homozygous cyp98A3 insertion plants compared to wild-type Ws. Two biological replicates were analyzed, with two technical replicates (dye swaps) each for a total of four microarrays. The CATMA Arabidopsis near-full genome array was employed, which contains 24,576 genespecific elements representing 21,371 annotated genes. Statistical analysis (see “Materials and Methods” for details) identified 1,944 elements representing 1,889 annotated genes to be differentially expressed (adjusted p [t test] < 0.05). Among these, 1,067 elements (1,038 genes) changed more than 2-fold, and 362 elements representing 352 annotated genes changed.
more than 3-fold (94 genes overexpressed and 260 genes repressed in the mutant).

Supplemental Table I lists normalized expression ratios and annotation details for all differentially expressed genes; the complete dataset was deposited into the ArrayExpress database (accession E-MEXP-346). Functional grouping of differentially expressed genes using the Functional Category Database at MatDB (Schoof et al., 2002; Fig. 8A) indicates a profound modification of plant metabolism. As expected in a mutant almost completely blocked in carbon utilization for lignin synthesis and with a strong phenotype, perturbations in primary metabolism are much more severe than in mutants such as pal1pal2, where lignin synthesis is only decreased to 30% (Rohde et al., 2004). Functional groups over-represented in the group of down-regulated genes, which change more than 3-fold, point to a strong repression of light- and oxygen-dependent energy production and carbon fixation, reflected by a decreased expression of plastidal genes involved in photosynthesis and respiration (Fig. 8A). Indeed, several genes encoding proteins involved in the light-harvesting complex (e.g. chlorophyll a/b-binding proteins At2g34430, At3g08940, and At3g47470), in photosynthetic electron transport (e.g. the putative ferredoxin At1g10960 and proton gradient regulation 5, At2g05620; Munekage et al., 2002), and in the Calvin cycle (e.g. Rubisco At5g38410 and the putative Rib-5-P isomerase At3g04790) are strongly down-regulated in the mutant compared to the wild type (Supplemental Table I). In contrast, genes expressed to higher levels in cyp98A3 insertion mutants compared to wild type were over-represented in functional categories covering carbohydrate metabolism and include genes encoding enzymes in glycolysis (e.g. the glyceraldehyde 3-P dehydrogenase, At3g04120), the tricarboxylic acid cycle (e.g. the phosphoenolpyruvate carboxylase, At3g14940), and other anaerobic metabolisms (alcohol dehydrogenase, At1g77120). Also, functional categories related to defense responses were over-represented (Fig. 8A). Among these were genes known to be transcriptionally induced by insect feeding (the β-glucosidase BGL1, At1g52400; and the lipoxygenase LOX2, At3g45140; Stotz et al., 2000) or wounding/jasmonate (e.g. jasmonate response JR1, At3g16470; Leon et al., 1998). Finally, genes involved in secondary metabolism are over-represented in the group of up-regulated genes (Fig. 8A). It is noteworthy that genes encoding enzymes involved in flavonoid and anthocyanin biosynthesis and transport are expressed to higher levels in the cyp98A3 insertion mutant; these include chalcone isomerase (CHI, At3g55120), dihydroflavonol 4-reductase (DFR, At5g42800), flavanone 3-hydroxylase (F3H, At3g51240), and glutathione transferase (TT19, At5g17220; Weisshaar and Jenkins, 1998; Kitamura et al., 2004). In contrast, but in agreement with quantitative RT-PCR results (see above), among genes involved in monolignol biosynthesis apart from CYP98A3, only C4H is differentially expressed, while the expression of all other genes of the phenylpropanoid pathway remains unaltered (ArrayExpress accession E-MEXP-346).

The higher expression of genes related to stress response, in particular mediated by jasmonate, prompted us to dissect the potential involvement of...
Upon normalization and statistical analysis, 1,889 annotated genes were found to be differentially expressed (Bonferroni-adjusted $p$-value $< 0.05$). A, Functional grouping of differentially expressed genes that differed in expression more than 3-fold between cyp98A3 insertion mutants and wild-type Ws using the Functional Category Database at MAtDB (School et al., 2002; http://mips.gsf.de/proj/functcatDB). The frequency of up-regulated genes in each functional category (shown as dark-gray bars) was compared to the frequency of all genes represented on the microarray in the same functional category (shown as light-gray bars). Only functional categories that are over-represented in the group of genes expressed to higher levels in the cyp98A3 mutant, compared to wild type (Ws); These include LOX2 (see above), LOX1, allene oxide synthase (CYP74A, At5g42650), and allene oxide cyclase 4 (At1g13280).

In addition, genes related to the abscisic acid cascade are over-represented in the group of genes expressed to higher levels in cyp98A3 insertion mutants (Fig. 8B). These include the gene responsive to dehydration 22 (RD22I, At5g25610), which is strongly induced by abscisic acid and drought (Yamaguchi-Shinozaki and Shinozaki, 1993), and the Arg decarboxylase 2 (ADC2, At4g34710) gene, which is strongly activated by abscisic acid, but also by methyl jasmonate and mechanical wounding, and is involved in polyamine biosynthesis (Perez-Amador et al., 2002).

Also expressed to higher levels in cyp98A3 insertion mutants were two homeodomain Leu-zipper transcription factors (ATHB5, At5g65310; and ATHB7, At2g46680) involved in abscisic acid-mediated signaling pathways related to drought and osmotic stress (Södeman et al., 1996) and seed germination (Johannesson et al., 2003), respectively.

None of the other plant hormones analyzed appears to have a similar relevance for the group of genes expressed to higher levels in the cyp98A3 insertion mutant. To a lesser extent, genes related to gibberellic acid and salicylic acid are more frequently found in the group of genes expressed to lower levels in cyp98A3 insertion mutants than expected by chance (Fig. 8B). Among genes related to salicylic acid signaling and expressed to lower levels in cyp98A3 insertion mutants, defective in induced resistance 1 (At5g48485) encodes a lipid transfer protein that, when mutated, results in specific loss of systemic acquired resistance (Maldonado et al., 2002).
Finally, in an attempt to explain the dramatic decrease in cell growth and plant development observed in the mutant, we further explored the expression of genes involved in cell expansion and cell division. This analysis revealed that no gene annotated in TAIR gene ontology (GO) term “cell division” is differentially expressed more than 2-fold. In addition, none of the genes involved in the core cell cycle machinery (Vandepoele et al., 2002) is differentially expressed between wild-type and cyp98A3 mutant plants. In contrast, 12.5% of genes annotated in the GO term “cell growth” are differentially expressed between cyp98A3 insertion and wild-type plants. Among genes in this group, putative expansins, which mainly act in loosening cell walls for regulating cell expansion (Lee et al., 2001), form the largest group. Six of the 28 expansin family members represented on the array used are differentially expressed, significantly more than expected by chance. However, some expansin isoforms (EXP, At1g69530; EXP10, At1g26770; and EXPR, At4g17030) are expressed to higher levels in cyp98A3 insertion mutants, whereas others (EXP, At2g40610; EXP3, At2g37640; and EXP11, At1g20190) are down-regulated compared to levels found in wild-type plants.

Genetic and Chemical Complementation

To ensure that the observed phenotype, in particular the drastic growth and developmental inhibition of the null mutant, was indeed the result of the CYP98A3 gene inactivation, a genetic complementation was performed by transformation of heterozygous plants with a construct containing the CYP98A3 coding region under control of the CaMV 35S promoter. Transformants were identified based on the BASTA resistance marker present in the construct, and homozygous cyp98A3 plants in the progeny of BASTA-resistant plants were identified using PCR (Fig. 9A). Homozygous cyp98A3 insertion plants containing the 35S::CYP98A3 constructs were morphologically indistinguishable from wild-type plants (Fig. 9B). They displayed the same phenotype, including growth and development, as wild-type and heterozygous plants. Based on chloroform staining, no signs of ectopic lignification were detected in roots of complemented plants (data not shown).

The observed growth inhibition in cyp98A3 insertion plants may result either from a depletion of 3-hydroxylated products or from the accumulation of potentially toxic or bioactive upstream or side products (e.g. flavonoids that were reported to modulate auxin transport [Peet et al., 2004]). In an attempt to dissect these hypotheses, chemical complementation tests were performed. CYP98A3 metabolizes with high efficiency both p-coumaroyl shikimate and p-coumaroyl quinate, both of which can be generated from p-coumaric acid by HCT. HCT is also expected to catalyze the conversion of the CYP98A3 product, caffeoyl shikimate/quinate, to caffeoyl-CoA (Hoffmann et al., 2003; Niggeweg et al., 2004). Complementation was therefore first attempted using the commercially available caffeic acid and caffeoyl quinate (chlorogenic acid). cyp98A3 insertion plants were grown on media containing these compounds, but no stimulation of plant growth was observed (data not shown). Given that caffeoyl shikimate is likely the major CYP98A3 product in vivo based on catalytic parameters (Schoch et al.,
DISCUSSION

Analysis of cyp98A3 Mutants Reveals the Existence of an Alternative meta-Hydroxylation Pathway of Phenolic Compounds

The genes belonging to the CYP98 family recently emerged as the cytochrome P450 monooxygenases catalyzing the meta-hydroxylation of phenolic compounds in several plant species. These enzymes catalyze the meta-hydroxylation of p-coumaric derivatives esterified to shikimate, quinate, or phenyllactate (Schoch et al., 2001; Matsuno et al., 2002). In Arabidopsis, CYP98A3 is required for the synthesis of lignin monomers and soluble sinapoyl esters (Franke et al., 2002a). A point mutation in the CYP98A3 gene was sufficient to completely suppress the production of G and S lignin monomers (as revealed by derivatization followed by reductive cleavage, nitrobenzene oxidation, and analytical pyrolysis) and to drastically reduce the level of soluble meta-hydroxylated phenolics in aerial parts of the ref8 mutant (Franke et al., 2002a, 2002b). It was thus expected that CYP98A3 was the sole 3-hydroxylation enzyme of the phenylpropanoid pathway in Arabidopsis, and that the phenylpropanoid pathway had evolved to exclusively channel the 3-hydroxylation step through p-coumaroyl esters and the highly conserved CYP98 enzymes (Schoch et al., 2001; Franke et al., 2002a, 2002b). Our present results challenge this hypothesis.

This analysis of Arabidopsis cyp98A3 insertion and cosuppressed lines confirms the essential role of CYP98A3 in the synthesis of G and S lignin monomers in aerial parts of the plant and, to some extent, also in the roots. It also confirms that, under normal growth and homeostasis conditions, CYP98A3 is the major contributor to the biosynthesis of soluble sinapoyl derivatives as previously reported by Franke et al. (2002a). However, the cyp98A3 insertion mutant displays a more dramatic phenotype compared to the ref8 mutant (Franke et al., 2002b), which suggests that the ref8 gene still had a low residual activity. In the T-DNA insertion mutant analyzed here, the CYP98A3 gene is totally inactivated. It nevertheless contains weak, but quantifiable, amounts of G and S units in the lignin of the aerial parts of the plants. G and S units are even more frequent in the ectopic root lignin. Our results, therefore, suggest the occurrence of an alternative meta-hydroxylation pathway in Arabidopsis, which seems to be more active in producing the ectopic lignin observed on the roots. It is likely that the same pathway is activated ectopically in the rachis of cosuppressed plants. The detection of SM in the seedlings of the insertion mutant further supports this hypothesis.

It is interesting to note that the alternative meta-hydroxylation pathway observed in cyp98A3 mutants appears to be activated when the prevalent pathway is not functional. However, this alternative pathway cannot complement the defects observed in cyp98A3 mutants (e.g. reduced growth, reduced lignin in stems, limp stems, and male sterility). It does not ensure normal growth and appears to be active in tissues that do not normally lignify, as judged from the ectopic phloroglucinol staining observed in cyp98A3 insertion and cosuppressed plants. This alternative pathway seems to be ectopically activated under conditions of perturbed metabolism, but the nature and normal physiological relevance, in specific tissues or at specific stages of plant development, remains to be elucidated. Interestingly enough, the ectopic CYP98A3 overexpression for complementation of the null mutant does not promote any ectopic lignification. It is worth mentioning that the analysis of precursor conversion by peltate glands isolated from two sweet basil (Ocimum basilicum) lines that differ in their ability to produce eugenol also led Gang et al. (2002) to propose the existence of an alternative meta-hydroxylation pathway. The line that produced methyl chavicol instead of eugenol had a strongly decreased ability to meta-hydroxylate p-coumaroyl esters, but an unaltered capability to convert p-coumaric acid into caffeic acid. The alternate pathway operating in sweet basil may, however, be different from that producing meta-hydroxylated phenolics in the cyp98A3 mutants from Arabidopsis.

Impact of CYP98A3 Gene Inactivation on Plant Growth and Metabolism

The ectopic activation of an alternative meta-hydroxylation pathway in cyp98A3 mutants is paralleled with strong morphological changes and with an accumulation of flavonoids. The accumulation of flavonoids (flavonol glycosides and anthocyanins) in null and cosuppressed cyp98A3 mutants is not surprising, since creating a bottleneck at the level of CYP98A3 leads to an accumulation of p-coumaroyl-CoA precursors that may subsequently simply overflow into the flavonoid pathway (Fig. 1).

The severe modification in plant development and the complete growth arrest observed in both cyp98A3
insertion and cosuppressed mutants, however, is more surprising. Given the reduced cell size, which is not accompanied by a reduction of the total number of cells per leaf, these morphological changes are likely due to an inhibition of cellular growth. Consistent with this hypothesis, our microarray analysis revealed no differences in transcript abundance of genes related to the cell cycle, while several genes related to cell expansion are expressed to different levels in wild-type and cyp98A3 insertion plants. Our data suggest that threshold amounts of meta-hydroxylated lignin monomers or of other caffeoyl shikimate-derived compounds are required for normal organization of the cell wall and plant growth. This conclusion is supported by the fact that external application of caffeoyl shikimate complements, at least partially, the growth phenotype of the null mutant. Such a meta-hydroxylated derivative might be needed as essential building blocks of the primary cell wall or might more indirectly promote cell wall modifications and growth (e.g. via Ca\(^{2+}\) chelation). Alternatively, it may directly act as a growth regulator. Phenolic compounds have been reported to play a role in cell division and expansion, either directly or as components of a cytokinin-dependent signaling cascade (Lynn et al., 1987). A major role in this respect was attributed to dehydrodiconiferyl alcohol glucoside (DCG), which was shown to directly activate cell growth and division in tobacco (Nicotiana tabacum) pith, leaves, or cell cultures (Binns et al., 1987; Tamagnone et al., 1998). DCG, as lignin G monomers, is expected to directly derive from CYP98A3 products (Fig. 1). DCG accumulation during plant growth so far has not been described in Arabidopsis. Its ability to restore growth of the null cyp98A3 mutant will be assessed.

Another possibility is that growth arrest simply results from global perturbations of plant metabolism. A block in lignin biosynthesis suppresses a major carbon sink and induces an accumulation of flavonoids and possibly of indolic derivatives. The latter are stress-related metabolites. Many of the differences in gene expression observed in the cyp98A3 insertion mutant compared to wild type are reminiscent of a stress response. These include a general downregulation of photosynthesis/chloroplastic genes, indicating reduced energy and carbon assimilation, and elevated expression levels of genes related to defense responses with activation of the jasmonate and abscisic acid signaling pathways. The observed ectopic lignification in roots of the cyp98A3 insertion mutant and in the rachis of cosuppressed plants can also be considered as indicative of a constitutive induction of a stress response. Ectopic lignification, resulting from mutations in genes encoding cellulose synthase, a chitinase-like enzyme, or a vacuolar ATPase (Schumacher et al., 1999; Caño-Delgado et al., 2000, 2003; Zhong et al., 2002), has been associated with activation of jasmonate and ethylene cascades, with defects in plant growth and cell wall biogenesis, and with stress response. The existence of a link between ectopic lignification and jasmonate cascade was further confirmed by the impact of chemical inhibition of cellulose synthesis with isoxaben, which phenocopies a mutation in cellulose synthase. Isoxaben treatment was induced to the jasmonate pathway as well as ectopic lignification (Ellis et al., 2002; Caño-Delgado et al., 2003). Conversely, the combined treatment of wild-type seedlings with jasmonate and 1-aminocyclopentane-carboxylic acid (an ethylene precursor) leads to both reduced cellulose synthesis and ectopic lignification (Caño-Delgado et al., 2003). These changes were correlated with increased expression of the genes involved in stress response and with pathogen resistance (Ellis et al., 2002).

Our data indicate that the defect in the biosynthesis of an essential phenolic component leads to a similar phenotype, including elevated expression levels of genes related to jasmonate signaling, reduced growth, and activation of several stress/defense-related genes. Phenolics thus seem to be one of the elements that relate cell wall biosynthesis with growth promotion and stress signaling. No significant activation of the ethylene signaling pathway was observed in our experiments, but expression profiling indicated higher transcript levels for genes related to abscisic acid signaling in cyp98A3 mutants. Abscisic acid has been implicated as a signal transduction component in mainly abiotic stress responses, in particular responses to salt, cold, and drought stress (Pastori and Foyer, 2002, and refs. therein), but it has also long been known that abscisic acid inhibits plant growth and is an inducer of dormancy (Koornneef et al., 2002; Xiong and Zhu, 2003). Therefore, it appears plausible that the observed higher levels of transcripts related to abscisic acid signaling may also be related to the observed growth phenotypes, including developmental arrest. In this context, it is noteworthy that cyp98A3 insertion mutants maintain in a state of developmental arrest and can survive for months without apparent signs of senescence.

What Is the Alternative meta-Hydroxylation Pathway?

The proposed alternative meta-hydroxylation pathway is not expressed during developmental stem lignification and cannot complement developmental lignification, because mutations in CYP98A3 lead to an almost complete lack of G and S lignin units in stem (Franke et al., 2002a; Table I). However, it is expressed in tissues of the cyp98A3 insertion mutant, which normally do not lignify. Together with the increased production of soluble phenolics and stress-related phenylpropanoids, this suggests that the proposed alternative pathway is active under stress conditions and that it is likely a defense-related pathway. Such a hypothesis is supported by the observed activation of genes related to the jasmonate and abscisic acid cascades. The existence of divergent isoforms with separate functions in developmental lignin biosynthesis and in stress-related production of phenolics has
been proposed for some enzymes of the phenylpropanoid pathway, e.g. for cinnamoyl-CoA reductase (Lauvergeat et al., 2001). However, it is unlikely that an alternative, stress-related pathway uses the same substrates as CYP98A3, because plants with reduced energy assimilation are not expected to accumulate shikimic or quinic acid in amounts sufficient for allowing their coupling to p-coumaric acid. The CoA ester of p-coumaric acid or its precursors are, however, expected to accumulate when CYP98A3 and/or shikimate become limiting (Fig. 1), which could also lead to the observed accumulation of flavonoids. Such an accumulation of precursor substrates under stress conditions, or in mutant plants, may allow the activity of an enzyme with low affinity for phenolics to become physiologically relevant. It is also possible that the expression of a specific hydroxylase is activated exclusively under stress conditions. The most obvious candidate genes encoding alternative 3-hydroxylases would be the other members of the CYP98 family. The genes most closely related to CYP98A3 in Arabidopsis, CYP98A8 and CYP98A9, do not catalyze the meta-hydroxylation of any free or conjugated phenolic substrates, including Glc and CoA derivatives (Schoch et al., 2001; M. Morant, G. Schoch, P. Ullmann, T. Ertunc, D. Little, C. E. Olsen, M. Petersen, J. Negrel, D. Wercz-Reichhart, unpublished data). However, many other enzymes were previously proposed to catalyze the meta-hydroxylation of various phenolic precursors (Schoch et al., 2001, and refs. therein; Franke et al., 2002b, and refs. therein). The production of meta-hydroxylated units under stress conditions may rely on one or several of them.

MATERIALS AND METHODS

Isolation of the cyp98A3 Mutant

An insertion mutant for CYP98A3 was isolated by screening the Alpha collection at the Arabidopsis Knockout Facility at the University of Wisconsin Biotechnology Center (http://www.biotech.wisc.edu/Arabidopsis/index2.asp; Krysan et al., 1999). Two primers were selected to identify insertions in the CYP98A3 coding sequences: 98A3F1 (5'-TGACGGACACACGAAATGTTCA-GAAATCAGGCTATCTGGTTTCCATAAGCGGTG-3') and anti-sense primer 98A3b1 (5'-GGGACATCGTTGAGGAAGGGTTGATG-3') and 98A3R2 (5'-AATACTACGATCCGATCATGTTCCATTTAC-3'). An insertion was identified in the first intron of CYP98A3, and plants heterozygous for the insertion were found in pool 177. The T-DNA contained left borders at both T-DNA junctions based on PCR and DNA sequence analysis.

Construction of the CYP98A3 Overexpression Vector for Genetic Complementation and Cosuppression

The CYP98A3 cDNA inserted in a pGEM-T vector was amplified using specific primers with added attB recombination sites (Gateway Technology; Karimi et al., 2002; sense primer 98A3b1 (5'-GGGGACACATTTTGAGGATATTCATATCTGAAGCAGTTCTCAGCTGAGTTTAAGGACTGTTATGCAACTTTAGCCAGGCTATCTGGTTTCCCTATAGCAGGTG-3') and anti-sense primer 98A3b2 (5'-GGGGACCATTTTGAGGATATTCATATCTGAAGCAGTTCTCAGCTGAGTTTAAGGACTGTTATGCAACTTTAGCCAGGCTATCTGGTTTCCCTATAGCAGGTG-3'). The PCR reaction was carried out using as template 200 ng of the plasmid DNA, 10 μM of each primer, and High Fidelity PCR master mix (Roche), according to the recommendations of the manufacturer. After 3 min of heating at 96°C, 18 cycles of amplification were carried out as follows: 1 min denaturation at 95°C, 1 min at 60°C, and 2-min extension at 72°C. The reaction was completed by a 10-min extension at 72°C. The PCR product was purified on a 1% agarose gel and cloned into the pDONR201 donor vector (Invitrogen, Gateway Technology). The ligation mix (20 μL) contained 300 ng of the PCR product, 300 ng of the pDONR201 vector, and BP Clonase reaction buffer, the volume being adjusted to 16 μL with sterile water, according to the instructions of the manufacturer. The reaction was started with 4 μL of BP Clonase enzyme mix. It was incubated at 25°C for 18 h and stopped by 10-min incubation at 37°C with proteinase K (4 μg/20 μL of ligation medium). Electrocompetent bacteria (DH5a) were transformed by electroporation with 1 μL of the ligation medium. Plasmid DNA of positive colonies selected on kanamycin (100 μg/mL) was prepared (NucleoSpin Plasmid) and sequenced for selection of a clone corresponding to the expected sequence. After extraction of the pDONR201 plasmid from the selected clone, the insert was transferred to the destination expression vector pB7WG2 (Karimi et al., 2002) for insertion of the CYP98A3 cDNA downstream of the CaMV 35S promoter. The reaction was carried out in a 20-μL mix containing 300 ng of pDONR201 with the CYP98A3 insert, 800 ng of pB7WG2, and LR Clonase buffer, the volume being adjusted to 16 μL with sterile water. Four microliters of LR Clonase enzyme mix were added before incubation at 25°C for 18 h, according to the protocol recommended by the manufacturer. The reaction was stopped by 10-min incubation at 37°C in the presence of proteinase K (4 μg/20 μL of reaction medium). The plasmid DNA (NucleoSpin Plasmid) prepared from the positive colonies selected on spectinomycin (100 μg/mL) was verified by digestion and PCR.

Plant Growth and Transformation

Seeds from Arabidopsis (Arabidopsis thaliana) Ws, Col-0, and heterozygous cyp98A3 mutants were kept at 4°C for 4 d and then washed for 5 min in 70% ethanol. After 20-min incubation with gentle shaking in 20% bleach supplemented with 0.1% Triton X-100, they were washed four times in sterile distilled water and germinated on Murashige and Skoog medium (Duchefa) containing 0.8% Pastagar (Sigma-Aldrich), 1% Suc (Carlo Erba Reagenzti), with or without 50 μg/mL kanamycin (Duchefa). Plates were placed in a 21°C growth chamber with a 16-h-light/8-h-dark photoperiod for 15 d.

For plant transformation (genetic complementation and cosuppression), some cyp98A3 heterozygous mutant or wild-type seedlings were grown on soil at 21°C under a 16-h-light/8-h-dark photoperiod. The pB7WG2 vector was introduced into Agrobacterium tumefaciens LBA 4404 virG by electroporation, and stable transformation of the construct in A. tumefaciens was confirmed by digestion of the plasmid isolated from the transformants and PCR using T-DNA-specific primers. The plants were then transformed using the floral-dip method (Clough and Bent, 1998).

Seeds from the T0 plants were collected and grown on soil at a density of 1,500 plants per 25 × 50 cm pot. Plants were sprayed with Basta (AgroÉvo), 250 mg/L, 5, 14, and 21 d after germination. Transgenic plants resistant to the herbicide were transferred into individual pots and seeds were collected as above. For in vitro selection, seeds of the T1 plants were grown on Murashige and Skoog medium supplemented with 0.8% Pastagar, 1% Suc, 10 μg/mL Basta, and 500 μg/mL carbenicillin.

Circadian rhythm analysis was performed with plants grown under a 12-h-light/12-h-dark cycle in vitro for 15 d for the insertion null mutants and on soil for 10 weeks for the cosuppressed plants.

Analysis of Transgenic Plants by PCR

Segregation analysis and selection of transgenic plants were performed using PCR. Approximately 100 mg of leaf tissue from T1 plants was bulked for DNA isolation using the method described by Fulton et al. (1995). The PCR mixture contained 600 ng of template, 20 pmol of primers, 200 μM dNTPs, 1 × DyNazyme buffer (Finnzyme), 0.5 μM betain (N,N,N-trimethyl-glycine; Sigma-Aldrich), and 0.6 units of DNA polymerase (DyNazyme) in a total volume of 25 μL. The following primers were used for testing for the presence of the T-DNA in the CYP98A3 gene (Fig. 2):

Tac2 (CYP98A3 promoter region) 5'-AAACTACGATCCGATCATGATTCTC-3'; LinC (T-DNA left border) 5'-CTTGTTGCTTGGATTAATGACGGATGTTGGC-3'; and P2 (3' end of the CDS) 5'-GCCCAAGATGTCCCTTCGATGTTGCT-3'. The primers used for testing for the presence of the T-DNA construction in A. tumefaciens and in the complemented cyp98A3 mutants were 98A5 (CYP98A3) CDS 5'-ACCCGAGGAGATGACATGTCATGC-3' and (Bar gene) 5'-CTAGGGTCAAAGAAGGCAG-3'.

RNA-Blot Hybridization

Total RNA was extracted from transgenic and wild-type plants using the TRizol method described by Chomczynski and Sacchi (1987). Total RNA

Lignin Histochemistry

Inflorescence stems, leaves, and roots of 10-week-old cosuppressed plants and roots of 3-week-old null seedlings were cleared in ethanol and sectioned with a razor blade (sections cut to approximately 200-μm thickness). The stem sections were stained with phloroglucinol-HCl (Wiesner reagent, 1% [w/v] phloroglucinol in 6 M HCl; Adler et al., 1948) and observed under a microscope.

LC-MS Analyses of Soluble Phenolics

Soluble phenolics from 15-d-old plate-grown seedlings were analyzed as described by Goujon et al. (2003). Briefly, phenolics from whole control (Ws) and homozygous or heterozygous cyp98A3 and control plants (six plants per assay carefully weighted and three assays per line) were extracted by a prolonged maceration in 2 mL of methanol/water (4:1; v/v) mixture in the presence of morin hydrate (5 μg; Fluka) as internal standard. The extracts were ultratitrated (0.45 μM; Millies) before injection (10 μL) into a HPLC-PDA/electrospray ionization-MS system comprising a Finnigan LCQ-DECA mass spectrometer (ThermoFinnigan). HPLC was carried out on a HYPURITY C18 column (Thermo-Hypersil; 4.6 mm × 150 mm, particle size 5 μm) at a flow rate of 1.1 mL/min. The elution was performed using a linear elution gradient from 5% to 65% solvent B (CH3CN + HCOOH, 0.1%) in solvent A (H2O + HCOOH, 0.1%) within 30 min. The mass spectral data were collected in both negative mode with the ion trap mass spectrometer equipped with a heated capillary electrospray interface. The sprayer needle voltage was set to 4 kV and the capillary was heated to 350°C. Product peaks were identified by their mass spectrometric fragmentation pattern and relative to data reported in the literature (Veit and Pauli, 1999; Bloor and Abrahams, 2002; Toghe et al., 2005).

The quantitative evaluation of the main identified p-hydroxycinnamoyl derivatives and flavonol glycosides was made from ion chromatograms reconstructed at their most specific ion (deprotonated molecule most often) relative to the morin internal standard and with a response factor arbitrarily set at 1 as each flavonol glycoside was not available as an authentic compound. The main flavonol glycosides identified and evaluated herein were kaempferol and quercetin derivatives (Figs. 4 and 5), together with trace amounts of isorhamnetin as detailed in supplemental text. Besides the flavonol glycosides, SM, SG, CG, and CM could be identified from their mass and UV spectra (see supplemental text).

The leaves of Col-0 and of the corresponding 10-week-old cosuppressed plants grown on soil at 21°C under a 16-h-light/8-h-dark photoperiod were also analyzed for their soluble phenolics by electrospray-MS run in the negative mode for the analyses of flavonol p-hydroxycinnamic derivatives and in the positive mode to detect anthocyanins. In addition to the major anthocyanin of Arabidopsis, cyanidin 3-O-[(2'-O-(6'-O-(sinapoyl) xylosyl) 6'"-O-(p-O-glucosyl-p-coumaroyl) glucoside) 5-O-(6'"-malonyl) glucoside, identified by Bloor and Abrahams (2002), we could observe a series of other cyanidin derivatives differing from the main one by their acylation mode, as reported by Toghe et al. (2005) in the case of an Arabidopsis mutant overexpressing a MYB transcription factor.
resoluibilized in 1 mL of CHCl₃ before silylation and GC-MS analysis as previously described (Lapierre et al., 1995). The quantitative determination of the main H, G, and S lignin-derived monomers, analyzed as their trimethylsilylated derivatives, was carried out from specific ion chromatograms reconstructed at m/z 239 for the H monomers, 269 for G monomers, and 299 for S monomers after an appropriate calibration relative to the docosane internal standard.

Chemical Complementation

Chlorogenic, p-coumaric, and caffeic acids were obtained from Sigma-Aldrich. Caffeoyl shikimate was synthesized enzymatically from enzymatically synthesized p-coumaroyl shikimate using recombinant CYP98A3 expressed in yeast (Saccharomyces cerevisiae; Schoch et al., 2001). The enzymatic conversion was carried out at 27°C for 45 min in several vials, with a final volume of 200 μL for each vial. The assay mixture contained 2 μM p-coumaroyl shikimate, 100 pmol of CYP98A3, 0.9 mM NADPH, 0.1 mM potassium phosphate, pH 7.4, and a regeneration system (1 mM Glc-6-P and 1 unit Glc-6-P dehydrogenase). The reaction was stopped by acidification with 12 μL of 4% HCL. The reaction medium was then extracted three times with 1 mL of ethyl acetate. The organic phases were evaporated under argon and the identity of the product was checked by UV spectroscopy and by HPLC analysis as described (Schoch et al., 2001).

To test chemical complementation, 2-week-old cyp98A3 mutant seedlings were transferred to plates with Murashige and Skoog medium containing 90 μM of the tested compounds for 2 more weeks.

Transcriptome Studies

The microarray analysis has been performed using the CATMA array containing 24,576 gene-specific tags from Arabidopsis (Crowe et al., 2003; Hilson et al., 2004). The gene-specific tag amplicons were purified on Multiscreen plates (Millipore) and resuspended in Tris-EDTA-diethyl sulfoxide at 100 ng/μL. The purified probes were transferred to 1,536-well plates with a Genesis workstation (Tecan) and spotted on UltraGAPS slides (Corn ing) using a Microgrid II (Genomic Solution). The current CATMA version printed at the Unité de Recherche Génomique Végétale consists of 36 metablocks. For transcriptome studies, total RNA from around 1 g of whole-plant material from 15- to 20-d-old wild-typeWs and cyp98A3 null plants (grown on Murashige and Skoog plates at 21°C with a 16-h-light/8-h-dark photoperiod) were extracted using the TRizol (Invitrogen) method as described by Chomczynski and Sacchi (1987) followed by two ethanol precipitations, then checked for RNA integrity with the Bioanalyzer from Agilent. Two biological replicates with independent dye swaps were performed for each comparison. cRNAs were produced from 2 μg of total RNA from each pool with the Message Amp aRNA kit (Ambion). Then 5 μg of cRNAs were reverse transcribed in the presence of 200 units of SuperScript II (Invitrogen), Cy3-dUTP, and Cy5-dUTP (NEN) according to Puska et al. (2002) for each slide. Samples were combined, purified, and concentrated with YM30 Microcon columns (Millipore). Slides were prehybridized for 1 h and hybridized overnight at 42°C in 25% formamide, 5 × SSC, 1% SDS. Slides were washed at room temperature in 2 × SSC + 0.1% SDS for 4 min, 1 × SSC for 4 min, 0.2 × SSC for 4 min, 0.05 × SSC for 1 min, and dried by centrifugation. Four hybridizations (two dye swaps) were carried out. The arrays were scanned on a GenePix 4000A scanner (Axon Instruments) and images were analyzed using the Genetix Pro 3.0 software (Axon Instruments).

Statistical Analysis of Microarray Data

Statistical analysis was performed as described in Lurin et al. (2004) based on two dye swaps, i.e., four arrays each containing the 24,576 gene-specific tags and 384 controls. The controls were used for assessing the quality of the hybridizations, but were not included in the statistical tests. For each array, the raw data comprised the logarithm of median feature pixel intensity at a wavelength of 635 nm (red) and 532 nm (green). No background was subtracted. First, we excluded spots that were considered badly formed features by manual inspection. Then we performed a global intensity-dependent normalization using the LOESS procedure (Yang et al., 2002) to correct the dye bias, thus generating four sets of log, ratios comparing mutant and control. Finally, on each block, the log-ratio median is subtracted from each value of the log ratio of the block to correct for print-tip effects in each metablock. To determine differentially expressed genes, we performed a paired t test on the log ratios. The number of observations per spot varies between 2 and 4 and is therefore inadequate for calculating a gene-specific variance. For this reason, we assume that the variance of the log ratios is the same for all genes and adjusted the parametric P-values by the Bonferroni method, which controls the family wise error rate. Differentially expressed genes (adjusted p [t test] < 0.05) between mutant and wild type were divided into groups of up- and down-regulated genes (mutant/wild type). Upon removal of duplicates and elements lacking a locus annotation (Atgxxxxx), these genes were used to search the Functional Category Database at MAIDB (mips.gsf.de/proj/functcatDB; Schoof et al., 2002). The frequency of differentially expressed genes that change more than 3-fold in each functional category was compared to the frequency of all genes represented on the microarray using a hypergeometric distribution and only categories that contain more than three gene entries and are over-represented in the group of differentially expressed genes (p [hyper] < 0.01) were retained. Using the same statistical analysis, the frequency of differentially expressed genes that have been placed in selected groups of GO categories was compared to the frequency of all genes on the array in these GO terms. Curator-annotated genes placed in the GO terms “involved in the metabolism of,” “involved in the signaling mediated by,” or “involved in the response to” the hormones auxin, abscisic acid, brassinosteroid, cytokinin, ethylene, gibberellic acid, jasmonic acid, and salicylic acid, respectively, were retrieved from TAIR database (Arabidopsis.org; Garcia-Hernandez et al., 2002). Gene lists related to each hormone were combined and duplicate entries in each group were removed prior to statistical analysis.

Immunofluorescence Visualization of the Microtubules

Whole 15-d-old seedlings were fixed in 1.5% (v/v) formaldehyde and 0.5% (v/v) glutaraldehyde in PEM buffer (50 mM PIPES, 2 mM MgSO₄, 0.05% [v/v] Triton X-100, pH 7.2) for 40 min, and rinsed in PEM buffer three times for 10 min. The samples were subsequently digested with 0.05% (w/v) Pectolyase Y-23 (Kikkoman) in PEM buffer (50 mM PIPES, 2 mM EGTA, 2 mM MgSO₄) with 0.4 μm mannitol for 20 min, rinsed in PEM buffer three times, treated with 220°C methanol for 10 min, and rehydrated in phosphate-buffered saline (PBS; 136 mM NaCl, 6 mM Na₂HPO₄, 2.7 mM KCl, 1.5 mM KH₂PO₄, pH 7.0) for 10 min. Autofluorescence caused by free aldehydes from glutaraldehyde fixation was reduced with 1 mg/ml NaBH₄ in PBS for 20 min, followed by a treatment with 50 mM Glys in PBS (incubation buffer) for 30 min. Seedlings were incubated with primary antibodies against tubulin at 4°C overnight, rinsed in incubation buffer three times for 10 min, and secondary antibodies were applied for 3 h at 37°C. After rinsing in PBS three times, roots were cut off from the rest of seedlings and mounted in 0.1% (w/v) paraformaldehyde in 1:1 PBS/glycerol, pH 9. Cut cover glasses were used to space slide and cover glasses so as to avoid crushing delicate root tips. Anti-tubulin (product N357; Amersham) was used at a dilution of 1:1,000 for this study. Fluorescein isothiocyanate-conjugated anti-mouse IgG (Silenus/Amrad Biotech), diluted 1:100, was used as a secondary antibody.

Immunofluorescence images were collected with an MRC-Bio-Rad 600 (Microscience Division) confocal laser-scanning microscope, coupled to a Zeiss Axiophot IM-10 inverted microscope. Excitation at 488 nm with an argon ion laser was used for fluorescein isothiocyanate fluorochromes. Images were collected using a Plan Neofluar 100× objective lens, N.A. 1.30, following Kalman averaging of six full scans. Images were processed with image-processing software programs including COMOS 7.0 (Microscience Division), confocal assistant 4.02 (written by Todd Clark Brelje), and Adobe Photoshop 4.0 (Adobe Systems).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_180006.

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