Activation Tagging Identifies a Conserved MYB Regulator of Phenylpropanoid Biosynthesis

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Plants produce a wide array of natural products, many of which are likely to be useful bioactive structures. Unfortunately, these complex natural products usually occur at very low abundance and with restricted tissue distribution, thereby hindering their evaluation. Here, we report a novel approach for enhancing the accumulation of natural products based on activation tagging by Agrobacterium-mediated transformation with a T-DNA that carries cauliflower mosaic virus 35S enhancer sequences at its right border. Among \( \sim 5000 \) Arabidopsis activation-tagged lines, we found a plant that exhibited intense purple pigmentation in many vegetative organs throughout development. This upregulation of pigmentation reflected a dominant mutation that resulted in massive activation of phenylpropanoid biosynthetic genes and enhanced accumulation of lignin, hydroxycinnamic acid esters, and flavonoids, including various anthocyanins that were responsible for the purple color. These phenotypes, caused by insertion of the viral enhancer sequences adjacent to an MYB transcription factor gene, indicate that activation tagging can overcome the stringent genetic controls regulating the accumulation of specific natural products during plant development. Our findings suggest a functional genomics approach to the biotechnological evaluation of phytochemical biodiversity through the generation of massively enriched tissue sources for drug screening and for isolating underlying regulatory and biosynthetic genes.

INTRODUCTION

Ethnobotany and limited screens of medicinal plants indicate that the huge repertoire of chemical diversity in plants contains many potentially useful bioactive structural principles for developing novel drugs, flavors, fragrances, and other specialty chemicals. Unfortunately, these complex natural products usually occur in very low abundance and with a restricted tissue distribution. Moreover, almost all of this phytochemical biodiversity resides in exotic, uncultivated species. Whereas drugs such as taxol, vinblastine, and vincristine illustrate the potential of plants as sources of new drugs, the development of rational approaches for the generation of useful amounts of complex natural products for industrial evaluation remains an unsolved problem. In particular, an intense 30-year effort using cell and tissue cultures from medicinal plants has failed to generate useful quantities of complex products for the commercial production of established drugs in vitro or for high-throughput, multiplex screening of phytochemicals (Facchini and Deluca, 1995; McCaskill and Croteau, 1998). This failure probably reflects the stringent spatial and temporal transcriptional controls governing the biosynthesis of specific natural products during plant development (Fowler, 1983; Robins, 1994; Facchini and Deluca, 1995). Transgenic manipulation to override these genetic controls thus may provide the key to enhancing natural product biosynthesis for industrial evaluation and exploitation.

Activation tagging with the enhancer from the cauliflower mosaic virus 35S transcript promoter (35Se) is an emerging technology in plant functional genomics (Weigel et al., 2000). This approach, based on Agrobacterium-mediated transformation, can create transgenic plants in which the T-DNA carrying 35Se at its right border is spliced into the plant genome at random sites. In each independent transgenic line, 35Se strongly activates the plant gene to which, by chance, it lies adjacent. Activation of a gene in this fashion may lead to observable effects on the modified plant, providing important clues about the function of the activated gene. Screening large collections of independent, activation-tagged lines thus represents a powerful way of surveying the genome and isolating genes that affect traits of interest.

Using activation tagging, we have isolated a bright-purple mutant, production of anthocyanin pigment 1-Dominant (pap1-D), in which genes encoding enzymes involved in the biosynthesis of phenylpropanoid natural products exhibit massive and widespread activation throughout plant development. The pap1-D phenotype, which is caused by...
Figure 1. *pap1-D* Phenotypes.

(A) *pap1-D* (left) and Col-0 (right) flowers.
(B) Roots of *pap1-D* (left) and Col-0 (right) plants.
(C) Six-week-old adult *pap1-D* (front) and Col-0 (back) plants.
overexpression of a gene encoding an MYB transcription factor, indicates that activation tagging can be used to overcome the stringent genetic controls regulating the developmental accumulation of specific natural products. These findings suggest a new approach for the systematic biotechnological evaluation of phytochemical biodiversity through the generation of massively enriched tissue sources for drug screening and for isolation of the underlying regulatory and biosynthetic genes.

RESULTS

Mutant Characterization

Approximately 5000 activation-tagged primary lines of Arabidopsis ecotype Columbia (Col-0) were generated by using pSKI015, which contains four copies of 35S at the right border of the T-DNA, pBluescript KS+ for plasmid rescue, and the BAR gene for Basta resistance as a selectable marker (Kardailsky et al., 1999; Weigel et al., 2000). A single bright-purple plant was observed in this collection, and its seed was collected for progeny analysis. T<sub>2</sub> plants segregated for the purple coloration characteristic of anthocyanins in a 3:1 ratio, which is consistent with this trait being determined by a single dominant allele, an allele we named pap1-D (see above). The whole plant, including the roots, stems, leaves, primary and secondary branches, and cauline leaves as well as sepals, anthers, and carpels, exhibited purple pigmentation (Figures 1B and 1C). The purple coloration was more pronounced when plants were grown under high-intensity light or other stress conditions, such as drought and pathogen infection (data not shown). Under these conditions, leaves and stems of wild-type plants also showed a slight pigmentation, suggesting that the pap1-D phenotype might in part reflect enhancement of an endogenous stress response. However, we never observed pigmentation in the roots of wild-type plants—in marked contrast to the strong pigmentation at the base of pap1-D roots (Figure 1B). Except for very weak pigmentation in flower petals (Figure 1A), enhanced pigmentation in pap1-D was observed throughout development. No other morphological phenotypes were observed.

Because anthocyanins are a subclass of flavonoid natural products derived from the phenylpropanoid skeleton, we examined the expression of phenylpropanoid biosynthetic genes and the accumulation of natural products. RNA gel blot analysis showed massive enhancement of the expression of phenylpropanoid biosynthetic genes in pap1-D plants (Figure 2). The amounts of transcripts encoding chalcone synthase (CHS), the entry point enzyme into the flavonoid branch pathway, and dihydroflavonol reductase, an enzyme of flavonoid biosynthesis specific for anthocyanins, were greater in pap1-D plants than in wild-type Col-0 plants.

Figure 2. Enhanced Expression of Phenylpropanoid Biosynthetic Genes in pap1-D.

RNA gel blot hybridization was conducted with total RNA isolated from 6-week-old pap1-D and Col-0 wild-type plants. DFR, dihydroflavonol reductase; GST, glutathione S-transferase; UBQ, ubiquitin.

Transcripts encoding glutathione S-transferase, which has been implicated in the transport of anthocyanins into the vacuole (Affenito et al., 1998), also were expressed in increased amounts. Moreover, the accumulation of transcripts that encode phenylalanine ammonia-lyase (PAL), the first enzyme of the overall phenylpropanoid biosynthetic pathway, also was markedly enhanced, indicating that transcriptional activation was not confined to the flavonoid branch.

To determine the extent of changes in anthocyanins and other phenylpropanoid-derived compounds in pap1-D, we extracted and analyzed soluble and cell wall–bound phenolic compounds by HPLC. Analysis of the soluble fraction, which was obtained by extraction in acetone, revealed several quantitative differences between pap1-D and wild-type Col-0 plants—in particular, increased concentrations of certain flavonol glycosides, including Glc-rhamnose (Rha)-quercetin, Glc-Rha-kaempferol, and unidentified conjugates of kaempferol and quercetin (Figures 3A and 3B). After alkaline hydrolysis of this residue that was obtained by acetone extraction, one portion was freeze-dried for analysis of anthocyanidins (anthocyanin aglycones); the remainder was partitioned into ethyl acetate for determination of cell wall–bound hydroxycinnamic acids. Anthocyanidins were present in greater concentrations in pap1-D than in Col-0 (Figures 3C and 3D), as were coumaric and sinapic acids measured in alkaline hydrolysates of the wall-bound phenolic fraction (Figures 3E and 3F). Thus, pap1-D is characterized by strongly increased concentrations of glycosylated anthocyanins, flavonols, and cell wall–esterified hydroxycinnamic acids in comparison with wild-type Col-0.
The observation of increased wall-bound hydroxycinnamic acids in pap1-D prompted us to measure the content and composition of lignin, which is derived from hydroxycinnamic acid precursors. Lignin was analyzed by derivatization followed by reductive cleavage, which helps to determine the absolute amounts of guaiacyl (G, monomethylated) and syringyl (S, dimethylated) lignin monomers (Lu and Ralph, 1997). The results in Table 1 indicate increases in both total G and total S residues in the cell wall fraction of pap1-D compared with those in Col-0, but the S/G ratio varied little. The change in lignin monomers could reflect an increase in lignin content or a change in composition that led to more efficient monomer extractability.

Changes in lignin content and composition have been engineered in transgenic plants by downregulation of PAL, caffeic acid O-methyltransferase, and caffeoyl-CoA O-methyltransferase, enzymes of the lignin branch of phenylpropanoid biosynthesis (Atanassova et al., 1995; Zhong et al., 1998). PAL activity in stems of pap1-D plants was approximately twice that found in stems of wild-type plants, whereas the activities of the two O-methyltransferases differed little between the two (Table 1). Thus, the changes in lignin composition and increased concentrations of wall-bound sinapic acid in pap1-D reflect the change in PAL activity but do not appear to be associated with increases in lignin O-methyltransferase activities.

The Arabidopsis transparent testa glabra1-1 (ttg1-1) mutation blocks anthocyanin accumulation and trichome formation (Koornneef, 1981). TTG1 encodes a WD40 repeat protein homologous with an AN11-encoded protein from petunia, which also controls anthocyanin production (de Vetten et al., 1997; Walker et al., 1999). To test the genetic
relationship between TTG1 and PAP1, we crossed ttg1-1 with pap1-D. The pap1-D allele was tracked by Basta resistance, and the ttg1-1 mutation was scored visibly. The double mutant F₂ plants failed to accumulate anthocyanins, indicating that TTG1 is required for the production of anthocyanins mediated by PAP1 overexpression and acts either downstream from or at the same step as PAP1.

**Cloning of PAP1**

In a population of >100 segregating T₂ plants, each plant that had the pap1-D phenotype showed resistance to Basta, and all plants with a wild-type phenotype (i.e., lacking purple pigmentation) were sensitive to Basta, indicating that the mutation was tightly linked to the T-DNA insert. Moreover, pigmentation (and all plants with a wild-type phenotype (i.e., lacking purple pigmentation) were sensitive to Basta, indicating that the mutation was tightly linked to the T-DNA insert. Moreover, pigmentation was scored visibly. The double mutant F₂ plants failed to accumulate anthocyanins, indicating that TTG1 is required for the production of anthocyanins mediated by PAP1 overexpression and acts either downstream from or at the same step as PAP1.

**Table 1. Enhanced PAL Activity and Lignin Levels in pap1-D Plants**

<table>
<thead>
<tr>
<th>Plant</th>
<th>PAL Activity (mkat/g FW)</th>
<th>COMT Activity (mkat/g FW)</th>
<th>CCOMT Activity (mkat/g FW)</th>
<th>Lignin Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stem</td>
<td>Stem</td>
<td>Stem</td>
<td>Total G (μmol/g)</td>
</tr>
<tr>
<td>Wild type</td>
<td>30.5</td>
<td>44.2</td>
<td>50.4</td>
<td>21.6</td>
</tr>
<tr>
<td>pap1-D</td>
<td>70.3</td>
<td>44.4</td>
<td>60.6</td>
<td>30.6</td>
</tr>
</tbody>
</table>

*aFW, fresh weight; kat, katal.

*bCOMT, caffeic acid O-methyltransferase.

*cCCOMT, caffeoyl-CoA O-methyltransferase.

Sequence alignments with the Arabidopsis databases showed that PAP1 is a member of the R2, R3 MYB family, which is estimated to have >100 members in Arabidopsis (Kranz et al., 1998; Romero et al., 1998) and >80 members in maize (Rabinowicz et al., 1999). PAP1 is identical to ATMYB75 (Kranz et al., 1998) except that ATMYB75 contains a sequencing error (1-bp deletion at position 695), creating an early stop codon. The PAP1 protein shares high homology with other MYB-like transcription factors that regulate anthocyanin production (Figures 4B and 4D). PAP1 is closely related to the product of the petunia AN2 gene, showing 82% identity through the MYB region and 50% identity overall. The products of the maize anthocyanin MYB genes C1 and PL are 64% identical through the MYB region, with 38% identity overall. The MYB transcription factor GLABAROUS1 from Arabidopsis and MIXTA from snapdragon both control trichome development (Oppenheimer et al., 1991; Glover et al., 1998) and are each 58% identical to PAP1 in the MYB domain and 33% identical overall. A phylogenetic tree constructed with these full-length MYB proteins shows that PAP1 belongs to a branch involved in anthocyanin biosynthesis (Figure 4D).

The PAP1 gene was mapped to 0.2 centimorgan (cM) up from mi303 at 83.7 cM on chromosome 1 by using an XbaI restriction fragment length polymorphism and the Col/Ler recombinant inbred lines (Nottingham University Stock Centre, Nottingham, UK). The sequencing project recently came to PAP1 on bacterial artificial chromosome F25P12 just below mi303 at 85 cM.

**PAP2**

Also discovered in the Arabidopsis database was 193M15, an expressed sequence tag with very high homology with
Figure 4. Molecular Characterization of PAP1.
**Activation Tagging of PAP1**

**Overexpression of PAP1 and PAP2 in Tobacco**

To test whether PAP1 and PAP2 could enhance pigmentation in other plants, we transformed tobacco *cv. xanthi* with pCHF3-PAP1 and pCHFS-PAP2. Both constructs generated purple tobacco plants, indicating that the Arabidopsis PAP1 and PAP2 genes could activate production of anthocyanin pigments in another species (Figures 5G and 5H). These transgenic tobacco plants also produced flowers with much more pigmentation than did the pCHF3 transgenic control plants (Figures 5I and 5J). Tobacco transformed with pCHF3 as an empty vector control did not have an increased pigmentation phenotype (Figures 5F and 5I).

**DISCUSSION**

Accumulation of phenylpropanoid products during development is under tight transcriptional regulation, and the *pap1-D* phenotype represents a striking override of these genetic controls. Thus, specific tranches of the overall pathway appear to be controlled by separate sets of transcription factors. For example, the maize `myb` genes *C1* and *pl* are involved in the regulation of anthocyanin synthesis from *CHS* onward but do not regulate *PAL* and other genes of the upstream central pathway (Cone et al., 1993a, 1993b; Mol et al., 1996), whereas *P* independently controls the 3-deoxyflavonoid branch pathway (Grotewold et al., 1994). In contrast, the *pap1-D* phenotype, which results from overexpression of the PAP1 MYB transcription factor, reflects massively enhanced expression of *PAL* as well as *CHS*, the gene encoding dihydroflavonol reductase, and the glutathione S-transferase gene. This broad transcriptional activation of the overall pathway is accompanied by a correspondingly broad pattern of increased product accumulation with increases in lignin, wall-bound hydroxycinnamic acid esters, flavonols, and anthocyanins. Moreover, pathway activation in *pap1-D* was observed in all vegetative organs and maintained throughout development, in marked contrast to activation in wild-type plants of individual branch pathways at defined developmental stages and with characteristic cell-type, tissue-type, and organ specificities (Graham, 1991; Grotewold et al., 1994). The relatively modest increase in lignin content probably reflects a major control point at the polymerization stage (Bate et al., 1994) with consequent spillover of lignin monomers and their precursors, which contributes to the marked accumulation of wall-bound hydroxycinnamic acid esters in *pap1-D*.

**MYB** genes contribute to the control of flavonoid biosynthesis in a wide range of plant species, often in combination with other regulatory genes. The extensive sequence similarity with *AN2*, *c1*, and *pl*, together with the overexpression phenotypes, suggests that PAP1 and PAP2 may be the Arabidopsis orthologs of these petunia and maize `myb` genes, with genetically defined functions in phenylpropanoid regulation. In maize, *c1* and *pl* but not *P* require *R* and *B*, encoding basic helix-loop-helix proteins, to activate transcription of maize flavonoid biosynthetic genes (Mol et al., 1996). Basic helix-loop-helix proteins and MYB proteins also function together in the control of flower pigmentation in snapdragon (Goodrich et al., 1992) and petunia (Quattrocchio et al., 1998). Moreover, the WD40 proteins TTG1 and AN11 are required for MYB control of flavonoid biosynthesis in both...
Arabidopsis and petunia (de Vetten et al., 1997; Larkin et al., 1999; Walker et al., 1999), and the pap1-D phenotypes require the WD40 gene TTG1. Despite the stringent and often complex genetic control of phenylpropanoid biosynthesis, strong overexpression of PAP1 in the pap1-D line was sufficient to hyperactivate the pathway, which is reminiscent of the enhancement of flavonoid biosynthesis by deliberate ectopic expression of P in suspension cultures of maize cells (Grotewold et al., 1999). The pap1-D phenotypes may reflect involvement of PAP1 as the limiting factor in a novel regulatory circuit with atypically broad control functions in phenylpropanoid biosynthesis or may indicate functional spillover from one regulatory circuit to related circuits when PAP1 is massively overexpressed.

A recent report describes the use of activation tagging in Catharanthus cell cultures to isolate ORCA3, a jasmonate-responsive transcriptional regulator of primary and secondary metabolism, the upregulation of which promotes biosynthesis of indole alkaloids (Van der Fits and Memelink, 2000). These data, along with the present findings, indicate that activation tagging can be used to generate novel gain-of-function mutations that affect complex biosynthetic pathways under polygenic control; as such, this presents a potentially powerful new approach for isolating the genes that regulate biosynthesis of plant natural products. Loss-of-function screens for transparent testa have been saturated, and no mutations map to the PAP1 or PAP2 loci (Shirley et al., 1995). Moreover, examination of >100 PAP1 antisense lines showed no visible phenotype (data not shown). The similar overexpression phenotypes of PAP1 and PAP2 suggest that these genes may be functionally redundant, such that only activation tagging or some other gain-of-function screen could have readily revealed their key attributes.

Activation tagging as a gene discovery tool based on gain-of-function is intrinsically oriented toward biotechnological utility, and the ability to activate a biosynthetic pathway that will lead to the enhanced accumulation of several distinct subclasses of natural products has several important potential applications. Thus, hyperactivated tissues or organs provide massively enriched sources for passage through multiplex drug screens with the potential for discovery of novel activities based on what combinatorial effects

Figure 5. Overexpression of PAP1 or PAP2 Enhances Pigmentation in Arabidopsis and Tobacco.

(A) to (E) Arabidopsis plants transformed with pMN20-2 ([A] and [D]), pMN-PAP1 ([B] and [E]), and pCHF3:PAP2 ([C]). (A) to (C) show six-week-old plants. (D) and (E) show flowers on 12-week-old plants.

(F) to (J) Tobacco plants transformed with pCHF3 ([F] and [I]), pCHF3:PAP1 ([G]), and pCHF3:PAP2 ([H] and [J]). Plantlets in (F) to (H) were photographed at age 4 weeks, and flowers in (I) and (J) at 10 weeks after transfer to soil. pCHF3-PAP1 plants had brilliant flower pigmentation, identical to that of pCHF3-PAP2 (data not shown).
might arise from complex mixtures as well as allowing convenient isolation and characterization of individual bioactive components. This approach in principle could be augmented by feeding studies using pathway intermediates or synthetic derivatives. Moreover, activation of phenylpropanoid biosynthesis in pap1-D reflects massively enhanced expression of genes encoding pathway enzymes; hence, these tissues provide a correspondingly enriched source for isolating the cDNAs that encode key biosynthetic enzymes not readily identified by biochemical approaches.

Although the plant kingdom has a remarkable diversity of natural products, the underlying pathway regulatory mechanisms appear to be at least partially conserved between species (Mol et al., 1996; Quattrocchio et al., 1998). For example, the maize anthocyanin regulatory gene R functions appropriately when expressed in Arabidopsis or tobacco (Lloyd et al., 1992). Likewise, ectopic expression of Pap1 or Pap2 in transgenic tobacco caused phenotypes similar to those observed in Arabidopsis. Therefore, convenient, readily transformed genetic model species, such as Arabidopsis, can be used to isolate candidate regulatory genes for direct evaluation in medicinal plants and other exotic species or as a platform for the identification of orthologs and potentially useful, related genes in target species.

The serendipitous discovery of pap1-D among a large collection of activation-tagged lines was possible because activation of Pap1 enhanced the accumulation of anthocyanin pigments, which was easily scored. Several other plant natural products, such as the isoprenoids lycopene and carotenoids and the alkaloid sanguinarine, also are colored. Hence, genetic activation of these tranches of plant metabolism also could be scored by visual inspection, but this is not a generally applicable approach. In principle, activation-tagged lines with enhanced accumulation of natural products of interest could be identified by high-throughput metabolic profiling. However, a more promising general strategy may be to make transgenic plants that express easily screened marker genes under the control of promoters from genes encoding enzymes involved in the biosynthesis of natural products of interest.

**METHODS**

**Plant Growth and Transformation**

*Arabidopsis thaliana* ecotype Columbia (Col-0) plants were grown in growth rooms at 22°C in long days (16 hr of light) or short days (9 hr of light) and received 250 μE from three 35-W cool white bulbs and one 35-W Sylvania GrowLux bulb (Osram Sylvania, Danvers, MA). *Nicotiana tabacum* cv xanthi plants were regenerated under 24-hr-light conditions at 25°C and then transferred to the greenhouse. Tobacco and Arabidopsis transformation was performed as previously described (Neff et al., 1999; Weigel et al., 2000) except that 0.02% Silwet-L77 (Lehle Seeds, Round Rock, TX) was used for the latter. Basta was obtained from AgrEvo (Montvale, NJ).

**Gel Blot Hybridization**

DNA and RNA gel blot hybridizations were performed according to standard procedures (Sambrook et al., 1989). RNA samples used in the gel blot analysis shown in Figure 2 were from vegetative leaves of *pap1-D* and Col-0 plants grown under short-day conditions for 4 weeks and long-day conditions for 2 weeks. Probes were full-length cDNA fragments of *PAP1*, the glutathione S-transferase gene, and the gene encoding ubiquitin. The chalcone synthase (CHS) probe was a polymerase chain reaction product amplified by using the primers 5'-TGCTGTCGCTTCCGTCCGTCAA and 5'-CCCTCAATTGCCGTCATGGAA. The phenylalanine ammonia-lyase (PAL) probe was amplified by using the primers 5'-CTATAAGTTACCTACCAACAAAC and 5'-TCTCCGATGAGAGTACACCAAC and the dihydroflavonol reductase probe was amplified with primers 5'-AAAAGATGACAGGATGGGT-3' and 5'-CCCTGTGTTGCTTCTGGTTA-3'.

**Enzyme Assays**

PAL activity was measured by using a microcuvette spectrophotometric assay (Blount et al., 2000). Caffeic acid O-methyltransferase and caffeoyl-CoA O-methyltransferase activities were assayed by standard methods (Inoue et al., 1998). Protein concentrations were determined by the procedure of Bradford (1976).

**Phenylpropanoid Analysis**

Soluble and wall-bound phenolics in whole-plant extracts as well as extracts of individual tissues were analyzed by HPLC (Blount et al., 2000). The aqueous phase, which remained after ethyl acetate extraction of the wall-bound phenolics, was lyophilized and resuspended in 70% methanol for analysis. The HPLC eluates were monitored by absorbance at 270, 310, and 550 nm, and the peaks were identified by comparing their retention times and UV light spectra with those of known standards. Lignin was assayed by derivatization followed by reductive cleavage (Lu and Ralph, 1997).

**pMN-PAP1, pCHFS-PAP1, and pCHFS-PAP2 Constructs**

A *PAP1* genomic fragment was amplified by using 5'-AACCTAGGCAGCTAGAGGATCCG-3' and 5'-TCAGAATGACAGAAGTACCA-3' to construct 5' and 3' Pet sites. This fragment was cloned into pmN20-2 (Weigel et al., 2000), which contains two copies of 35S to create pMN-PAP1. pCHFS-PAP1 was created by amplifying the *PAP1* cDNA with primers 5'-ACTGTACCTTTTCAATTGTTTA-3' and 3'-AGGGGATCTTACACACCAGC-5' and cloning it into the KpnI and BamHI sites of pCHFS, a pZP211-based plant expression vector carrying the cauliflower mosaic virus 35S promoter and a pea ribulose 1.5-bisphosphate carboxylase/oxygenase terminator (C. Fankhauser, K. Hanson, and J. Chory, unpublished data). The *PAP2* cDNA was excised from expressed sequence tag clone 193M15 with KpnI and BamHI and was cloned into pCHFS to create pCHFS-PAP2.

**ACKNOWLEDGMENTS**

We thank the following (all at Salk Institute unless otherwise noted): Tsegaye Dabi for help with transgenic tobacco; Mary Anderson at
the Nottingham University Stock Centre for help with restriction fragment length polymorphism mapping; David Huhman (Noble Foundation) for lignin measurements; Michael Neff, Christian Fankhauser, Kim Hanson, and Joanne Chory for pMN20 and pCHF3 vectors; and Igor Kardailsky, Sioux Christensen, and Detlef Weigel for pSK1015. J.O.B. thanks Joanne Chory for providing laboratory facilities for completion of this work.

Received June 6, 2000; accepted October 3, 2000.

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