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ORIGINAL ARTICLE

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Overexpression of a fungal laccase gene induces nondehiscent anthers and morphological changes in flowers of transgenic tobacco

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Abstract Laccases play important roles in the development of fruiting bodies and in lignin degradation by basidiomycetes. In this study, we present novel phenotypes of transgenic tobacco plants with a chimeric gene for fungal laccase under the control of the cauliflower mosaic virus 35S promoter. At the flowering stage, the transgenic plants that produced recombinant laccase had brownish anthers instead of the greenish anthers of wild-type plants. The brownish anthers exhibited male sterility with a nondehiscent phenotype at varying frequencies. The frequency of nondehiscence depended on the temperature at which plants were cultivated and it was higher at 24°C than at 29°C. The cell wall structures of transgenic anther tissues were almost the same as in the wild type, but the stomium was severely deformed, and abnormal components were apparent in cells of the endothecium and epidermis. Furthermore, the pattern of deposition of flavonoids in the transgenic anther epidermis differed from the wild-type pattern. The expression of laccase also induced other phenotypic changes in the flowers of transgenic plants, namely, increased petal number, fused and petaloid stamens, and doubling of floral organs. These results indicate that the ectopic expression of laccase influences various aspects of flower development.

Key words Laccase · Nondehiscent anther · Petaloid stamen · *Trametes versicolor* · Transgenic tobacco

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Introduction

Laccase (EC 1.10.3.2) belongs to the family of multicopper oxidases that are found in animals, plants, bacteria, and fungi. The enzyme catalyzes the one-electron oxidation of a variety of substrates, which include the aromatic hydroxyl group and both aromatic and aliphatic amines.[1,2] The enzyme then reduces oxygen to generate water and phenolic radicals, which can, in turn, form dimers, oligomers, and polymers. The enzyme from fungi has been of interest as a catalyst for bleaching pulp, fibers, and textiles and for the transformation and detoxification of natural and industrial phenolics. Fungal laccases play important roles in the development of the fruiting bodies of basidiomycetes.[3] In plants, laccases are encoded by a diverse group of genes and appear to be important for the oxidative coupling of lignin monomers, for oxidative browning of proanthocyanidins, for maintenance of the structure of plant cell walls, for the regulation of plant hormones, and for responses to stress (Liang et al.).[4–10]

Efforts have been made to produce recombinant laccases in plants. For example, Wang et al.[11] succeeded in overproducing laccase derived from cotton in transgenic *Arabidopsis*, and the resultant plants had the potential ability to contribute to the bioremediation of phenolic compounds. Dean et al.[12] produced transgenic yellow poplar plants that overexpressed cDNA for a laccase of the sycamore maple. Transgenic embryos that expressed the cDNA tended to fuse to one another and were significantly more rigid and harder than wild-type embryos. The regenerated transgenic yellow poplars derived from such embryos were also more rigid than wild-type yellow poplars; they were

dwarf and had fused organs. Wang et al.[13] also produced transgenic poplar plants that overexpressed cDNA for cotton laccase. In contrast to the transgenic yellow poplar described above, the growth rate and morphological characteristics of these transgenic poplar plants were similar to those of control plants, with the exception that levels of lignin were elevated in the transgenic stem tissues. These results indicate that the overexpression of endogenous plant genes for laccase in plant cells influences plant growth and development.

Disruption of genes for laccase also has physiological effects. For example, seeds of the *Arabidopsis* mutant *tt10*, with a mutation in one of the genes for laccase, contained higher levels of soluble proanthocyanidin and of condensed tannins than wild-type seeds.[8] This specific gene for laccase also plays an important role in the biosynthesis of lignin in *Arabidopsis* seeds.[14] Reduced expression of a gene for laccase in transgenic poplar resulted in a two- to threefold increase in levels of total soluble phenolic compounds and dramatic changes in the cell walls of xylem fibers, with severe deformation.[4] Brown et al.[6] also reported collapsed xylem cells in the *Arabidopsis irx12* mutant, in which a putative gene for laccase had been mutated. These observations imply that plant laccases play a variety of roles in secondary metabolism and in the formation of cell walls in plants.

In addition to transgenic plants with altered expression of plant-derived genes for laccase, transgenic plants that overexpress fungal genes for laccase have been reported. Hood et al.[15] reported the expression of chimeric genes for a laccase from fungi under the control of an embryo- or endosperm-specific promoter in transgenic maize. Some of the transgenes used for transformations had negative effects on plant growth, and one of the transgenes failed to generate any transgenic lines. Furthermore, replacement of signal sequences and improvements in codon usage of fungal genes for laccase have been shown to enhance gene expression in transgenic plants.[16,17]

In a previous study, we isolated a cDNA for laccase from *Trametes versicolor* and generated 20 independent transgenic tobacco plants, each of which had been transformed with a chimeric gene for laccase under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter.[18] These transgenic plants produced recombinant laccase and exhibited remediation activity against pentachlorophenol that had been dissolved in a liquid medium. In the present study, we selected from our transgenic lines four lines with elevated laccase activities and cultivated them in soil until they flowered. In this report, we focus on the abnormal phenotypic features of the transgenic plants and discuss them in relation to the gene for laccase.

Materials and methods

Transgenic plants with a chimeric gene for laccase

Tobacco plants were transformed by *Agrobacterium tumefaciens* LBA4404 that harbored the binary vector pWPflacIII, into which we had ligated a full-length cDNA for laccase from *Trametes versicolor* (accession no. D13372) under the control of the CaMV35S promoter, as described previously.[18] We selected four transgenic lines (FL4, FL9, FL20, and FL22) from the first generation of 20 transformants on the basis of elevated laccase activity and cultivated them at 24°C (lower-temperature condition, LTC) or at 29°C (higher-temperature condition, HTC) in pots, with soil, after in vitro clonal propagation of each transgenic plant.

Laccase activity in anther tissue

Crude protein from each line of plants, as indicated, was extracted by incubating frozen ground powder of anther tissue in 2 ml of 10 mM Tris–HCl (pH 4.0) on ice for 120 min (from 40 to 70 anthers were used from each line). After extraction, each solution of crude proteins was dialyzed on a spin column to concentrate the protein and to remove any H_2O_2 from the solution. The reaction mixture (pH 4.0) included acetate buffer (8.2 g/l sodium acetate, 0.25 mg $CuSO_4$/l) and contained 200 μg of crude protein and 2.3 mM guaiacol in a total volume of 1.0 ml. After incubation of the mixture at 30°C for 1 h, absorbance of the mixture was recorded at 436 nm by spectrophotometry. Laccase activity was calculated using the extinction coefficient (6400 $M^{-1}cm^{-1}$) of oxidized guaiacol, and activity was expressed as definitive units (1 unit = 1 mol guaiacol oxidized per min).[19]

Histochemical localization of laccase activity

For histochemical detection of laccase activity, thin sections (30 μm) of anther tissues were stained as described by Galuszka et al.[7] To avoid the simultaneous detection of peroxidase activity, catalase was added to the reaction mixture to remove endogenously formed H_2O_2. The staining solution contained 0.05% (w/v) 3,3′-diaminobenzidine (Wako Pure Chemical, Osaka, Japan) and 0.1% (w/v) bovine liver catalase (Wako Pure Chemical) in 0.1 M potassium phosphate buffer, pH 7.0. Sections were incubated for 40 min at 37°C in darkness and were then examined. Control experiments were carried out with the same staining solution prepared without 3,3′-diaminobenzidine. For inhibition of laccase activity in certain control sections, sodium diethyldithiocarbamate (DIECA) was added to the reaction mixture.

Quantification of lignin in anther tissue and of anthocyanin in petals

Lignin was quantified by the micro-Klason method, as described previously[20] with slight modifications. Pulverized air-dried anther tissues were prepared by a modified version of the procedure described by Casler and Hatfield.[21] Weighed pulverized air-dried anthers (approximately 5 mg) were transferred to a screw-capped tube (2 ml) and then

70 µl of 72% sulfuric acid was added. After 2 h of incubation at room temperature, 23 volumes of distilled water was added to the tube. The capped tube was then transferred to an autoclave and heated at 121°C for 30 min. After cooling, the solid material in the tube was washed with distilled water, dried in oven at 105°C, and then weighed as lignin. Quantification of anthocyanins in petals was performed as described by Giusti and Wrolstad.[22]

Microscopic analysis

For staining with phloroglucin–HCl, anther tissue was fixed overnight in formalin–acetic acid solution (formalin : acetic acid : ethanol : water, 1 : 1 : 9 : 9, v/v). Fixed samples were dehydrated gradually in an ethanol plus *tert*-butanol series (ethanol, *tert*-butanol, and water) and embedded in paraffin (Paraplast Plus, Okenshoji, Tokyo, Japan). Thin sections (approximately 20 µm thick) were cut on a sliding microtome (LS-113; Yamato Kohki, Saitama, Japan) and then paraffin was removed from sections with xylene and an ethanol series. For lignin staining, the resultant sections were treated with a solution of phloroglucin in ethanol (2%, w/v) for 2 min and then acidified with 12 M HCl for 40 s.

Safranin staining of anther sections was performed as described by Chaffey[23] and modified by Begum et al.[24] For staining of proanthocyanidins in anther tissue, intact anthers were incubated overnight in a solution of vanillin–HCl [1% (w/v) vanillin in 6 M HCl] at room temperature. Then each anther was placed on a glass slide and covered with a cover slip, which was pushed down slowly to attach the anther to the glass. Vanillin turns red upon binding to flavan-3,4-diols (leucoanthocyanidins) and flavan-4-ols (catechins), which are present either as monomers or as terminal subunits of proanthocyanidins.[25]

Analysis of gene expression by semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR)

We performed sqRT-PCR with total RNA from anther tissues of transgenic tobacco plants that had been cultivated under HTC or under LTC. Prior to synthesis of first-strand cDNA, total RNA was treated with RNase-free DNase I (Takara Bio, Otsu, Japan) for removal of contaminating DNA. Reverse transcription was performed with a Transcriptor First-Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) using the oligo(dT) primer supplied with the kit. We used 3 µg of total RNA for the synthesis of first-strand cDNA in a 20.8-µl reaction mixture and then we used 1 µl of the resultant reaction mixture as the template for subsequent PCR with gene-specific primers. For the analysis of the expression of the fungal gene for laccase, we used CVL-f (5′-AGTACTGTGATGGTCT GAGG-3′) and CVL-r (5′-GAAGTTGGTGCCGTT GAAGT-3′) as primers. We also monitored the expression of the gene for 18S ribosomal RNA by RT-PCR using Nt18SrRNA-f (5′-CGGGGAGGTAGTGACAATAA-3′) and Nt18SrRNAr-r (5′-GGTATCTGATCGTCTTCGAG-3′) as primers. The conditions for PCR were as follows: incubation for 1 min at 95°C and then 26 cycles for the gene for laccase or 18 cycles for the gene for 18S rRNA of 30 s at 95°C, 30 s at 58°C, and 1 min at 72°C. Amplified products were visualized after agarose-gel electrophoresis by subsequent staining with ethidium bromide.

Results

Male sterility associated with the nondehiscent anther phenotype

When we cultivated four transgenic lines (FL4, FL9, FL20, and FL22) in soil, plants were somewhat shorter than wild-type plants at the flowering stage (Fig. 1a). In addition, all four transgenic lines exhibited male sterility at various frequencies due to nondehiscent anthers. Wild-type anthers were normally dehiscent just before flower buds opened, but most of the transgenic anthers failed to dehisce after blooming (Figs. 1b,c). In contrast to the greenish anthers of wild-type plants, the nondehiscent anthers of the transgenic lines changed gradually from pale brown to brown or to dark brown during anther development (Figs. 1h–k). Observations of the surface of transgenic anthers under the stereomicroscope revealed brown pigmentation and a rough epidermis (Figs. 1d,f). No brown pigmentation was found in the wild-type anthers and their epidermis was smooth (Figs. 1e,g).

Dependence on temperature of the nondehiscent phenotype

Normally, we cultivate tobacco plants in a culture room in which the temperature is set at 24°C (lower-temperature condition, LTC). Under LTC, four transgenic lines had nondehiscent anthers at various frequencies (Fig. 2). The frequency in FL20 (50%) was relatively low compared to those in the other transgenic lines, in which frequencies exceeded 80%.

When transgenic plants were cultivated at a higher temperature (HTC, 29°C), the frequency of nondehiscent anthers decreased dramatically in all four transgenic lines (Fig. 2). The decreased frequencies varied among the lines, as indicated by results of an analysis of more than 800 anthers from more than five plantlets of each line. The nondehiscent phenotype almost disappeared in the case of FL20 and FL22 (less than 1%), but anthers and stamens remained abnormal, as described below. By contrast, in FL4 and FL9, frequencies of nondehiscent anthers under HTC were still relatively high (20% and 54%, respectively). In most cases, transgenic anthers appeared to contain much less brown pigment after growth under HTC than under LTC (data not shown).

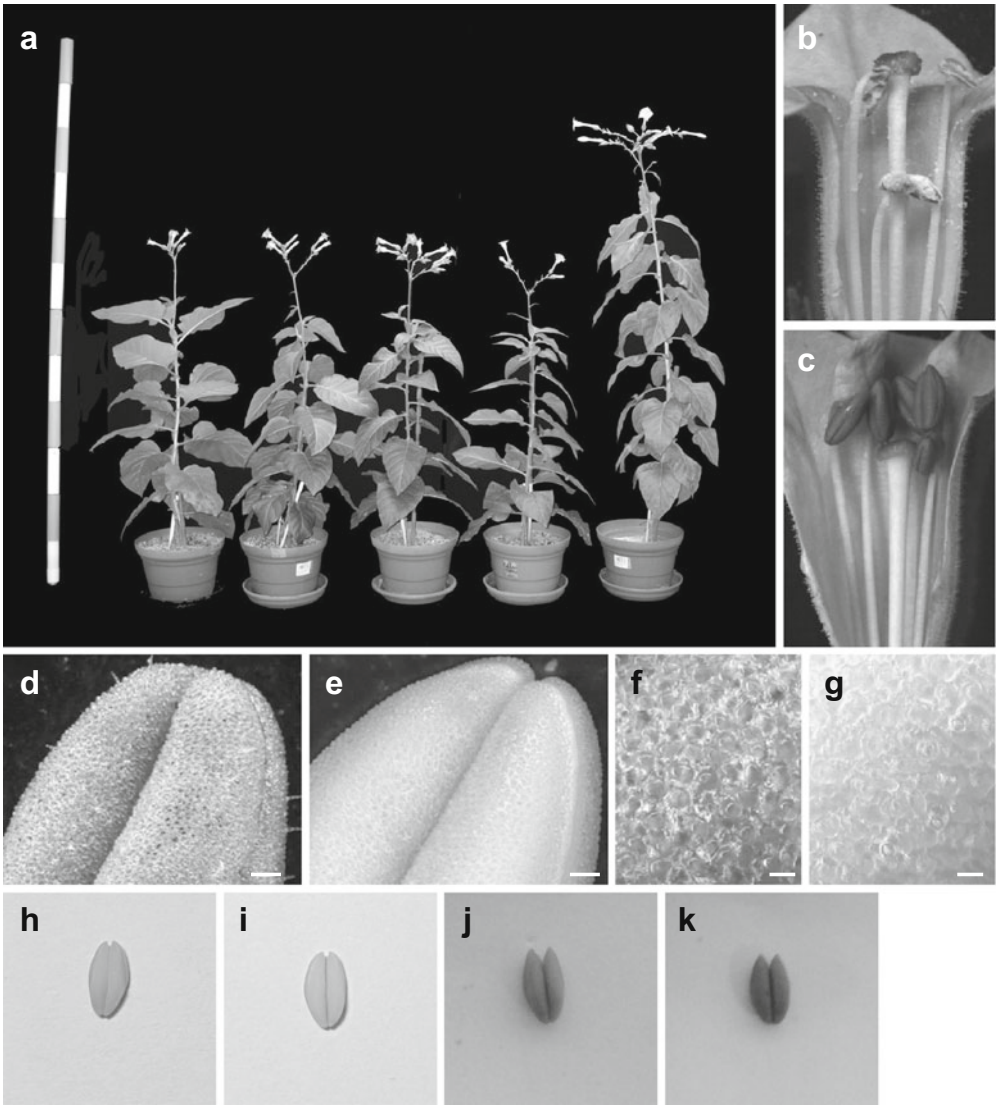

Fig. 1a–k. Phenotypes of transgenic plants that harbored a chimeric gene for fungal laccase and were grown under lower-temperature conditions (24°C). **a** Mature FL4, FL9, FL20, FL22, and wild-type plants (from *left* to *right*). *Bar* 10 cm per segment. **b** Wild-type flower with normal anther. **c** Transgenic (FL9) flower with nondehiscent anther. **d** Stereomicroscopic view of a transgenic (FL9) anther. **e** Stereomicroscopic view of a wild-type anther. **f** Magnified view of d. **g** Magnified view of e. **h**, **i** Intact anthers of wild-type flowers at the immature stage and just before opening, respectively. **j, k** Intact anthers of FL9 flowers at the immature stage and just before opening, respectively. *Bars* 200 μm in **d** and **e**, 50 μm in **f** and **g**

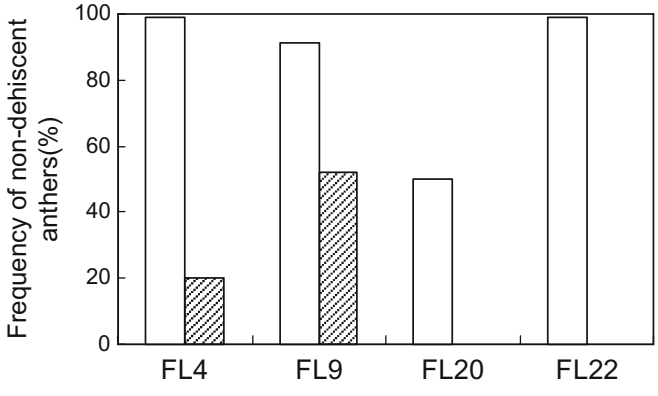

Fig. 2. Frequencies of nondehiscent anthers in transgenic plants grown under lower- (LTC, 24°C) and higher- (HTC, 29°C) temperature conditions. Since wild-type plants never exhibited the nondehiscent phenotype, data for wild-type anthers are not included. Each frequency was calculated from observations of more than 800 anthers from each transgenic line. Indicated values are ratios of nondehiscent to dehiscent anthers. *Open* and *shaded bars* indicate growth under LTC and HTC, respectively

Relationship between the level of laccase activity and the nondehiscent phenotype

To examine the relationship between laccase and male sterility, we analyzed the expression of the gene for laccase by sqRT-PCR using total RNA prepared from anthers of each line. As indicated in Fig. 3, expression of the gene for laccase under both LTC (24°C) and HTC (29°C) was detected in the transgenic anthers. The level of expression of the gene in the FL20 line, with its lower frequency of nondehiscent anthers, was lower than the levels in the other three transgenic lines. No transcripts of the gene for laccase were detected in the wild-type anthers.

We measured laccase activity in anthers from transgenic plants grown under both LTC and under HTC. After preparation of crude proteins from anthers that had been harvested from flowers before they had opened, we measured laccase activity with guaiacol as the substrate. Figure 4 shows the activity in anthers from each transgenic line. Under LTC, in comparison with wild-type plants, stronger

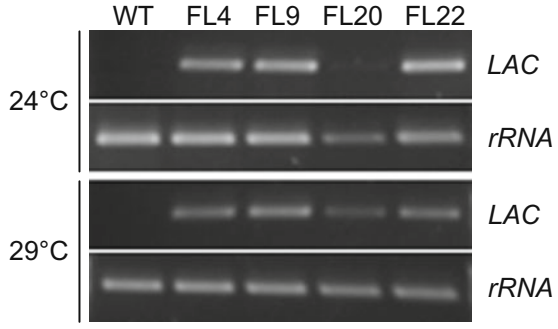

Fig. 3. Analysis of the expression of the transgene for laccase (*LAC*) in each line by semi-quantitative reverse transcription/polymerase chain reaction. Total RNA was prepared from each line after growth under LTC (24°C) or HTC (29°C). Transcription of the gene for 18S ribosomal RNA (*rRNA*) was analyzed as a control. *WT*, wild type

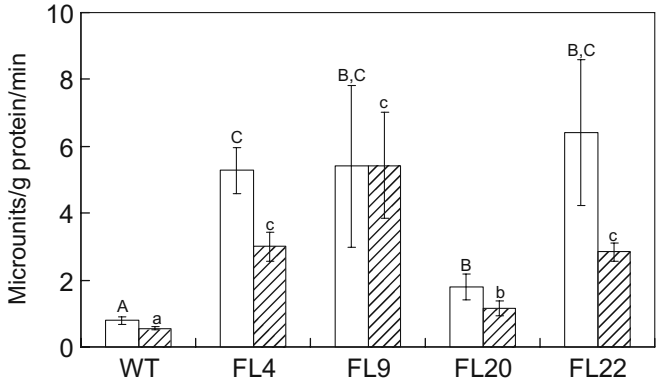

Fig. 4. Laccase activity in anther tissues prepared from transgenic and wild-type plants after growth under LTC (24°C) or HTC (29°C). *Open* and *shaded bars* indicate the activities for growth under LTC and HTC, respectively. Results shown are means ± SDs from the analysis of three independent biological samples in each case. Different letters indicate significant differences at $P = 0.1$, as compared with values for wild-type anthers grown under the same conditions

activity was detected in FL4, FL9, FL22, and FL20, although the activity of FL20 was lower than that for the other three. Activity was also detectable in wild-type plants but was much lower than in the transgenic lines. Laccase activity roughly correlated with the frequency of nondehiscent anthers in each line under LTC.

Although we found almost no nondehiscent anthers in FL20 and FL22 under HTC, laccase activities in these two lines were still higher than that in wild-type anthers. For anthers of FL9, even though laccase activity was similar under HTC to that under LTC, the frequency of nondehiscent anthers in HTC FL9 plants was lower than that in LTC FL9 plants. In addition, while the activity in FL4 anthers was similar to that in FL22 anthers under HTC, the frequency of nondehiscent anthers in the former case was much higher than in the latter case. These results indicate that the nondehiscence of transgenic anthers under HTC might be controlled by unidentified factors in addition to the expression of the gene for laccase.

Histochemical and chemical analyses of anther tissue

The differentiation of anther tissues such as stomium and endothecium, and the formation of secondary cell walls in endothecium and their lignification, are important for the normal dehiscence of anthers.[26–28] The secondary cell walls are deposited in the tangential wall of the cells of the endothecium, generating the driving force for the opening of anthers. To examine the structural differences between anther tissues of wild-type and transgenic plants, we prepared thin sections of anther tissues and examined them by light microscopy after staining with phloroglucin–HCl and, separately, with safranin to visualize lignin deposition and the morphology of anther tissues, respectively.

The structures of the lignified cell walls in endothecium cells and the extent of their lignification were similar in stained sections of wild-type and transgenic anthers (Figs. 5a,b). The absence of changes in the lignification of transgenic anthers was confirmed by quantitative chemical analysis of lignin in the anthers (Fig. 6a). In contrast to the normal features of the endothecium cells, we observed considerable deformation of the stomium at the site at which dehiscence occurs on safranin-stained sections (Fig. 5d). Such deformation might contribute to the nondehiscence of transgenic anthers because this tissue plays a critical role in the dehiscence of tobacco anthers.[26]

Our microscopic analysis also revealed the presence of abnormal components only in the epidermal and endothecium cells of transgenic anthers (arrows in Figs. 5b–d), as also observed by stereomicroscopy (Figs. 1d,f). The presence of these abnormal features in transgenic anthers was confirmed at higher magnification (arrows in Fig. 5f). Abnormal components seemed to have accumulated in vacuoles with numerous granule-like compartments (Fig. 5f).

In contrast to the normal lignification, altered patterns of deposition of proanthocyanidins were revealed in the transgenic anthers by vanillin staining of anther walls. The intercellular layer of normal anther epidermal cells was stained red after exposure to vanillin–HCl (Fig. 5h). No similar staining was observed in the transgenic epidermis (Fig. 5g). These results indicate that the expression of laccase might have interfered with the localization of flavonoids in the transgenic anthers.

Histochemical localization of laccase activity

We confirmed the activity of laccase in anther tissues by visualizing the distribution of colored reaction products that were generated by laccase as a result of oxidation of 3,3′-diaminobenzidine.[7] In transverse sections of anthers, laccase activity was detectable in cells of the endothecium and epidermis of our transgenic lines (Fig. 7d). Although a slight change in color, derived perhaps from the oxidation of endogenous compounds, was also evident when we omitted 3,3′-diaminobenzidine from the reaction mixture (Fig. 7c), staining was much stronger when we included the substrate (Fig. 7d). Laccase activity was detected in cells of the transgenic endothecium and epidermis, in which we also

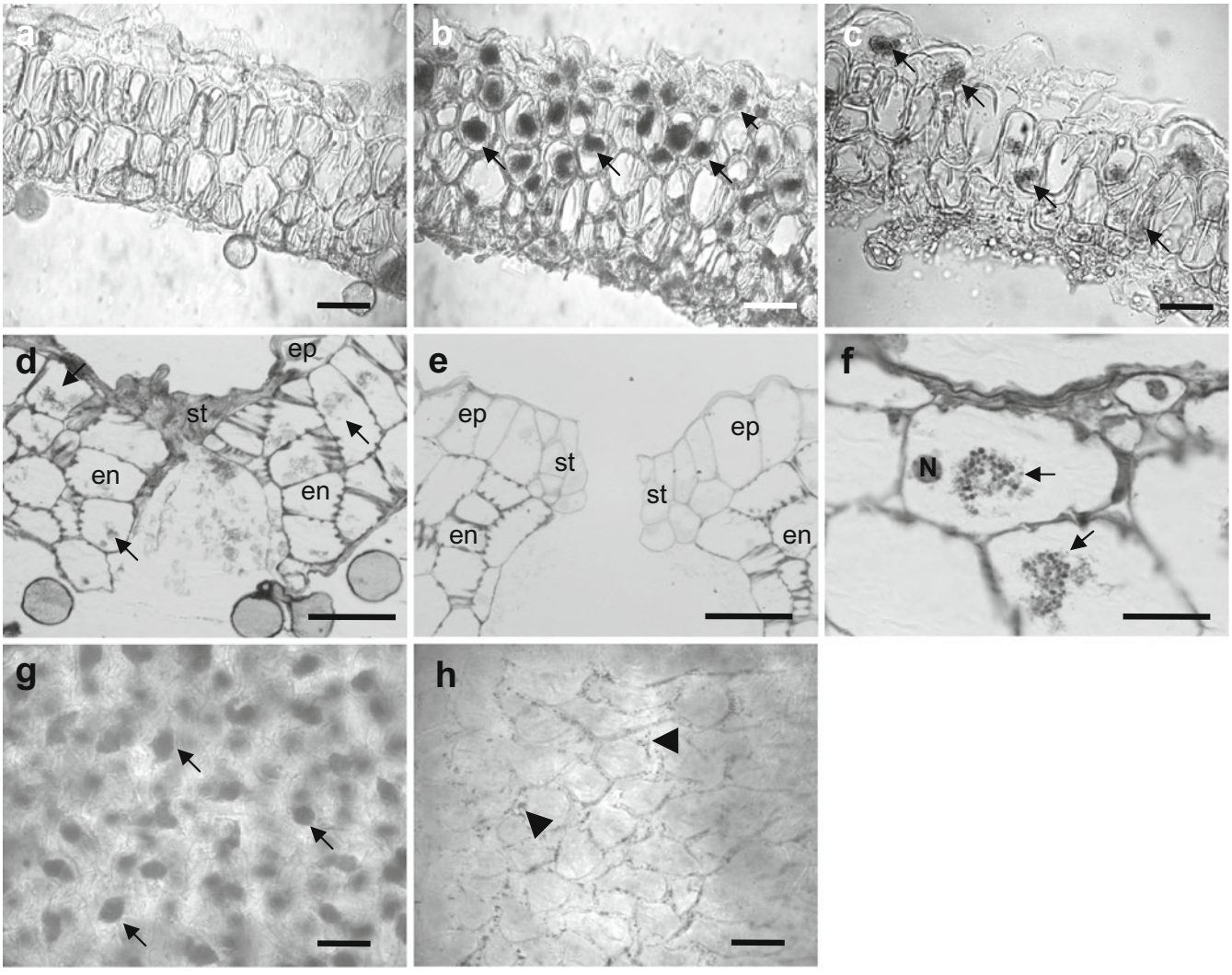

Fig. 5a–h. Histochemical analysis of anther tissues after growth under LTC (24°C). **a**, **b** Staining of lignin with phloroglucin–HCl in wild-type and transgenic (FL9) anthers, respectively, prepared from flowers just before opening. **c** Staining of lignin with phloroglucin–HCl in a transgenic anther (FL9) prepared from an immature flower. **d**, **e** Safranin-stained thin sections of a mature anther from a transgenic (FL9) and a wild-type plant, respectively. **f** Magnified view of transgenic endothecium cells (FL9) after staining with safranin. *Arrows* indicate abnormal components in cells of the endothecium and epidermis. **g** Surface of a mature transgenic anther (FL9) after staining with vanillin–HCl. *Arrows* indicate abnormal components in epidermal cells. **h** Thin section of a mature wild-type anther after staining with vanillin–HCl. *Arrowheads* indicate stained flavonoids in the compound middle lamella. *ep*, epidermis; *en*, endothecium cell; *N*, nucleus; *st*, stomium. *Bars* 50 µm in **a**–**e**, **g**, **h**; 20 µm in **f**. *Arrows* indicate abnormal components in epidermal and endothecium cells

observed abnormal intracellular components and changes in cell shape. Active staining of transgenic anther section was inhibited in the presence of the metal chelator DIECA, which acts as an inhibitor of laccases (Fig. 7e). In contrast, staining was also observed in wild-type anthers in the presence of the substrate, but it was weaker than that in the transgenic anthers (Fig. 7b). These results indicate that expression of the fungal gene resulted in the expression of laccase and abnormal structures.

Other morphological and metabolic changes in flowers of transgenic plants

In addition to the nondehiscent anthers, other aspects of flower development were affected by the expression of laccase. Petals and filaments of stamens of transgenic plants were more deeply pigmented than those of wild-type plants (Figs. 8a–d,h). Deep-pink transgenic petals were observed under both LTC and HTC, due, apparently, to higher levels of anthocyanins in the petals of the four transgenic lines, in which levels were more than double those in wild-type petals (Fig. 6b).

With respect to morphology, duplicated and petaloid stamens were often observed in all transgenic lines grown under HTC (Figs. 8h,i). When plants were cultivated under LTC, these defects were also evident. The petaloid stamens were mainly of two types: in some cases, most of the stamen had been changed to petal-like tissue (arrow in Fig. 8i) and the anther had disappeared; in other cases, petal-like tissue partially surrounded anther tissue (arrowhead in Fig. 8i).

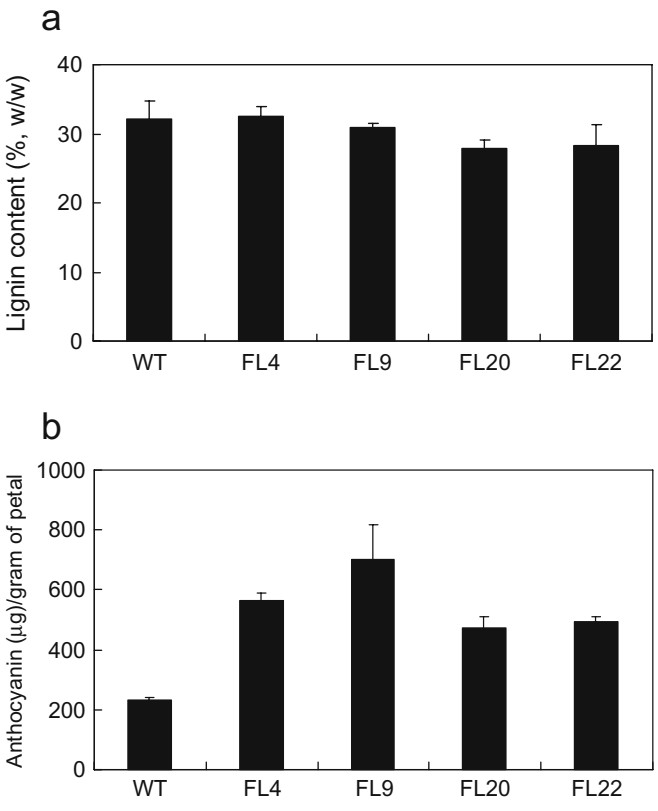

Fig. 6. **a** Levels of lignin in anther tissues. Lignin was quantified by a modified version of the Klason method. **b** Levels of anthocyanin in anther tissues. Experiments were performed with triplicate samples for each line. *Error bars* indicate SDs

Changes in numbers of other floral organs, such as petals and anthers, were also observed in the transgenic lines (Figs. 8e–g). Such abnormalities occurred in all transgenic lines. Furthermore, fused flowers with an abnormal shape were very occasionally seen in the FL4 line (less than 0.1% of all flowers: Fig. 8j). The fused flowers occurred only in FL4 under HTC. These changes in floral organs were probably the result of fusion or separation of floral and/or organ meristems during flower development.

Discussion

In transgenic tobacco plants that overexpressed a chimeric gene for fungal laccase, the anthers exhibited an abnormal nondehiscent phenotype and turned brown (Fig. 1c). In contrast to the greenish anthers in wild-type plants, the brownish color of the mature anthers in the transgenic plants was deeper in color than young anthers in the same plant (Figs. 1h–k). This may have been due to changes in the level of oxidized material reacted with the fungal laccase during anther development in the transgenic plants. Abnormal browning of plant organs was also observed in seeds from transgenic maize that strongly expressed a fungal gene for laccase.[15] Since we were able to produce hybrids from crosses between our transgenic lines and wild-type plants, it was clear that the expression of laccase did not have a lethal effect on pollen grains, even though it inhibited the normal dehiscence of anthers.

In tobacco, the stomium plays a critical role in the normal dehiscence of anthers. Tissue-specific ablation in the stomium induced failure to dehisce in transgenic tobacco plants.[26] One explanation for the nondehiscent phenotype is that our transgenic anthers might have been abnormal with severely deformed stomium (Fig. 5d). The nondehiscent phenotype has also been reported in several other lines of transgenic plants. Deluc et al.[29] reported that overexpression of a grapevine gene for Myb transcription factor induced copious accumulation of flavonoids, such as anthocyanins, flavonols, and condensed tannins, in the stamens and petals of transgenic tobacco plants. This accumulation was accompanied by nondehiscent anthers that were the result of limited development of secondary cell walls and depressed lignification of radial walls of endothecium cells. Failures in anther dehiscence were also observed in *Arabidopsis* mutants plants that lacked intact genes for transcription factors such as MYB26, NST1, and NST2, which are required for the formation of lignified secondary cell walls in anther tissue.[27,30,31] In addition to lignification, the direction of reinforcement of secondary walls in endothecium cells is important for the rigidity of the wall frame, which is essential for normal dehiscence.[28] Despite the importance of the formation and lignification of secondary walls, histochemical observations of our transgenic plants indicated that the secondary walls in endothecium cells in both wild-type and transgenic anthers were similar in terms of size, shape, and extent of lignification (Figs. 5a,b). The absence of differences in lignin content was confirmed by chemical analysis (Fig. 6a). Our results indicate that the nondehiscence of the transgenic anthers of our four transgenic lines was not due to changes in the structure of the secondary walls of endothecium cells or in the extent of their lignification.

Vanillin staining of anther tissues revealed that deposition of anthocyanidins in the cell wall of the anther epidermis was reduced in transgenic plants (Figs. 5f,g). In addition, the abnormal deposition of abnormal components, which apparently seemed to accumulate in the vacuoles of epidermal and endothecium cells, was observed in transgenic anthers (arrows in Figs. 5b–d,f,g). Deposition of the abnormal components was markedly increased during maturation of anthers (Figs. 5b,c). This increase was correlated with developmental changes in the color of transgenic anthers (Figs. 1j,k). It is generally accepted that flavonoids accumulate in vacuoles and are then transferred in part or in toto to cell walls.[32–34] Some flavonoids, such as flavonols, catechins, and proanthocyanidins, can be oxidized by laccases from both plants and fungi, with subsequent formation of dimers, oligomers, and polymers.[8,35–38] The deposition of flavonoids plays an important role in determining the structure of the plant cell wall.[39,40] Furthermore, colorless and light-colored flavonoids can turn brown upon oxidative transformation, suggesting that the changes in anther color in our transgenic plants might have been due, in part at least, to the abnormal oxidation of flavonoids. Our present data and earlier findings together suggest that, in our trans-

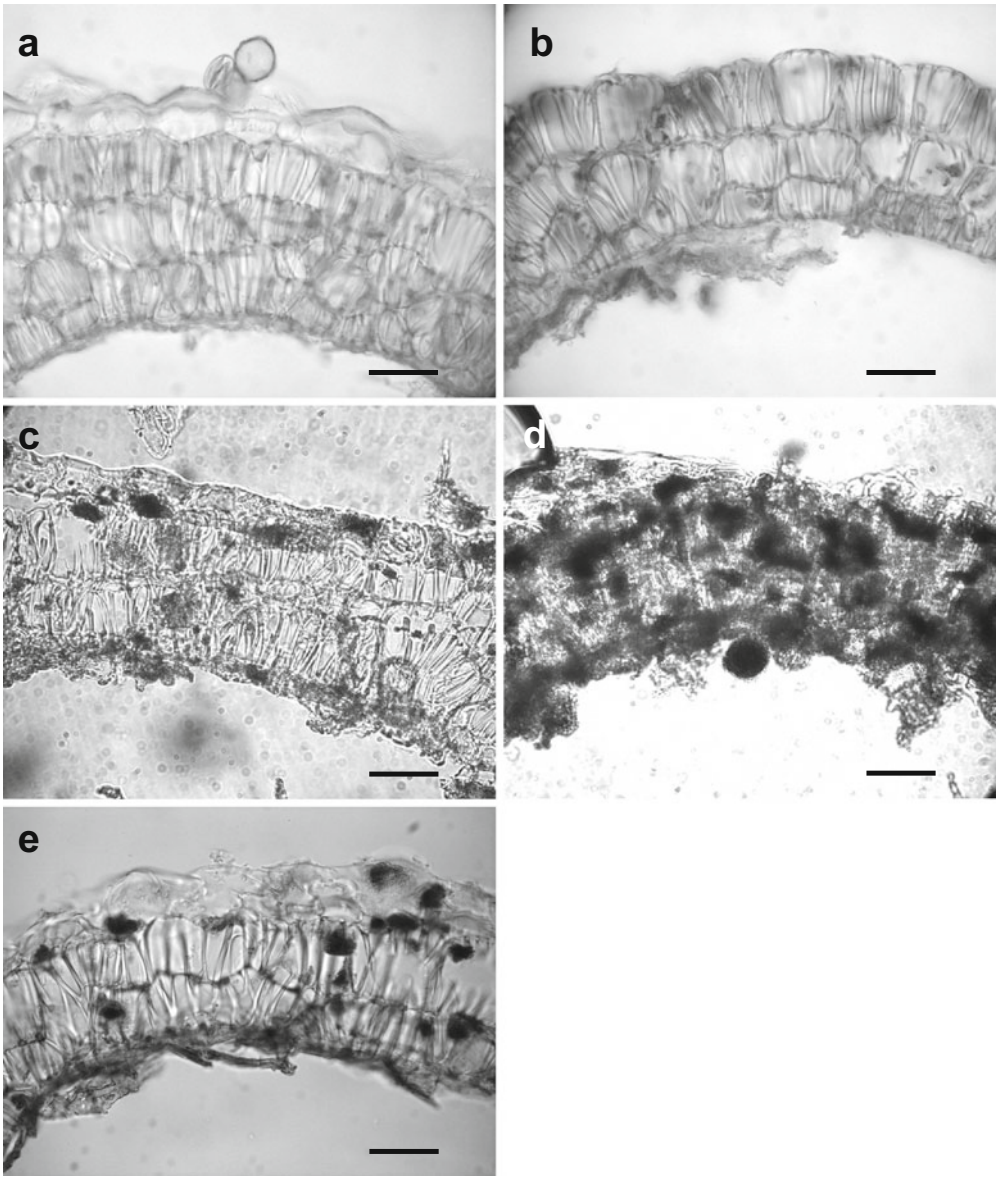

Fig. 7a–e. Histochemical localization of laccase activity in thin sections of anthers with 3,3′-diaminobenzidine as substrate. To eliminate endogenous peroxidase activity, each reaction mixture contained catalase. **a**, **c** Thin sections of wild-type and transgenic anther after incubation with catalase but no substrate, respectively. **b**, **d** Thin sections of wild-type and transgenic anther after incubation with catalase and substrate, respectively. **e** Thin section of transgenic anther after incubation with catalase, substrate, and laccase inhibitor (1 mM sodium diethyldithiocarbamate). Wild-type and transgenic (FL9) anthers were prepared from respective plants after growth at 24°C. *Bars* 50 µm

genic plants, flavonoids that are normally deposited in cell walls are not targeted correctly to cell walls and remain, to some extent, in vacuoles. This alteration in the deposition of flavonoids might be related to the nondehiscent phenotype of the transgenic anthers, via decreases in the rigidity of the wall frame of anther tissue and/or changes in the structure of stomium tissue (Fig. 5d).

As illustrated in Fig. 2, the frequency of anther nondehiscence depended on growth temperature. Lines FL20 and FL22 exhibited the nondehiscent phenotype at high frequency under LTC (25°C), but the abnormal phenotype was almost completely lost under HTC (29°C). Although the frequency was roughly correlated with laccase activity under both LTC and HTC, the nondehiscent phenotype cannot be explained solely in terms of the level of expression of laccase in each plant. The nondehiscent phenotype of our transgenic lines was closely related to the change in color of anther tissue and deformation of the stomium (Figs. 1, 5d). Changes in the color of transgenic anther were more prominent under LTC than under HTC in all of our transgenic lines. It is possible that cytochemical localization and/or levels of phenolic compounds, such as flavonoids, which can be oxidized by laccase, might differ between plants grown under different temperature conditions and that the differences might influence, to varying extents, both coloration and deformation of the stomium under HTC and LTC. This hypothesis remains to be proven in future study.

We often observed petaloid stamens in our transgenic plants (Fig. 8i). Blee et al.[41] also reported that downregulation of an endogenous gene for peroxidase in tobacco induced formation of petaloid stamens and increased the number of petals. Petaloid stamens are typically observed in transgenic plants with altered expression of floral-identity genes.[42–45] These observations indicate that changes in the activities of phenol oxidases, such as laccases and peroxidases, might influence the expression of genes for the

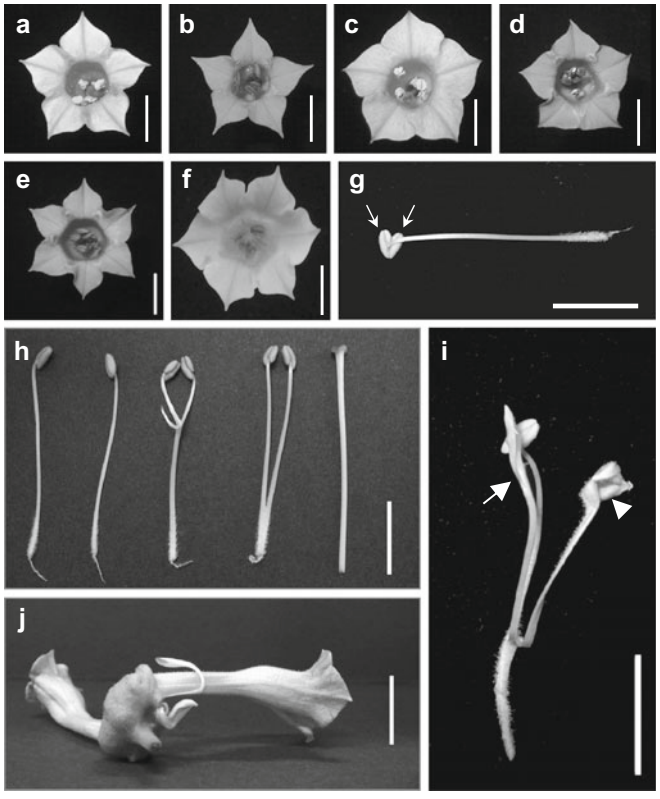

Fig. 8a–j. The abnormal phenotype of transgenic flowers. **a** Flower from a wild-type plant after growth under LTC (24°C). **b** Flower from an FL9 plant (LTC). **c** Flower from a wild-type plant after growth under HTC (29°C). **d** Flower from an FL9 plant (HTC). **e, f** Flowers with six petals and six stamens from a first-generation FL9 plant and from the T3 generation of the FL20 plant, respectively. **g** Twin-anther phenotype (*arrows*) observed in an FL4 plant after growth under HTC. **h** Six stamens from an FL4 flower after growth under HTC. Filaments of transgenic stamens had more deep-pink pigmentation than wild-type filaments. **i** Petal-like stamen (*arrowhead*) and petal-like tissue (*arrow*) from an FL22 flower after growth under HTC. **j** Fused flower from an FL4 plant after growth under HTC. *Bars* 1 cm

organization of flowers and/or floral identity. We observed separated and fused stamens in our transgenic plants (Fig. 8h). Similar fusion of organs was noted in transgenic poplar embryos in which a gene for laccase from sycamore maple had been overexpressed.[12] Transgenic poplar plants generated from these embryos had abnormally rigid tissue, a dwarf phenotype, and fused organs of the type observed in our transgenic lines. Phenol oxidases, such as laccases and peroxidases, and phenolic compounds are known to act as regulators of levels of plant hormones, such as auxins and cytokinins.[7,46] These findings suggest that changes in the extent of oxidation of phenolic compounds by phenol oxidases might have critical effects on the morphogenesis of plant organs.

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