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TRANSPARENT TESTA10 Encodes a Laccase-Like Enzyme Involved in Oxidative Polymerization of Flavonoids in *Arabidopsis* Seed Coat [W]

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The *Arabidopsis thaliana transparent testa10* (*tt10*) mutant exhibits a delay in developmentally determined browning of the seed coat, also called the testa. Seed coat browning is caused by the oxidation of flavonoids, particularly proanthocyanidins, which are polymers of flavan-3-ol subunits such as epicatechin and catechin. The *tt10* mutant seeds accumulate more epicatechin monomers and more soluble proanthocyanidins than wild-type seeds. Moreover, intact testa cells of *tt10* cannot trigger H_2O_2-independent browning in the presence of epicatechin and catechin, in contrast with wild-type cells. UV–visible light detection and mass spectrometry revealed that the major oxidation products obtained with epicatechin alone are yellow dimers called dehydrodiepicatechin A. These products differ from proanthocyanidins in the nature and position of their interflavan linkages. Flavonol composition was also affected in *tt10* seeds, which exhibited a higher ratio of quercetin rhamnoside monomers versus dimers than wild-type seeds. We identified the *TT10* gene by a candidate gene approach. *TT10* encodes a protein with strong similarity to laccase-like polyphenol oxidases. It is expressed essentially in developing testa, where it colocalizes with the flavonoid end products proanthocyanidins and flavonols. Together, these data establish that TT10 is involved in the oxidative polymerization of flavonoids and functions as a laccase-type flavonoid oxidase.

INTRODUCTION

Flavonoids are plant secondary metabolites derived from the phenylpropanoid pathway. In seeds, they act in defense against predators and pathogens (Dixon et al., 2002), increase seed coat (testa)–imposed dormancy (Debeaujon et al., 2000), and protect against UV radiation (Winkel-Shirley, 2002a). *Arabidopsis thaliana* seeds accumulate two types of flavonoid pigments during their development. Flavonols (yellow pigments) are localized in the seed envelopes and in the embryo, mainly as glycosylated quercetin derivatives (Routaboul et al., 2005). Proanthocyanidins (PAs), also called condensed tannins, accumulate exclusively in the inner integument (essentially the endothelium cell layer) and in the chalaza zone (Debeaujon et al., 2003). In *Arabidopsis* testa, PAs consist of oligomers of the flavan-3-ol 2,3-cis-(−)-epicatechin, also called procyanidins (Abrahams et al., 2003). PAs are synthesized as colorless polymers in vesicles derived from the endoplasmic reticulum, which further coalesce into the central vacuole (Stafford, 1988). In dead cells in which the cytoplasm has disintegrated, PAs are oxidized into brown complexes that cross-bond within the cell. These modifications contribute the typical brown color of *Arabidopsis* wild-type testa during seed desiccation.

The flavonoid pathway in *Arabidopsis* (Figure 1) has been characterized mainly using mutants called *transparent testa* (*tt*) and *tannin-deficient seeds* that are affected in seed coat pigmentation (Winkel-Shirley, 2002b; Abrahams et al., 2003). At least 20 loci have been identified. They correspond to enzymes, regulatory factors, and transporters (Winkel-Shirley, 2002b; Debeaujon et al., 2003; Baxter et al., 2005).

In plants, browning reactions are usually caused by the oxidation of phenolic compounds and result in the formation of quinones, which are unstable molecules prone to form brown polymers (Guyot et al., 1996). These reactions may be catalyzed by polyphenol oxidases such as laccase (EC 1.10.3.2), catechol oxidase (EC 1.10.3.1), and tyrosinase (EC 1.14.18.1) and by peroxidases (EC 1.11.1.7). Oxidation proceeds in the presence of molecular oxygen for polyphenol oxidase and with H_2O_2 for peroxidase (Dehon et al., 2002; Lopez-Serrano and Barcelo, 2002). In addition, quinones can be obtained by autoxidation or chemical oxidation (Guyot et al., 1996). Although valued in tea and wine processing, browning reactions are particularly detrimental for fresh fruits and vegetables (Fraignier et al., 1995). Moreover, the coupling of phenols in the presence of catechol oxidase is responsible for browning and cloudiness in beverages (Guyot et al., 1996).

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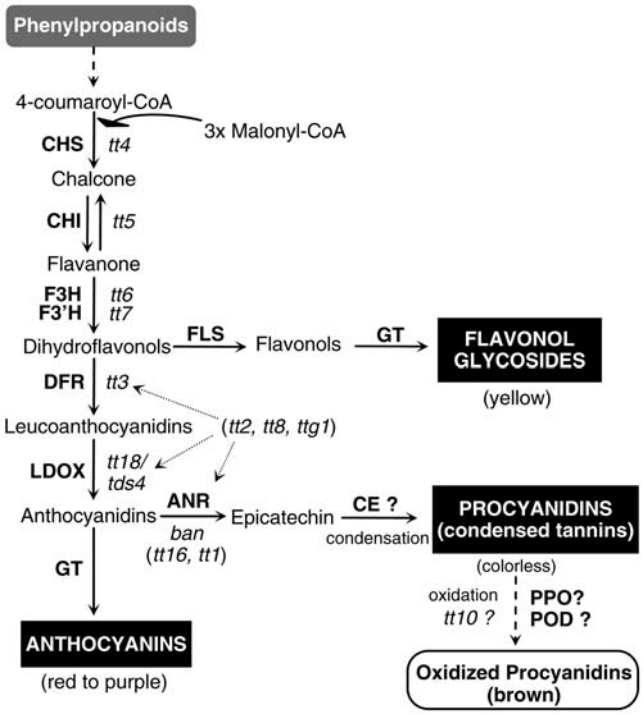


Figure 1. Scheme of the Flavonoid Pathway in *Arabidopsis.*

The flavonoid pathway leads to the formation of three major compounds: PAs that get brown after oxidation, flavonol glycosides, and anthocyanins. Enzymes are represented in uppercase boldface letters, the corresponding mutants in lowercase italic letters, and the regulatory mutants in parentheses. ANR, anthocyanidin reductase; CE, condensing enzyme; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol reductase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; FLS, flavonol synthase; GT, glycosyltransferase; LDOX, leucoanthocyanidin dioxygenase; POD, peroxidase; PPO, polyphenol oxidase; *tt*(*g*), *transparent testa* (*glabra*). (Adapted from Debeaujon et al., 2003.)

Laccases (*o*- and *p*-diphenol:dioxygen oxidoreductases) are part of a larger group of enzymes called blue copper oxidases that includes, among others, ascorbate oxidase and ceruloplasmin. These enzymes are copper-containing glycoproteins that catalyze the reduction of molecular oxygen to water with concomitant oxidation of the phenolic substrate, which results in the formation of quinones (Messerschmidt and Huber, 1990). Laccases are widely present in eukaryotes (higher plants, fungi, and insects) and in prokaryotes (Claus, 2004). They generally have a low specificity with regard to the reducing substrate and can oxidize *o*- and *p*-diphenols, monophenols, and ascorbic acid (Mayer and Staples, 2002). In some reports, plant laccase activities have been associated with lignification (Bao et al., 1993; Chabanet et al., 1994; Ranocha et al., 2002), but a direct activity of the enzyme on the polymerization of monolignols within the cell wall matrix has not been demonstrated (Mayer and Staples, 2002). Plant laccases were also reported to play a role in iron metabolism (Hoopes and Dean, 2004). The expression of a fungal laccase gene in transgenic maize was associated with kernel browning and limited germination (Hood et al., 2003).

In this study, we report the isolation and functional characterization of the *TT10* gene from *Arabidopsis*. Browning of the *tt10* mutant seed coat is delayed compared with that of the wild type. On the other hand, *tt10* showed wild-type levels of flavonoids in all its tissues, as established by thin layer chromatography (Shirley et al., 1995). This phenotype suggested that TT10 may act at the level of the unknown step(s) leading to PA oxidative browning (Figure 1). *TT10* encodes a predicted protein exhibiting strong similarity with polyphenol oxidases of the laccase type. The activity of its promoter colocalized with PA- and flavonol-accumulating cells of the testa. Moreover, TT10 is involved in the oxidative polymerization of flavonoids. We propose that a function of TT10 would be to oxidize epicatechin into the corresponding quinones, initiating subsequent polymerization and the formation of brown epicatechin oligomers different from procyanidin oligomers.

RESULTS

Phenotypic and Genetic Characterization of the *tt10* Mutant

A first *tt10* allele (*tt10-1*) was characterized previously (Koornneef, 1990). Shirley et al. (1995) genetically mapped the mutation and showed that it was recessive and that the seed phenotype was determined by the maternal genotype. We isolated five new independent alleles, *tt10-2* to *tt10-6*. They display the same seed phenotype at harvest, which is a pale-brown seed coat with a dark-brown chalaza zone (Figures 2A and 2B). After 6 to 12 months of storage, the *tt10* seed coat goes brown until it resembles the wild type. The presence of flavan-3-ols and flavonols in *tt10* seeds was detected histochemically using vanillin and diphenylboric acid 2-aminoethyl ester staining, respectively. No difference was observed between mutant and wild-type seeds with these techniques. Moreover, no modification in seed coat structure was detected after cytological observations.

Identification of the *TT10* Gene by a Candidate Gene Approach

To explain the delay in browning of the *tt10* testa, we proposed that early seed coat browning could be caused by the enzymatic oxidation of phenolic compounds by a laccase, a catechol oxidase, or a peroxidase. Chemical oxidation by air molecular oxygen (autoxidation) would explain the late browning observed in *tt10* mutant seeds.

The *tt10* mutation had been located previously on chromosome 5, between the *TT3* and *LEAFY* (*LFY*) loci, corresponding to the two molecular markers DFR (for dihydroflavonol reductase) and LFY located at 16.8 and 24.5 Mb, respectively (Shirley et al., 1995). Consequently, in silico analyses were performed to identify candidate genes that mapped around this region. The *Arabidopsis* genome was screened for genes annotated as laccase, catechol oxidase, diphenol oxidase, and peroxidase. Eleven genes were identified in the LFY to DFR interval, corresponding to two laccases and nine peroxidases.

The expression pattern of the candidate genes was established by RT-PCR analyses with flowers and immature siliques of

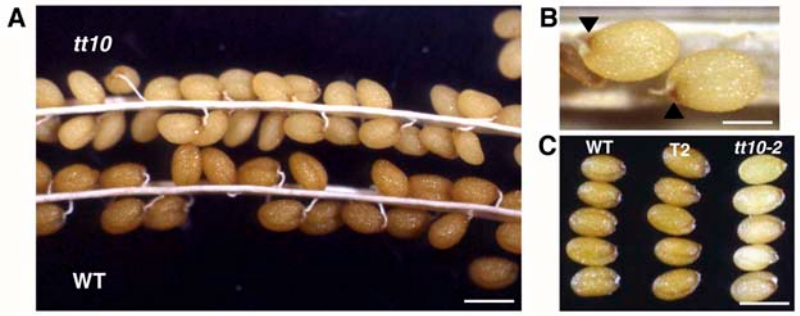


Figure 2. Seed Phenotypes.

(A) and **(B)** Phenotype of a *tt10* null mutant allele. At harvest, *tt10* seeds present a pale-brown seed coat color with a dark-brown chalaza zone (arrowheads).

(C) Complementation of the *tt10* mutant. Genotypes are *tt10-2* seeds; T2 progeny of *tt10-2* homozygous plants transformed with the wild-type *TT10* genomic region; and wild-type seeds. All seeds were obtained under the same conditions and observed at harvest.

Bars = 600 μm in **(A)**, 240 μm in **(B)**, and 480 μm in **(C)**.

the wild type and then with flowers of the *tt10-1* and *tt10-2* alleles. The results revealed that only one gene, At5g48100, was strongly expressed in siliques and flowers of the wild type and not detected in the *tt10-2* mutant. To provide further evidence that At5g48100 was *TT10*, we analyzed its expression and DNA sequence in the six *tt10* alleles.

Semiquantitative RT-PCR did not detect the *TT10* cDNA in all of the alleles except *tt10-1* and *tt10-3* (Figure 4A). DNA gel blot analyses revealed a restriction length polymorphism between the alleles and the corresponding wild types, except for *tt10-1* (see Supplemental Figure 1 online). However, sequencing of the cDNA amplified in the *tt10-1* allele showed the presence of two point mutations leading to modifications of two amino acids (152 D→G and 509 G→D). Finally, by sequencing the At5g48100 gene in *tt10-6*, a T-DNA insertion was found in the 3′ untranslated region (UTR), whereas no mutation appeared in the coding region. These data suggest that the 3′ UTR is important for *TT10* expression. The localization of the T-DNA insertion in the *tt10-4* and *tt10*-5 alleles was confirmed by PCR analysis (Figure 3B).

To confirm that the phenotype conferred by *tt10* is caused by a mutation in the At5g48100 gene, we conducted a functional complementation of the *tt10-2* mutant with an 8-kb genomic clone containing the At5g48100 gene with a 2.8-kb promoter and a 1.6-kb 3′ flanking region (Figure 3A). The T2 seed progeny originating from 15 independent T1 hygromycin-resistant transformants exhibited a reversion of the phenotype to that of the wild type on the basis of seed color (Figure 2C) and flavonoid composition (see Supplemental Figure 2 online). In addition, hygromycin resistance cosegregated with wild-type seed color in the progeny from selfing of T2 plants. Together, these data demonstrate that the mutation in At5g48100 is responsible for the phenotype conferred by *tt10*. Thus, the At5g48100 gene is called *TT10* below.

Structure of *TT10*

The structure of *TT10* was inferred from comparison of a 1698-bp full-length cDNA sequence with the genomic DNA sequence. It contains six exons and five introns. The UTRs were determined by rapid amplification of cDNA ends to be 34 bp for the 5′ UTR and 50 bp for the 3′ UTR (Figure 3B). Functional complementation of the *tt10-2* mutant with the TT10 cDNA (*cTT10*) confirmed its structure. The reversion to the wild-type phenotype was observed in the T2 progeny of T1 hygromycin-resistant transformants expressing the $Pro_{35Sdual}$*:5′UTR-cTT10-3′UTR* construct but not in those expressing $Pro_{35Sdual}$*:cTT10*. Ectopic expression of *TT10* did not have any obvious detrimental effect on plant development and growth (see Supplemental Figure 3 online).

Expression of the *TT10* Gene

The expression pattern was investigated in various vegetative and reproductive tissues (Figure 4B). The *TT10* mRNA was detected predominantly in developing siliques, with an increase during seed development. We observed an increase of accumulation at ~3 to 4 d after fertilization (DAF). *TT10* mRNA was also detected at low levels in stems, seedlings, and flowers but not in roots. Our results are consistent with data from in silico microarrays (https://www.genevestigator.ethz.ch/) and EST databases (http://www.tigr.org/) showing that TT10 is expressed mainly in developing siliques and seeds. We reproducibly observed that *TT10* was expressed at a lower level in Landsberg *erecta* (L*er*) ecotype than in Wassilewskija-2 (Ws-2) and Columbia (Col) ecotypes (Figure 4A).

Analysis of *TT10* Promoter Activity

To investigate further the regulation of *TT10* at the transcriptional level, a 2.0-kb promoter was translationally fused to the *uidA* reporter gene encoding a β-glucuronidase (GUS) protein and to the *mGFP5-ER* reporter gene. Wild-type plants were transformed with the constructs. In seed coat, at early stages of embryo morphogenesis (1 to 3 DAF), *TT10* promoter activity was detected in the endothelium and in the pigment strand at the chalaza zone (Figures 5A, 5C, 5D, and 5G). Later, the activity increased and spread to the outer integument, mostly in the oil

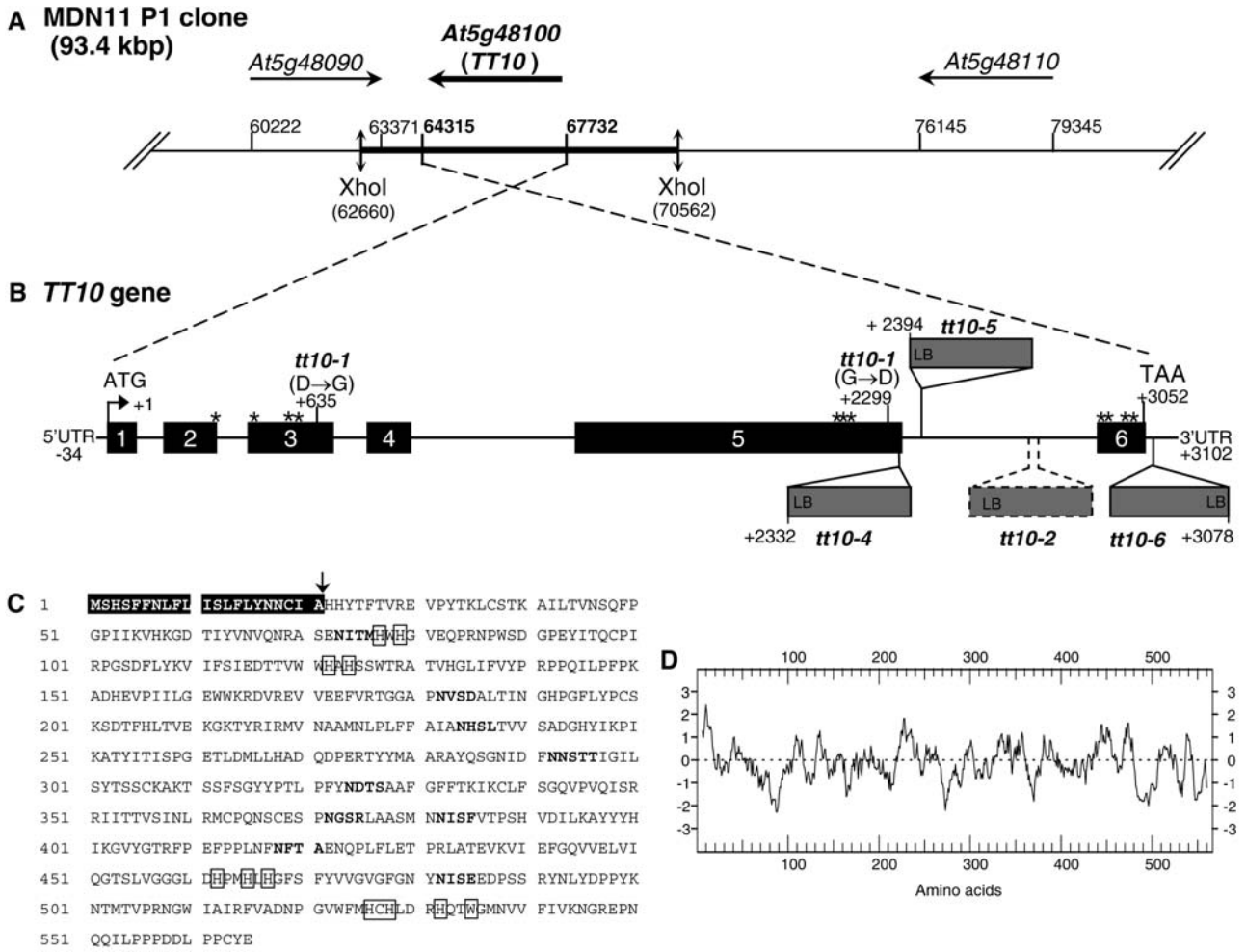


Figure 3. Molecular Analysis of the *TT10* Locus, and Structure of the Putative TT10 Protein.

(A) Scheme of the *TT10* gene on the MDN11 P1 clone. The arrows represent the orientation of gene transcription. An 8-kb *Xho*I genomic fragment (thick line) containing *TT10* was used for *tt10-2* complementation.

(B) Structure of the *TT10* gene and localization of the mutations in the *tt10* alleles. Gray rectangles represent T-DNA insertions, with the left border (LB) indicated inside. The positions of the conserved copper binding residues are indicated by asterisks. Lengths are in base pairs.

(C) *TT10* encodes a putative laccase with 565 amino acids. The N-terminal predicted signal peptide is shaded in black. The cleavage site is indicated with an arrow. The 10 putative N-glycosylation sites are shown in boldface. The amino acids involved in copper binding are boxed.

(D) Analysis of the hydrophobicity profile using the method of Kyte and Doolittle (1982). The hydropathy value was calculated over a window of 11 amino acids (DNA Strider 1.3. program).

penultimate cell layer (Figures 5E, 5F, and 5H to 5K). Therefore, promoter activity colocalizes first with PA-producing cells and afterwards with flavonol-producing cells of the testa (Figures 5L to 5N). The *uidA* gene was also strongly expressed in early aborted seeds (Figure 5B) and in the transmitting tissue of the silique (see Supplemental Figure 4 online). Three regulatory mutants, *tt2*, *tt8*, and *ttg1* (Nesi et al., 2000), were also transformed with the Pro_{TT10}:*uidA* construction. Resulting transformants did not show any difference in GUS activity compared with the wild type, suggesting that these regulators do not affect *TT10* promoter activity. Consistently, no difference in *TT10* mRNA accumulation was observed in regulatory mutant backgrounds.

The TT10 Protein Belongs to the Laccase-Like Multigene Family

The *TT10* gene encodes a putative laccase protein with 565 amino acids (Figure 3C), a predicted molecular mass of 64 kD, and a pI of 7.09 (http://www.expasy.org/tools/protparam/). The TT10 protein sequence contains four His-rich copper binding domains, L1 to L4 (Kumar et al., 2003), corresponding to the putative catalytic sites (http://www.expasy.ch/tools/scanprosite/). These domains are characteristic of the multi-copper oxidase family and are highly conserved in laccases of various organisms (Figure 6A). The protein harbors many putative glycosylation sites (Figure 3C). A putative cleavage site was predicted in the

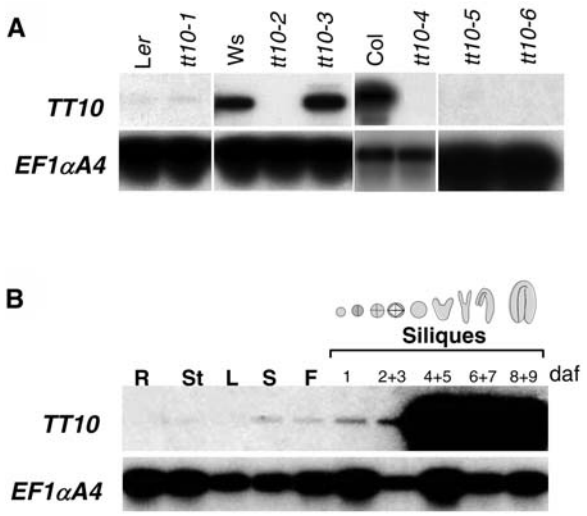


Figure 4. Expression Pattern of the *TT10* Gene in Mutant Alleles and the Wild Type.

(A) *TT10* gene expression in the *tt10* alleles was detected in flowers (*tt10-1* to *tt10-3* alleles, L*er*, and Ws) and siliques at 2 to 3 DAF (*tt10-4* to *tt10-6* and Col) by semiquantitative RT-PCR (amplification of *cTT10* from ATG to STOP). Hybridization was performed using the *TT10* cDNA as a probe. *EFαA4* gene expression was used as a control.

(B) The *TT10* mRNA accumulation pattern was investigated in various wild-type Ws-2 tissues by semiquantitative RT-PCR: roots (R), stems (St), leaves (L), seedlings (S), flowers (F), and siliques at different stages of development (1, 2+3, 4+5, 6+7, and 8+9 d after fertilization), as described above. Drawings represent corresponding developmental embryo stages.

N-terminal region (http://www.cbs.dtu.dk/services/SignalP/), defining a hydrophobic signal peptide of 21 amino acids (Figures 3C and 3D). According to PSORT website predictions (http://psort.ims.u-tokyo.ac.jp/), the TT10 protein would be secreted in the apoplast.

A search for homologous proteins in the *Arabidopsis* genome identified 16 other putative laccases (http://www.arabidopsis.org/Blast/). The corresponding genes are mostly localized on chromosome 2 and at the extremities of chromosome 5. We named them At LAC1 to At LAC17 according to their positions on the genome (from the top of chromosome 1 to the bottom of chromosome 5) (Figures 6B and 6C). A multialignment of the 17 full-length *Arabidopsis* proteins showed that TT10 (At LAC15) was close to the At5g09360 protein (At LAC14), with 49% identity at the amino acid level. From in silico microarray data (https://www.genevestigator.ethz.ch/) and EST databases (http://www.tigr.org/), we inferred that TT10 is by far the most abundant laccase in *Arabidopsis* seeds.

An analysis of the distance relationships between plant laccase sequences was performed and is illustrated by the simplified tree presented in Figure 6D (for the complete tree, see Supplemental Figure 5 online). It is fully consistent with the recent phylogenetic analysis of McCaig et al. (2005), proposing the existence of six groups. TT10/At LAC15 belongs to group 4, with four dicotyledon laccases: At LAC14, *Rhus vernicifera* Rv LAC2 (47% identity), *Acer pseudoplatanus* Ap LAC1 (49% identity), and *Gossypium arboreum* Ga LAC1 (43% identity), and one monocotyledon laccase, *Oryza sativa* Os LAC1 (44% identity). In addition, the use of ascorbate oxidase sequences as an outgroup suggests that the TT10 group diverged early from the other groups of plant laccases.

Flavonoid Composition of *tt10* Seeds

To investigate further the function of TT10, we extracted flavonoids from freshly harvested seeds. Epicatechin monomers and PA polymers were measured, combining acid-catalyzed hydrolysis and liquid chromatography–mass spectrometry (LC-MS) analysis. Upon cleavage and oxidation under strongly acidic conditions, PAs yield pink-colored anthocyanidin monomers that can be easily quantified (Porter et al., 1986). The *tt10-2* mutant contained 4.6-fold more soluble PAs than did the wild-type control (Figure 7A). These results were confirmed by detecting epicatechin and procyanidin polymers by LC-MS analysis of the soluble fraction (Figure 7B). Additionally, we measured insoluble PAs by performing acid-catalyzed hydrolysis directly on the pellet remaining after solvent extraction. The difference between *tt10-2* and wild-type content was smaller in this PA fraction (Figure 7A).

LC-MS also allowed the analysis of flavonols. Quercetin-3-*O*-rhamnoside was the main flavonol present in seeds and was 50% more abundant in *tt10-2* mutants than in wild-type controls (Figure 7C). On the other hand, dimers from quercetin rhamnoside were 12-fold less in *tt10-2* seeds (Figure 7D). The abundance of other flavonols was not modified by the mutation of *TT10*.

In Situ Localization and Analysis of TT10 Activity in Immature Seed Coat

A histochemical assay was developed to search for any acceleration of seed coat browning after adding different phenolic substrates exogenously (Figure 8). Immature seeds at 7 to 8 DAF were used because, at this time, the testa is still colorless and the *TT10* gene is strongly expressed. The genotypes tested were the wild type Ws-2, the *tt10-2* mutant, and a control *tt4-8* without flavonoids (chalcone synthase knockout mutant). After an overnight incubation in control buffer, the seed coat stayed transparent for all of the genotypes. When epicatechin or catechin (flavan-3-ol monomers) was added with or without the addition of catalase (preventing H_2O_2 accumulation and therefore any peroxidase activity), wild-type as well as *tt4-8* mutant seed coats went brown (Figures 8A and 8C). On the contrary, *tt10-2* stayed colorless. A *tt4-8* seed section after reaction showed that the brown color was localized in the endothelium and the oi1 cell layers of the seed coat, where *TT10* is expressed (Figure 8B). The addition of quercetin and quercetin rhamnoside did not modify seed coat color in any of the tested genotypes (Figure 8C). Peroxidase activity in the chalaza region of wild-type seeds was revealed by brown staining in the presence of H_2O_2, whereas no reaction was observed in the absence of H_2O_2.

To investigate further the activity of the TT10 enzyme, we performed LC-MS/MS analysis of flavonoid products extracted from wild-type, *tt10-2*, and *tt4-8* immature seeds after incubation overnight with epicatechin. The wild-type and *tt4-8* extracts

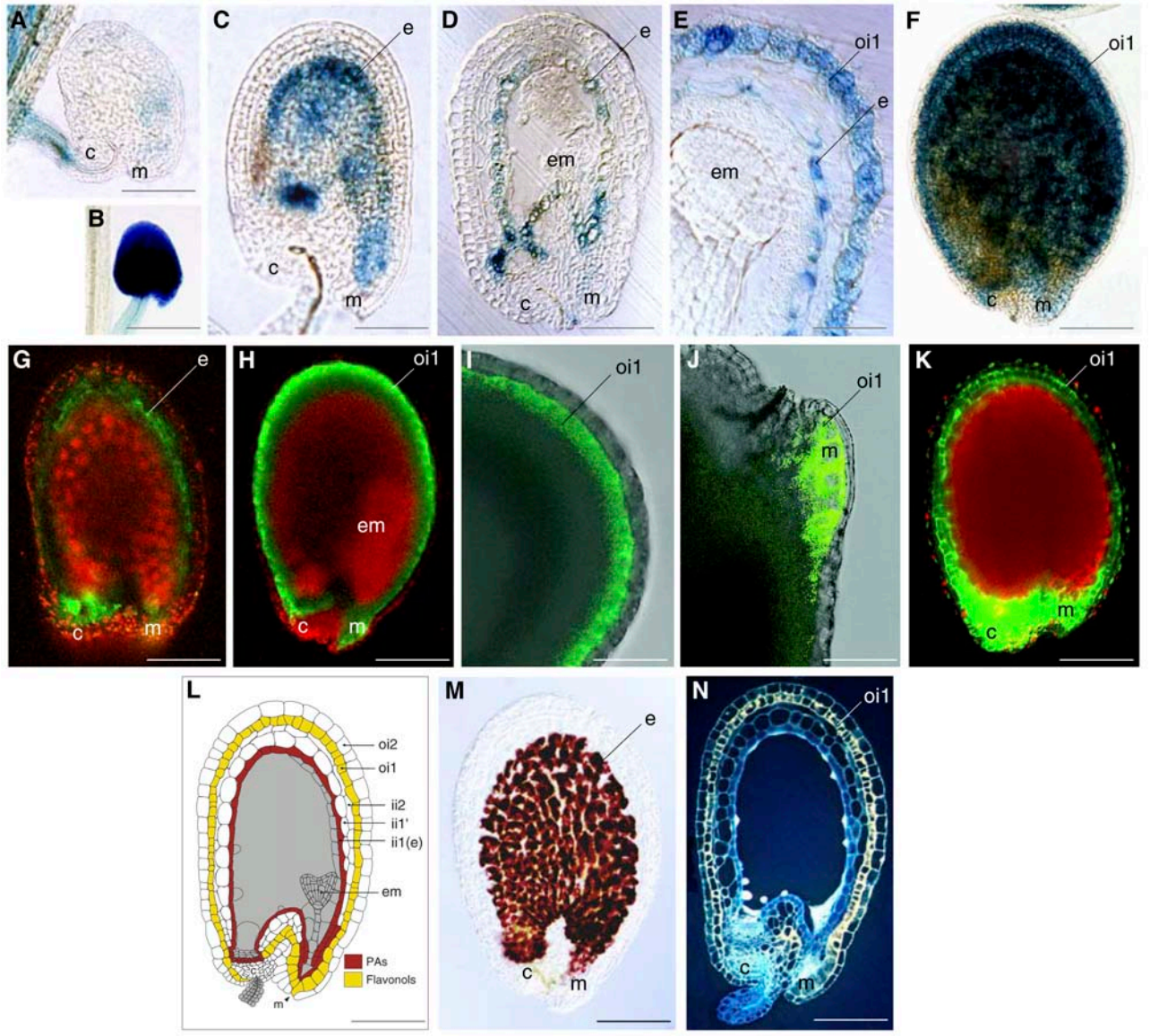
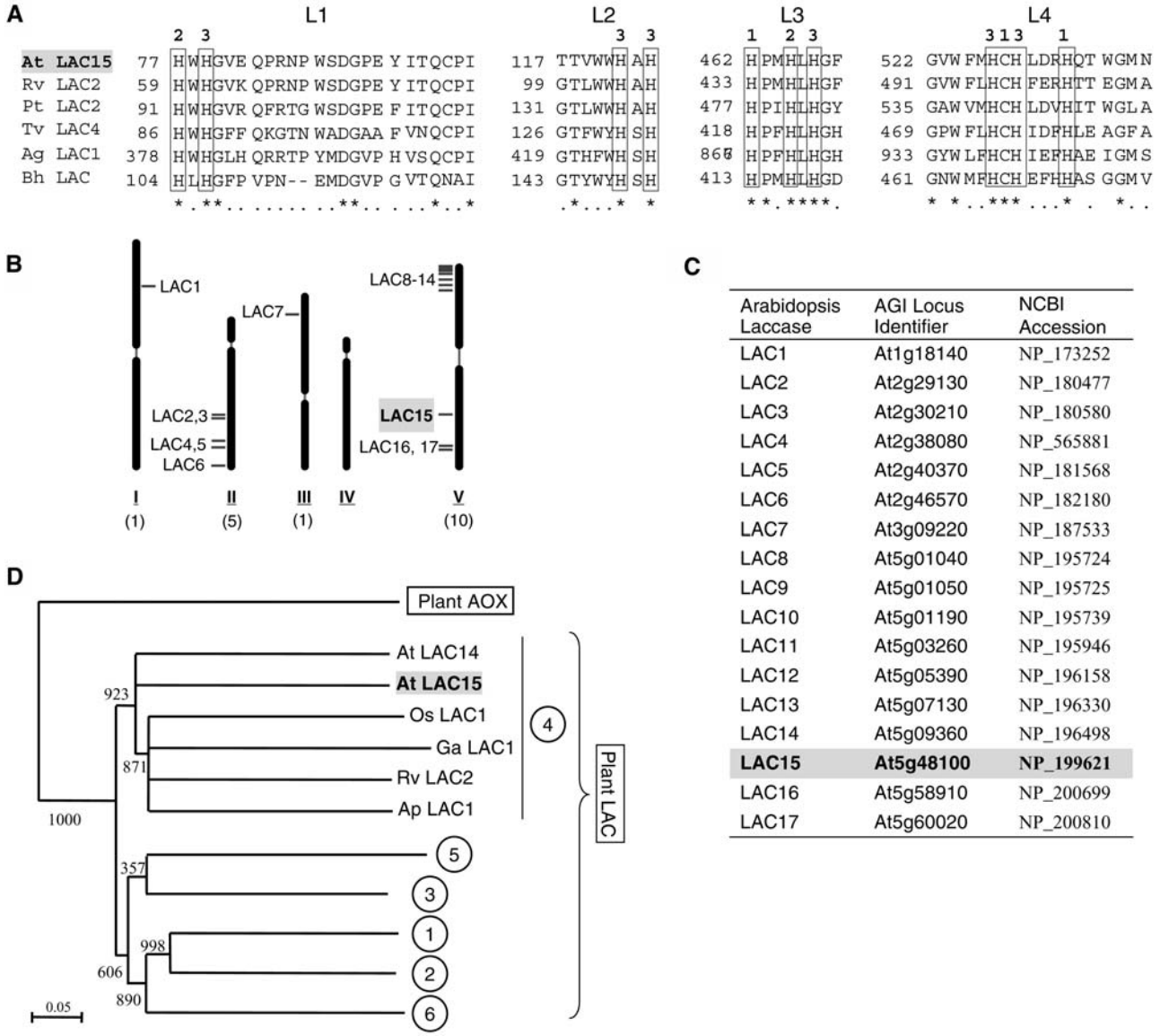


Figure 5. Pattern of *TT10* Promoter Activity in Wild-Type Seeds.

(A) to **(F)** Expression of the Pro_{TT10}:*uidA* cassette in developing seeds at 1 DAF **(A)**, 3 DAF (**[C]** and **[D]**), 8 DAF (**[E]** and **[F]**), and in an aborted seed **(B)**. GUS activity was observed with Nomarski optics on whole mounts for **(A)** to **(C)** and **(F)** and on sections for **(D)** and **(E)**.
(G) to **(K)** Expression of the Pro_{TT10}:*mGFP5-ER* cassette in developing seeds at 2 DAF **(G)**, 3 to 4 DAF (**[H]** to **[J]**), and 6 DAF **(K)** visualized on confocal cross sections showing GFP activity. **(I)** to **(J)** show overlays.
(L) Scheme of an immature seed transverse section showing the location of flavonoid pigments at the seed coat level (integuments and chalaza).
(M) Accumulation of PAs and precursors in an immature seed at 3 to 4 DAF (whole mount vanillin staining).
(N) Accumulation of flavonols in an immature seed at 3 to 4 DAF (diphenylboric acid 2-aminoethyl ester staining on seed section).
c, chalaza; e, endothelium; em, embryo; ii, inner integument; m, micropyle; oi, outer integument. Bars = 24 μm in **(F)** and **(K)**, 70 μm in **(A)** to **(D)** and **(G)**, 80 μm in **(M)**, 90 μm in **(H)**, **(L)**, and **(N)**, 110 μm in **(I)** to **(J)**, and 150 μm in **(E)**.

exhibited a bright yellow color with absorbance maxima at 280 and 380 nm, whereas the *tt10-2* extract was weakly colored and displayed a single absorbance maximum at 280 nm. The *tt4-8* extract yielded no red color when submitted to acid-catalyzed hydrolysis, showing that it did not contain procyanidins. HPLC analysis at 380 nm (Figure 8D) revealed eight major peaks present in the wild type and *tt4-8*, but not in *tt10-2*, that were denoted 1 to 8 in increasing retention time order. These peaks were absent from extracts of wild-type and mutant seeds incubated in control buffer. Importantly, all of the peaks detected in *tt4-8* represent only oxidation products of epicatechin by TT10, because this mutant does not have endogenous flavonoids. Interestingly, these oxidation products were more abundant in *tt4-8* than in the wild type.



Arabidopsis Laccase	AGI Locus Identifier	NCBI Accession
LAC1	At1g18140	NP_173252
LAC2	At2g29130	NP_180477
LAC3	At2g30210	NP_180580
LAC4	At2g38080	NP_565881
LAC5	At2g40370	NP_181568
LAC6	At2g46570	NP_182180
LAC7	At3g09220	NP_187533
LAC8	At5g01040	NP_195724
LAC9	At5g01050	NP_195725
LAC10	At5g01190	NP_195739
LAC11	At5g03260	NP_195946
LAC12	At5g05390	NP_196158
LAC13	At5g07130	NP_196330
LAC14	At5g09360	NP_196498
LAC15	**At5g48100**	**NP_199621**
LAC16	At5g58910	NP_200699
LAC17	At5g60020	NP_200810

Figure 6. Relationships between TT10 and Other Laccase-Like Proteins.

(A) Alignment of the amino acid sequences of TT10 (At LAC15) and other laccases around the four His-rich copper binding domains. Rv LAC2, *Rhus vernicifera* laccase; Pt LAC2, *Pinus taeda* laccase; Tv LAC4, *Trametes versicolor* laccase; Ag LAC1, *Anopheles gambiae* laccase; Bh LAC, *Bacillus halodurans alkaline* laccase. The amino acids involved in binding copper are numbered in boldface (types 1, 2, and 3) and boxed. Identical amino acids are indicated with asterisks.

(B) Genetic localization of the putative *Arabidopsis* laccases (LAC). Chromosome numbers are written below in roman numerals. The number of laccase genes on each chromosome is shown in parentheses.

(C) References of *Arabidopsis* laccases in the Arabidopsis Genome Initiative and National Center for Biotechnology Information databases.

(D) Simplified phylogenetic relationships between TT10/At LAC15 and other plant putative laccases. Alignment was performed between TT10 and the 100 most homologous protein sequences using the ClustalX program (version 1.8; Thompson et al., 1997) and optimized manually. The distance matrix was subjected to clustering using the neighbor-joining method. Bootstrap values with 1000 repetitions were used for statistical analysis and are indicated at each branch point. The tree is rooted by ascorbate oxidase (AOX) sequences as an outgroup. Circled numbers represent the six laccase groups established by McCaig et al. (2005). Only the composition of group 4, to which *Arabidopsis* TT10/At LAC15 laccase belongs, is presented (for complete tree, see Supplemental Figure 5 online). Protein sequences used in this analysis are as follows. The two letters preceding the protein name (LAC and AOX) describe the organism from which the sequence is derived: Ap, *Acer pseudoplatanus*; At, *Arabidopsis thaliana*; Ga, *Gossypium arboreum*; Os, *Oryza sativa*; Rv, *Rhus vernicifera*. The bar at bottom indicates relative branch length.

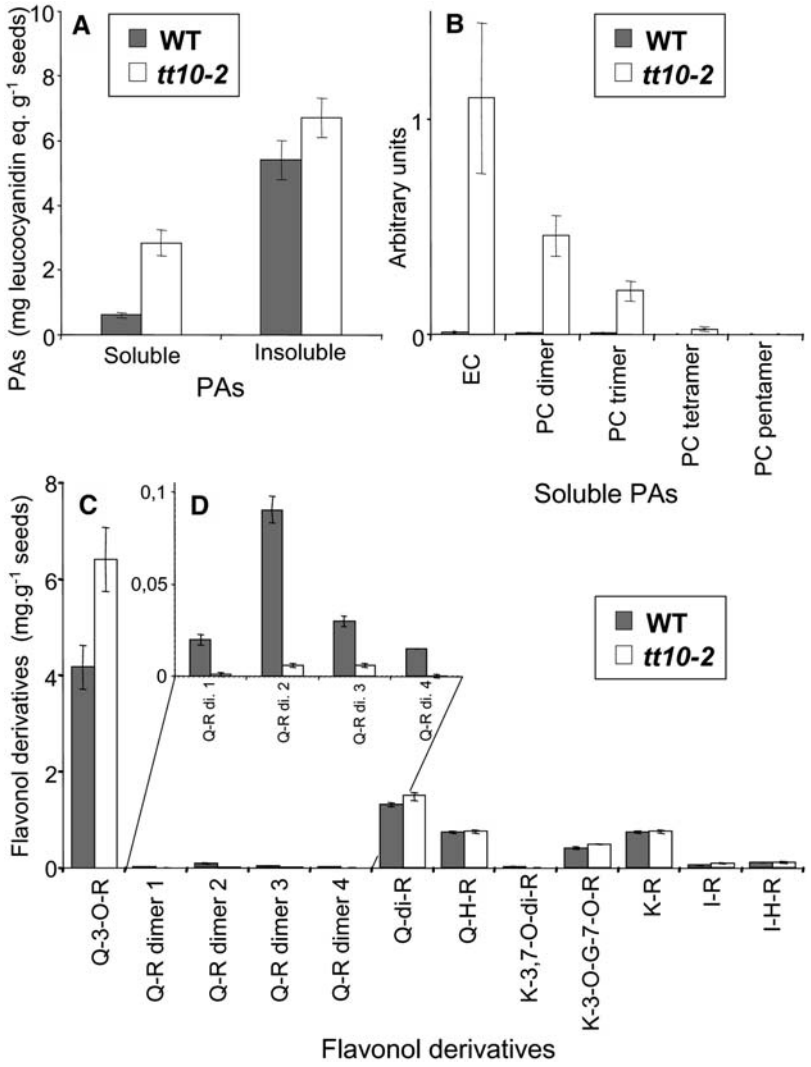


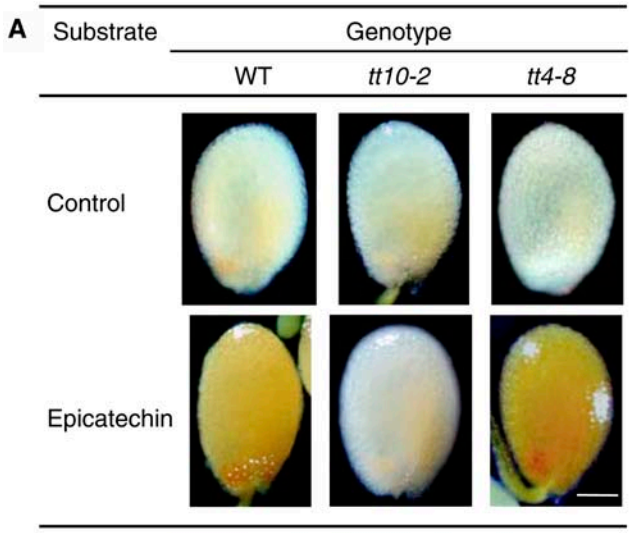
Figure 7. Flavonoid Composition of *tt10-2* and Wild-Type Mature Seeds.

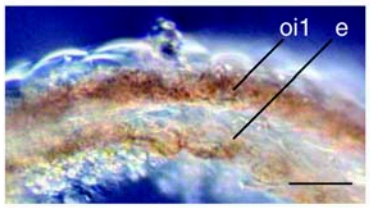
(A) Analysis of soluble and insoluble PAs measured after acid-catalyzed hydrolysis.
(B) Detection of epicatechin and soluble PAs by LC-MS.
(C) and **(D)** Detection of flavonol derivatives by LC-MS.
Values represent averages ± SE of three independent measurements. EC, epicatechin; G, glucoside; H, hexoside; I, isorhamnetin; K, kaempferol; PC, procyanidin; Q, quercetin; R, rhamnoside.

The electrospray mass spectra (in both positive and negative modes) gave molecular masses of 576 (for peaks 2, 4, and 6), 862 (for peaks 3, 5, 7, and 8), and 864 (for peak 1). Thus, compounds 2, 4, and 6 may correspond to epicatechin dimers with one additional unsaturation (two interflavan linkages and/or one additional unsaturation), peaks 3, 5, 7, and 8 may correspond to epicatechin trimers with two additional unsaturations, and compound 1 may correspond to an epicatechin trimer with one additional unsaturation. Fragments of the MS/MS spectrum of compound 6 (Figure 8E) were also found in the MS spectrum of dehydrodicatechin of the A type characterized previously after catechin oxidation by grape (*Vitis vinifera*) polyphenol oxidase in vitro (Guyot et al., 1996). By analogy with these previous results, we propose that compound 6 is a dehydrodiepicatechin A (see Supplemental Figure 6 online). The similarity between the MS/MS spectra obtained for the eight oxidation products (result not shown for products 2 to 4, 7, and 8), especially at $m/z < 575$, suggests that they all derive from epicatechin through similar oxidative mechanisms. Notably, if these oxidation products are isomers of procyanidin dimers and trimers, they differ from them by the nature and position of their interflavan linkage.

DISCUSSION

The biology of fungal laccases has been a subject of intense research for some time; however, analyses of the physiological





C

Substrate	Genotype		
	WT	*tt10-2*	*tt4-8*
Control	(-)	(-)	(-)
Epicatechin ± Catalase	(+)	(-)	(++)
Catechin ± Catalase	(++)	(-)	(+++)
Quercetin / Q-3-O-R	(-)	(-)	(-)
DAB	(- / -)	(- / -)	(- / -)
DAB + H_2O_2	(- / ++)	(- / ++)	(- / ++)

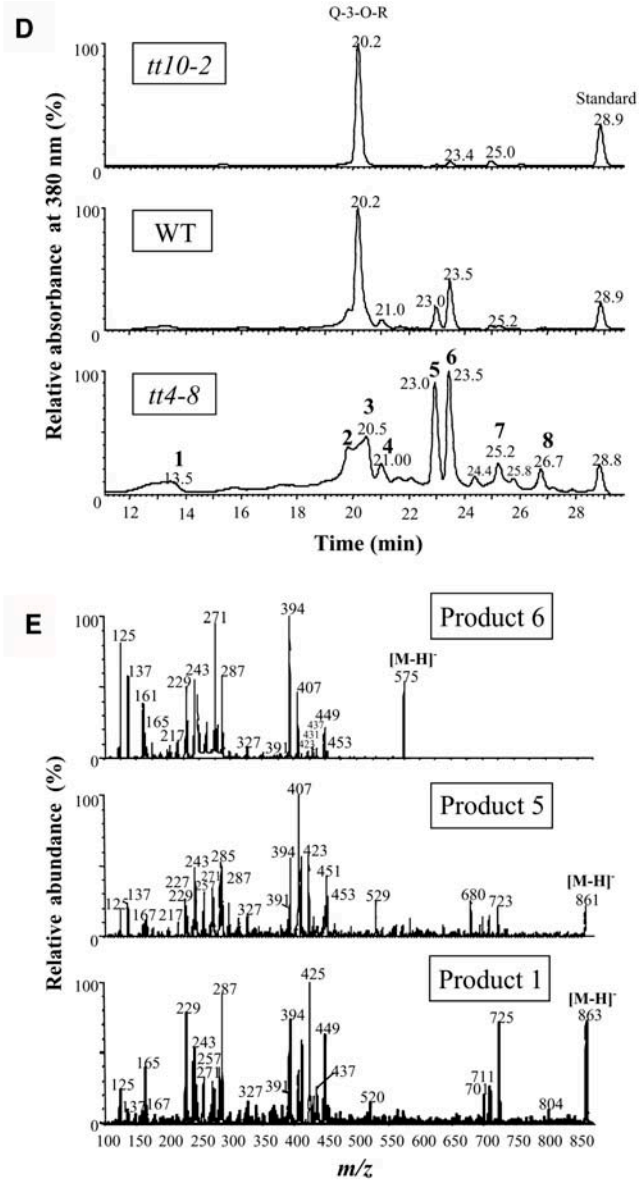


Figure 8. Analysis of in Situ Enzymatic Activity.

(A) Detection of browning in immature seed coats (7 to 8 DAF) from the wild type, *tt10-2*, and *tt4-8* after incubation in epicatechin overnight. Incubation in phosphate buffer was used as a control. Bar = 90 μm.

(B) Vibratome section of a *tt4-8* seed after incubation in epicatechin overnight. e, endothelium; oi, outer integument. Bar = 25 μm.

(C) Observation of seed coat color after the addition of various phenolic substrates. −, colorless; + < ++ < +++, browning intensity. The chalaza is brown only with the peroxidase substrate 3,3′-diaminobenzidine (DAB) + H_2O_2. Color observations are for seed coat color or seed coat color/chalaza color. Q-3-O-R, quercetin 3-*O*-rhamnoside.

(D) HPLC analysis of flavonoid products extracted from wild-type, *tt10-2*, and *tt4-8* immature seeds after incubation overnight with epicatechin. The seeds were thoroughly rinsed in control buffer before extraction to eliminate residues of exogenous epicatechin.

(E) MS/MS spectra in the negative mode of products 6 (at 30 eV), 5 (at 40 eV), and 1 (at 35 eV) formed from epicatechin.

functions of higher plant laccases are scarce (Mayer and Staples, 2002). Experimental approaches based on enzyme purification are rendered difficult because plant extracts contain many oxidative enzymes with overlapping substrate specificities (Ranocha et al., 1999). Genetic strategies based on the use of mutants, antisense knockouts, and overexpressors represent powerful alternatives. For instance, Ranocha et al. (2002) analyzed populations of transgenic poplar (*Populus trichocarpa*) showing reduced levels of a laccase and demonstrated alterations in phenolic metabolism and cell wall structure. In this

study, we have characterized several *Arabidopsis tt10* mutant alleles affected in seed coat browning and demonstrated that the corresponding protein is involved in the oxidative polymerization of flavonoids. We provide additional evidence that the spectrum of physiological functions of plant laccases extends beyond their potential role in the oxidative polymerization of monolignols.

The TT10 protein contains the four conserved copper binding domains characteristic of the multi-copper oxidase family, which were thoroughly described for fungal laccases (Bertrand et al., 2002; Kumar et al., 2003). From both of these motifs and the overall sequence similarity with laccases of other organisms, particularly with the prototype laccase enzyme from lacquer tree (Nitta et al., 2002), we infer that TT10 is a laccase-like protein.

Semiquantitative RT-PCR experiments and analysis of promoter activity with reporter genes showed that *TT10* is expressed mainly in the testa during seed development, although it is also detected in a few other tissues. Our results contradict those of McCaig et al. (2005), who detected a strong expression of At5g48100 in *Arabidopsis* roots and leaves by RT-PCR. One possible explanation for this discrepancy could be that expression of the gene was induced by stressful environmental conditions that were not present in our experiments. These results suggest the need to examine further the response of *TT10* to various stresses. The detection of a strong GUS activity driven by the *TT10* promoter in early aborted seeds strongly suggests that *TT10* may be transcriptionally induced by senescence. It is well known that oxidative browning is observed during the aging of various tissues of plants as a process preventing infections through the production of antimicrobial components and reactive oxygen species. Some peroxidases oxidize vacuolar and apoplastic phenolics to produce *o*-quinones that react further to produce brown components (Takahama, 2004). Our data suggest that the TT10 laccase may also be involved in such a process, albeit at the apoplast level.

From the analysis of the *tt10-6* allele and functional complementation with the *TT10* cDNA, we deduced that the 3′ UTR may be important for *TT10* expression. The lack of mRNA in the *tt10-6* background suggests that the 3′ UTR would exhibit regulatory sequences increasing the levels of *TT10* expression, possibly by stabilizing mRNA. Such a situation has been described for plant genes (Gutierrez et al., 1999).

TT10 expression is lower in the L*er* ecotype than in Ws-2 and Col. This reduction fits a reduction in enzyme activity, because L*er* exhibits twice the amount of soluble PAs compared with Ws-2 and Col (Routaboul et al., 2005).

TT10 promoter activity appears to be tightly regulated in time and space. The activity appears first in the endothelium and then in the oi1 cell layer of the outer integument. This pattern probably requires the action of specific regulatory factors. However, in contrast with the *BAN* gene expressed essentially in PA-producing cells (Debeaujon et al., 2003), *TT10* does not seem to be regulated by the TT2, TT8, and TTG1 regulatory factors.

Importantly, the cellular pattern of *TT10* promoter activity correlates with PA- and flavonol-producing cells, which is consistent with a role for TT10 in flavonoid metabolism in the seed coat. Whether TT10 may also play a role in lignin metabolism remains to be investigated. It appears from phloroglucinol-HCl staining that lignin in the *Arabidopsis* seed coat is restricted to the xylem vessels at the chalaza (data not shown), where no *TT10* promoter activity was detected.

The TT10 protein, which is predicted to be glycosylated, is likely to transit within the secretory pathway and be delivered into the apoplast after cleavage of the transit peptide. *TT10* mRNA accumulation increases during seed development at the same time as epicatechin biosynthesis, whereas seed browning occurs concomitantly with seed desiccation, at 10 DAF. The difference in intracellular compartmentation between substrate and enzyme may explain this delay.

Kitamura et al. (2004) showed that epicatechin and PAs accumulate in the vacuole in early developmental stages and migrate to the cell wall in senescing testa, until they are not visible in the vacuole any longer. Epicatechin and PA secretion into the apoplast can be explained by a vacuole burst caused by cell death during the seed desiccation period. Then, epicatechin and PAs would interact with TT10 and become oxidized and polymerized. Interactions between laccases and phenolic compounds in the cell wall have been observed in the case of monolignol polymerization (Liu et al., 1994). TT10 would act in the same way with epicatechin and PAs. Similar to *tt10*, the *tt19* mutant exhibits a delay in seed coat browning compared with the wild type. The corresponding gene encodes a glutathione *S*-transferase putatively involved in the transport of flavan-3-ols (Kitamura et al., 2004). The delay in enzymatic browning in this mutant may be explained by a late interaction between flavan-3-ols and TT10 in the cell wall. A better knowledge of the PA secretory pathway will be necessary to understand the complete mechanisms of seed coat browning.

LC-MS analyses of mature seed extracts revealed a role for TT10 in the oxidative polymerization of flavan-3-ols. During seed desiccation, the initially colorless PAs form oxidized complexes with cell wall polysaccharides and other phenolics, a process that causes the tissue to darken (Marles et al., 2003). Epicatechin does not bind to cell walls, in contrast with procyanidins, which form noncovalent interactions with polysaccharides (Renard et al., 2001). The higher amount of extractable PAs detected in the *tt10* mutant than in the wild type may be attributable to the absence of such interactions.

As expected, the browning process observed during the in situ activity assay was triggered by providing flavan-3-ol monomers, subsequently oxidized by the resident enzyme expressed in the seed coat. The major products resulting from TT10 activity are yellow quinone-methide epicatechin dimers and trimers. They are similar to those reported by Guyot et al. (1996), which are formed in vitro when a grape polyphenol oxidase acts on catechin (an epimer of epicatechin) to form the corresponding quinones. As established in model oxidation experiments, the *o*-quinones generated are generally colored and are highly reactive: for instance, they may polymerize and also oxidize other compounds. Some of the secondary reaction products are colored or may be oxidized to colored quinoid compounds. Therefore, the color of enzymatically oxidized phenols depends mainly on the nature and relative importance of their subsequent reactions, although it may be influenced by that of the primary quinones (Nicolas et al., 1993). The brown color observed in seed coats during maturation probably results from successive oxidation reactions leading to more complex oxidation levels.

Dixon et al. (2005) proposed a model for PA formation from flavan-3-ols. They speculated that epicatechin or catechin may be converted to the corresponding *o*-quinones by a polyphenol oxidase enzyme. The quinones would then be converted to carbocations through coupled nonenzymatic oxidation. Nucleophilic attack on the carbocations by epicatechin or catechin would produce dimers and then oligomeric PAs linked through C_4–C_8 or C_4–C_6. In our experimental system, if the yellow polymers are isomers of the unoxidized, colorless PA polymers, they differ from them by the interflavan linkages (for structural details, see Supplemental Figure 6 online). No PAs were detected in *tt4-8* seeds incubated with epicatechin, which suggests that TT10 is not a condensing enzyme that catalyzes the polymerization of flavan-3-ols into the typical unoxidized PAs. We show here that TT10 is a laccase-like flavonoid oxidase that is able to catalyze the oxidative polymerization of epicatechin into yellow to brown pigments different from the colorless PAs. Whether TT10 can also oxidize PAs remains to be investigated (see Supplemental Figure 6 online). Feeding PAs to immature seeds in the frame of our in situ activity assay will help answer this question. Dissecting the activity of the TT10 recombinant protein toward given substrates in vitro will also be essential before definitive conclusions on the function of this enzyme can be drawn.

Dirigent proteins have been demonstrated to ensure the stereoselective coupling step leading to the formation of (+)-pinoresinol, which is a specific coniferyl alcohol dimer and the only product formed after the oxidation of coniferyl alcohol by a still unknown one-electron oxidase (Halls and Lewis, 2002). Whether such proteins are responsible for some linkage specificity in the polymerization process initiated by TT10 requires further investigation. Comparing the oxidation products obtained in situ and in vitro may help answer this question.

The fact that more oxidation products are made in the *tt4-8* background than in the wild-type background during the in situ activity assay may be attributable to an inhibitory effect of procyanidins on TT10 activity in the wild type, as described previously for apple polyphenol oxidase (Le Bourvellec et al., 2004).

TT10 activity on flavan-3-ols is H_2O_2-independent, demonstrating that TT10 does not act as a peroxidase. We have detected a peroxidase browning activity in the chalaza zone using a peroxidase-specific substrate (DAB). This result may explain the presence of dark-brown chalaza in *tt10* mutant seeds at harvest. A peroxidase activity leading to the formation of brown products was also reported in the chalaza of developing barley (*Hordeum vulgare*) grain (Cochrane et al., 2000). The authors suggest that the enzyme may be involved in mechanisms of defense against pathogen invasion, which may also be the situation in *Arabidopsis*. During seed development, the presence of several oxidases may be needed to ensure different functions.

TT10 is also involved in quercetin rhamnoside oxidative polymerization. The products formed are quercetin rhamnoside dimers, which are apparently not brown, in contrast with oxidized epicatechin derivatives. A mutant without flavonols would allow a better understanding of the role of TT10 in quercetin rhamnoside dimerization in relation to browning. Protection against UV light–induced damage is an important function of flavonols in plants (Winkel-Shirley, 2002a).

The 16 other *Arabidopsis* laccase-like proteins cannot compensate for the loss of TT10 activity in *tt10* seeds. This finding suggests either that they are not localized in similar tissues or that they have a different activity or substrate specificity. To date, none of these laccases has been functionally characterized. We also infer from the *tt10* mutant seed coat phenotype that no other oxidase enzyme, such as catechol oxidase or peroxidase, carries out epicatechin oxidation in the absence of TT10 during seed development. The seeds of *tt10* plants do finally turn brown, probably by autoxidation. This takes more time than enzymatically, which explains why *tt10* seeds are pale at harvest but darken with storage time and not during seed maturation.

The mechanism of oxidative polymerization involving a laccase has been suggested in the case of monolignols (Sterjiades et al., 1992; Boerjan et al., 2003). TT10 displays strong similarity with *Rhus vernicifera*, *Acer pseudoplatanus*, and *Gossypium arboreum* laccases (Rv LAC2, Ap LAC1, and Ga LAC1, respectively), the activities of which have been linked to polyphenol metabolism. Rv LAC2 was found to catalyze the polymerization of urishiol into lacquer for wound healing in response to pathogen attack (Nitta et al., 2002). Ap LAC1, purified from cell suspension, was able to oxidize monolignols to form water-insoluble polymers in vitro (Sterjiades et al., 1992). The Ga LAC1 secretory laccase expressed in *Arabidopsis* was responsible for the conversion of sinapic acid into a monolactone-type dimer and for the transformation of 2,4,6-trichlorophenol (Wang et al., 2004). Therefore, phenolic polymerization may be a common function for plant laccases. The biochemical step catalyzed by these enzymes is the formation of highly reactive quinones, which afterward spontaneously polymerize to give yellow to brown products according to the extent of polymerization (Rouet-Mayer et al., 1990). Therefore, polymerization is an indirect consequence of quinone formation.

The plant laccases can be divided into at least six groups present in both monocots and dicots (McCaig et al., 2005). TT10 belongs to group 4, which probably diverged early from the other group. Laccases may have acquired specific functions very early in evolution, such as the induction of resistance to biotic and abiotic stresses, on the model of tomato catechol oxidase (Thipyapong et al., 2004). The *Arabidopsis* genome does not contain any typical catechol oxidase, as assessed by searching for *Arabidopsis* sequences homologous with those of a poplar catechol oxidase protein (Constabel et al., 2000). Laccases may fill the absence of catechol oxidase in the sense of a specialization toward *o*-diphenol oxidation.

The biological role of TT10 during seed development may be to form flavonoid derivatives that strengthen the testa to protect the embryo and endosperm from biotic and abiotic stresses. Werker et al. (1979) showed that quinones reinforced the barrier to water permeation of *Pisum* seed coats. The physiological role of *TT10* in the seed coat may be also to create a barrier against pathogens by triggering the formation of antimicrobial quinones, thus protecting the seed during storage and germination. Expression data (https://www.genevestigator.ethz.ch/) indicated that *TT10* is significantly induced by nematode attack and by heat and osmotic stresses that can be related to drought. Moreover, putative binding sites for regulatory factors induced by desiccation, pathogens, and elicitors are present in the *TT10*

promoter (http://www.dna.affrc.go.jp/PLACE/signalscan.html). An in-depth functional study of the promoter, as well as an analysis of *tt10* seed longevity, germination, and resistance to pathogens, will have to be performed to determine the precise roles of TT10 in seed biology.

METHODS

Plant Materials, Growth Conditions, and Genetic Analyses

The *tt10-1* allele (CS110) comes from an ethyl methanesulfonate mutagenesis of the L*er* ecotype of *Arabidopsis thaliana* (Koornneef, 1990). The *tt10-2* and *tt10-3* alleles (CPI13 and CQK31 lines, respectively) are T-DNA insertion lines in the Ws-2 ecotype, generated at the Institut National de la Recherche Agronomique Versailles (Bechtold et al., 1993). They were visually screened from T3 seed lots for an altered seed color. These lines were untagged by T-DNA. The *tt10-4*, *tt10-5*, and *tt10-6* alleles (N502972, N128292, and N114753 lines, respectively) are T-DNA insertion lines in the Col ecotype obtained from the Salk Institute (http://signal.salk.edu/). They were identified by reverse genetics based on the At5g48100 gene sequence. The *tt4-8* allele, corresponding to the DFW34 line, is a T-DNA line in the Ws-2 ecotype from the Versailles collection (Debeaujon et al., 2003). The *tt4-8* allele is completely devoid of flavonoids (Routaboul et al., 2005). In vitro and greenhouse culture conditions were described previously by Debeaujon et al. (2001). Crosses were performed between *tt10-1* and the other alleles. In all cases, F2 seeds exhibited a mutant phenotype, indicating that the mutations were allelic. Reciprocal crosses with the corresponding wild types were also realized to assess the maternal determinism of the mutations.

Nucleic Acid Analyses

DNA and RNA experiments were performed according to Nesi et al. (2000) and Debeaujon et al. (2001). The 5′ and 3′ ends of the mRNA transcript were identified with the Ambion kit FirstChoice RLM-rapid amplification of cDNA ends. Nucleic acids were sequenced using the Applied Biosystems DNA sequencing kit (Bigdye Terminator version 3.0) and the ABI Prism 310 genetic analyzer. RT-PCR for amplification of the 1.6-kb *TT10* cDNA in wild-type and mutant tissues was realized using primers TT10-ATG and TT10-STOP defined in the coding region (see Supplemental Table 1 online).

Constructs for Functional Complementation

PCR amplifications were performed using the proofreading Pfu Ultra DNA polymerase (Stratagene) with the primer sequences presented in Supplemental Table 1 online. Complementation was performed with a genomic 8-kb *Xho*I subclone of the MDN11 Mitsui P1 clone, containing the *TT10* gene. The subclone was ligated into the *Sal*I site of the pBIB-HYG binary vector (Becker, 1990) to generate the *TT10*/pBIB-HYG plasmid. Complementation was also performed with *cTT10*, either with or without the 5′ and 3′ UTRs, and placed under the control of the dual *35S* promoter. A full-length cDNA (clone RAFL15-04-D21) isolated from an *Arabidopsis* siliques/flowers library (Seki et al., 2004) was obtained from RIKEN. The cDNA was amplified from 5′ to 3′ UTR using primers *TT10*-5′UTR-*Sal*I and *TT10*-3′UTR-*Sal*I and from ATG to STOP using primers *TT10*-ATG-*Sal*I and *TT10*-STOP-*Sal*I. Finally, the cDNA was *Sal*I-digested and cloned at the *Sal*I sites of the pMagic plasmid (Nesi et al., 2002) to generate the $Pro_{35Sdual}$:*cTT10*/pBIB-HYG construction.

Constructs for the Analysis of Promoter Activity

To construct the Pro_{TT10}:*uidA*:*t35S* cassette, a 2.0-kb *TT10* promoter was amplified by PCR from the *TT10*/pBIB-HYG plasmid using primers p*TT10-5′Xho*I and p*TT10-3′Nco*I. The *Xho*I-*Nco*I–digested fragment was cloned into the *Nco*I and *Xho*I sites of the pBS-*GUS* vector described previously (Debeaujon et al., 2003). The Pro_{TT10}:*uidA*:*t35S* cassette was cloned as a *Sma*I-*Kpn*I fragment into the pBIB-HYG binary vector.

To build the Pro_{TT10}:*mGFP5-ER* cassette, a 2.0-kb *TT10* promoter was amplified with primers p*TT10-3′Bam*HI and p*TT10-5′Sal*I, generating a *Bam*HI-*Sal*I PCR product that was cloned at the *Bam*HI-*Sal*I sites of the pBI101-*mGFP5-ER* plasmid. To generate pBI101-*mGFP5-ER*, the *mGFP5-ER* cassette was released from pBIN-*mGFP5-ER* plasmid with *Bam*HI-*Sst*I and cloned into a *Bam*HI-*Sst*I–digested pBI101 binary vector (Clontech).

Stable transformation of *Arabidopsis* plants was performed as described previously (Nesi et al., 2000).

Microscopy

GUS staining, together with sample mounting in a chloral hydrate solution and inclusion in resin for the realization of sections, were performed according to Debeaujon et al. (2003). Samples were observed with a fluorescence Axioplan microscope (Zeiss) equipped with Nomarski differential interference contrast optics. GFP observations of living seeds were made as described by Debeaujon et al. (2003). For the histochemical detection of flavan-3-ols, vanillin staining was handled as described previously (Debeaujon et al., 2000). Flavonol localization was assayed with diphenylboric acid 2-aminoethyl ester staining according to Sheahan and Rechnitz (1993).

In Situ Enzyme Activity

The accelerated browning assay was performed on opened immature siliques (7 to 8 DAF) in 100 mM phosphate buffer, pH 6.6, with 50 mM (+)-catechin hydrate, 50 mM (−)-epicatechin, 10 mM quercetin dihydrate, or 50 mM quercetin rhamnoside hydrate (Sigma-Aldrich for all products). Assays were run with or without catalase (125 units/mL) to remove any possible endogenous H_2O_2 and therefore inhibit peroxidase activity. Siliques were preincubated in the catalase solution for 1 h before the addition of substrate. Histochemical staining for peroxidase activity was performed with 2.5 mM DAB in the presence of 0.02% H_2O_2. Control staining was performed without H_2O_2. For all assays, vacuum was applied for 1 h before incubation at 37°C in the dark, overnight. Tissues were observed directly with a microscope.

Flavonoid Extraction and Analysis

Fifteen milligrams of seeds was ground in a 2-mL homogenizer (Potter) with 1 mL of acetonitrile:water (75:25, v/v) for 5 min at 4°C and were sonicated for 20 min. Four micrograms of apigenin-7-*O*-glucoside was used as an internal standard. After centrifugation, the pellet was re-extracted with 1 mL of acetonitrile:water (75:25, v/v) overnight at 4°C. The two extracts were pooled and concentrated under nitrogen and the dry extract was dissolved in 200 μL of acetonitrile:water (75:25, v/v) to give the final extract used for the analysis of flavonols and soluble PAs. The pellet was preserved for insoluble PA analysis.

Analyses of soluble and insoluble PAs were performed after acid-catalyzed hydrolysis (acid butanol assay) according to Porter et al. (1986) on aliquots of the final extracts and on the entire remaining pellets, respectively. They are expressed in milligrams per gram of leucocyanidin equivalent assuming that the effective $E^{1\%,1cm,550nm}$ of leucocyanidin is 460. Five grams of seeds was ground in a 40-mL conical glass (Potter) with 30 mL of acetonitrile:water (75:25, v/v) at 4°C and sonicated for 20 min. After centrifugation, the pellet was extracted overnight at 4°C, with an additional 30 mL of acetonitrile:water (75:25, v/v). To minimize PA oxidation, solutions were saturated with nitrogen and kept in the dark at 4°C. The two extracts were pooled, concentrated under reduced

pressure at 40°C, and dissolved in 1 mL of methanol:water (1:1, v/v). The crude PA extract was purified using Toyopearl TSK HW 40-F (Sigma-Aldrich) as described previously (Kennedy and Jones, 2001). We checked that epicatechin monomer was removed after this purification step using LC-MS. The purified PA extract was dissolved in 1 mL of 0.1 N HCl in methanol containing 50 mg of phloroglucinol and 10 mg of ascorbic acid and reacted for 20 min at 50°C. The reaction was stopped with 5 mL of 40 mM sodium acetate. Reaction products were analyzed using LC-MS and quantified at 280 nm using published values for the molar response of epicatechin and epicatechin-phloroglucinol (Kennedy and Jones, 2001).

Mass analysis was conducted on an aliquot of the final extract using a Quattro liquid chromatograph with an electrospray ionization Z-spray interface (MicroMass), MassLynx software, an Alliance 2695 reversed-phase HPLC system (Waters), and a Waters 2487 UV detector set at 280 nm. An Uptisphere C_{18} column (150 × 2 mm, 5 μm; Interchrom) was used with a mix comprising solvent A acetonitrile:water (95:5, v/v; 0.2% acetic acid) and solvent B acetonitrile:water (5:95, v/v; 0.2% acetic acid) with a gradient profile (starting with 10:90 A/B [v/v] for 5 min; linear gradient up to 70:30 A/B over 35 min; a washing step of 100:0 A/B for 15 min, and a final equilibration at 10:90 A/B for 20 min) at a flow rate of 200 μL/min. Quantification was based on the area of major MS signals ($[M+H]^+$ and fragment). Flavonoid content was expressed relative to quercetin 3-*O*-rhamnoside, rutin, and epicatechin (Extrasynthèse) for monoglycosylated flavonols, diglycosylated flavonols, and procyanidin derivatives, respectively.

Assignment of epicatechin itself was made by comparison with an authentic standard (same retention time at 6 min, instead of 3.5 min for catechin, and same MS/MS fragmentation pattern for ion $[M+H]^+$ at *m/z* 291). Procyanidins (up to 4 units) were characterized during LC-MS analysis by their $[M+H]^+$ mass signals at *m/z* 579, 867, and 1155 and characteristic fragments resulting from losses of 289/290 neutrals. Assignment of quercetin-3-*O*-rhamnoside was made by comparison with an authentic standard; it was characterized by its retention time at 23.1 min, ions at *m/z* 449 $[M+H]^+$ and 303 $[M+H\text{-rhamnose}]^+$ in the positive mode. Quercetin rhamnoside dimer characteristics were 17.5, 19.7, 20.5, and 21.5 min, *m/z* 895 $[M+H]^+$, 749 $[M+H\text{-rhamnose}]^+$, and 603 $[M+H\text{-rhamnose rhamnose}]^+$

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: Ag LAC1 (AAN17505), Ap LAC1 (AAB09228), Bh LAC (AY228142), Ga LAC1 (AAR83118), MDN11 P1 clone (AB017064), Os LAC1 (NP_918753), Pt LAC2 (AAK37824), RAFL15-04-D21 cDNA clone (BT002919), Rv LAC2 (BAB63411), and Tv LAC4 (Q12719).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Primers Used for PCR and RT-PCR Analysis of the *TT10* Gene, cDNA, and Promoter.

Supplemental Figure 1. Molecular Characterization of the *tt10* Mutant Alleles.

Supplemental Figure 2. Flavonoid Composition of Mature Seeds from Complementing Lines Expressing the Wild-Type Genomic Region.

Supplemental Figure 3. Analysis of *TT10* Gene Expression in Overexpressing Lines.

Supplemental Figure 4. Pattern of *TT10* Promoter Activity in the Transmitting Tissue of the Silique.

Supplemental Figure 5. Phylogenetic Relationships between TT10/AtLAC15 and Other Plant Putative Laccases.

Supplemental Figure 6. Partial *Arabidopsis* Flavonoid Pathway Showing the Various Levels of TT10 Action: A Working Model.

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