

A *DEFICIENS* Homolog from the Dioecious Tree Black Cottonwood Is Expressed in Female and Male Floral Meristems of the Two-Whorled, Unisexual Flowers¹

Lorraine A. Sheppard², Amy M. Brunner, Konstantin V. Krutovskii³, William H. Rottmann⁴, Jeffrey S. Skinner, Sheila S. Vollmer⁵, and Steven H. Strauss*

Genetics Program (L.A.S.) and Department of Forest Science (A.M.B., K.V.K., W.H.R., J.S.S., S.S.V., S.H.S.), Oregon State University, Corvallis, Oregon 97331-5752

We isolated *PTD*, a member of the *DEFICIENS* (*DEF*) family of MADS box transcription factors, from the dioecious tree, black cottonwood (*Populus trichocarpa*). In females, in situ hybridization experiments showed that *PTD* mRNA was first detectable in cells on the flanks of the inflorescence meristem, before differentiation of individual flowers was visually detectable. In males, the onset of *PTD* expression was delayed until after individual flower differentiation had begun and floral meristems were developing. Although *PTD* was initially expressed throughout the inner whorl meristem in female and male flowers, its spatial expression pattern became sex-specific as reproductive primordia began to form. *PTD* expression was maintained in stamen primordia, but excluded from carpel primordia, as well as vegetative tissues. Although *PTD* is phylogenetically most closely related to the largely uncharacterized *TM6* subfamily of the *DEF/APETELA3(AP3)/TM6* group, its spatio-temporal expression patterns are more similar to that of *DEF* and *AP3* than to other members of the *TM6* subfamily.

One of the greatest challenges in plant developmental biology is ascertaining the genetic mechanisms responsible for the great variety of floral forms. We are studying floral developmental genetics in black cottonwood (*Populus trichocarpa*), a dioecious tree with unusual two-whorled flowers. Black cottonwood is a fast-growing tree native to the northwestern United States and Canada (Fig. 1A). It is the dominant species in many riparian ecosystems and is commonly used in breeding hybrids for use in fiber and energy plantations. Several factors render cot-

tonwoods (black cottonwood and *Populus deltoides*) attractive as model tree species: their small genome size (approximately 1.1 pg per diploid cell and approximately 500 mbp per haploid genome [Bradshaw and Stettler, 1993]), the existence of an efficient transformation system (Han et al., 1996), ease of propagation, and a relatively short time from germination to flowering (≤ 5 years).

The flowers of black cottonwood develop on pendulous inflorescences (aments and catkins) that begin to develop in spring (late April to early May in Corvallis, OR) of the year before the spring in which they will open. Inflorescence buds develop as axillary accessory buds on short shoots and as axillary lateral buds near branch tips (Fig. 1, B and C). After anthesis, the new inflorescences begin to elongate rapidly within the bud scales. Concurrently, bract primordia develop acropetally along the inflorescence flanks. As the bract primordia enlarge, cells in the axils of the bracts become organized into flattened floral discs (Boes and Strauss, 1994). Continued growth at the perimeter of each floral disc produces a raised ring of tissue that will develop into an unusual structure known as the perianth cup. Vascular traces and developmental morphology indicate that the perianth cup is derived from the fusion of perianth parts (Fisher, 1928; Stoehr et al., 1988; Kaul, 1995). However, it remains unclear if the perianth cup is derived from fused sepals, fused petals, or adnation of petals and sepals. In females, three carpel primordia arise from the floral meristem and later unite to form a unilocular ovary. The mature female inflorescence bears 20 to 40 flowers, with each flower consisting of

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² Present address: Institute of Forest Genetics, U.S. Department of Agriculture Forest Service, Department of Environmental Horticulture, One Shields Avenue, University of California, Davis, CA 95616.

³ Permanent address: Laboratory of Population Genetics, N.I. Vavilov Institute of General Genetics, Russian Academy of Sciences, 117809 GSP-1, Moscow B-333, Russia.

⁴ Present address: Westvaco Forest Science and Technology, P.O. Box 1950, Summerville, SC 29484.

⁵ Present address: U.S. Department of Agriculture-Agriculture Research Service National Forage Seed Production Research Center, 3450 SW Campus Way, Corvallis, OR 97331-8539.

* Corresponding author; e-mail Steve.Strauss@orst.edu; fax 541-737-1393.

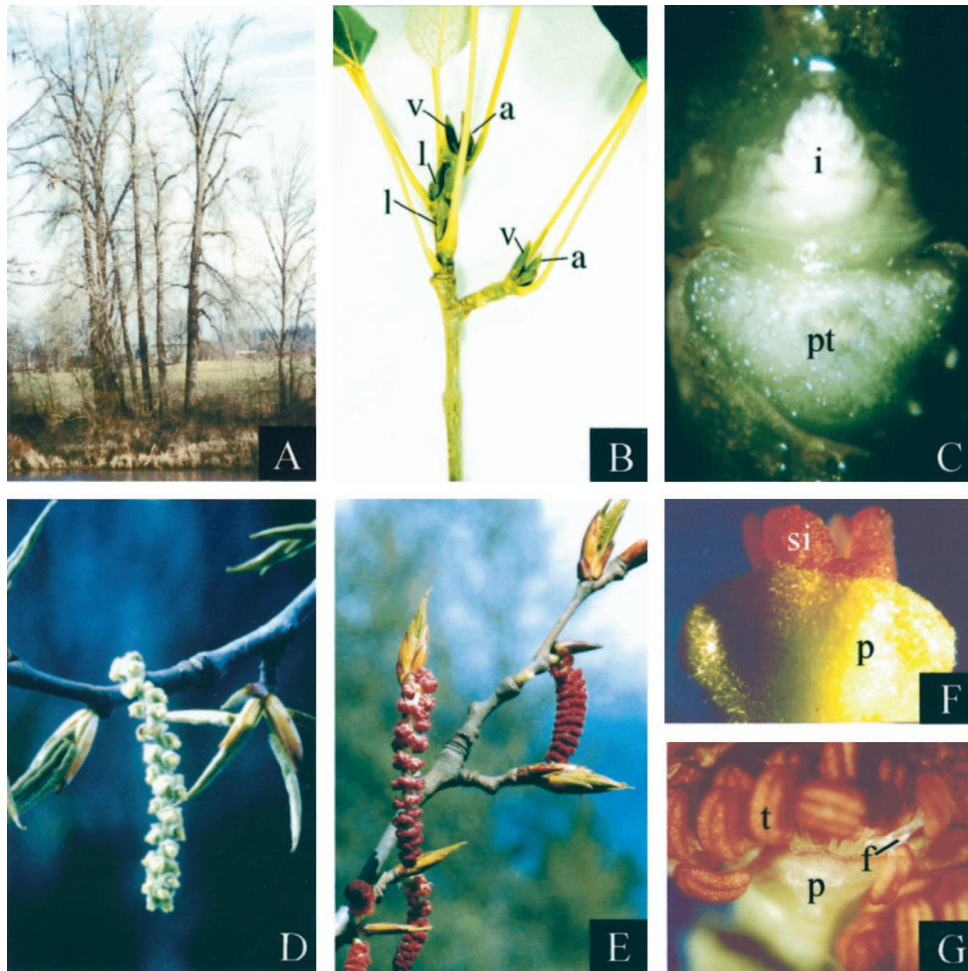


Figure 1. Black cottonwood floral morphology. Images are digitized. A, Mature black cottonwood trees on a riverbank during winter in Corvallis, Oregon. B, Developing inflorescence and vegetative buds in early summer. C, Developing inflorescence bud in early summer. Bud scales and the adjacent leaf petiole were removed. D, Mature female catkin in early spring. E, Mature male catkins in early spring. F, Mature female flower in early spring. G, Mature male flower in early spring. a, Axillary accessory inflorescence bud; f, filament of stamen; i, developing inflorescence; l, lateral axillary inflorescence bud; p, perianth cup; pt, petiole attachment (petiole removed); t, stamen; si, stigma; v, vegetative bud.

a stigma surrounded by a perianth cup (Figs. 1, D and F, and 2A). In males, stamen primordia begin to arise in the center of the floral disc and organogenesis proceeds centrifugally. The mature male flower consists of 40 to 60 stamens surrounded by a perianth cup (Figs. 1, E and G, and 2B).

Dioecy is estimated to occur in less than 4% of the angiosperms and is achieved by different means (for review, see Ainsworth et al., 1998). Most commonly, male and female organs are initiated, and then production of either ovules or pollen is blocked at a later stage. More rarely, only organs of a single sex are initiated and the floral meristems are thus unisexual. Detailed light and electron microscopy studies indicate that cottonwoods belong to the latter group in which initiation of stamen development does not occur in female flowers and initiation of carpel development does not occur in male flowers (Boes and Strauss, 1994; Kaul, 1995; Sheppard, 1997). The cot-

tonwood flower thus consists of only two whorls of organs: the perianth cup and either stamens or a pistil.

Bulked segregant analyses of randomly amplified polymorphic DNA data indicate that sex determination in cottonwoods may be controlled by multiple loci acting in an additive or epistatic manner (McLetchie and Tuskan, 1994). However, perfect flowers and flowers of inappropriate sex have been noted on rare occasions (Larsen, 1970; Stettler, 1971) and environmental factors such as infestation by Eriophyid mites have been implicated in sex modification (Heslop-Harrison, 1924). The exact mode of sex determination in cottonwoods remains unclear.

We are studying floral homeotic genes in black cottonwood to better understand the genetic factors involved in floral development and to facilitate genetic engineering of reproductively sterile trees for the purpose of transgene containment. Most floral

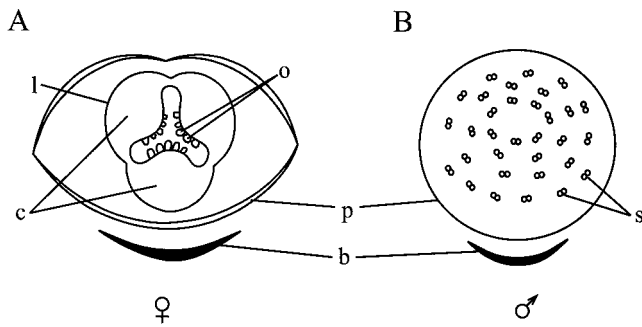


Figure 2. Diagrams of female and male black cottonwood flowers. Each flower consists of only two whorls: the perianth cup and either stamens or a pistil. A, Female flower with three fused carpels. The double line denoting the perianth cup indicates infolding that occurs at the rim. B, Male flower with 40 to 60 stamens. b, Bract; c, carpel; l, pistil; o, ovule; p, perianth cup; s, stamen.

homeotic genes isolated to date belong to the MADS box family of transcription factors (for review, see Riechmann and Meyerowitz, 1998). Because MADS box genes are known to play fundamental roles in floral development, variations in floral MADS box gene sequences are likely to play a significant role in specifying the great diversity of floral forms. Recent phylogenetic analyses based on DNA sequences (Soltis et al., 1999), fossil evidence, and traditional classifications based on morphology (for review, see Eckenswalder, 1996) suggest that the two-whorled black cottonwood flowers evolved from a four-whorled flower typically found among the higher eudicots. The establishment of the four organ types of the typical eudicot flower are largely explained by the ABC model of flower development, which describes the combinatorial action of three classes of homeotic genes (for review, see Riechmann and Meyerowitz, 1998). From the standpoint of understanding how regulation of cottonwood floral development deviates from this model, the B-class organ identity genes are of particular interest. The combined action of A and B class genes specify petals, whereas B and C class genes specify stamens. The study of B-class genes in cottonwood may therefore provide insight into the derivation of the perianth cup and unisexuality. In *Arabidopsis* and snapdragon, two MADS box genes belonging to sister clades are necessary to specify B-function. The *Arabidopsis* genes are *AP3* and *PISTILLATA (PI)*, whereas the corresponding snapdragon orthologs are *DEF* and *GLOBOSA (GLO)*.

We describe the isolation of *PTD*, a MADS box gene from black cottonwood that has homology with the floral homeotic transcription factors *DEF* (Sommer et al., 1990) and *AP3* (Jack et al., 1992). Although phylogenetic analyses indicate that *PTD* is a member of the largely uncharacterized *TM6* clade of the *DEF/AP3/TM6* family, we report that the expression patterns of *PTD* are more similar to those of *DEF* and *AP3* than to other members of the *TM6* group. *PTD* is not expressed in vegetative tissues and its spatial and temporal expression patterns are sex-specific.

RESULTS

cDNA and Genomic Clone Isolation and Structure

To identify black cottonwood MADS box genes potentially involved in floral development, an early female inflorescence cDNA library was probed at low stringency with the MADS box region of the *Arabidopsis* *AGL1* gene. One of the cDNA sequences subsequently isolated had high homology with the *DEF* gene from snapdragon and is referred to here as *PTD* (black cottonwood *DEF*-like). The *PTD* cDNA was 917 bp in length with an open reading frame corresponding to 224 amino acid residues. An initiating Met was not present in the cDNA clone.

The *PTD* genomic clone was isolated from a black cottonwood genomic library. The genomic sequence (GenBank accession no. AF057708) matched the cDNA sequence exactly. An ATG was present 10 bases upstream from the truncated 5' end of the cDNA thus the deduced *PTD* protein sequence consists of 228 amino acid residues.

Gel blots of male and female genomic DNA probed with the 3' region of *PTD* revealed one major band in lanes containing DNA digested with *DraI* or *EcoRI*, and two bands in lanes containing DNA digested with *HindIII* or *XbaI*, indicating that *PTD* is a single copy gene (data not shown). No differences were observed between the hybridization patterns of DNA from male and female trees.

The *PTD* gene consists of seven exons, and the number and positions of introns are nearly identical with those of the *DEF* gene. *PTD* introns have a slightly larger average size than those of *AP3* (Schwarz-Sommer et al., 1992), but are smaller than those of *DEF* (Irish and Yamamoto, 1995) and *ST-DEF* (Garcia-Maroto et al., 1993).

A TATA-like sequence [TATTTA] was present 30 bases upstream from an Inr motif [TTCACCCTT], and CCAAT motifs were present at -215 and -245 relative to the putative translational start site. A homeobox protein binding consensus site [ATTTAAT-TGA] is present 878 bases upstream from the putative translational start site. Sequences matching the CArG box consensus sequence [CC(A/T)₆(A/G)] are present at -1,031 to -1,021 [CCTATAATAG], -920 to -910 [CCTTTTAAAG], and -159 to -149 [CCT-TATTTAG]. The latter site encompasses the TATA motif. Statistical inference (see "Materials and Methods") indicated that the -807 to -407 region has high similarity to known matrix attachment regions.

Phylogenetic Analyses

Forty-four deduced amino acid sequences belonging to the *DEF/AP3/TM6* and *GLO/PI* groups (Table I) were analyzed by three methods: neighbor-joining (NJ) with Poisson correction, NJ using a Dayhoff-weighted genetic distance matrix, and maximum parsimony (MP; see "Materials and Methods"). All three

Table 1. Genes included in the phylogenetic analyses

Gene	Species	Accession No. or Reference
AP3	<i>Arabidopsis thaliana</i>	A42095
BobAP3	<i>Brassica oleraceae</i> var. botrytis	BOU67456
Boi1AP3	<i>B. oleraceae</i> var. italica	BOU67453
Boi2AP3	<i>B. oleraceae</i> var. italica	BOU67455
CMB2	<i>Dianthis caryophyllus</i>	L40405
CUM26	<i>Cucumis sativus</i>	AAD02250
DeAP3	<i>Dicentra exima</i>	AF052875
DEF	<i>Antirrhinum majus</i>	S12378
GDEF1	<i>Gerbera hybrida</i>	CAA08802
GDEF2	<i>G. hybrida</i>	CAA08803
GGM13	<i>Gnetum gnemon</i>	CAB44459
GLO	<i>A. majus</i>	S28062
HPI2	<i>Hyacinthus orientalis</i>	AF134115
LeAP3	<i>Lycopersicon esculentum</i> cv Celebrity	AF052868
LtAP3	<i>Liriodendrum tulipifera</i>	AF052878
MADS 16	<i>Oryza sativa</i>	AAD19872
MfAP3	<i>Michelia figo</i>	AF052877
NMH7	<i>Medicago sativa</i>	L41727
NTDEF	<i>Nicotiana tabacum</i>	X96428
NTGLO	<i>N. tabacum</i>	X67959
OsMADS2	<i>O. sativa</i>	AAB52709
PcAP3	<i>Papaver californicum</i>	AF052872
PD2	<i>Solanum tuberosum</i>	Garcia-Maroto et al., 1993
PhAP3	<i>Peperomia hirta</i>	AF052879
PI	<i>A. thaliana</i>	D30807
PMADS1	<i>Petunia hybrida</i>	X69946
PnAP3-1	<i>Papaver nudicaule</i>	AF052873
PnAP3-2	<i>P. nudicaule</i>	AF052874
PtAP3-1	<i>Pachysandra terminalis</i>	AF052870
PtAP3-2	<i>P. terminalis</i>	AF052871
PTD	<i>Populus trichocarpa</i>	AAC13695
RAD1	<i>Rumex acetosa</i>	X8913
RAD2	<i>R. acetosa</i>	X89108
RbAP3	<i>Ranunculus bulbosus</i>	AF052876
RbAP3-2	<i>R. bulbosus</i>	AAD31697
RfAP3-1	<i>R. ficaria</i>	AF052854
RfAP3-2	<i>R. ficaria</i>	AF130870
RfPI2	<i>R. ficaria</i>	AAD31700
ScPI	<i>Sanguinaria canadensis</i>	AAD31699
SLM3	<i>Silene latifolia</i>	X80490
STDEF	<i>S. tuberosum</i>	X67511
SvAP3	<i>Syringa vulgaris</i>	AF052869
TAMADS51	<i>Triticum aestivum</i>	AB007506
TM6	<i>L. esculentum</i> cv Tiny Tim	X60759

methods were employed in the analysis of two different data sets: the complete deduced amino acid sequences and a slightly edited data set, for a total of six analyses. We applied a successive approximations approach to character weighting in the MP analysis.

Three major, moderately well-supported groups within the *DEF/AP3/TM6* family were revealed by our phylogenetic analyses: the *TM6* group, the *DEF* group, and the *AP3* group (Fig. 3). In all tests, *PTD* resolved as a member of the *TM6* group containing *GDEF1* from *Gerbera hybrida*, *TM6* from tomato, and *PD2* from potato. The *TM6* group had an 87% boot-

strap value in all MP trees. We used PAUP (Phylogenetic Analysis Using Parsimony, version 4.0b, Sinaur Associates, Sunderland, MA) to further test support of the *PTD/TM6* group by imposing a *PTD/TM6*-constrained tree, then calculating the MP trees and keeping only trees that were not compatible with the constrained tree. Then these trees were compared with the strict consensus unconstrained tree. The results of this test confirmed that the *PTD/TM6* group is well supported: all of the best trees that were not compatible with the *PTD/TM6*-constrained tree were always longer by at least two steps. The amino acid residues present at 15 positions are diagnostic of the *TM6* clade, and five amino acids of the *PaleoAP3* motif described by Kramer et al. (1998) are common among *PTD*, *PD2*, and *TM6*, but not *GDEF1* (Fig. 4).

A second group (*DEF*) with weaker bootstrap support (67%) consisted of *DEF*, *SvAP3*, *LeAP3*, *NTDEF*, *PMADS1*, and *STDEF*. A third, well-supported group (*AP3*; 93%) contained the brassicaceous genes *AP3*, *Boi1AP3*, *BobAP3*, and *Boi2AP3*. Twenty-two additional genes resolved within the *DEF/AP3/TM6* family, but did not associate strongly with any of the three groups just described nor did they form significant relationships with each other except for several strongly associated pairs from species of close taxonomic affinity.

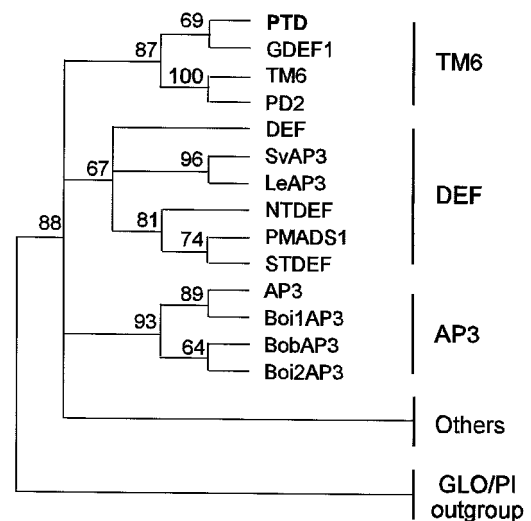


Figure 3. Phylogenetic tree of the *DEF/AP3/TM6* gene family derived from MP analysis. Major groups are indicated at the right. The numbers above the nodes are percentages of bootstrap confidence levels from 1,000 replicates. Nodes with values less than 60% are collapsed. "Others" consists of sequences associated with the *DEF/AP3/TM6* family (bootstrap value 88%) that are not members of any well-defined group within the family. Sequences included in "others" are *CMB2*, *DEAP3*, *GDEF2*, *GGM13*, *LtAP3*, *MADS16*, *MfAP3*, *NMH7*, *PcAP3*, *PhAP3*, *PnAP3-1*, *PnAP3-2*, *PtAP3-1*, *PtAP3-2*, *RAD1*, *RAD2*, *RbAP3*, *RbAP3-2*, *RfAP3-1*, *RfAP3-2*, *SLM3*, and *TaMADS51*. Sequences included in the *GLO/PI* outgroup are *CUM26*, *GLO*, *HPI2*, *NTGLO*, *OsMADS2*, *PI*, *RfPI-2*, and *ScPI*.

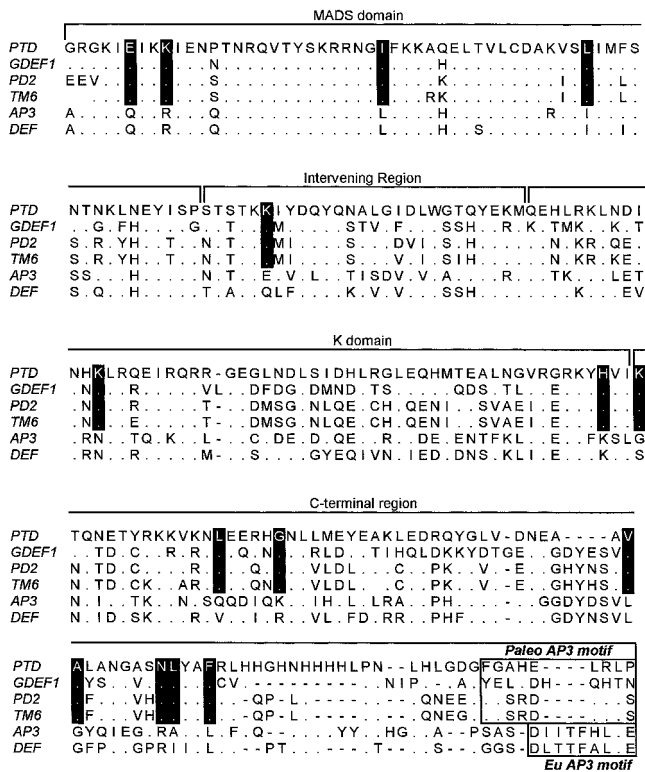


Figure 4. Alignment of the deduced amino acid sequence of *PTD* with *GDEF1*, *PD2*, *TM6*, *AP3*, and *DEF*. Fifteen residues diagnostic of the *TM6* group are shown with a black background. The Paleo AP3 and Eu AP3 motifs (Kramer et al., 1998) are boxed. Dots indicate identical residues. Dashes represent gaps introduced to maximize the alignment.

RNA Gel Blot

Inflorescences were collected from wild black cottonwood trees at two stages of maturity: in late spring when the inflorescences that will bear mature flowers the following year are developing, and in early spring when the flowers are nearly mature just prior to anthesis. Black cottonwood flowers mature acropetally on inflorescences, thus the early inflorescences bore flowers in various stages of development: the least mature "flowers" were cells on the flanks of the inflorescence meristem that would develop into floral meristems; the most mature flowers (at the proximal end of the inflorescence) had carpel or stamen primordia emerging from the floral meristem. A 1.1-kb *PTD* transcript was present in developing and mature inflorescence buds of both sexes, but not in vegetative buds or roots (Fig. 5). Quantitation of PhosphorImager scans (see "Materials and Methods") showed that the signal intensity in early male inflorescences is approximately four times greater than that of early female inflorescences. In mature inflorescences, the signal from male inflorescences was approximately twice that of female inflorescences.

In Situ Hybridization Analyses

We used in situ hybridization analysis to determine the cell-specific expression patterns of *PTD* in developing and mature inflorescences of both sexes. Black cottonwood tissue sections were probed with a fragment from the non-conserved 3' region of the *PTD* cDNA. In developing male inflorescences, *PTD* expression was strong at its onset, which occurred well after the floral meristem had differentiated from the inflorescence meristem, as the perianth cup differentiated, but prior to any indications of stamen organogenesis (Fig. 6, A–C). At this stage, *PTD* signal was present across the entire male floral meristem excluding the perianth cup (Fig. 6, D and E). In slightly more mature male flowers with developing stamens, hybridization signal was visible in the stamens, but appeared more diffuse (Fig. 6F). In mature male flowers a strong hybridization signal was present in the sporophytic tissues of the stamen, particularly the tapetum and filament (Fig. 6, G and H). No signal was detected in the perianth cup, pedicel, bracts, or peduncle of either sex at any stage of development.

In contrast to male inflorescences, female inflorescences first showed a weak hybridization signal in the cells on the flanks of the inflorescence meristem that would develop into floral meristems (Fig. 6I). A weak signal was present in the earliest floral meristems and became stronger as the perianth cup differentiated (Fig. 6, J and K). As carpel organogenesis began, *PTD* mRNA became excluded from the cells differentiating into carpel primordia (Fig. 6L). Later,

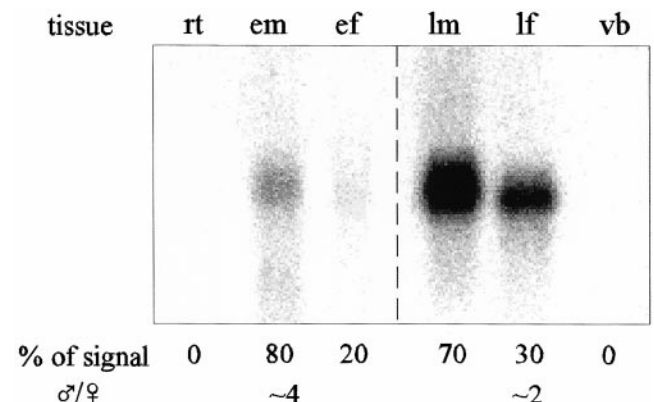


Figure 5. *PTD* mRNA expression in various tissues. The RNA gel blot was probed with the *NdeI/XhoI* fragment of *PTD* and visualized using a phosphorimager as described in "Materials and Methods." The first three lanes each contained 30 μ g of total RNA. The last three lanes each contained 2 μ g of poly(A)⁺ RNA. Values in the first row are the percentage of total signal contributed by each tissue after correction following ribosomal RNA hybridization (see "Materials and Methods"). Values in the final row are the proportion of signal from male inflorescences as compared with female inflorescences at the same stage of maturity. rt, Root; em, early male inflorescence; ef, early female inflorescence; lm, late male inflorescence; lf, late female inflorescence; vb, vegetative bud.

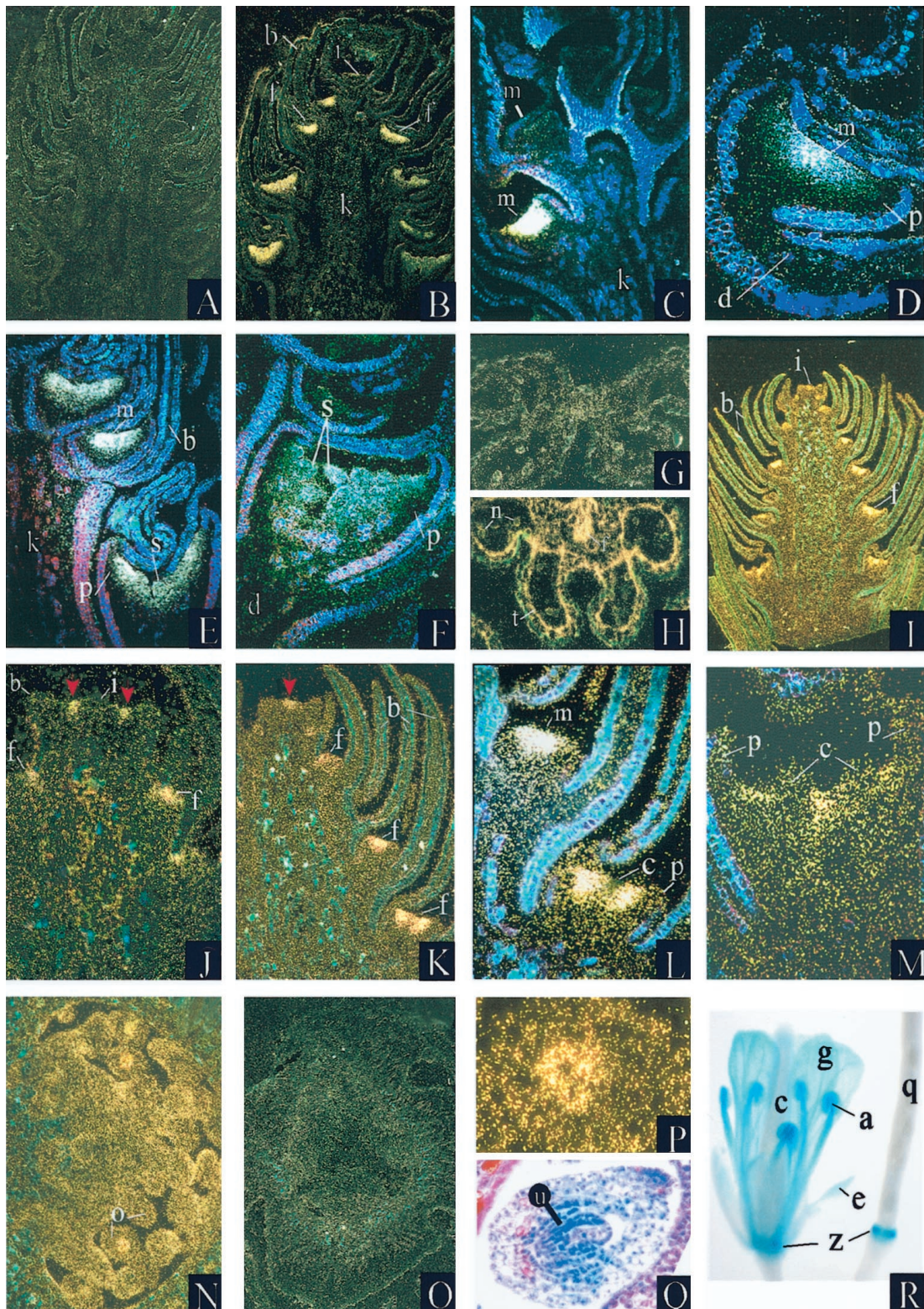


Figure 6. Analysis of *PTD* expression. A through Q, In situ hybridization analysis of *PTD* expression in black cottonwood. R, Expression pattern of *pPTD:GUS1* in transgenic *Arabidopsis*. A, G, and O were hybridized with sense control probe; B through F, H, I through N, and P were hybridized with antisense *PTD* probe. G and H depict cross sections. All others are longitudinal sections. White and gold dots indicate areas of *PTD* hybridization. Images are digitized. A and B, Developing male inflorescences. Developing flowers along the inflorescence represent a gradient of maturational stages with the most (Legend continues on facing page.)

when carpel primordia emerged, hybridization occurred in the areas of the floral meristem between and around the carpel primordia, but was excluded from the carpel primordia (Fig. 6M). In mature female flowers, hybridization signal was present in the nucellus of the ovules (Fig. 6, N–Q). No expression was detected in vegetative buds and seedlings of either sex (not shown).

Histochemical Analysis of *pPTD:GUS1* Expression in Transgenic Arabidopsis

pPTD:GUS1 was generated by fusing approximately 1.9 kb of sequence upstream of the *PTD* coding region, including 30 bp of 5'-untranslated sequence, with the *uidA* reporter gene. T1 seeds of transformed Arabidopsis were selected on kanamycin-containing medium and grown to maturity under 16-h days. Leaf, root, pedicel, and floral tissues of 10 independent lines were evaluated for β -glucuronidase (*GUS*) activity by histochemical analysis. Visible *GUS* activity was detected only in floral tissues, even when entire plants were cleared and stained (Fig. 6R). In flowers, strong expression was observed in the petals and stamens. Two lines displayed expression in sepals; however, it was weak. Staining was typically stronger in stamens than in petals, and strongest in the anthers. In older flowers, expression was maintained in a discrete band at the base of the developing silique, probably corresponding to the floral abscission zone.

DISCUSSION

PTD Is a Member of the *TM6* Subgroup

We performed phylogenetic analysis of *PTD* along with 31 other members of the *DEF/AP3/TM6* family and eight members of the *GLO/PI* family. Our analyses indicate that *PTD* belongs to a subgroup of the *DEF/AP3/TM6* family that includes the largely uncharacterized genes *TM6*, *PD2*, and *GDEF1* (Fig. 3). The four genes formed a well-supported clade (87%) in all of our MP trees. Placement of *PTD* within the *TM6* group was further supported by PAUP analysis. Black cottonwood is placed in the rosoid clade (Soltis

et al., 1999) thus *PTD* is the first nonasteroid gene in the *TM6* subgroup to be described.

Two other groups were resolved: one (*DEF*) comprised largely of genes from the *Solanaceae*; the other (*AP3*) comprised of four *Brassicaceae* genes. Twenty-two other genes were clearly members of the *DEF/AP3/TM6* family, but did not form strong relationships with the *TM6*, *AP3*, or *DEF* subgroups nor with each other. The existence of two paralogous lineages within the *DEF/AP3/TM6* family, one containing *DEF* and *AP3* and their orthologs, the other containing *TM6* and its orthologs, has been suggested (Doyle, 1994; Kramer et al., 1998). It is possible that two *DEF*-like genes are present in black cottonwood and that *PTD* is orthologous to *PD2*, *TM6*, and *GDEF1*, whereas an as yet unidentified gene is orthologous to *DEF* and *AP3*. However, the results of our RNA gel-blot and in situ hybridization studies indicate that although *PTD* is a member of the *TM6* group, its expression pattern is unlike that of *GDEF1* and *TM6*, and is more similar to that of *DEF* and *AP3*. Whereas *GDEF1* is expressed primarily in leaves, bracts, and scapes (Yu et al., 1999), *PTD* is not expressed in vegetative tissues. In addition, although *PTD* is expressed early in floral development (discussed above), expression of *GDEF1* is not detectable at this early stage. Although *GDEF1* is expressed only weakly in the corolla and stamens of mature flowers, *PTD*, like *DEF* (Schwarz-Sommer et al., 1992) and *AP3* (Jack et al., 1992), is strongly expressed in the portion of the floral meristem that gives rise to stamen primordia.

In the hermaphroditic floral meristems of snapdragon and Arabidopsis, this area excludes the central fourth whorl where carpel primordia will develop, whereas in male black cottonwood it encompasses the entire floral meristem excluding the perianth cup. In mature stamens, *PTD* expression, like *DEF*, is localized to the tapetum and filament. In developing carpels, *PTD* expression, like *AP3* and *DEF*, is absent or low, whereas *TM6* expression is high (Pneuli et al., 1991). *PTD* and *AP3* are expressed in ovules (Jack et al., 1992). Indeed, the expression patterns of all three members of the *TM6* phylogenetic group that have been studied differ from each other significantly. Thus

Figure 6. (Legend continued from facing page.)

mature flowers at the proximal end (lower portion of photograph); C, distal (i.e. upper) portion of male inflorescence. Four floral meristems are visible; D, developing male flower; E, medial portion of male inflorescence showing three developing flowers; F, developing male flower with stamens beginning to differentiate. Expression is evident in the stamen primordia; G and H, mature anther; I, developing female inflorescence; J, female inflorescence meristem and the two distal-most developing flowers. The area of expression in the cells at the flanks of the inflorescence meristem is perpendicular to the plane of the section and is indicated by arrows; K, distal portion of female inflorescence. The area of hybridization in the inflorescence meristem is parallel to the plane of the section and is indicated by an arrow; L, medial portion of developing female inflorescence; M, female flower with developing carpels; N and O, ovary of mature female flower; P, mature ovule. Hybridization signal is concentrated in the nucellus; Q, mature ovule, brightfield micrograph. R, Arabidopsis mature flower (left) and developing silique (right). a, Anther; b, bract; c, carpel; d, pedicel; e, sepal; f, flower; g, petal; i, inflorescence meristem; k, peduncle; l, filament; m, floral meristem; n, pollen; o, ovule; p, perianth cup; q, developing silique; s, developing stamens; t, tapetum; u, nucellus; z, floral abscission zone.

although they belong to the same phylogenetic group, it is doubtful that members of the *TM6* subfamily are orthologs with similar functions. Given the similar expression patterns of *PTD*, *DEF*, and *AP3*, it seems more likely that *PTD* performs a function similar to that of *DEF* and *AP3*.

The Temporal and Spatial Expression Patterns of *PTD* Are Sex Specific

Black cottonwood is well suited to the study of sex-specific developmental programs. In black cottonwood, no morphological indications of carpel initiation occur in male flowers; stamen primordia first appear in the center of the floral meristem and their emergence proceeds centrifugally (Boes and Strauss, 1994; Kaul, 1995). Likewise, no morphological indications of stamen initiation are present in developing female flowers. The unisexual nature of black cottonwood floral meristems allows sex-specific differences in gene expression patterns to be discerned early in floral development.

The temporal expression pattern of *PTD* in male black cottonwood is similar to that observed in *DEF* and *AP3*: expression is not detected until after the floral meristem has formed and the initiation of sepal (or perianth cup, in black cottonwood) primordia have occurred. However in black cottonwood females, *PTD* expression is first detectable earlier in cells on the flanks of the inflorescence meristem. The hybridization signal in female black cottonwood became stronger as the floral meristem expanded, then more diffuse as carpel primordia developed. The area of expression initially encompassed the entire floral meristem exclusive of the perianth cup, but later became excluded from those cells that were differentiating into carpel primordia. When carpel primordia developed, *PTD* was excluded from them, yet remained present in the cells of the meristem that surrounded them. The presence of *PTD* mRNA in stamen primordia and the lack of *PTD* expression in carpel primordia are consistent with the expression pattern predicted for B-function genes by the ABC model. In several hermaphroditic species, one B-class gene is initially expressed in the whorl that will form carpels. In *Arabidopsis*, *PI* is initially expressed in the fourth whorl, whereas in snapdragon and the Solanaceae, *DEF* or *DEF* orthologs are initially expressed (for review, see Irish, 1999). Studies indicate that *PI* or *DEF* expression is not maintained in the fourth whorl because the second B-function gene (*AP3* or *GLO*) is not expressed in this whorl, and both gene activities are needed for maintenance of expression. The similarity of *PTD*'s expression pattern suggests the possibility that an analogous B-class gene autoregulatory mechanism may prevent the maintenance of *PTD* expression in developing carpels. Isolation and analysis of additional poplar B-class gene homologs would indicate if this is the case.

The early onset of *PTD* expression in female versus male inflorescence meristems and the initially high intensity of expression in developing female floral meristems suggest that *PTD* may play a role in female floral organogenesis, perhaps in cell proliferation. In snapdragon, the initiation of fourth whorl organogenesis in L1 chimeras indicated that *DEF* activity in epidermal cells promotes cell proliferation in the center of the meristem (Perbal et al., 1996). These studies also suggested that a high level of *DEF* expression in the L1 cell layer allows the growth and expansion of petal lobes by stimulating L1 cell division and/or cell shape and elongation. In a similar manner in the *Arabidopsis* floral meristem, *AP3* and *PI* appear to have a role in cellular proliferation. Ectopic expression of *AP3* and *PI* results in the production of extra whorls of stamens, and ectopic expression of *AP3/PI* rescues missing second whorl organs in class A mutants (Krizek and Meyerowitz, 1996). In petunia, ectopic expression of the *DEF* homolog *PMADS1* causes delayed petal fusion and additional lateral growth of petal tissues, suggesting that *PMADS1* might be involved in stimulation of directed cell proliferation (Halfter et al., 1994). Alternatively, *PTD* may have no function in the black cottonwood female inflorescence; *PMADS1* is present in the third whorl of petunia flowers, yet plays no discernable role there (van der Krol et al., 1993; Angenent et al., 1995).

We observed no consistent differences between the banding patterns of DNA isolated from male and female trees on our genomic blots, thus it seems unlikely that *PTD* is directly involved in sex determination. However it is possible that sex-specific regulation of *PTD* expression is involved in sex determination as a consequence of the action of upstream sex determination genes.

Evidence indicates that the perianth cup of black cottonwood is derived from a fusion of perianth parts (Fisher, 1928; Stoehr et al., 1988; Kaul, 1995), however, it remains unclear which particular parts were incorporated into the structure. If we assume that the perianth cup is derived from fused sepals, then the lack of *PTD* expression in the cup is consistent with the predicted expression patterns of B-function genes such as *DEF* and *AP3*. On the other hand, it is possible that the perianth cup is derived from fused petals, or from adnation of petals and sepals, and that the sepal-like nature of the black cottonwood perianth results, at least in part, from the evolutionary loss of *PTD* expression in that organ. Experiments to determine the exact function of *PTD* by suppression of *PTD* expression in transgenic cottonwood trees are under way.

The *PTD* Promoter Contains Consensus Sites for Binding Regulatory Proteins

MADS box gene products are believed to regulate gene expression by binding to sites present in the

promoters of target genes (Riechmann et al., 1996). These binding sites, known as CARG boxes, are found in the promoter regions of plant MADS box genes, including *DEF* (Schwarz-Sommer et al., 1990; Tröbner et al., 1992) and *AP3* (Riechmann et al., 1996), and are thought to be targets for autoregulation. The *AP3* promoter contains three CARG boxes that regulate different aspects of *AP3* expression (Hill et al., 1998; Tilly et al., 1998). We found three sites in the *PTD* promoter region that match the CARG box consensus motif. One site encompasses the TATA box; the other two are approximately 1 kb upstream from the initial ATG. When a construct consisting of the *PTD* 5'-flanking region fused with the *uidA* coding region was introduced into Arabidopsis, we observed *GUS* expression in the organs where *AP3* is normally expressed, namely petals and stamens. This conserved B-function expression pattern suggests that the transcription factors responsible for regulation of *AP3* expression in Arabidopsis are able to bind to sites present in the *PTD* promoter region and supports the hypothesis that *PTD* performs a function similar to that of *AP3*.

The *PTD* gene promoter also contains a motif that exactly matches the consensus site for binding homeodomain proteins. Homeodomain proteins are known to be expressed in inflorescence and floral meristems (Jackson et al., 1994) and in ovules (Reiser et al., 1995), and are involved in the regulation of floral homeotic MADS box gene expression (Ray et al., 1994). The interaction of homeodomain and MADS domain transcription factors has been reported in animals (for review, see Duprey and Lesens, 1994). It is possible that a homeodomain protein binds to the consensus site present in the *PTD* promoter and is involved in regulating *PTD* gene expression. Consensus sites for binding homeodomain proteins are not present in the promoters of *DEF*, *AP3*, or the three *ST-DEF* promoters.

The *PTD* Promoter Should Be Useful for Engineering Reproductive Sterility in Trees

Genetic engineering shows potential for the improvement of qualitative and quantitative traits in trees (Tzfira et al., 1998). Transgenic cottonwoods with engineered insect and herbicide resistance have been produced (Meilan et al., 2000). However, there is a high potential for escape of transgenes in trees into wild populations because of their long distance movement of seeds and pollen and their ubiquitous wild relatives. Genetically engineered sterility has been proposed as the best method for transgene containment in trees (Strauss et al., 1995). Fusion of reproductive tissue-specific promoters with coding regions from genes that encode ablatable proteins has been used to engineer male and female sterility in a variety of agronomic and model plants (e.g. Mariani et al., 1990; Goldman et al., 1994). If deleterious ef-

fects on vegetative growth are to be avoided, this approach requires a promoter that limits expression specifically to reproductive tissues. Because *PTD* is expressed early in the floral meristems of male and female trees and does not appear to be expressed in vegetative buds, seedlings, or roots, its promoter should be useful for engineering of the complete male and female sterility desirable for gene containment. Studies to test the effectiveness of this approach in transgenic trees are under way.

MATERIALS AND METHODS

Plant Materials

Plant materials were collected from wild black cottonwood (*Populus trichocarpa*) trees near Corvallis, Oregon. Cut branches were partially submerged in aerated water to produce the roots used in the RNA gel blot. Seedlings used for in situ hybridization experiments were sprouted in potting soil in the greenhouse.

Nucleic Acid Isolation

RNA was isolated using the method of Hughes and Galau (1988) with the following modifications: Tissues were ground in liquid nitrogen in a blender then homogenized in homogenization buffer using a polytron (Brinkman Instruments, Westbury, NY); after thawing the homogenates, 0.5 volume of 5 M potassium acetate, pH 6.5, was added; the phenol/chloroform extraction was performed prior to the LiCl precipitation.

Poly(A)⁺ RNA was isolated using oligo(dT) spin columns (CLONTECH, Palo Alto, CA). Genomic DNA was isolated from vegetative buds using the CTAB method of Wagner et al. (1987).

cDNA and Genomic Clone Isolation

Floral buds were collected from a native female black cottonwood tree in late May. The bud scales were removed and poly(A)⁺ RNA isolated from the developing inflorescences was used to construct a cDNA library in λ Zap (Stratagene, La Jolla, CA). The *PTD* cDNA was obtained by probing 1 × 10⁶ clones of the amplified library at low stringency with an *Eco*RI fragment of pCIT2241 that contained the highly conserved MADS box region of *AGL1* (Ma et al., 1991). Plasmids containing putative MADS box cDNA clones were excised from λ ZAP as suggested by the supplier.

Construction of the black cottonwood genomic library has been described (Rottmann et al., 2000). Isolation of the *PTD* genomic clone was accomplished by screening 6 × 10⁵ genomic clones with a *BSU361/Xho*I restriction fragment that contained the 3' portion of the *PTD* cDNA, but not the highly conserved MADS box region.

DNA Sequencing and Analysis

Sequencing was performed by the Oregon State University Central Services Laboratory using fluorescent primer

dye/dideoxy chain termination and an automated DNA sequencer (ABI, Sunnyvale, CA). Both strands of the templates were sequenced. Sequence analysis was conducted using the database of motifs in the GeneRunner version 3.0 program (Hastings Software, Hastings, NY) supplemented with the CARG box consensus sequence [CC(^A/_T)₆^A/_GG]. The location of a putative matrix attachment region was statistically inferred using the computer program MAR-Finder (Kramer et al., 1997).

Sequence Alignment and Phylogenetic Analyses

The sequence of *PD2* was obtained from Garcia-Maroto et al. (1993). All other sequences were obtained from GenBank (Table I). Alignment of protein sequences was accomplished using the GeneDoc software program (Nicholas and Nicholas, 1997). *GLO* and its homologs were chosen as outgroup (least related) sequences because as members of the *DEF* sister-clade (Doyle, 1994) their sequences were similar enough to allow alignment within the minimally conserved C-terminal region.

A total of six phylogenetic trees were derived from two different data sets using three different methods. One data set employed the complete deduced amino acid sequences of all genes. For the other, sequences were edited to exclude those residues where corresponding positions in all other sequences were gaps. The NJ method (Saitou and Nei, 1987) of the MEGA computer program (Molecular Evolutionary Genetic Analysis, version 1.0, Pennsylvania State University, University Park) was used for heuristic distance-based phylogenetic analyses of both data sets. Poisson distribution-correction distance was employed to estimate the number of amino acid substitutions per site. Two additional distance-based trees were constructed, based on the weighted genetic distance matrix computed from deduced protein sequences using the PROTDIST program in the PHYLIP software package (Phylogeny Inference Package, version 3.57, Department of Genetics, University of Washington, Seattle), where amino acid substitutions were scaled using Dayhoff's PAM 001 empirical matrix of mutation rates (Dayhoff et al., 1978). Consensus trees and estimates of statistical confidence were inferred from 1,000 bootstrapped (randomly sampled with replacement) data sets.

Parsimony analysis (derivation of a phylogenetic tree requiring the smallest number of evolutionary changes) was performed using the PROTPARS program in the PHYLIP package (Felsenstein, 1995). The SEQBOOT program was used to generate 1,000 data sets. Majority-rule and strict consensus trees were generated using CONSENSE. The computer program PAUP was used to apply a successive approximations approach to character weighting in the MP analysis (Farris, 1969). To begin, a heuristic search with 10 randomizations of sequence input order was used to find the most parsimonious trees. A strict consensus tree was calculated from the equally most parsimonious trees obtained in the search. This tree was used in successive approximations weighting with the characters reweighted by maximum value of rescaled consistency indices in consecutive successive approximations weighting runs until identical trees

were found in two consecutive searches. We used PAUP to test support of the *PTD/TM6* group by imposing the *PTD/TM6*-constrained tree, then calculating the maximum parsimony trees and keeping only those trees that were not compatible with the constrained tree. These trees were then compared with the strict consensus unconstrained tree.

DNA and RNA Gel Blots

For the RNA gel blot, 2 μ g of poly(A)⁺ RNA from vegetative buds and mature male and female catkins of black cottonwood collected in February, or 30 μ g of total RNA from roots and from developing male and female floral buds collected in May, were applied to a formaldehyde agarose gel, subjected to electrophoresis, blotted onto a nitrocellulose membrane, hybridized, and washed at high stringency (0.1 \times SSC; 1% [w/v] SDS, 69°C). For the DNA gel blot, 10 μ g of black cottonwood genomic DNA were digested with restriction enzymes, blotted onto a nylon membrane, hybridized, and washed according to established procedures (Sambrook et al., 1989). The blots were probed with a 595-bp *NdeI/XhoI* fragment that contained only the 3' portion of the *PTD*-coding region to avoid cross-hybridization with other MADS box sequences present in the black cottonwood genome. The RNA gel blot was stripped and reprobed with 18S rDNA from *P. deltoides* (D'Ovidio, 1992) as control. Both RNA and DNA gel blots were exposed to a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) plate and digitized using a SI PhosphorImager (Molecular Dynamics). The values in Figure 5 were obtained by quantifying signals on digitized images of an RNA gel blot probed sequentially with *PTD* and an 18S rDNA from *P. deltoides* (D'Ovidio, 1992) as a control using ImageQuant software (version 4.2, Molecular Dynamics), correcting for background, and adjusting for variation on the assumptions that rRNA was present at equal amounts in all tissues and that the oligo(dT) columns removed equal proportions of rRNA from the three poly(A)⁺ samples.

In Situ Hybridizations

In situ hybridizations were performed as described by Kelly et al. (1995) with the following modifications. Eight- to 10- μ m sections were probed with either antisense *PTD* cRNA transcribed using the T7 promoter in the pBlueScript vector or with sense (control) *PTD* cRNA transcribed from the T3 promoter. The antisense cDNA template was digested with *NdeI* at a site 380 bases from the 5' end to terminate transcription within the K box, yielding a probe that lacked the highly conserved MADS box region. Control sections were probed with a sense transcript generated from a T3 promoter flanking the 5' end of the cDNA. The sense template was digested with *XhoI* directly at the 5' end of the cDNA (thus the control probe included the entire *PTD* cDNA sequence). Probe was applied at a concentration of 10 to 30 ng/mL of hybridization solution; higher probe concentrations resulted in unacceptable levels of non-specific hybridization. Silver grains were visualized with a Axioskop (Zeiss, Jena, Germany) microscope

equipped with a darklight illuminator and/or a darkfield stop in the substage condenser. Tissue sections were photographed with a camera (Contax, UK) mounted on the microscope using slide film (Sensia ISO 100, Fuji Phot Film, Tokyo). The ovule depicted in the brightfield micrograph was stained with hematoxylin and eosin. The images in Figures 1 and 6 were digitized using a Microtek Scanmaker III. Images were adjusted to match the original slides and prints using Adobe Photoshop 4.0.1 (Adobe Systems Inc., Mountain View, CA, <http://www.adobe.com>) and composites were assembled using Presentations 3 (Corel, Ottawa, ON, Canada).

pPTD:GUS1 Construction and Analysis of Expression in Arabidopsis

pPTD:GUS1 was constructed by subcloning a approximately 1.9-kb *HindIII*/*AvrII* fragment of the *PTD* gene upstream region into *HindIII*/*XbaI*-digested *pBI101* (CLONTECH) that had been modified by replacing the existing *uidA* gene with an intron-containing version of *uidA* (Vancanneyt et al., 1990). The *AvrIII* site, which constituted the 3'-terminus of the subcloned *PTD* fragment, occurred approximately 90 bp upstream of the presumed translational start site. Transformation of Arabidopsis ecotype Columbia with the *pPTD:GUS1* fusion construct as well as selection and growth of transgenic plants were performed as previously described (Rottmann et al., 2000). Histochemical GUS staining was accomplished as described by Jefferson et al. (1987). Digital images of stained tissues were produced using a digital documentation system (UVP Inc., Upland, CA).

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