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HIGH LEVEL OF α-DIOXYGENASE IN SHORT STYLES OF DISTYLOUS *TURNERA* SPECIES

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To identify proteins responsible for self-incompatibility and/or other dimorphisms in distylous species of *Turnera*, we used SDS-polyacrylamide gel electrophoresis to compare protein profiles of long and short styles. We detected a prominent 68-kD protein in styles of short- but not long-styled plants. This result was consistent in all five of the distylous species examined, except in a more divergent species, *Piriqueta caroliniana*, in which short styles did not express the protein but long styles may exhibit a low level of expression. Sequencing of tryptic peptides, genomic DNA, and phylogenetic analyses revealed the 68-kD protein to be an α-dioxygenase. Immunoblotting using antiserum against a pathogen-induced α-dioxygenase from tobacco further confirmed that the 68-kD protein is an α-dioxygenase. Using immunoblotting, we could not detect the α-dioxygenase in other floral or vegetative organs, in styles of homostylous species, or in three mutants. The α-dioxygenase reaches detectable levels in short styles 1 d before anthesis and high levels at flowering. Immunocytochemical analysis localized the α-dioxygenase to the transmitting tissue of short styles in all three species examined. An α-dioxygenase assay using crude style extracts revealed twice the activity for short compared with long styles. The tissue-specific and temporal patterns of expression, as well as its consistent appearance in short-styled plants of all *Turnera* species examined, indicates that the α-dioxygenase plays some as yet unspecified role in distyly.

Keywords: distyly, *Turnera*, α-dioxygenase.

Introduction

Antiselfing mechanisms in the angiosperms range from spatial separation of male and female reproductive organs, e.g., herkogamy, to interactions between pollen and pistil cells resulting in the cessation of growth of self pollen (Herrero and Hormaza 1996). This latter selective rejection of self-pollen is referred to as self-incompatibility (SI). It is the most effective system in promoting outbreeding in hermaphroditic plants and results in the maintenance of heterozygosity in natural populations (de Nettancourt 1977; Rudd and Franklin-Tong 2003). While considerable progress has been made on the mechanisms of some SI systems, little is known of the mechanisms involved in distyly.

A majority of species in the genus *Turnera* are distylous, having two genetically determined mating types in populations, the long- and short-styled morphs (Urban 1883; Shore and Barrett 1985; Barrett and Shore 1987). The morphs differ in the lengths of styles and stamens and have a reciprocal positioning of these organs. Compatible pollinations involve crosses between morphs (Barrett and Cruzan 1994). Flowers of the morphs may possess other dimorphic traits, including pollen size, pollen production, and stigma morphology (Shore and Barrett 1986; Dulberger 1992).

The SI system and all the floral dimorphisms in distylous species commonly appear to be controlled by a single Mendelian locus with two alleles. In *Turnera scabra* and *Turnera subulata*, short-styled plants are heterozygous (*Ss*), and long-styled plants are homozygous recessive (*ss*) (Shore and Barrett 1985). Studies of *Primula* species have provided evidence that distyly is controlled by a series of tightly linked genes forming a gene complex, or supergene, that is rarely disturbed by crossing over (Ernst 1955; de Nettancourt 1977; Lewis and Jones 1992). Lloyd and Webb (1992) suggest that the suite of characters distinguishing the distylous morphs may result from a mix of morph-linked genes as well as genes that are not linked but show morph-limited expression. They also suggest that the mix of morph-linked and morph-limited genes might vary among species.

At present there is no conclusive evidence for a gene complex determining distyly in *Turnera* species. The occurrence of homostylous *Turnera* species (self-compatible species having anthers and stigmas in close proximity), their compatibility behavior in crosses with long- and short-styled plants, and the inheritance of homostyly is consistent with the hypothesis that a gene complex might underlie the genetic basis of distyly (Shore and Barrett 1985; Barrett and Shore 1987;

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Tamari et al. 2001). We have recently investigated the inheritance of two homostylous mutants in *T. scabra* and in a synthetic hybrid, *T. subulata* × *Turnera krapovickasii* (Tamari et al. 2004). The results are consistent with the gene complex model for *Primula* but cannot be distinguished from a model where the homostyle phenotype is the result of a mutation to a new allele at the distyly locus. Interestingly, Athanasiou et al. (2003) have shown that a short-specific polygalacturonase is not a component of a distyly gene complex and exhibits morph-limited expression. Taken together, these results indicate that distyly in *Turnera* species may well be determined by a mix of genes that are morph linked and others that show morph-limited expression.

To date, little molecular evidence is available to account for SI and the floral dimorphisms of distylous species (de Nettancourt 1997). Investigations have been initiated to detect proteins specific to either long- or short-styled plants (Golynskaya et al. 1976; Shivanna et al. 1981; Stevens and Murray 1982; Wong et al. 1994); however, little progress has been made. In distylous *T. subulata* and other species in series Turnera (Turneraceae), a 35-kD protein identified as polygalacturonase (PG) based on sequence homology was localized to the transmitting tissue of short styles and stigmas (Athanasiou et al. 2003). The PG first appears in short styles 3 d before flowering. The protein is absent from self-compatible homostylous species and homostylous mutants (Khosravi et al. 2003; Tamari and Shore 2004).

The role of the style PG in distyly in *Turnera* is unknown at present. PGs are generally involved in hydrolyzing pectins, a class of complex polysaccharides in plant cell walls and the middle lamella (Hong et al. 2000). PGs are associated with growth and development, and their roles in fruit ripening, organ abscission, pod dehiscence, and pollen maturation have been reasonably well established (Hadfield and Bennett 1998). In addition, PGs may be involved in signaling plant defense responses to wounding by herbivores and attack by pathogens and parasites (Bergey et al. 1999; Hong et al. 2000; Dal Degan et al. 2001; Hermsmeier et al. 2001; Orozco-Cárdenas et al. 2001). Interestingly, the short-style-specific PG discovered by Athanasiou et al. (2003) falls into a clade with a PG that is induced in *Glycine max* in response to nematode attack (Mahalingam et al. 1999).

Digestion of pectins from plant primary cell walls by PGs yields biologically active oligogalacturonides (OGAs) (Fry et al. 1993; Ridley et al. 2001). OGAs act as signal molecules, and a wide array of biological responses to OGAs have been documented (Ridley et al. 2001). These responses have been categorized into those involved in plant defense and those involved in plant growth and development. OGAs influence plant growth via inhibition of auxin-stimulated growth (Branca et al. 1988; Fry et al. 1993; Ridley et al. 2001). In plant defense, the initial response to OGAs is the production of reactive oxygen species, which may play a role in plant cell death resulting from the hypersensitive reaction (HR). Reactive oxygen species and OGAs are believed to induce the expression of defense-related genes (Côté and Hahn 1994; Ridley et al. 2001).

The single, short-style-specific PG in *Turnera* species (Athanasiou et al. 2003) is insufficient to account for SI of both the long- and short-styled morphs as well as the other dimorphic traits. Its occurrence in only the short-styled morph raises the possibility that the mechanisms of SI differ among the morphs (Athanasiou et al. 2003). By exploiting pollen size variation between two populations of *T. scabra*, Tamari et al. (2001) used control pollinations and observations of pollen tubes in styles to reject the hypothesis that insufficient pollen-tube nutrition is responsible for SI of the long-styled morph. They speculate that there must be a protein (or proteins) specific to long-styled plants that is involved in the SI response.

To detect proteins involved in distyly, we have initiated a proteomics approach. Total proteins from both long and short styles will be separated on one- and two-dimensional polyacrylamide gels, and their profiles will be compared. Proteins that are specific to one morph will be identified to aid in discovering their role in SI or other floral dimorphisms. We expect such proteins to be conserved within the Turneraceae because distyly would probably have had a single origin within this family. Indeed, SI proteins identified from three well-studied systems in the Brassicaceae, Papaveraceae, and Solanaceae are conserved within each family (McCubbin and Kao 1999, 2000; Igic and Kohn 2001; Nasrallah et al. 2002; Kemp and Doughty 2003; Wheeler et al. 2003). The mechanisms of SI, however, differ among these families (Nasrallah et al. 1985; Anderson et al. 1986; Foote et al. 1994; Igic and Kohn 2001).

Here we identify a 68-kD protein expressed to a high level in short styles of five distylous *Turnera* species. We show that the protein is an α-dioxygenase based on peptide and genomic sequence data as well as immunoblotting and immunocytochemical studies. Temporal and tissue localization experiments reveal that the protein first appears 1 d before anthesis and is restricted to the style and stigma transmitting tissue. The putative α-dioxygenase was not detected in self-compatible homostylous species or in three mutants. An α-dioxygenase assay revealed that oxygen consumption was almost twice as high in short styles compared to long styles. The gene encoding the α-dioxygenase is not closely linked to the distyly locus.

Material and Methods

Plants

The species used are listed in tables 1 and 2, and further details of the localities may be found in Tamari et al. (2001). With one exception, all the species investigated are in series Turnera (=Canaligerae; Urban 1883). *Piriqueta caroliniana* belongs to a different genus within the Turneraceae. Plants were grown in a greenhouse, and floral materials were obtained within a few hours after anthesis unless indicated otherwise. Pollinations were conducted a short time after anthesis using a pair of fine forceps.

Three mutants were included in this investigation as follows. (1) A somatic mutant homostyle (Mhomo-H) arose on a single branch of a short-styled plant (Mhomo-S). The mutant is self-compatible and has long styles and long stamens. The nonmutant short-styled plant was generated from a cross between two diploid distylous species (*Turnera subulata* × *Turnera krapovickasii*). The inheritance of the mutation and the

Table 1

Distribution of the Style α-Dioxygenase (S68) in Distylous Species Using SDS-PAGE and Coomassie Blue Staining

	Short		Long	
Species	No. with S68	*N*	No. with S68	*N*
Turnera grandiflora (Gran)	9	9	0	8
Turnera joelii (Joli)	7	7	0	6
Turnera krapovickasii (Krpo)	10	10	0	10
Turnera scabra (Cosr)	3	3	0	3
T. scabra (Mrgd)	4	4	0	4
T. scabra (Nica)	3	3	0	4
T. scabra (Dor4)	7	7	0	10
T. scabra (Dor7)	5	5	0	5
Turnera subulata (Subu)[a]	10	10	0	10
T. subulata (E)	6	6	0	5
Piriqueta caroliniana (Pird)	0	5	0	7

Note. Species (code), number of plants with the style α-dioxygenase (S68), and sample sizes (*N*) are provided for long- and short-styled plants.

[a] Selfed progeny of a single plant termed BRY (Athanasiou and Shore 1997).

compatibility behavior of the mutant has been determined by Tamari et al. (2004). (2) Two x-ray-generated mutants of diploid *T. subulata*, one of which is homostylous and the other long styled, were produced. The mutants were generated by irradiating pollen of a short-styled plant with ca. 45 Gy and then pollinating a long-styled plant. The pollen parent was homozygous *SS* at the distyly locus and at two isozyme marker loci (*Aco-1* and *Pgd-c*) that lie on either side of the distyly locus (Athanasiou and Shore 1997). The long-styled (ovule) parent was homozygous *ss* at the distyly locus and homozygous for alternative alleles at the two marker loci. Over 1000 progeny were screened for floral morphology. Two mutants were obtained (one homostylous, one long styled) and verified to ensure they were heterozygous at both marker loci. The pattern of inheritance of the mutations is presently unknown.

Protein Purification and Internal Amino Acid Sequencing

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described below. Gels were stained with CBB (0.3 mM Coomassie brilliant blue, 40% methanol, 7% acetic acid) for 30 min and destained in destaining solution (5% methanol, 10% acetic acid) until the band of interest was visible. The gel was washed with deionized water (DIW, 2 × 10 min). The protein band of 68 kD (S68) was excised from the 10% (w/v) SDS-PAGE gel and cut into ca. 1-mm gel fragments on a glass plate using a surgical blade.

The following procedures were performed at room temperature. To remove SDS and CBB, gel fragments were washed with DIW (200 μL, 2 × 10 min) and dehydrated with a mixture of 25 mM ammonium bicarbonate (ABC) in 50% acetonitrile (100 μL) for 15 min. During washing and dehydration steps, the gel fragments were vortexed. This procedure was repeated two to four times until the blue color of CBB was removed.

To reduce the protein in the gel fragments, 10 mM dithiothreitol (DTT) in 25 mM ABC (75–100 μL) was added to a microfuge tube containing the gel fragments and incubated in a 50°–56°C water bath. The fragments were S-alkylated by adding 75–100 μL of 55 mM iodoacetamide in 25 mM ABC and were incubated at room temperature in the dark with occasional vortexing. The gel fragments were washed with 25 mM ABC (100 μL) for 10 min and dehydrated with 25 mM ABC in 50% acetonitrile for 5 min. The wash and dehydration steps were repeated, the liquid phase was removed, and the gel fragments were dried in a vacuum centrifuge for 20 min. The gel fragments were rehydrated in trypsin solution (25 mM ABC at pH 8, 12.5 ng/μL trypsin) by vortexing for 5 min, followed by storage for 40 min at 4°C. Excess trypsin solution was removed, and gel fragments were overlaid with a minimum amount of 25 mM ABC at pH 8 (10 μL) and incubated 12–16 h at 37°C.

After trypsin digestion, the supernatant was transferred to a new tube, and peptides were extracted with 20 μL of DIW by vortexing for 10 min at least twice, once with 20 μL of 5% formic acid. All recovered peptides were pooled. The final volume was reduced to 10–20 μL in a vacuum centrifuge; the concentrate was used immediately or stored at −20°C. Peptide mass fingerprints (PMFs) were generated by means of matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (Applied Biosystems [AB] Perceptive Voyager DE-STR). For sequencing, peptides were subjected to MALDI or nano-electrospray ionization and tandem mass spectrometry on hybrid quadrupole-TOF mass spectrometers (AB MDS Sciex, QSTAR-XL and MDS Sciex Centaur [Qstar prototype]).

N-Terminal Sequencing

Style proteins were resolved using 10% (w/v) SDS-PAGE and transferred onto Sequi-Blot PVDF membrane (BioRad) as described below, with minor modification of the manufacturer's protocol. Membranes were stained with Ponceau S (0.2% Ponceau S, 1% acetic acid) for 5 min and destained with 5% acetic acid or distilled water until bands were visible. The 68-kD band was excised from the membrane, and the protein was eluted from the membrane and subjected

Table 2

Distribution of the Style α-Dioxygenase (S68) in Homostylous Species Using SDS-PAGE and Coomassie Blue Staining

Species	No. with S68	*N*
Turnera orientalis (Orie)	0	4
Turnera ulmifolia (Ulm3)	0	3
T. ulmifolia (Ulm33)	0	3
Turnera velutina (Velu)	0	3
Turnera aurelii (Ar1)	0	3
Turnera cuneiformis (Cune)	0	3

Note. Species (code), number of plants with the style α-dioxygenase, and sample size (*N*) are provided.

to N-terminal sequencing using Edman degradation (performed by the Protein Chemistry Core Facility of the Baylor College of Medicine).

Protein Quantification, SDS-PAGE, and Immunoblotting

Protein quantification was based on BSA standards using the Bradford (1976) assay. SDS-PAGE and immunoblotting were performed according to Khosravi et al. (2003) and Athanasiou et al. (2003) with modifications. Styles were collected a few hours after anthesis and used immediately or stored at −80°C for later use. Styles were ground to fine powder in liquid nitrogen using a mortar and pestle. Once the powder reached room temperature, it was suspended in extraction buffer (0.25 M Tris-HCl at pH 7.5, 50 mM EDTA, 2 mM phenylmethyl sulfonyl fluoride [PMSF]). The extract was mixed with SDS-PAGE sample buffer (10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.0625 M Tris-HCl at pH 6.8, 0.0004% bromophenol blue), incubated at room temperature for 30 min, placed in boiling water for 6 min, and centrifuged for 3 min (15,000 *g*), and the supernatant was collected and used immediately. After transferring, the membranes were blocked with blocking solution (Tris-buffered saline [TBS], 5% skim milk, 0.01% Thimerosal [Sigma]). The primary antibody was diluted to 1/8000 and the secondary antibody to 1/10,000 in blocking solution. The primary antibody was a polyclonal antibody raised against a pathogen-inducible α-dioxygenase from tobacco (Sanz et al. 1998). The secondary antibody was a monoclonal goat antirabbit antibody conjugated to alkaline phosphatase (Sigma). As a control, we used a source of preimmune serum from a rabbit used by Athanasiou et al. (2003) to produce an antibody against a PG. Secondary antibody alone was used as an additional control.

Gel Staining

Gels were either stained with CBB R250 according to Sambrook et al. (1989) or silver stained following the fast silver staining protocol of Rabilloud (1999).

Immunolocalization of S68

The procedure described in Athanasiou et al. (2003) and Khosravi et al. (2003) was used for immunolocalization. The primary antibody (above) was diluted 1 : 200. The secondary antibody was goat antirabbit Cy3 conjugated and was diluted 1 : 400.

α-Dioxygenase Assay

The procedure of Koeduka et al. (2002) was used to monitor the rate of oxygen consumption using a Clark-type oxygen electrode. Fresh styles were ground in 100 mM Hepes buffer (pH 7.2) with a mortar and pestle and used immediately. The reaction was initiated by addition of the crude extract of styles to the reaction mixture (0.55 mM linoleic acid [Sigma] and 0.002% IGEPAL CA-630 [Sigma] in 100 mM Hepes buffer at pH 7.2). Two controls were used. For the first, all components were included except the substrate (linoleic acid). For the second control, the crude extract was omitted from the reaction mixture.

Primer Design and DNA Sequencing

The amino acid residue sequences obtained from mass spectrometry were used in MS-BLAST searches. Because of the lack of genomic sequence information for *Turnera* spp., protein identification relied on an MS-BLAST protocol developed for identification of proteins by sequence similarity searches using partial peptide sequences produced by the interpretation of tandem mass spectra (Shevchenko et al. 2001; http://dove.embl-heidelberg.de/Blast2/msblast.html). A number of α-dioxygenases were identified that showed high sequence identity to the peptide sequences. These sequences were aligned, and degenerate primers were made to regions of DNA sequences that were highly conserved across plant families. Primers were made using an Internet-based program, Prime3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi).

Genomic DNA was extracted from freshly collected flower buds or leaves (0.5 g) following Doyle and Doyle (1987). PCR was performed as follows: 13 μL DIW, 1–2 μL (ca. 50 ng) of genomic DNA template, 5 μL of each primer (10 pmol/μL), and 25 μL of Jumpstart REDTaq ReadyMix PCR Reaction Mix (Sigma) to a total reaction volume of 50 μL. Two PCR programs were used to amplify the sequence depending on the primers used. Touchdown PCR was according to Roux (2002) for degenerate primers; the thermal cycler was set to denature for 2 min at 94°C and anneal for 2 min and primer extend for 3 min at 74°C. The annealing stage was set for two cycles per 1°C beginning at 55°C, decreasing in 1°C increments to 41°C, giving 30 cycles in total, followed by 10 additional cycles at 40°C. The program concluded with a 7-min primer extension (74°C) and was then held at 4°C. The second program followed the procedure described in the Jumpstart REDTaq ReadyMix PCR Reaction Mix manual (Sigma) for nondegenerate primers. PCR-amplified DNA was run on ethidium bromide–stained 1% agarose gels and purified using the QIAGEN gel purification kit (QIAGEN). DNA was sequenced using cycle sequencing on an ABI373A sequencer (Applied Biosystems) at the York University Molecular Core Facility.

Phylogenetic Analysis

We used BLAST searches of NCBI databases (nr, est, and swissprot) to obtain sequences similar to the *Nicotiana tabacum* PIOX gene. Six protein sequences were obtained, and we used the *Lycopersicon esculentum* feebly gene (van der Biezen et al. 1996) to root the phylogeny. Sequences were aligned using Clustal X (Thompson et al. 1997), and we used the protein parsimony routine of PHYLIP (Felsenstein 2001) to obtain a phylogeny. We used 1000 bootstrap replicates to place confidence intervals on the phylogeny. We reran this analysis using only the sequence data corresponding to the sequence data from *Turnera scabra* and *T. subulata* (corresponding to amino acid residues 282 through 617 of the *N. tabacum* α-dioxygenase PIOX gene; see fig. 3). BLAST searches also revealed a number of partial sequences and expressed sequence tags (ESTs). We carried out additional analyses using a number of these partial and/or EST sequences as above.

Linkage Analysis

To determine whether the gene encoding the S68 protein is linked to the distyly locus, we identified a cleaved amplified polymorphism following methods of Athanasiou et al. (2003). We amplified and sequenced a 392 bp portion of the S68 gene in *T. krapovickasii* using the primers 5′-acctggcagctcttgaaagt-3′ and 5′-gtaaaagcggtctcgctgat-3′ and compared the sequence to that of *T. subulata*. There is one NlaIII restriction site difference between the two sequences. It is therefore possible to distinguish two alleles of the S68 gene using NlaIII (New England Biolabs) restriction digestion (following the manufacturer's protocol). Restriction digestion of the PCR-amplified sequence of *T. krapovickasii* yields three fragments (179 bp, 166 bp, 47 bp), while the *T. subulata* sequence yields two fragments (345 bp, 47 bp). We analyzed 18 progeny produced by selfing a mutant homostyle, Mhomo-H, and followed segregation of the cleaved amplified polymorphism, the distyly locus, and an aconitase isozyme locus, Aco-1, known to be linked to the distyly locus (Athanasiou et al. 2003). Aconitase was assayed following the methods of Athanasiou et al. (2003). The products of the restriction digest were run on 2% agarose gels stained with ethidium bromide (Athanasiou et al. 2003). We estimated the recombination frequency between the distyly locus and the S68 gene and between the Aco-1 locus and the S68 gene using maximum likelihood estimation.

Results

Short-Style Specificity of a 68-kD Protein

Crude extracts of styles from *Turnera subulata* were separated using SDS-PAGE, and protein profiles from long and short styles were compared to detect proteins that are differentially expressed in the morphs. A heavily stained protein band with a molecular mass of ca. 68 kD (referred to as S68) was observed in styles of the short-styled morph (but not the long-styled morph) when gels were stained with CBB or silver stain (fig. 1*A*, 1*B*). The S68 protein appears to be abundant, as it is readily detected with CBB stain using only 20% of the total protein extract (ca. 1 μg) from one short style

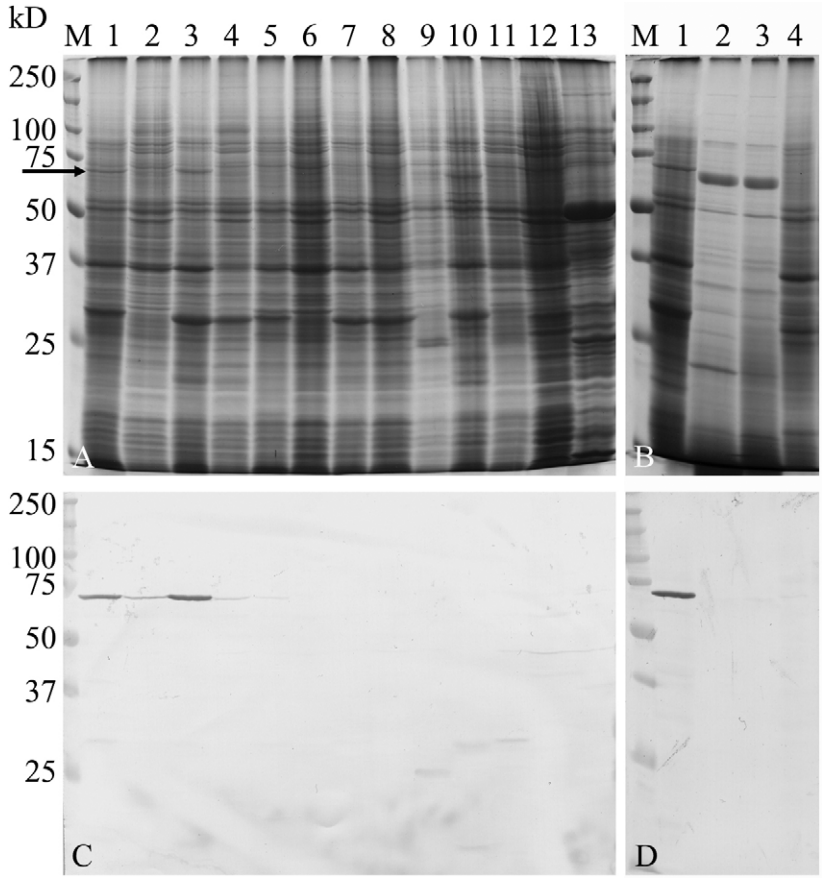

Fig. 1 SDS-PAGE gels stained with Coomassie brilliant blue (*A, B*) or replicate gels (*C, D*) immunoblotted and stained with an antibody against the *Nicotiana tabacum* α-dioxygenase. *A*, *C*, Lane 1: *Turnera scabra* short style; lane 2: *T. scabra* short style 1 d before anthesis; lane 3: *Turnera subulata* short style; lane 4: *T. subulata* short style 1 d before anthesis; lane 5: *T. scabra* long style; lane 6: *T. scabra* long style 1 d before anthesis; lane 7: *T. subulata* long style; lane 8: *T. subulata* long style 1 d before anthesis; lane 9: styles of *Turnera ulmifolia* var. *acuta*; lane 10: styles of *Turnera velutina*; lane 11: styles of *T. ulmifolia*; lane 12: ovary of *T. scabra*; lane 13: leaf of *T. scabra*. *B*, *D*, Lane 1: *T. scabra* short style; lane 2: *Piriqueta caroliniana* short style; lane 3: *P. caroliniana* long style; lane 4: *T. scabra* long style. Lanes M: molecular mass markers. Arrow in *A* indicates location of the 68-kD protein band.

following SDS-PAGE (data not shown). Total protein extracts of long and short styles were compared 1 to 4 d before anthesis. Some variation can be seen in protein profiles of styles from different developmental ages; however, no morph-specific proteins were detected for these immature styles (fig. 2*A*).

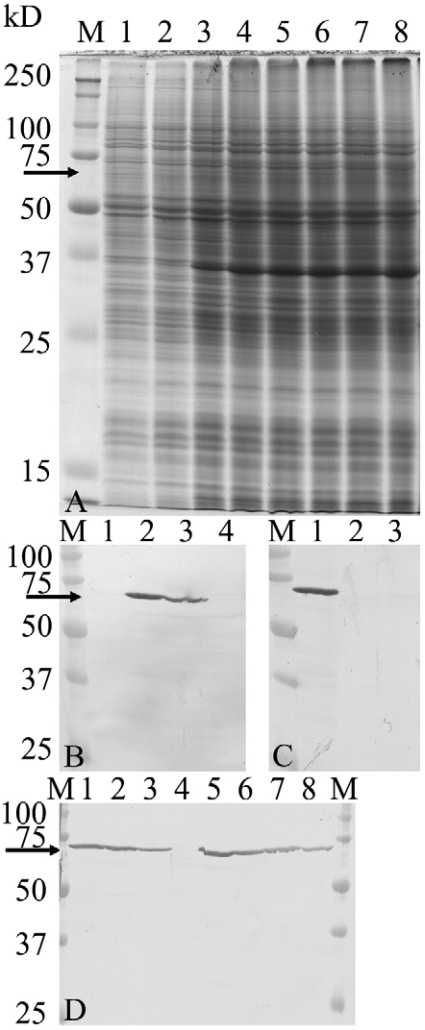

Fig. 2 *A*, SDS-PAGE gel stained with Coomassie brilliant blue to investigate the time of appearance of the 68-kD protein in styles of *Turnera scabra*. Odd-numbered lanes contain long styles; even-numbered lanes contain short styles. Lanes 1, 2: 4 d before anthesis; lanes 3, 4: 3 d before anthesis; lanes 5, 6: 2 d before anthesis; lanes 7, 8: 1 d before anthesis. *B–D*, Immunoblots using antibodies against the *Nicotiana tabacum* α-dioxygenase. *B*, Lane 1: *T. scabra* long style; lanes 2, 3, 4: *T. scabra* short styles at flowering, 1 and 2 d before flowering, respectively. *C*, Immunoblotting of styles from two x-ray-generated mutants. Lane 1: *T. scabra* short style control; lane 2: long-styled mutant; lane 3: homostylous mutant. Lanes M: molecular mass markers. *D*, Immunoblotting for *T. scabra* following self- (lanes 1–3) or cross-pollination (lanes 5–8) of short styles. Lanes 1–3: 80, 60, and 20 min after pollination, respectively; lane 4: unpollinated long style; lanes 5–7: 80, 60, and 20 min after compatible pollination, respectively; lane 8: unpollinated short style. Arrows in *B* and *D* indicate location of the 68-kD protein band.

We investigated a minimum of 10 plants from four additional distylous species of series *Turnera*, including *Turnera krapovickasii*, *Turnera joelii*, *Turnera grandiflora*, and *Turnera scabra* (five populations of the latter species). All of these species showed a prominent 68-kD protein band specific to the styles of the short-styled morph (table 1), although there appears to be some variation in staining intensity among species (fig. 1*A*). When a more distantly related distylous species, *Piriqueta caroliniana*, was examined, S68 was not observed in styles of either morph. Protein profiles from these species are different from the *Turnera* species, and we could not detect any morph-specific proteins using SDS-PAGE (fig. 1*B*).

We explored the occurrence of the S68 protein in five self-compatible homostylous species (table 2). Flowers of homostylous species have long styles and long stamens, although there is variation in stigma-anther separation within and among some homostylous species. S68 was not detected in styles of any of the homostylous species (fig. 1*A*; table 2). We also investigated the occurrence of S68 in a spontaneous homostylous mutant (Mhomo-H), a homostylous x-ray-generated mutant (X-homo), and a long-styled x-ray-generated mutant (X-long). None of these mutants possessed the S68 protein; however, the short-styled plants from which the mutants were derived did possess the S68.

Identity of the S68 Protein

We sequenced 11 peptides obtained from a tryptic digest of the S68 protein by means of tandem mass spectrometry (fig. 3). Mass spectrometry cannot differentiate between two isobaric amino acids pairs, lysine and glutamine and leucine and isoleucine. However, because trypsin reliably cuts C-terminal to lysine, any C-terminal residue that can be either lysine or glutamine will usually be lysine.

We assembled a search file containing all permutations of the leucine/isoleucine and the internal lysine/glutamine residues for the 11 peptide sequences to be used in MS-BLAST. This approach does not compromise the search result because multiple peptide sequences are needed to assure the identification of protein homologues. There were six high-scoring pairs produced out of the 11 unique peptides from MS-BLAST search results. These indicated that S68 is an α-dioxygenase, initially referred to as pathogen-induced oxygenase in *Nicotiana tabacum* (PIOX). We list (fig. 3) the sequence bearing lysine/glutamine and leucine/isoleucine assignments that agree with our inferred amino acid sequence based on DNA sequencing. In two instances, the peptides lie outside of the range of the DNA sequence we obtained, and we have resolved these ambiguities by indicating the amino acids that are shared with the *N. tabacum* PIOX gene (fig. 3). We also obtained the N-terminal sequence of the protein, which is composed of the following 10 amino acids residues: MLSTILLPLK. We have not included this peptide as a result of difficulties with alignment because of the low level of conservation of the N-terminal region of the proteins (fig. 3).

Using the peptide sequences and alignments of amino acid and DNA sequence data from α-dioxygenases of *Oryza sativa* (AF229813), *N. tabacum* (AJ007630), *Capsicum annuum*

```
TSCA     --------------------------------------------------
PIOX     MSLVMDSLKNLLLSPLRGFIHKDLHDIFERMTLLSKLLFLIVHLVDKLNL

TSCA     --------------------------------------------------
PIOX  51 WHRLPVLLGLLYLGARRHLHQEYNLINVGKTPIGVRSNPADHPYRTADGK

TSCA     ---PFNEVAGSQGTFFGR-------------------------------
PIOX 101 YNDPFNEGAGSELSFFGRNMLPVDQHNQLKKPDPMVVATKLLARRNFVDT

TSCA     --------------------------------------------------
PIOX 151 GKQFNMIAASWIQFMIhDWIDHLEDTKQIELKAAEEVASQCPLKSFRFFK

TSCA     ------------------TPWWDGSAIYGS------------------
PIOX 201 TKEIPTGFYEIKTGHLNTRTPWWDGSAIYGSNAEVLKKVRTFKDGKLKLS

TSCA     ------------------------------FVQEhTAVCDPLKEEYPDL
PIOX 251 ADGLLEIDKNGKIISGDVRNTWAGLSALQALFVQEhNSVCDALKKEYPEL

TSCA     GDEELYRHARLVTSAVIAKVHTIDWTVELLKTDTLYAGMRANWYGLLGKK
PIOX 301 EEEDLYRHARLVTSAVIAKVHTIDWTVELLKTDTLLAGMRANWYGLLGKK

TSCA     FKDTFGHVGGAILGGLVGLKKPENHGVPYSLTEEFVGVyRMhSLLPDDLQ
PIOX 351 FKDTFGHVGGSILGGFVGMKKPENYGVPYSLTEEFTSVyRMhQLLPDKLQ

TSCA     LRDISSTSGPDKTPPATEKVPLQNLIGHKGEKTLSEIGFSKVMVSMGHQA
PIOX 401 LRNIDATPGPNKSLPLTNEIPLEDLIGGKGEKNLSKIGFTKQMVSMGHQA

TSCA     SGALELWNYPNWLRDLVVQDVDGVDRPDHVDLAALEIYRDRERKVARYNE
PIOX 451 CGALELWNYPVWMRDLIPQDVDGTDRPDHIDLAALEIYRDRERSVARYNE

TSCA     FRRGLLLIPISKWEDLTDDKEAIATLREVYGDDVEALDLLVGLMAEKKIS
PIOX 501 FRRGMLQIPISKWEDLTDDEEVINTLGEVYGDDVEELDLMVGMAAEKKIK

TSCA     GFAISETAFTIFLLMATRRLEADRFFTSNFNEETYTKKGLKWVNTTETLK
PIOX 551 GFAISETAFFIFLVMAsRRLEADRFFTSNYNEETYTKKGLEWVNTTESLK

TSCA     DVIDRHYPTMTKKWMNST-------------------------
PIOX 601 DVLDRHYPEITEKWMNSSSAFSVWDSTPQPHNPIPLYFRVPPQ
```

Fig. 3 Amino acid sequence alignment of the S68 partial sequence of *Turnera scabra* (TSCA) against the α-dioxygenase gene (PIOX) of *Nicotiana tabacum*. Identical amino acids residues (80.7%) are shaded. Underlined sequences indicate the internal amino acid sequences obtained directly using tandem mass spectrometry. Amino acid residues in lowercase bold print indicate those that have been shown to be important for catalytic activity of α-dioxygenase in *N. tabacum* and/or *Oryza sativa*.

(AY040869), and *Arabidopsis thaliana* (AF334402), we designed and used primers to amplify the corresponding gene from *T. scabra* and *T. subulata*. Using this approach, we were able to obtain 1864 bp of genomic DNA sequence from *T. scabra*, which included much of the 3′ end of the gene. We obtained comparable sequence data for *T. subulata*. Using alignments of DNA sequence data against those of *A. thaliana* α-dioxygenase (AF334402) and the consensus splice sites for intron excision, we inferred the amino acid sequence for most of the C-terminal region of the protein (337 amino acid residues). There are five introns in the sequence we obtained. Exon lengths are identical to those of *A. thaliana* (AF334402), and the intron positions appear to be conserved in number and location relative to those of *A. thaliana* (fig. 4). There is some variation in intron length between the *A. thaliana* compared with the *T. scabra* and *T. subulata* sequences as well as between the two *Turnera* sequences for the third intron (fig. 4). The DNA sequences and inferred amino acid sequences will be deposited in GenBank.

An alignment of *T. scabra* versus *N. tabacum* α-dioxygenase shows 80.7% identity (excluding the N-terminal region) at the amino acid level over the range of sequences we have obtained (fig. 3). The *T. scabra* and *T. subulata* amino acid sequences differ by four amino acid substitutions.

Phylogenetic Analysis

A phylogenetic analysis revealed that the S68 sequences of *T. scabra* and *T. subulata* fall within a clade containing the α-dioxygenases originally described in *N. tabacum* and in *O. sativa* (fig. 5). The phylogeny is well supported by bootstrap analysis. We also carried out an analysis by truncating the aligned sequences so that only sequences overlapping the *Turnera* spp. data were used, and we obtained an identical phylogeny. We carried out analyses using additional species (table 3) for which partial amino acid sequence data were available. These data were obtained by BLAST searches and subsequent translation of EST data for a number of species (table 3). These analyses revealed that the *Turnera* sequences remain embedded within a clade containing what appear to be α-dioxygenases that occur in both monocot and dicot families, including the Poaceae, Fabaceae, Brassicaceae, Rubiaceae, and Solanaceae.

Given the high degree of homology between the *T. scabra* sequence and the *N. tabacum* α-dioxygenase (fig. 3), we used a polyclonal antibody raised against the *N. tabacum* α-dioxygenase to verify further the identity of the S68 protein and to study its organ and tissue localization. On SDS-PAGE immunoblots, the immune serum reacted with a 68-kD protein from short but not long styles of *T. subulata* (fig. 1*C*). Similarly, for short styles of *T. scabra*, a prominent S68 band was immunostained (fig. 1*C*). The S68 protein may be weakly expressed in long-styled plants of *T. scabra* (a faint band that is barely visible occurs on the immunoblot in fig. 1*C*). In *T. subulata* and *T. scabra*, S68 was observed in short but not long styles 1 d before anthesis (fig. 1*C*). For styles taken from flowers 2 d before anthesis, neither short nor long styles possessed S68 at a detectable level (fig. 2*B*, 2*C*). These results indicate that the S68 protein reaches a detectable level, using antibody, 1 d before flowering. Using CBB, we could only detect S68 at anthesis. The organ specificity of S68 was examined by running extracts of ovaries, pollen, and leaves. S68 was not detected in any of these organs (fig. 1*A*, 1*C*).

Styles of neither the homostylous species nor the mutants (Mhomo-H, X-homo, and X-long) possessed the S68 protein at anthesis or 1 d prior to anthesis (fig. 2*C*). Styles of Mhomo-S showed the S68 protein typical of short styles. As aforementioned, S68 was not detected in styles of *P. caroliniana* using silver or CBB staining. When styles from open flowers or those sampled 1 d before anthesis were tested against α-dioxygenase

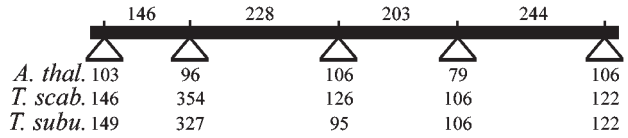

Fig. 4 Schematic showing the size (bps) and positions of exons (heavy lines) and introns (triangles) for *Arabidopsis thaliana* (*A. thal*) α-dioxygenase, as well as that of *Turnera subulata* (*T. subu*) and *Turnera scabra* (*T. scab*).

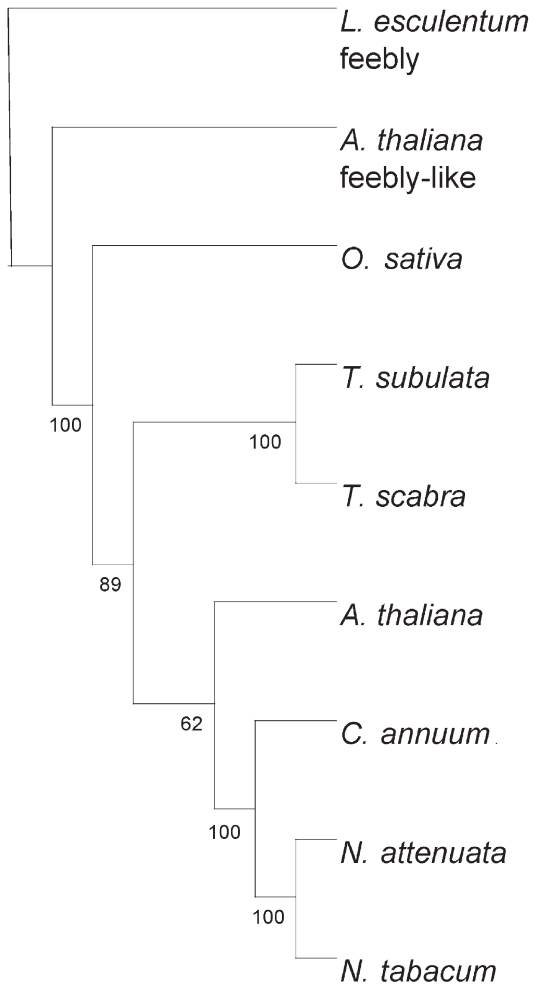

Fig. 5 Gene tree of α-dioxygenases based on a protein parsimony analysis. The tree was rooted using the *Lycopersicon esculentum* feebly gene. The numbers adjacent to each node represent the percentage of bootstrap samples in which that particular node occurred.

immune serum, only mature long styles of *P. caroliniana* showed a very faint 68-kD band (fig. 1*B*, 1*D*).

We explored the possibility that the S68 protein might be expressed after pollination of long styles (i.e., whether it is induced by pollination) and whether changes in expression of the S68 protein in short styles might occur after pollination. Short styles pollinated with compatible or incompatible pollen for different periods of time (20, 60, and 80 min) possess the S68 protein, and its abundance might increase with the time (fig. 2*D*). For long styles, we did not detect S68 following the pollination scheme used above (data not shown).

Immunolocalization of S68

Immunocytochemistry studies localized the S68 protein to the transmitting tissue of short styles of *T. subulata*. No staining was observed for long styles of this species. For *T. scabra*, staining is intense in short styles, and slight staining can also be seen in the transmitting tissue of long styles (fig. 6). Some staining of vascular bundles occurs for both long and short styles. Analysis of cross sections of short styles made throughout the length of the style confirmed the presence of the S68 protein in the transmitting tissue in all style sections and in the stigma transmitting tissue (data not shown). Preimmune serum and secondary antibody alone were used as controls, and only slight immunostaining was observed for vascular bundles in these controls. In agreement with the immunoblotting results, no staining was observed for short styles of *P. caroliniana*, but there appeared to be some staining of the transmitting tissue of long styles for this species (fig. 6*F*–6*H*).

α-Dioxygenase Assay

The addition of the linoleic acid substrate to crude extracts of long and short styles of *T. scabra* resulted in an increase in oxygen consumption. The rate of oxygen consumption was almost twice as high in short styles (131.6 nmol O_2/min/mg) compared to long styles (67.8 nmol O_2/min/mg). For controls, extracts without the substrate showed a very small amount of oxygen consumption. When the substrate and assay buffer without the style extracts were investigated, no oxygen consumption occurred.

Linkage Analysis

We used a cleaved amplified polymorphism to explore whether the gene encoding the α-dioxygenase was linked to and/or a component of the distyly locus in *Turnera*. We assayed F_2 progeny obtained by selfing the mutant plant, Mhomo-H. While the sample is small ($n = 18$), there is no evidence for close linkage between the distyly locus and the gene encoding the S68 protein (recombination frequency $= 0.43 \pm 0.15$). Likewise, the Aco-1 locus does not appear to be linked to the gene encoding the S68 gene (recombination frequency $= 0.50 \pm 0.12$).

Discussion

We have shown that a protein expressed to a high level in short-style transmitting tissue of five species in series *Turnera* appears to be an α-dioxygenase. The evidence for this conclusion is based on peptide and DNA sequence data, immunoblotting and immunocytochemistry using antibodies against a tobacco α-dioxygenase, and finally on an assay for α-dioxygenase activity in crude style extracts. BLAST searches and subsequent phylogenetic analyses revealed that the *Turnera scabra* and *Turnera subulata* α-dioxygenases are homologous to a number of α-dioxygenases that are induced in response to attack by various plant pathogens and/or may be expressed in response to oligogalacturonide as well as glucan elicitors or during salt stress (table 3).

There are four amino acid residues that are thought to be important in the activity of α-dioxygenase, and these are shared by both *Nicotiana tabacum* and *Oryza sativa* (fig. 3; Sanz et al. 1998; Koeduka et al. 2002). Both the *T. scabra* and *T. subulata* sequences possess three of these amino acid

Table 3

Species, Accession Numbers, and Identity of Sequences Used in Analysis and/or Cited

Species	Accession no.	Protein identity or tissue or means of induction
Nicotiana tabacum	AJ007630	Pathogen-induced α-dioxygenase
Nicotiana attenuata	AF229926	Pathogen-induced α-dioxygenase
Capsicum annuum	AY040869	Cyclo-oxygenase-like
Oryza sativa	AF229813	Fatty acid α-oxidase
Arabidopsis thaliana	AF334402	Fatty acid α-dioxygenase
A. thaliana	NM_106027	Feebly related
Lycopersicon esculentum	U35643	Feebly
Cicer arietinum	AJ487467	α-dioxygenase
Coffea arabica	AF343970	Oxygenase
L. esulentum	BI422699	EST callus
Zea mays	AY108782	EST
Medicago trunculata	CB895193	EST oligogalacturonide treated
M. trunculata	BG645762	EST *Sinorhizobium meliloti* inoculated roots
Solanum tuberosum	BI432698	EST *Phytophthora infestans*–challenged leaf
Triticum aestivum	BQ744531	EST salt-stressed roots
Lactuca sativa	BQ850613	EST
Glycine max	BQ743020	EST glucan elicitor from mycelial walls of *Phytophthora sojae*

Note. EST = expressed sequence tags.

residues. The fourth amino acid residue, a serine at position 567, is not conserved in *T. scabra*, *T. subulata*, or *Arabidopsis thaliana* (AF334402) α-dioxygenase.

Interestingly, we were not able to detect the α-dioxygenase using nondenaturing isoelectric focusing or two-dimensional gel electrophoresis. This is likely because the solubility of the α-dioxygenase is low in the absence of an effective detergent such as SDS. In fact, the appearance of the α-dioxygenase was sensitive to the concentration of SDS and the time of denaturation in SDS-PAGE buffer (data not shown). Previous reports have indicated the need for a detergent to solubilize α-dioxygenase from rice (Koeduka et al. 2002).

A recently identified enzyme of the oxylipin pathway, α-dioxygenase catalyzes the dioxygenation of fatty acids such as linolenic, linoleic, or oleic acids (Hamberg et al. 1999). It is believed to be involved in the generation of cellular signals that mediate a plant's response to pathogens, and α-dioxygenases show similarity to animal cyclo-oxygenases, which are involved in the synthesis of lipid signal molecules (Sanz et al. 1998; Kim et al. 2002).

Tobacco α-dioxygenase expressed in insect cells showed oxygen uptake in the presence of substrates such as linoleic, linolenic, and arachidonic acids (Sanz et al. 1998). A recombinant gene encoding α-dioxygenase from rice with high sequence similarity to tobacco α-dioxygenase also showed

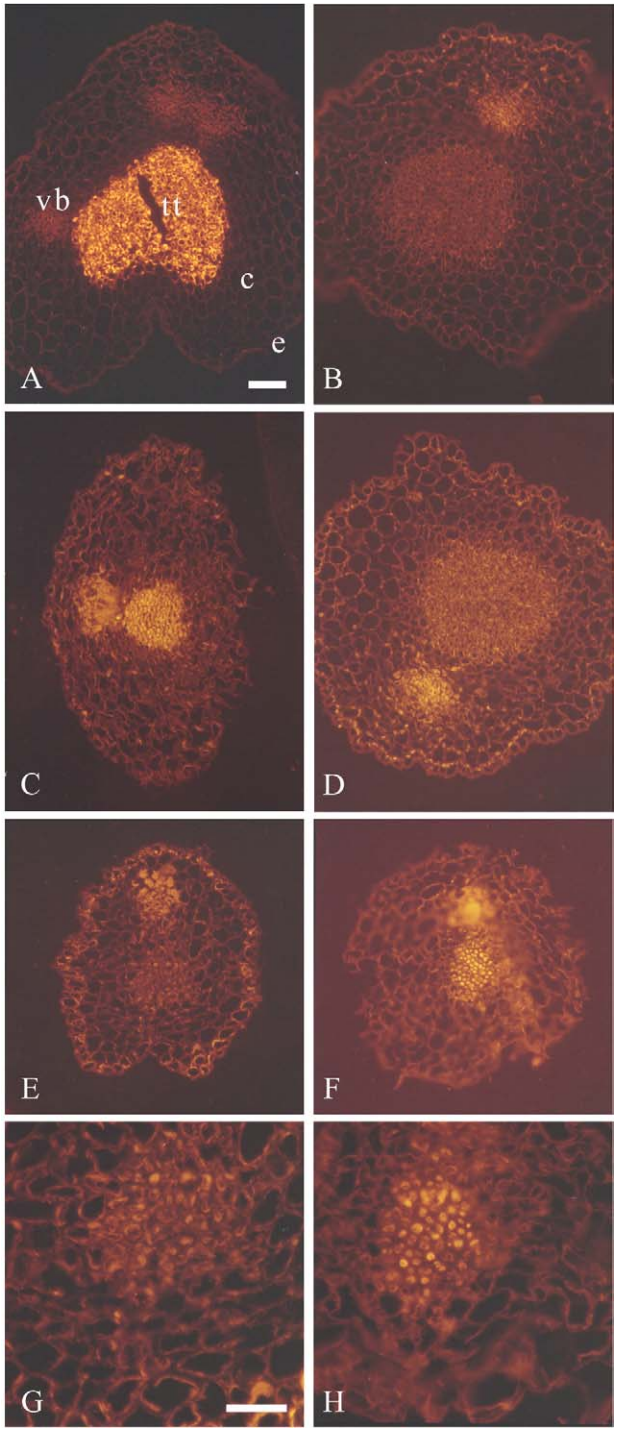

Fig. 6 Immunocytochemical analysis of cross sections of styles stained with an antibody against the *Nicotiana tabacum* α-dioxygenase. *A*, *C*, *E*, *G*: short styles; *B*, *D*, *F*, *H*: long styles. *A*, *B*: *Turnera subulata*; *C*, *D*: *Turnera scabra*; *E*, *F*: *Piriqueta caroliniana*. *G*, *H*: greater magnification of styles in *E*, *F*. *tt*, transmitting tissue; *vb*, vascular bundles; *e*, epidermal cells; *c*, cortical cells. Scale bars represent 150 μm. Scale bar in *A* applies to *A–F*. Scale bar in *G* applies to *G*, *H*.

oxidation of a number of these substrates (Koeduka et al. 2002). In the presence of linolenic acid, crude extracts of short styles of *T. scabra* showed twice the oxygen uptake compared with long styles. An enzyme assay carried out on a purified enzyme would provide more direct evidence for α-dioxygenase activity.

Pathogen-induced α-dioxygenase was first observed in tobacco (*N. tabacum*) leaves, where it is induced in response to inoculation with the HR-inducing pathogen *Erwinia amylovora*, injection of the harpin HrpN protein of *E. amylovora* (Sanz et al. 1998), or attack by the herbivore *Manduca sexta* (Hermsmeier et al. 2001). Hot pepper leaves (*Capsicum annuum*) inoculated with *Xanthomonas campestris* pv. *glycine* 8ra expressed an α-dioxygenase homologous to that of tobacco (Kim et al. 2002). In tobacco, the α-dioxygenase was induced by signal molecules including salicylic acid, jasmonic acid, and hydrogen peroxide; however, these molecules did not induce α-dioxygenase expression in hot pepper leaves. In *A. thaliana*, α-dioxygenase is induced in response to inoculation with compatible and incompatible strains of *Pseudomonas syringae* DC3000, but greater levels of α-dioxygenase are induced when infection results in a HR (Ponce de León et al. 2002). The α-dioxygenase is also expressed in roots, senescing leaves, and anthers of untreated plants of *A. thaliana*. There was no induction of expression following jasmonic acid treatment, but salicylic acid and chemicals generating nitric oxide, intracellular superoxide, or singlet oxygen induced the α-dioxygenase (Ponce de León et al. 2002).

The role that an α-dioxygenase might play in the style transmitting tissue of short-styled plants of distylous *Turnera* species is unclear. Distylous *Turnera* species have ephemeral flowers (remaining open for no more than 4–8 h depending on species and temperature), and pollen germination and pollen tube growth occurs rapidly (Tamari et al. 2001). Both compatible and incompatible pollen germinates, and pollen tubes penetrate the stigma tissue. Compatible pollen tubes grow rapidly through styles. Incompatible pollen tubes in short-styled plants are largely inhibited in the stigma, while inhibition for long-styled plants occurs in the upper portion of styles (Tamari et al. 2001). This suggests that pollen tubes most likely interact with SI factor(s) in the stigmatic and/or style transmitting tissue (depending on morph) rather than on the stigma surface, as in *Brassica* spp. (Goring 2000; Nasrallah 2000).

The *Turnera* α-dioxygenase is the second protein discovered that is specific to the style transmitting tissue of short-styled plants of species in series *Turnera* (Athanasiou et al. 2003). It shares some features in common with the short-specific PG investigated by Athanasiou et al. (2003) and Khosravi et al. (2003), including its occurrence in transmitting tissue of short styles (and absence or low level of occurrence in long styles) of all distylous species in series *Turnera*, its absence from short styles of *P. caroliniana*, its absence from styles of self-compatible homostylous *Turnera* species and homostylous mutants, and, finally, that both proteins may be involved in the generation of signals in plant pathogen or wounding interactions. The temporal pattern of expression of the two proteins differs as the short-style-specific PG is first detected 3 d prior to anthesis, while the α-dioxygenase is first detected 1 d prior to anthesis. It is possible that oligogalacturonide signals generated by PG activity are responsible for the induction of the α-dioxygenase. The presence of α-dioxygenase-expressed sequence tags from tissues treated with oligogalacturonide elicitors (table 3) and its induction in response to wounding (Hermsmeier et al. 2001) support this possibility. The role of both proteins in distyly, however, remains unclear.

The gene encoding the α-dioxygenase does not appear to be closely linked to the distyly locus in *Turnera* spp., unlike the gene encoding the short-specific PG, which is 4.6 cM distal to the distyly locus (Athanasiou et al. 2003). This indicates that the α-dioxygenase is not a component of the distyly locus and that its expression must be regulated in some as yet unknown way by the dominant *S* allele of the distyly locus.

We have not explored the induction of α-dioxygenase in *Turnera* species using any chemical elicitors or pathogens. However, we did not detect α-dioxygenase induction in long styles in response to self- or compatible pollination, and short styles showed little variation from their control expression levels following these pollinations. These results indicate that the expression of α-dioxygenase is independent of pollination. The only other report of expression of an α-dioxygenase in flowers is for anthers of *A. thaliana* (Ponce de León et al. 2002). A 2-oxoglutarate-dependent dioxygenase is also known to be induced in pistils by pollination, wounding, jasmonates, and salicylic acid in self-incompatible *Solanum chacoense* (Lantin et al. 1999).

Functional similarity between SI systems and host plant–pathogen interactions has been previously suggested (Bushnell 1979; Hodgkin et al. 1988). For example, callose formation was thought to be associated with SI (Hodgkin et al. 1988), and callose is believed to play an important role in plant response to pathogens (Beffa et al. 1996; Hammond-Kosack and Jones 1996). Similarities between the early signaling events in both the hypersensitive response of plants to pathogens and the SI response of *Papaver rhoeas* have been pointed out by Rudd and Franklin-Tong (2003). While PG and α-dioxygenase association with plant responses to pathogens have been reasonably well established, no such connection has been observed for SI systems (Sanz et al. 1998; Hong et al. 2000; Federici et al. 2001; Kim et al. 2002; Nasrallah et al. 2002; Cruz-Garcia et al. 2003; Rudd and Franklin-Tong 2003). We speculate that the PG and α-dioxygenase might be members of a signal pathway responsible for the behavior of compatible and/or incompatible pollen tubes in short-styled plants of *Turnera* species. The use of methods to knock out the expression of these proteins should aid in testing this hypothesis and/or in clarifying the role of these proteins in distyly.

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