

CHARACTERIZATION AND LOCALIZATION OF SHORT-SPECIFIC POLYGALACTURONASE IN DISTYLOUS *TURNERA SUBULATA* (TURNERACEAE)¹

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We describe for distylous *Turnera subulata* a polygalacturonase specific to short-styled plants that is localized to the style transmitting tissue (the tissue through which pollen tubes grow). The polygalacturonase gene is linked to and may be upregulated by the *S* allele of the distyly locus. Because of its tissue-specific location, the polygalacturonase may be involved in the self-incompatibility response, acting in a complementary or antagonistic manner, or possibly in signalling downstream events. A pollen-specific polygalacturonase was also identified and may be a member of a small multigene family of pollen polygalacturonases. The role, if any, played by the pollen polygalacturonase in distyly, is presently unknown.

Key words: distyly; heterostyly; immunocytochemistry; polygalacturonase; transmitting tissue; *Turnera*; Turneraceae.

Distyly, a genetic polymorphism that occurs in a wide range of flowering plant families, has evolved independently at least 28 times (Arroyo and Barrett, 2000). Distylous populations possess two mating types (long- vs. short-styled morphs) that have a reciprocal positioning of male and female reproductive organs (reciprocal herkogamy), the anthers and stigmas. Both morphs commonly possess a self- and intra-morph incompatibility system that enforces inter-morph mating and prevents self-fertilization. Despite a long history of use as a model system in genetics and evolutionary biology (Ornduff, 1992) beginning with Darwin (1877), nothing is known of the molecular genetic basis of the polymorphism including dimorphisms in reproductive organ lengths, self-incompatibility (SI), or ancillary characters.

Genetic studies of distylous species indicate that all dimorphic characters, including the SI system, commonly appear to be determined by a single diallelic locus, where short-styled plants are usually heterozygous, *Ss*, and long-styled plants are homozygous recessive, *ss* (Lewis and Jones, 1992). The dominance relationship is reversed in two families (Baker, 1966; Ornduff, 1979). Studies of *Primula* spp. (Primulaceae) indicate that the distyly locus is actually composed of at least three tightly linked loci that are held in extreme linkage disequilibrium and comprise a supergene (Ernst, 1955; Dowrick, 1956; Lewis and Jones, 1992; Richards, 1997). In fact, distyly is one of the best examples of a supergene, yet the molecular genetic basis of the system is unknown, although candidate proteins/molecules have been proposed (Golynskaya et al., 1976; Shivanna et al., 1981; Wong et al., 1994; Athanasiou and Shore, 1997).

Athanasiou and Shore (1997) discovered proteins specific to the styles and pollen of the short-styled morph of *Turnera subulata* Smith, *T. scabra* Millsp., and a few plants of *T. kra-*

povickasii Arbo. In the present study, we extend the work of Athanasiou and Shore (1997) by sequencing, identifying, and localizing the proteins in *T. subulata*. The style-specific protein is a polygalacturonase (PG) localized to the transmitting tissue of the short-styled morph of *T. subulata*. Linkage analysis reveals that the style PG gene is linked to and its expression may be upregulated by the *S* allele of the distyly locus. The pollen-specific protein is also a polygalacturonase.

MATERIALS AND METHODS

Protein purification and sequencing—Extracts of styles from short-styled plants of *T. subulata* were run on isoelectric focusing (IEF) gels (Athanasiou and Shore, 1997) and stained with coomassie brilliant blue. The most heavily stained protein band with an isoelectric point (pI) of 6.5 was excised from the gel and digested with Lys-C. The resulting peptides were fractionated by high-performance liquid chromatography. Individual fractions were subjected to Edman degradation and four amino acid sequences were obtained (performed by the Protein Chemistry Core Facility of the Baylor College of Medicine). Using extracts of anthers, an identical protocol was followed for the pollen protein (Athanasiou and Shore, 1997).

DNA sequencing—Degenerate primers were designed based on partial peptide sequences from the style protein. These primers were used to amplify a genomic DNA sequence using the polymerase chain reaction (PCR). The amplified fragment was sequenced. We designed new primers based upon this initial DNA sequence and used 3' RACE (rapid amplification of cDNA 3' end) to obtain much of the coding sequence (starting with amino acid residue 107). Genomic DNA was extracted according to Doyle and Doyle (1987). Total RNA was extracted from styles (Jones et al., 1985). We used walking PCR (Katz et al., 2000) to obtain the remainder of 5' end of the sequence from genomic DNA, because 5' RACE failed to yield any new sequence data. Sequencing of both strands was performed on the PCR-amplified DNA using cycle-sequencing on an ABI373A sequencer (Applied Biosystems, Foster City, California, USA) at the York University Molecular Biology Core Facility.

For the pollen protein, we amplified a fragment from genomic DNA using degenerate primers designed from partial peptide sequences. This DNA fragment was used to screen a pollen cDNA library (Athanasiou, 2001). A single clone was isolated and both strands of the cDNA were sequenced. The sequence data have been submitted to GenBank and accession numbers may be found in the Appendix (<http://ajbsupp.botany.org/v90>).

The PCR amplifications were generally performed using the following conditions with minor modifications depending upon the primers used: to 1 μ L

¹ Manuscript received 5 September 2002; revision accepted 5 December 2002.

The authors thank André Bédard, Barrie Coukell, Daphne Goring, and Mohan Subramanian for helpful advice, Maria Mercedes Arbo for seeds, and Wendy Lezama and Lee Wong for technical assistance. This research was supported by a National Sciences and Engineering Research Council of Canada grant to J.S.S.

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(approximately 50 ng) of genomic DNA was added 40.75 μ L nuclease-free water, 1 μ L dNTP mix (10 mmol/L each dATP, dCTP, dGTP, dTTP), 1 μ L 5' primer (5 pmol/ μ L), 1 μ L 3' primer (5 pmol/mL), 5 μ L 10 \times buffer (100 mmol/L Tris-HCl pH 8.8, 500 mmol/L KCl, 15 mmol/L MgCl₂, 1% Triton X-100), and 1.25 units of AmpliTaq DNA polymerase (Perkin Elmer/Applied Biosystems, Foster City, California, USA). The samples were processed in a PE-9600 thermal cycler (Perkin Elmer, Boston, Massachusetts, USA) for 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, with a final extension at 72°C for 5 min. The PCR products were run on ethidium bromide-stained agarose gels (0.8% in TBE buffer) to verify the specificity of the PCR reaction and for further gel purification and sequencing. Detailed information on PCR, 3' RACE, cloning, library construction, screening, and sequencing protocols are found in Athanasiou (2001).

Phylogenetic analysis—Amino acid sequences of both the style and pollen proteins were deduced from the cDNA sequences. The amino acid sequences we obtained were compared to plant PG sequences recently used in the phylogenetic analyses (Hadfield et al., 1998; Hong and Tucker, 2000; Toriki et al., 2000; Markovič and Janeček, 2001; Appendix at <http://ajbsupp.botany.org/v90>). Amino acid sequences were aligned using Clustal X (Thompson et al., 1997). The N-terminal region (corresponding to the first 85 amino acids of the style PG, Fig. 1A) and the last 15 amino acids of the C-terminal region were removed from all sequences because of considerable gap formation. The truncated sequences were then realigned. Gaps in the sequences were coded as unknowns. The gene tree was constructed using ordinary protein parsimony analysis of PHYLIP (Felsenstein, 2001) and rooted using a fungal outgroup (*Aspergillus flavus*, Fig. 2, AspflB.pg). The percentage of bootstrap samples in which a particular node occurred was calculated based upon 1000 random samples of the sequences with replacement (Felsenstein, 1985).

Immunoblotting—Polyclonal antibodies were made against the style and pollen PGs by generating fusion proteins that were injected into New Zealand White rabbits. The style fusion protein was made by subcloning the region of cDNA corresponding to amino acids 180 through to the 3' end of the coding region of the style gene (Fig. 1A) into a pTrcHisB expression vector (Invitrogen, Burlington, Ontario, Canada). For the pollen gene, a region beginning with amino acid 105 through to the 3' end (Fig. 1B) of the coding region was cloned into the expression vector. Plasmids were transformed into *E. coli* (strain BL21(DE3)) which was induced to express the protein (following the manufacturer's protocol).

Three styles from a single flower were ground on ice in 20 μ L of phosphate-buffered saline (pH 7.4). Loading buffer (54% glycerol, 8% sodium dodecylsulfate, 25% mercaptoethanol, 0.024% bromophenol blue) was added to each sample, vortexed briefly, heated in a boiling water bath for 3 min, and centrifuged at 13 000 g for 5 min. The extracts and a molecular weight ladder (Kaleidoscope or Precision marker proteins; BIO-RAD, Mississauga, Ontario, Canada), were run using discontinuous (5% stacking, 10% resolving) sodium dodecylsulfate-polyacrylamide gels (Hames and Rickwood, 1987). Proteins were stacked at 50 V and then resolved at 140 V. Separated proteins were transferred electrophoretically (approximately 16 h at 4°C, 30 V, followed by 1 h at 100 V), in Towbin-buffered saline (25 mmol/L Tris-HCl, 192 mmol/L glycine, 20% methanol, pH 8.3), to 0.2 μ m Immuno-blot polyvinylidene difluoride (PVDF) membranes (BIO-RAD), according to manufacturer's instructions.

Crude extracts of pollen, using all anthers from a single flower, were run on IEF gels (Athanasiou and Shore, 1997). Following electrophoretic separation, the pollen proteins were transferred electrophoretically in 0.7% acetic acid to PVDF membranes (overnight at 30 V).

Immunostaining of proteins transferred to PVDF membranes was according to Riggs and Hasenkampf (1991), with minor modification. Membranes were first blocked for 30 min and then incubated with a 1 : 2000 dilution of the primary antibody (2 h) in tris-buffered saline containing 0.05% Tween 20, pH 7.5 (TBST). After three 10-min washes with TBST, the membranes were incubated with a 1 : 10000 dilution of the secondary antibody (monoclonal goat anti-rabbit antibody conjugated to alkaline phosphatase) in TBST (1 h). Following three additional washes in TBST, the membranes were incubated

in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBP-BCIP) at 37°C in the dark. All immunostaining procedures, with the exception of the final colorimetric reaction, were carried out with gentle agitation.

Immunocytochemistry—Styles were vacuum-infiltrated for 45 min and fixed for an additional 4 h in (3 : 1) ethanol : glacial acetic acid. Styles were dehydrated through a graded series of ethanol : tertiary butyl alcohol (TBA) and were finally equilibrated to 100% TBA and infiltrated with Tissueprep wax (Fisher, Nepean, Ontario, Canada) at 62°C. Wax-embedded styles were cross-sectioned to 7- μ m thickness. Sections were stored at 4°C for several weeks without any activity loss. Sections were expanded by floating at 37°C in double-distilled water (ddH₂O) and placed on Biobond-coated (Cedarlane, Hornby, Ontario, Canada) glass slides. Slides were then placed on a warming tray at 35°C (overnight) to adhere the sections to the slides. Slides were passed through two 15-min changes of histoclear (Sigma, Oakville, Ontario, Canada), followed by washes in a graded series of ethanol and ddH₂O ending with ddH₂O and a final wash in buffer (Tris-HCl-NaCl, 100 mmol/L Tris, 120 mmol/L NaCl, 30 min). Sections were blocked for 30 min by incubation in 200 μ L of blocking solution (normal goat serum diluted 1/20 in wash buffer). Blocking solution was shaken gently from the slides, which were then incubated with primary antibody diluted 1/100 in wash buffer. Sections were washed for 3 \times 10 min and then incubated with secondary antibody (CY3-conjugated affinipure goat anti-rabbit IgG, H+L; Jackson ImmunoResearch, West Grove, Pennsylvania, USA), for immunodetection. Sections were washed again, and aqueous mounting medium (ProLong Antifade Kit, Molecular Probes, Eugene, Oregon, USA) was applied. Sections were viewed and photographed using a Lietz Dialux UV fluorescence microscope and appropriate filters (TRITC/Dil filters, exciter: D540/25, emitter: D605/55, beam-splitter: 565dcp; Chroma Technology, Brattleboro, Vermont, USA).

Linkage analysis—We mapped four loci using a test cross between a long- and a short-styled plant. The long-styled plant was from *T. subulata*. The short-styled plant was of hybrid origin, from a backcross between a short-styled plant of *T. subulata* and a hybrid plant we had produced from a cross between *T. subulata* and *T. krapovickasii*. The hybrid plant carried a somatic mutation that made it self-compatible and phenotypically homostyled, having both long stamens and long styles (F. Tamari and J. S. Shore, unpublished data).

The short-styled plant was heterozygous at four loci including Aconitase-1 (*Aco-1*, heterozygous for the *Aco-1M* and *Aco-1S* alleles), cytosolic 6-phosphogluconate dehydrogenase (*Pgd-c*, heterozygous for the *Pgd-cF* and *Pgd-cS* alleles, Athanasiou and Shore [1997]), *Distyly* (*SS**, in which *S** is the mutant allele conferring the homostyled phenotype in *S*S** and *S*s* genotypes, while the dominant *S* allele yields the short-styled phenotype; F. Tamari and J. S. Shore [unpublished data]), and two *TsPG* alleles (of the style PG gene) were identified by segregation of a cleaved amplified polymorphism (CAP marker, described later). The other parental plant was long-styled and homozygous at all four loci (homozygous for the *Aco-1F* allele, *Pgd-cS* allele, *s* at *Distyly*, and homozygous at *TsPG*). The *distyly* locus, *Aco-1*, and *Pgd-c* were all known to be linked (Athanasiou and Shore, 1997).

We amplified a 419-bp region of *TsPG* from genomic DNA of the parental plants and 169 progeny using the primers 5'-CAGTACTTCCATAGAACCTCAA-3' and 5'-GCCCTGTTAGTCCCAAGATT-3' to detect alleles of the style PG gene. The 419-base pair (bp) region was gel purified and digested with *KpnI* and separated on 1.5% agarose gels containing ethidium bromide (0.5 μ g/mL). The homozygous long-styled parent possessed a single *KpnI* restriction site cutting the amplified DNA into two fragments of 92 bp and 327 bp. This allele was derived from *T. subulata*. The short-styled parent was heterozygous possessing one allele yielding the 92-bp and 327-bp fragments, while the other allele (derived from *T. krapovickasii*) possessed an additional *KpnI* restriction site resulting in the cleavage of the 327-bp fragment into two additional fragments of approximately equal size. Recombination frequencies and their standard errors among pairs of loci were determined using maximum-likelihood estimation.

A Style polygalacturonase

		Signal peptide
TsPG	1	MKTGGFNKKSLLTMLLIALCLWC ^{SR} FETCOAREGKYWTQSEAGPATSALV
GmPG	1	MKNMNMKLNIALIIATAFVWSS---SCTAARVSHWRKLAAS-----
TsPG	51	KKENGYGGNDGRRLSKFMGDMWNGEAQATQQYGKPS ^S FNVL ^{DY} GAKGDGH
GmPG	43	-----ATSFNVL ^{DY} GAKGDGH
TsPG	101	TDDTKAWTDAG---KVARSKILVPAGSVFFVGPV ^S FLGSGCGANIELQL
GmPG	59	ADDTKAFEDAWAAACKVEGSTMVVP ^S SGSVFLV ^{KPI} S ^S SGPNCEPNIVFQL
TsPG	147	DGTILAPVSSKIWGSGLLOWIEFKELNGFTTQGGKTIDGRGSV ^W W ^N -DLP
GmPG	109	DGKIIAPT ^S SEAWGSGTLQWLE ^F SKLNTITIRGKGVIDGQGSV ^W W ^N NDSP
TsPG	196	SYGSEWESEVGNQLDGKMPSTKPTALRFYGSTGVTVTGITIQNSPQTHLK
GmPG	159	TYN--PTEVMLESNGRLPSTKPTALRFYGS ^D GVTVTGITIQNSQ ^Q THLK
TsPG	246	FDSCTGVQVSDFTTSS ^{FP} NSPNTDGIHLQNSQNVLIYSSDLACGDDCIS ^I
GmPG	206	FDSCTNVQVSGISVSS ^{FP} GDSPNTDGIHLQNSQNVVIYSSTLACGDDCVS ^I
TsPG	296	QTGCSNVFIHN ^V NCGPGHG ^S ISIGGLGKDN ^T KACVSNVTVRDVSMHNTLTG
GmPG	256	QTGCSDIYVHN ^V NCGPGHG ^S ISIGSLGRENTKACVRNVTVRDVTIQNTLTG
TsPG	346	VRIKTWQGGSGSVQGITFSNVQVSGVGTPI ^M IDQFYCDGSKCKNDSSAVA
GmPG	306	VRIKTWQGGSGSVQIN ^M FSNVQVSGVQTPISIDQYYCDGGRCRNESSAVA
TsPG	396	LTGINYLGIKGTYTEKPIHFACSDSMPCVGVSLSTIELTASEED---SPP
GmPG	356	VSGIHVNVKGTYYTKEPIYFACSDNLPCSGITLDTIQLES ^A QETKNSNVF
TsPG	443	LCWNAYGTVKTTTLPV ^D CLRTGKSRYPTEC-----
GmPG	406	FCWEAYGELKTTTVPV ^E CLQRGNPSKEGGINSNIDSC

B Pollen polygalacturonase

		Signal peptide
TsPP	1	M---VLGVEISAI ^S LLLLLASSASAQ-DGV ^P DIKKYNPKADIAEALSAAF
SgPP	1	MGLKVVSSA ^I ISFSL ^L LL ^L LASTAKA ^Q NSGV ^F VDVTKY ^G CKEDITEALNNAW
TsPP	47	QRDACASATPSKVVV ^P AGEYTMGPVDMKGPCKSTVEVQVDG ^N LKAPAE ^{PK}
SgPP	51	-KDACASTNPSKVLIPSGTYS ^L RQVTLAGPCKAA ^I ELQVNGILKAPV ^{NP} D
TsPP	97	GE ^P GP--WVSFEYIDGLTFSGKGVFDGQGHIAWANN ^C NKNPNCKSFPL ^N F
SgPP	100	QFSGSHWV ^N FRYIDQLT ^L SGSGTFDQGNVAWSK ^T CSK ^N KNCEGLPM ^N I
TsPP	145	RFTSLKHALIKDLTSKDSKNFHV ^N VINCENVT ^F QNFVIDAPAESL ^N TDGI
SgPP	150	RFDFITNGLVRDITTRDSKNFHV ^N VLGCKN ^L TFQHFTVTAPAES ^I NTDGI
TsPP	195	HMGRSKGIKI ^I DSKIGTGDDCISIGDGTEQVTVTGVT ^C GP ^H G ^S ISIGSLG
SgPP	200	HIGRSTGIY ^I LDSKIGTGDDCISVGDGTEELHVTGVT ^C GP ^H G ^S ISVGS ^L G
TsPP	245	RYDNEQPVRGVLVKNCILKNTDNGVRIKSWPAMK ^G GEASDIHFEDITMEN
SgPP	250	RYPNEKPVSGIFVKNCTISNTANGVRIKSW ^P DLYGGV ^A SNMHPEDIV ^M NN
TsPP	295	VTNPVIDQ ^E YCPWNQCTKDAPSKVKISNV ^S FKNIKGT ^N TPEAVK ^I ICS
SgPP	300	VQNPILLDQVYCPWNQCSLKAPSKVKIS ^D V ^S FKNIRGTSATPVV ^K LACS
TsPP	345	SALPCEQVQLNGIDLKYTG ^T QGP ^A KSECKNAKLTVTGH-----
SgPP	350	SGIPCEKVELANINLLYSGSEGPAKSQCSNVKPKISGIMSASGC

Fig. 1. Amino acid sequence alignments of the polygalacturonases from *Turnera subulata*. (A) Short style polygalacturonase (TsPG) aligned against a polygalacturonase from *Glycine max* (GmPG). (B) Short pollen polygalacturonase (TsPP) aligned against a polygalacturonase from *Salix gigliana* (SgPP). Solid bars above the sequences indicate the four amino acid sequences obtained directly from protein fractionation and sequencing (the five dots above the bars in [B] indicate differences between the peptide and deduced amino acid sequence). The four boxes indicate highly conserved domains common to plant polygalacturonases. A line above the beginning of each sequence indicates the predicted signal peptide.

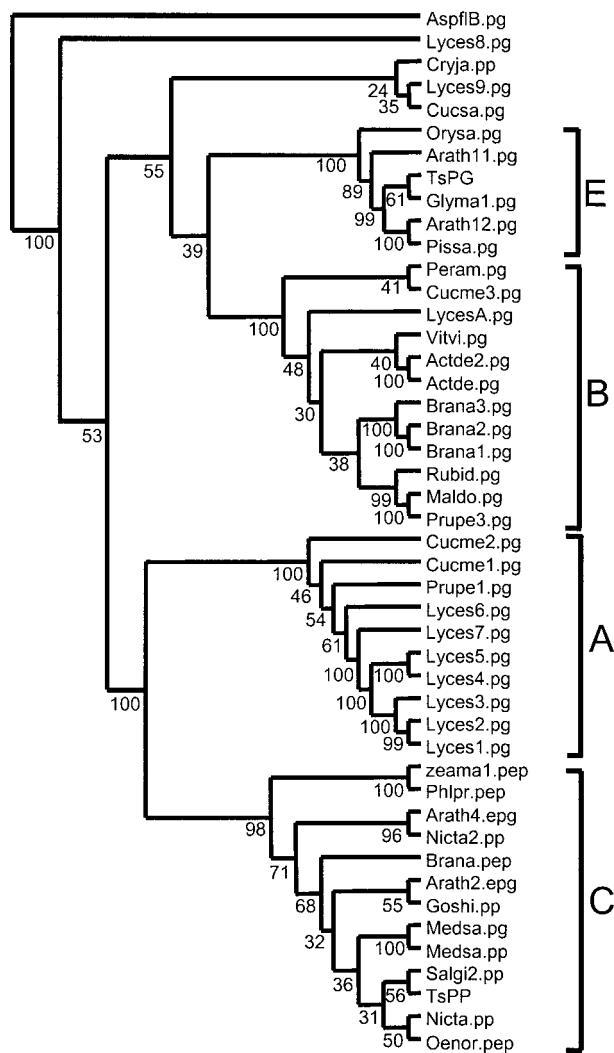


Fig. 2. Gene tree of plant polygalacturonases (PGs) based upon protein parsimony analysis. The numbers adjacent to each node represent the percentage of bootstrap samples in which that particular node occurred. Four clades (A, B, C, E) of PGs are indicated following Markovič and Janeček (2001).

RESULTS

Athanasiou and Shore (1997) used nondenaturing isoelectric focusing (IEF) to discover proteins specific to styles or pollen of the short-styled morph of *Turnera subulata*, *T. scabra*, and *T. krapovickasii*. The style PG protein sequence (Fig. 1A) is predicted to have 473 amino acid residues, with a molecular mass of approximately 50.4 kD and a pI of 6.6. Without the 31-amino-acid-predicted signal peptide (Nielsen et al., 1997), the molecular mass would be 46.9 kD and pI 6.0. The predicted pI is in close correspondence with the pIs of 6.1–6.5 of the style proteins reported in Athanasiou and Shore (1997). The identity between the amino acid sequence inferred from cDNA and those based upon the four peptide sequences we obtained indicates that we have amplified and sequenced the gene corresponding to the style protein discovered by Athanasiou and Shore (1997). The style amino acid sequence contains four domains highly conserved among plant polygalacturonases (Torki et al., 2000) and appears most similar to a

PG from *Glycine max* (Mahalingam et al., 1999) with 59.2% identity at the amino acid level (Fig. 1A).

Remarkably, the short-specific pollen protein also appears to be a polygalacturonase based upon its sequence similarity and possession of conserved amino acid sequences around the putative active site (Torki et al., 2000; Fig. 1B). The pollen PG has 61% identity to a PG from *Salix gigliana* (Fig. 1B). The pollen PG has a predicted molecular mass of 40.8 kD and a pI of 6.6 or 6.8 with or without the predicted signal peptide (Nielsen et al., 1997; Fig. 1B), in agreement with an earlier report (Athanasiou and Shore, 1997).

Phylogenetic analysis—A gene tree of plant polygalacturonases (Fig. 2) places the style PG (TsPG) in clade E and the pollen PG (TsPP) in clade C, based on previous analyses of plant polygalacturonases (Hadfield et al., 1998; Hong and Tucker, 2000; Torki et al., 2000; Markovič and Janeček, 2001). The style PG is closely allied with PGs of *G. max* (Glyma1.pg, Fig. 2; Mahalingam et al., 1999), Arath11.pg (Fig. 2; Torki et al., 2000), and Arath12.pg (Fig. 2; Torki et al., 2000), both of *Arabidopsis thaliana* (Appendix, <http://ajbsupp.botany.org/v90>). Interestingly, the style PG does not cluster with any of the pistil-specific PGs of *Lycopersicon esculentum* (Lyces1.pg, Lyces2.pg, Lyces4.pg, Lyces7.pg, Fig. 2, Appendix [<http://ajbsupp.botany.org/v90>]; Hong and Tucker, 2000). The pollen PG is in a clade with PGs of dioecious *S. gigliana* (Salgi2.pp, male flower-specific) and many pollen-specific PGs (Fig. 2, Appendix [<http://ajbsupp.botany.org/v90>]; Markovič and Janeček, 2001). Analyses using distance and maximum-likelihood methods also resulted in placement of the style PG and pollen PG in the clades detailed earlier.

Immunoblotting and immunocytochemistry—Immunoblotting of proteins was carried out for both the style and pollen proteins (Fig. 3). Using antibodies against the style PG fusion protein, we revealed a protein of approximately 35 kD in styles of short-styled plants, but not in the long-styled (Fig. 3A–B). We made antibodies to the style PG in two different rabbits and both show that the 35-kD protein is specific to styles of short-styled plants (Fig. 3A–B). One antibody source also shows a 120-kD protein that occurs in both morphs (Fig. 3B). The protein is not morph- or tissue-specific and occurs in all floral and vegetative tissues we have examined (D. Khosravi, F. Tamari, and J. S. Shore, unpublished data). This 120-kD protein does, however, provide a convenient internal marker for protein loading levels.

We have analyzed styles from 58 short-styled plants of two species (*T. scabra*, $n = 33$; *T. subulata*, $n = 25$), and all possessed the 35-kD protein, while none of the 59 long-styled plants did (*T. scabra*, $n = 33$; *T. subulata*, $n = 26$). The 35-kD style protein also occurs in styles of short-styled plants of all five species within the series *Turnera* (series *Canaligeræ* Urb.) of the genus *Turnera* that we have investigated (Tamari, 2001).

Immunocytochemical analyses indicate that the style PG is in the transmitting tissue of short styles and in tissues within the stigma of short- but not long-styled plants (Fig. 4). In additional surveys, we have shown that this pattern of staining appears only in styles of short-styled plants for all five species of series *Turnera* that we have investigated (Khosravi, 2000). Some staining of vascular tissue is also apparent in the style sections of both morphs (Fig. 4).

Isoelectric focusing immunoblotting of the pollen PG re-

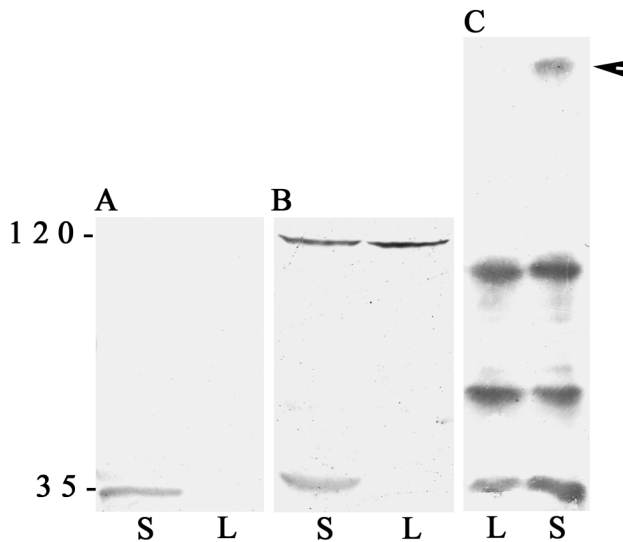


Fig. 3. Immunoblots of style and pollen polygalacturonases (PGs) of *Turnera subulata* (Lane S, extracts of styles or pollen of short-styled, and Lane L, long-styled plants). (A) SDS PAGE immunoblot of the short-specific style PG, approximate molecular mass of 35 kD. (B) As in A, but using a second source of antibody showing the 35-kD PG as well as a 120-kD protein that is neither morph- or organ-specific, but provides an internal control. (C) Isoelectric focusing immunoblot of pollen PGs. The protein initially identified as short specific is indicated by the arrow. A number of proteins are common to both pollen of short- and long-styled plants.

vealed a number of protein bands common to pollen of both long- and short-styled plants (Fig. 3C). Pollen of short-styled plants possessed the pI 6.8 protein band initially described by Athanasiou and Shore (1997). More recent studies of two species have revealed that 23 of 27 short-styled plants of *T. scabra* possess the pI 6.8 protein, while none of the 26 long-styled plants possessed it (F. Tamari and J. S. Shore, unpublished data). For *T. subulata*, all 40 short-styled plants possessed the pI 6.8 protein, while one of 36 long-styled plants showed weak staining of this protein band (F. Tamari and J. S. Shore, unpublished data).

Linkage analysis—A cleaved amplified polymorphism in the style PG gene that was segregating in a test cross we had made was identified. We mapped the style PG gene in this cross relative to the position of the distyly locus and two flanking isozyme loci (Athanasiou and Shore, 1997). Single locus segregation ratios at all four loci in this cross are comparably distorted (Table 1; F. Tamari and J. S. Shore, unpublished data) and show a considerable excess of progeny carrying alleles derived from the *T. subulata* recurrent parental species (all ratios are approximately 3.6 : 1). These distorted ratios were common in crosses into the *T. subulata* genetic background (but not into *T. krapovickasii* background). Despite these distorted ratios, recombination events occurred on both chromosomes, and the map of *Aco-1*, *Distyly*, and *Pgd-c* is comparable to one obtained previously (Athanasiou and Shore, 1997). The mapping reveals that the style PG gene, while linked to the distyly locus, is 4.6 cM distal to it, lying outside the *Pgd-c* marker locus (Fig. 5). This finding indicates that although the style PG gene is linked to the distyly locus, it is perhaps not linked closely enough to be considered a component of a supergene.

DISCUSSION

We have shown that both proteins discovered to be specific to styles or pollen of short-styled plants (Athanasiou and Shore, 1997) are divergent polygalacturonases based upon their sequence homology to known polygalacturonases. Determination of their activity in vivo and in vitro using biochemical methods should aid in clarifying their roles in nature. The pollen PG identified may not be involved in distyly. A wider analysis of species within the genus *Turnera*, series *Turnera*, led to the finding of short-styled plants of *T. krapovickasii*, *T. joelii*, and *T. grandiflora* that did not possess this specific band of pI 6.8 (Tamari, 2001). The pollen PG gene might be very closely linked and in extreme linkage disequilibrium with the *S* allele of the distyly locus in *T. scabra* and *T. subulata*. The pollen PG is likely a member of a small multigene family based upon banding patterns on IEF immunoblots having multiple bands, most of which are shared by long-styled plants (Fig. 3C). Small multigene families of highly homologous pollen PGs occur in both monocots and dicots (Brown and Crouch, 1990; Allen and Lonsdale, 1992; Hadfield and Bennett, 1998; Toriki et al., 1999). Furthermore, we note that five amino acid differences in the sequence deduced from sequencing cDNA vs. the peptide sequences occur (Fig. 1B), suggesting that we may have cloned and sequenced a different member of a putative multigene family. The affinity purification of antibodies recognizing specifically the pI 6.8 PG may aid in clarifying the discrepancy among sequences. Currently, we suggest that while the pollen PG is likely involved in the reproductive process (Hadfield and Bennett, 1998), we do not have sufficient evidence to claim that it plays a role in distyly.

In contrast, the style PG is morph-specific, style-specific, localized to the style transmitting and stigmatic tissues, and appears 24 h prior to anthesis (Athanasiou and Shore, 1997), coinciding with the time at which an SI system should begin to be functional. Furthermore, the style PG is not possessed by the five self-compatible long-homostyled species we have investigated, including *T. aurelii*, *T. cuneiformis*, *T. orientalis*, *T. ulmifolia*, and *T. velutina* (Khosravi, 2000; Tamari, 2001). A self-compatible long-homostyle somatic mutant that arose on a branch of an otherwise short-styled plant also lacks the style PG (F. Tamari, D. Khosravi, and J. S. Shore, unpublished data). These features strongly indicate that the style PG plays a role in distyly.

The SDS-PAGE analysis of the style PG revealed a discrepancy between the observed 35-kD molecular mass based upon immunoblotting (Fig. 3A–B) vs. the predicted 46.9-kD molecular mass based upon sequence data (Fig. 1A). This finding indicates that a second peptide, in addition to the signal peptide, may be cleaved from the immature style PG. An acidic prosequence peptide is known to be cleaved from a PG of *L. esculentum* (DellaPenna and Bennett, 1988). The occurrence of prosequences has been proposed for *Cucumis melo* and all members of clade B (Fig. 2; DellaPenna and Bennett, 1988; Hadfield and Bennett, 1998). Post-translational processing of the C-terminus is also known to occur for some plant PGs (Hadfield and Bennett, 1998), which could account for the discrepancy in observed vs. predicted molecular mass. Recently, we have used two-dimensional gel electrophoresis, immunoblotting, and peptide sequencing using a mass spectrometer to provide evidence that a 10-kD protein is homologous to a portion of the N-terminal region of the style polygalacturonase (D. Khosravi, unpublished data). This provides further

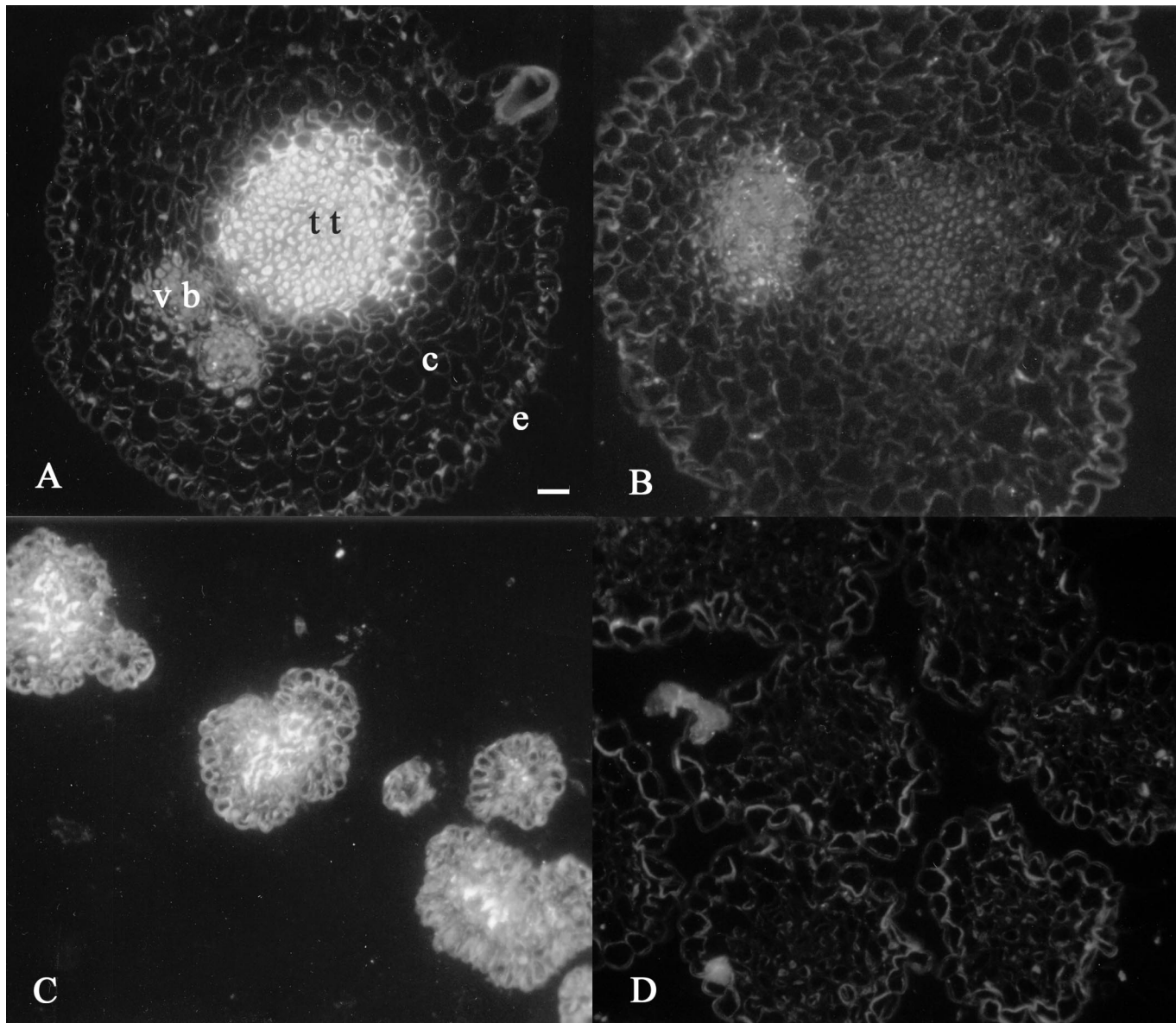


Fig. 4. Immunocytochemical staining of style (A, B) and stigma (C, D) cross sections, using antibodies made against the style polygalacturonase. Scale bar is 0.07 mm. (A) The transmitting tissue (tt) is heavily stained in this short style section. Vascular bundles (vb) show some staining, but there is no staining of the cortex (c) or epidermis (e). (B) Cross section of long style with some staining of the vascular bundle but no staining of transmitting or other tissues. (C) Staining of cells in a cross section of a short stigma. (D) Lack of staining in cross section of a long stigma.

TABLE 1. Single locus segregation ratios for four linked loci. Ratios were tested against the expected 1 : 1 backcross segregation using the G statistic for goodness of fit. A heterogeneity G statistic was used to compare segregation distortion among the loci. *TsPGS* and *TsPGK* represent two alleles of the style polygalacturonase, identified by CAP analysis, in which the *TsPGS* allele is derived from *Turnera subulata*, while the *TsPGK* allele is from *T. krapovickasii*.

Locus	Progeny ratio	G , df = 1
<i>Aco-1</i>	129 <i>Aco-1F/Aco-1S</i> : 40 <i>Aco-1F/Aco-1M</i>	49.3***
<i>Distyly</i>	133 short : 36 homostyle	59.2***
<i>Pgd-c</i>	134 <i>Pgd-cF/Pgd-cS</i> : 35 <i>Pgd-cS/Pgd-cS</i>	61.8***
<i>TsPG</i>	129 <i>TsPGS/TsPGS</i> : 40 <i>TsPGS/TsPGK</i>	49.3***

Note: $G_{\text{heterogeneity}} = 0.7$, df = 3, $P > 0.05$.

*** $P < 0.001$.

support for the possibility that a prosequence is cleaved from the immature PG polypeptide.

Interestingly, the style PG appears to belong to a relatively recently identified class of PGs (Markovič and Janeček, 2001; Fig. 2). The only known sites of expression of these PGs are in *Glycine max* roots in response to nematode attack (Mahalingam et al., 1999) and in *A. thaliana*, in which it is expressed

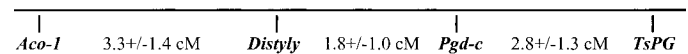


Fig. 5. Genetic map of loci linked to the distyly locus (*Distyly*). Numbers between loci indicate the map distances in centimorgans (cM) and standard errors. *Aco-1*, aconitase-1; *Pgd-c*, cytosolic 6-phosphogluconate dehydrogenase; *TsPG*, short-specific style polygalacturonase gene.

in roots and seedlings (Torki et al., 2000). The occurrence of this class of PG in style transmitting tissue appears to be novel.

While PGs are involved in cell growth processes (Hadfield and Bennett, 1998), the style PG is unlikely to determine the length of the short style, but it may play a role in the SI system, given its restriction to the transmitting tissue. That role, however, is uncertain. We suggest that the style PG may operate in at least one of three ways: First, it may operate in an oppositional manner preventing self-pollen tube growth in short-styled plants. The style PG may break down pollen tube walls, leaving open the question of how pollen from long-styled plants remains impervious to this enzymatic activity. In support of this possibility, Tamari et al. (2001) demonstrated an asymmetry in the lengths of pollen tubes; pollen tubes from short-styled plants were generally inhibited in the stigma. Furthermore, because callose plugs do not form during self-pollination of short-styled plants, these pollen tubes might be inhibited very soon after germination and penetration of the stigma (Tamari et al., 2001). Second, the style polygalacturonase may operate in a complementary manner, enabling pollen from long-styled plants to grow through the styles of short-styled plants. Under this model, incompatibility of short-styled plants would have to be determined by another, as yet unidentified, protein(s). Finally, oligogalacturonides, which are products of the action of PGs, are known to be active elicitors of plant defenses (Hadfield and Bennett, 1998; Mahalingam et al., 1999), leading to the possibility that they may be involved in signalling SI responses of short-styled plants.

Self-incompatibility proteins in flowering plants are diverse and include functionally unrelated proteins (Anderson et al., 1986; Nasrallah et al., 1987; Foote et al., 1994). Polygalacturonases have not been shown to be SI proteins but are known to enhance and suppress rates of pollen tube growth in vitro, depending on their concentration (Roggen and Stanley, 1969). Proof of the involvement of the style PG in the SI system or in some other aspect of distyly will need to be obtained, perhaps using antisense strategies coupled with functional assays of polygalacturonase activity on pollen tube growth in vitro.

Studies of inheritance and compatibility relationships in the *Turnera ulmifolia* complex (Shore and Barrett, 1985; Barrett and Shore, 1987; Tamari et al., 2001) are consistent with the possibility that distyly may be determined by a supergene. A direct test of this hypothesis involves the discovery and mapping of the genes determining distyly. Athanasiou and Shore (1997) found no evidence for recombination between the style PG gene and the distyly locus and estimated that the recombination frequency must be less than 0.87% (0.87 cM). They could not, however, discount the possibility that the style PG protein exhibited morph-limited (short-limited) expression rather than extremely tight linkage. To distinguish between these hypotheses, we have mapped the style PG gene using a cleaved amplified polymorphism (CAP marker), showing that the style PG is 4.6 cM distal to the distyly locus (Fig. 5). This finding indicates that style PG gene is not a component of a supergene but rather its expression is morph-limited and may be upregulated by the dominant *S* allele of the distyly locus.

With a recombination frequency of 4.6% between the distyly locus and the style PG gene, any initial linkage disequilibrium between the style PG gene and the *S* allele of distyly would have decayed rapidly. Thus, the complete association between the presence of the style PG in short-styled plants from across a number of Central and South American populations, two ploidy levels and two species, and its absence

from long-styled plants (Athanasiou and Shore, 1997) cannot be explained by linkage disequilibrium. Furthermore, we have shown that this relationship holds in three additional species, *T. krapovickasii*, *T. joelii*, and *T. grandiflora* (Khosravi, 2000; Tamari, 2001). Finally, Athanasiou (2001) produced two long-styled plants that were homozygous recombinants at the *Pgd-c* marker locus, which implies they were also homozygous for the style PG gene linked to the *S* allele of distyly. Athanasiou (2001) did not detect the presence of the style PG protein in these long-styled plants, nor did Athanasiou and Shore (1997) detect the style PG protein in any of the 13 *Pgd-c* recombinant long-styled plants, at least half of which should be expected to possess the style PG protein. Thus, we believe this lack of expression of the style PG (derived from short-styled plants) in recombinant long-styled plants supports the possibility that the *S* allele of distyly upregulates the expression of the style PG gene. If so, this report is the first example of a gene regulated by the distyly locus and indicates that in *T. subulata* the distyly locus (or supergene) possesses a regulatory capacity.

The occurrence of genes with morph-limited expression is consistent with a model of the evolution of distyly by Lloyd and Webb (1992). The population genetic model of Charlesworth and Charlesworth (1979) is predicated upon the occurrence of a supergene, because of linkage constraints involved in the establishment of the polymorphism. Interestingly, the style PG gene we have mapped is sufficiently closely linked to the distyly locus to have been one of the SI genes initially driven to increase in frequency under the model of Charlesworth and Charlesworth (1979). What is unexpected about the gene is its apparent morph-limited expression. A detailed analysis of the molecular genetics of distyly in *Turnera* spp., as well as in species where distyly has evolved independently, should provide a means of distinguishing among, and/or in refining, these and other evolutionary models.

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