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ALLELIC VARIATION FOR A SHORT-SPECIFIC POLYGALACTURONASE IN *TURNERA SUBULATA*: IS IT ASSOCIATED WITH THE DEGREE OF SELF-COMPATIBILITY?

Farshad Tamari[1] and Joel S. Shore[2]

Department of Biology, York University, 4700 Keele Street, Toronto, Ontario M3J 1P3, Canada

We demonstrate the occurrence of allelic variation at the *TSPG* locus that encodes a polygalacturonase localized to the transmitting tissue of styles of short-styled plants of *Turnera* spp. Using DNA sequencing, we infer and compare amino acid sequences of two alleles of *TSPG* and show that they differ by an amino acid substitution in the putative mature polygalacturonase, resulting in different predicted isoelectric points (pI). The predicted pI differences approximate those estimated using isoelectric focusing. An assay of F_2 progeny reveals a complete correspondence between allelic variation at the *TSPG* locus, as detected by a cleaved amplified polymorphism, and the pI profile of the polygalacturonase. We determine whether there is an association between allelic variation at *TSPG* and the strength of self-incompatibility by generating F_2 progeny segregating for the two alleles of *TSPG*, and we also introduce variation for self-compatibility into the family by ensuring that one of the parental plants is self-compatible. We screen 138 F_2 progeny for their degree of self-compatibility (selfed seed set) and genotype at the *TSPG* locus. The results of two replicate experiments indicate that the strength of self-compatibility is weakly associated with the *TSPG* genotype of the F_2 progeny, but we cannot conclude that *TSPG* is necessarily causal.

Keywords: *Turnera*, distyly, self-incompatibility, polygalacturonase, isoelectric focusing gel electrophoresis, cleaved amplified polymorphic DNA.

Introduction

Distyly is a genetic polymorphism characterized by the occurrence of two approximately equally frequent floral morphs (long- and short-styled) having reciprocal arrangements of reproductive organs as well as ancillary dimorphisms in pollen size and stigma morphology (Ganders 1979; Dulberger 1992; Richards 1997). The morphs usually possess a dimorphic self-incompatibility system (Ganders 1979; Barrett 1992). While the Mendelian inheritance of distyly has been known for some time and in diverse species (Ornduff 1979; Lewis and Jones 1992), little is known of the molecular genetic basis of distyly in any species. Recent research efforts are focusing on this lacuna (Athanasiou et al. 2003; Khosravi et al. 2004; Miljuš-Đukic et al. 2004; Yasui et al. 2004; Manfield et al. 2005).

Distyly, including all the characters included in the floral syndrome, is determined by a single locus (Ganders 1979; Lewis and Jones 1992). The dominant *S* allele usually determines the short-styled phenotype, while the homozygous recessive genotype *ss* gives rise to long-styled plants (Shore and Barrett 1985; Lewis and Jones 1992; Richards 1997). Evidence from *Primula* spp. indicates that the *S* locus might be a gene complex composed of a number of tightly linked genes (Ernst 1955; Dowrick 1956; Lewis and Jones 1992; Kurian and Richards 1997). Studies of the inheritance of homostyly in *Turnera* are also consistent with this model (Shore and Barrett 1985; Barrett and Shore 1987; Tamari et al. 2005).

Recent studies of *Turnera* spp. have led to the discovery of candidate proteins that might be involved in distyly (Athanasiou and Shore 1997; Athanasiou et al. 2003; Khosravi et al. 2004). One such protein is a polygalacturonase specific to the transmitting tissue of short-styled plants in all six distylous species of *Turnera* (in the taxonomic series Turnera) investigated (Athanasiou et al. 2003; Khosravi et al. 2003; Tamari and Shore 2004). This polygalacturonase is not found in long-styled plants or in long-homostyled (self-compatible plants with long styles and long stamens) species or mutants (Khosravi et al. 2003; Tamari and Shore 2004). Evidence from linkage analysis revealed that while the gene encoding the style polygalacturonase (*TSPG*) is linked to the *distyly* locus, it is 4.6 cM distal to it, lying outside a flanking cytosolic 6-phosphogluconate dehydrogenase (*Pgd-c*) isozyme marker locus (Athanasiou et al. 2003). Athanasiou et al. (2003) suggested that the dominant *S* allele of *distyly* likely upregulates the expression of the polygalacturonase in short-styled plants because long-styled plants carry the allele but do not possess the protein. The function of the polygalacturonase in distyly, if any, remains unknown.

A transformation system is not available for *Turnera*, and therefore investigations aimed at finding direct evidence for the involvement of the polygalacturonase in distyly cannot yet be performed. One indirect approach to exploring the

[1] Current address: Department of Human Genetics, Mount Sinai School of Medicine, 1425 Madison Avenue, Box 1498, New York, New York 10029, U.S.A.

[2] Author for correspondence; phone 416-736-2100, ext. 33492; fax 416-736-5698; e-mail shore@yorku.ca.

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function of the polygalacturonase is to discover naturally occurring alleles of *TSPG* and explore their phenotypic effects. This is the approach we pursue in this article.

Both long- and short-styled plants of *Turnera* typically possess a rigid self-incompatibility system, but partially self-compatible plants can be found (Shore and Barrett 1986; Tamari et al. 2001). In this article, we exploit the occurrence of a self-compatible short-styled plant (designated Bry) of *Turnera subulata* (Shore and Barrett 1986; Athanasiou and Shore 1997) as well as a long-styled plant (SL8-1L) derived from a second short-styled plant (SL8-18S) that exhibits a different isoelectric point (pI) profile of the style polygalacturonase (Athanasiou and Shore 1997; Khosravi et al. 2003; Tamari and Shore 2004). More specifically, we (1) show that the short-specific polygalacturonase from SL8-18S has a lower pI than that observed for other plants of *T. subulata* and *Turnera scabra*, (2) provide evidence that the pI variant is encoded by an allele of *TSPG*, (3) infer the amino acid substitution responsible for the change in pI of the polygalacturonase in the plant SL8-18S, and (4) explore the function of the polygalacturonase in distyly by looking for an association between the genotype of F_2 progeny at the *TSPG* locus and the degree of self-compatibility.

Material and Methods

A limited number of plants of *Turnera subulata* Smith (Turneraceae) were available for this investigation. We exploited two plants originally sampled from different populations of *T. subulata*. It was necessary to use a long-styled plant from the lineage of the short-styled plant SL8-18S because the pI variant of the polygalacturonase has only been detected in this single short-styled plant and its progeny. SL8-18S is a somewhat self-compatible short-styled plant (see below), and we have no information as to whether its self-compatibility can be transmitted through its long-styled progeny. Long-styled plants obtained by selfing SL8-18S are thoroughly self-incompatible.

It was also important to use the short-styled plant, termed Bry, because it possesses the high-pI polygalacturonase, it is self-compatible, and its self-compatibility is transmitted to its short-styled progeny (Shore and Barrett 1986; Athanasiou and Shore 1997). It was necessary to introduce self-compatibility into the F_2 progeny in order to carry out this investigation in which we look for an association between the degree of self-compatibility and the *TSPG* genotype of progeny (see below).

We carried out a set of crosses to generate the required F_2 progeny (fig. 1). The short-styled plant SL8-18S was selfed, and a number of progeny were grown. We chose a single long-styled plant, SL8-1L, from among these selfed progeny and used it in further crosses. We ensured, using gel electrophoresis, that SL8-1L carried alternative alleles of isozyme marker loci, aconitase-1 (*Aco-1*) and cytosolic 6-phosphogluconate dehydrogenase (*Pgd-c*), that lie on either side of the *distyly* locus (Athanasiou and Shore 1997; Athanasiou et al. 2003). We then crossed SL8-1L to the short-styled plant Bry. We assayed a number of progeny and selected one self-compatible short-styled plant that was heterozygous at both of the flanking isozyme marker loci. This plant, termed SB2, was the F_1 parent used in generating F_2 progeny. We selfed SB2 to produce an F_2 family of 138 progeny.

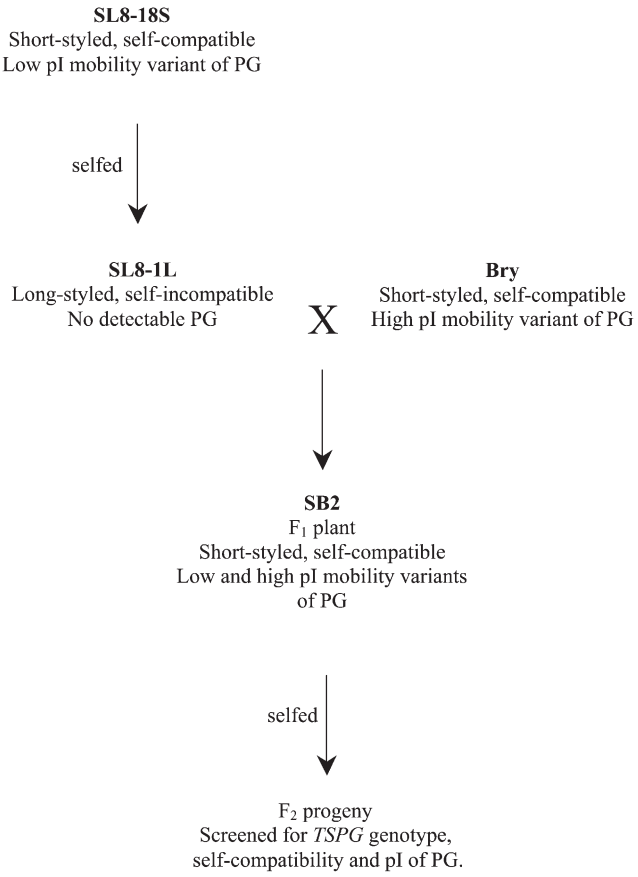

Fig. 1 Schematic including parental plants and crosses performed to produce 138 F_2 progeny used for this investigation. The morph (long- or short-styled), genotype at *TSPG*, and the isoelectric points (pI) of the polygalacturonase (PG) are provided.

Protein Electrophoresis

Details of starch gel electrophoresis and isozyme assays follow the methods in Shore and Barrett (1987), while SDS-PAGE, isoelectric focusing (IEF) gel electrophoresis, electrophoretic transfer of proteins, and immunoblotting follow those in Tamari and Shore (2004). Briefly, SDS-PAGE and immunoblotting, using a polyclonal antibody raised against the style polygalacturonase, were performed to detect the short-specific polygalacturonase in style extracts, following methods of Athanasiou et al. (2003). Styles were extracted on ice in 20 μL of phosphate-buffered saline (PBS, pH = 7.4). We added 5 μL of loading buffer to the extracts and then heated the samples in a boiling water bath (3 min) and centrifuged them at 13,000 *g* for 5 min. Approximately 20 μL of supernatant was loaded on gels for electrophoretic separation, and one of two protein ladders was run on each gel (kaleidoscope or precision marker proteins [Bio-Rad]). Samples were subjected to SDS-PAGE (discontinuous gel system, C = 5%, resolving C = 10%, MINI-PROTEAN gel system, Bio-Rad), initially at 50 V (stacking) and then at 140 V

(resolving), for electrophoretic separation. Electrophoretic transfer of proteins to 0.2-μm polyvinylidene difluoride (PVDF, Bio-Rad) membranes was conducted overnight at 30 V, then at 100 V for 1 h, in Towbin buffered saline. The immunostaining procedure is described below.

IEF gel electrophoresis and immunoblotting were used to detect pI polymorphisms for the style polygalacturonase. IEF Ready Precast Gels (Ampholine PAGplate, pH 5.5–8.5, T = 5%, C = 3%, Amersham Pharmacia) were prefocused at 10°–12°C for 1 h at 12 W, using 0.4 M HEPES buffer at the anodal end and 1 M NaOH buffer at the cathodal end (applied with the supplied wicks). Nine short styles or four long styles (to balance total protein loading levels) for each sample were extracted on ice in 30 μL of PBS (pH = 7.4) and applied to the gel using precut Miracloth wicks. Protein separation was conducted at 3 W for ca. 1.5 h. Electrophoretic transfer to PVDF membranes was conducted overnight at 30 V in a 0.7% (v/v) acetic acid solution (direction of transfer was from anode to cathode). Immunostaining was performed as described below. We ran pI standards (Broad Range pI 4.45–9.6 IEF standards, Bio-Rad) on IEF gels to estimate the pI of protein bands.

Details of the immunostaining protocol may be found in Tamari and Shore (2004). Membranes were incubated in Tris-buffered saline (TBS) containing 0.05% Tween20 (TBST, pH = 7.5) (30 min), followed by incubation with a 1/4000–1/2000 dilution of the 1° antibody in TBST (120 min). The membrane was washed in TBST (3×10 min) and then incubated (1 hr) with a 1/10,000 dilution of the 2° antibody (monoclonal goat antirabbit antibody conjugated to alkaline phosphatase, Sigma). Three additional 10-min washes with TBST were performed, and the membrane was then bathed in the colorimetric substrate solution (at 37°C, no agitation) 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBP-BCIP). Immunostaining procedures were performed using gentle agitation at room temperature.

Inheritance and Linkage Analysis

We followed the simultaneous segregation of alleles at two isozyme loci (*Aco-1* and *Pgd-c*), the *distyly* locus, and the *TSPG* locus in 138 F_2 progeny. The genotypes of the parental (Bry and SL8-1L) and F_1 plants (SB2) are as follows:

Bry: $TSPG^{B}Pgd\text{-}c^{F}S\ Aco\text{-}1^{S}/TSPG^{B}Pgd\text{-}c^{S}s\ Aco\text{-}1^{F}$;
SL8-1L: $TSPG^{S}Pgd\text{-}c^{S}s\ Aco\text{-}1^{M'}/TSPG^{S}Pgd\text{-}c^{S}\ s\ Aco\text{-}1^{M'}$;
SB2: $TSPG^{S}Pgd\text{-}c^{S}s\ Aco\text{-}1^{M'}/TSPG^{B}Pgd\text{-}c^{F}S\ Aco\text{-}1^{S}$.

Pgd-c and *Aco-1* are two isozyme loci flanking the *distyly* locus. The superscripts *F*, *M′*, and *S* specify whether the alleles encode fast-, intermediate-, or slow-migrating forms of these enzymes, respectively. Note that the $Aco\text{-}1^{M'}$ allele migrates slightly more slowly than the $Aco\text{-}1^{M}$ allele of *Turnera krapovickasii* studied by Athanasiou et al. (2003). Alleles segregating at the *distyly* locus are referred to as *S* or *s*. $TSPG^{B}$ and $TSPG^{S}$ refer to alleles of the style polygalacturonase gene (*TSPG*) derived from the plants Bry and SL8-18S, respectively. All 138 F_2 progeny (fig. 1) were assayed for their genotypes at the isozyme and *TSPG* loci (see below). IEF gel electrophoresis was performed to determine the polygalacturonase phenotypes (i.e., pI profiles) for 38 of these progeny.

To distinguish alleles of the *TSPG* locus, we used a cleaved amplified polymorphism (CAP), following Athanasiou et al. (2003). We sequenced and compared *TSPG* from both Bry (GenBank accession AY185765) and SL8-18S to identify a restriction site polymorphism. We sequenced both strands of *TSPG*, using cycle sequencing on an AB1373A sequencer (Applied Biosystems, Foster City, CA) at the York University Molecular Biology Core Facility.

Once a polymorphism in *TSPG* was identified, we extracted DNA from all progeny, following Doyle and Doyle (1987). We used the following primers to amplify a portion of the *TSPG* gene from all progeny: forward, 5′-GCCCCTGTTAGTTCCAAGATT-3′, and reverse, 5′-TCAAAGCAACAGCTGAAGA-3′. PCR was performed using the JumpStart ReadyMix REDTaq DNA polymerase (Sigma). A Mastercycler Gradient thermal cycler (Eppendorf) was used for all amplifications. The cycle parameters were as follows: initial denaturation at 94°C (2 min), followed by 35 cycles of 94°C (30 s), 55°C (30 s), 72°C (2 min), and a final extension at 72°C (5 min). After amplification, the samples were run on ethidium bromide–stained agarose gels (1%, TAE system). DNA bands were gel purified using the QIAquick Gel Extraction Kit (QIAGEN).

DNA sequence data indicated that there is a *ScaI* restriction site difference between DNA sequences of *TSPG* from Bry and those from SL8-18S and SL8-1L. We digested the gel-purified DNA with *ScaI* (following the manufacturer's protocol). The digested DNA samples were run on 1% agarose gels (as above), and progeny were scored for their genotype at the *TSPG* locus.

The *G* statistic for goodness of fit was used to compare observed and expected single-locus ratios for the four loci investigated. Two-locus ratios were used to test for linkage, and maximum likelihood estimates of recombination frequencies and their standard errors were obtained (Allard 1956; Athanasiou and Shore 1997). A genetic map was constructed using Kosambi's mapping function (JoinMap 3.0; Stam 1993).

Degree of Self-Compatibility

To assess the degree of self-compatibility of progeny and parental plants, we conducted a controlled pollination experiment under glasshouse conditions, where short-styled F_2 progeny with different *TSPG* genotypes were self-pollinated 10–13 times and crossed to a long-styled plant 10–13 times. The experiment was conducted in a paired manner whereby on each day one flower from each plant was selfed and a second flower was crossed using compatible pollen from a long-styled plant. The number of seeds per pollination was counted 2–3 wk after the pollinations were performed.

In the first experiment, we chose 10 short-styled plants of each of the three *TSPG* genotypes for pollination. After several weeks, we repeated the entire experiment a second time, using a greater number of short-styled plants (although we were limited to only 10 recombinant $TSPG^{S}/TSPG^{S}$ plants). We analyzed the results of both experiments separately, using a one-way analysis of variance (ANOVA) comparing the genotypic means. We first calculated the mean seed set of each plant based on 10–13 replicate pollinations and then used these means in the one-way ANOVA. The main effect term (the *TSPG* genotype effect) and its associated *F* value and

probability from this analysis are the same as the main effect (the *TSPG* genotype effect), *F* value, and probability from a nested ANOVA in which the sources of variation were genotype and plants within genotype. We used a one-way ANOVA because we were most interested in the effect of genotype on seed set and not that on plant variation within genotype. We also analyzed the difference between the paired selfed and compatible seed sets in a similar analysis, to control for daily environmental variation and differences in plant vigor. Thus, for each plant, we first calculated the difference between selfed and crossed seed sets for each pair of flowers pollinated on a particular day and on a particular plant. We then calculated the mean of the paired differences for each plant and conducted the one-way ANOVA as above. Analyses were conducted using SAS (SAS Institute 1996).

Results

Inheritance and Linkage Analysis

Single-locus segregation ratios do not differ statistically from Mendelian expectations for any of the loci studied, including *Aco-1* ($G_{2df} = 2.66$ [ns]), *Pgd-c* ($G_{2df} = 3.47$ [ns]), *TSPG* ($G_{2df} = 3.08$ [ns]), and *distyly* ($G_{1df} = 0.81$ [ns]). We estimated recombination frequencies between all pairs of loci and tested two-locus segregation ratios, all of which deviate from those expected under independent assortment (table 1). A linkage map based on these data was constructed (fig. 2).

pI Polymorphism and CAPs

IEF, followed by immunoblotting, revealed differences in the pIs of the style polygalacturonase from two short-styled plants of *Turnera subulata* (Bry and SL8-18S) (fig. 3). Bry has two bands with pIs of ca. 6.5 and 6.3 (fig. 3, lane 3), while SL8-18S has two bands with pIs of ca. 6.3 and 6.0 (fig. 3, lane 4). The F_1 plant, SB2, has all three bands, covering the range of pIs exhibited by the parental plants (fig. 3, lane 1).

ScaI digestion of the amplified region of the *TSPG* gene from Bry yields three fragments of ca. 550, 415, and 200 bp (designated the $TSPG^B$ allele), while the same region of the gene from SL8-1L yields two fragments of 750 and 415 bp (designated the $TSPG^S$ allele). Using this CAP, we can readily distinguish three genotypes among the F_2 progeny, $TSPG^S$/$TSPG^S$, $TSPG^S$/$TSPG^B$, and $TSPG^B$/$TSPG^B$ (fig. 4). The ratios of these genotypes do not deviate statistically from the expected 1:2:1 segregation ratio (above).

Table 1

Goodness-of-Fit Tests for Two-Locus Segregation Ratios, Recombination Frequencies (*r*), and Their Standard Errors (SE)

Locus pair	*G*	*r*	SE
Pgd-c, *Aco-1*	157.9^{***}	0.080	0.017
Aco-1, *TSPG*	122.9^{***}	0.124	0.021
Pgd-c, *TSPG*	190.0^{***}	0.048	0.013
Distyly, *Pgd-c*	109.6^{***}	0.043	0.018
Distyly, *Aco-1*	122.6^{***}	0.023	0.013
Distyly, *TSPG*	75.2^{***}	0.110	0.028

Note. Expected ratios for the codominant pairs of loci were 1 : 2 : 1 : 2 : 4 : 2 : 1 : 2 : 1, while those involving the *distyly* locus were tested against 3 : 6 : 3 : 1 : 2 : 1 ratios.

*** $P < 0.001$.

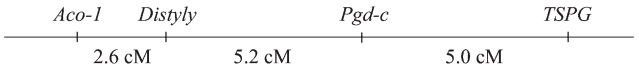

Fig. 2 Genetic map of four loci including *Aco-1*, *Distyly*, *Pgd-c*, and *TPSG*. Map distances (cM) were estimated using Kosambi's mapping function (JoinMap 3.0; Stam 1993).

In the absence of recombination between the *distyly* and *TSPG* loci, long-styled plants should have the SL8-1L parental genotype ($TSPG^S$/$TSPG^S$). Twenty-seven of the 30 long-styled progeny were of this genotype; however, we recovered three recombinant long-styled plants that were genotypically $TSPG^B$/$TSPG^S$. Likewise, in the absence of recombination, short-styled plants should have one of two genotypes at the *TSPG* locus ($TSPG^B$/$TSPG^B$ or $TSPG^S$/$TSPG^B$). While most short-styled progeny were nonrecombinants (we obtained 26 $TSPG^B$/$TSPG^B$ and 70 $TSPG^S$/$TSPG^B$ genotypes), we recovered a number of recombinants having the $TSPG^S$/$TSPG^S$ genotype (fig. 4, lanes 5, 6).

We assayed 38 of the F_2 progeny, using IEF gel electrophoresis and immunoblotting, to verify the association between allelic variation at *TSPG* as detected by CAP and the pI profile of the polygalacturonase. Long-styled plants do not possess the polygalacturonase, independent of their *TSPG* genotype (table 2; fig. 5), and so they provide no information. Of the 33 short-styled progeny assayed, the results show a complete correspondence between the genotype at *TSPG* and the pI profile of the style polygalacturonase (table 2). The *TSPG* genotypes derived from Bry ($TSPG^B$/$TSPG^B$) always show the IEF profile of Bry (e.g., fig. 3, lane 3), while the *TSPG* genotypes derived from the SL8-18S lineage ($TSPG^S$/$TSPG^S$) always show the IEF profile of SL8-18S (e.g., fig. 3, lane 4). Finally, *TSPG* heterozygotes ($TSPG^S$/$TSPG^B$) show the three-banded phenotype of the F_1 parent (e.g., fig. 3, lanes 1, 2).

Does TSPG *Alone Result in the Presence of the Polygalacturonase?*

The occurrence of long recombinants for *TSPG* (i.e., long-styled progeny that are genotypically $TSPG^B$/$TSPG^S$) is of interest because it illustrates that the $TSPG^B$ allele originally derived from the short-styled plant, Bry (in which *TSPG* is "coupled" to the *S* allele of the *distyly* locus), is not expressed in long-styled progeny (fig. 5, lanes 2–4). Likewise, short-styled recombinants homozygous for the $TSPG^S$ allele originally derived from the long-styled parental plant SL8-1L (in which *TSPG* is linked to the recessive *s* allele of the *distyly* locus) do express the style polygalacturonase (e.g., fig. 5, lane 9). All nine short-styled plants examined that were $TSPG^S$/$TSPG^S$ homozygotes possessed the polygalacturonase. This indicates that the presence of the polygalaturonase gene (*TSPG*) alone does not result in the expression of the polygalacturonase but that its expression only occurs in the presence of the dominant *S* allele of distyly.

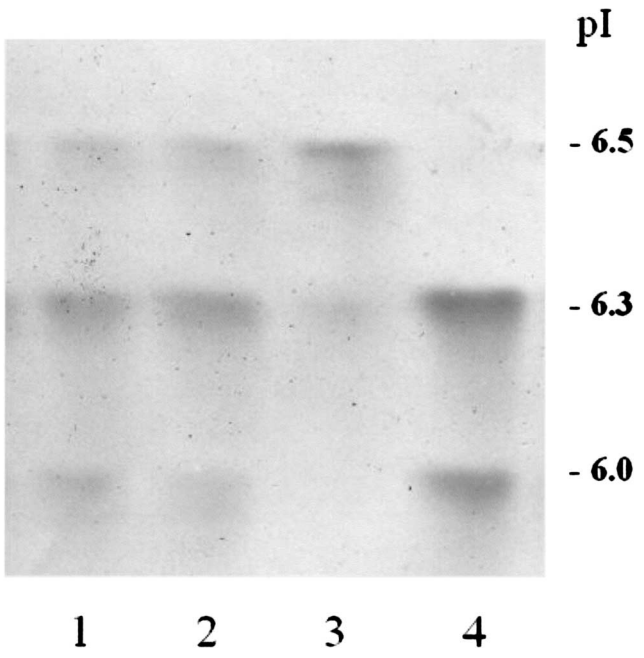

Fig. 3 Isoelectric focusing immunoblot of the style polygalacturonase. Lanes 1 and 2 show three-banded patterns for the F_1 parent and F_2 progeny that are $TSPG^S/TSPG^B$; lane 3 shows a two-banded pattern of the parental plant Bry and F_2 progeny that are $TSPG^B/TSPG^B$; lane 4 shows the two-banded pattern of the parental plant SL8-18S and F_2 progeny that are $TSPG^S/TSPG^S$. The approximate isoelectric points (pI) are indicated.

Sequence Comparison

To determine the cause of the observed differences in the pI of the polygalacturonase, we PCR-amplified and sequenced *TSPG* from both SL8-18S and Bry. A total of 22 nucleotide substitutions occur between the sequences of *TSPG* (the sequence of SL8-18S will be deposited in GenBank). Sixteen of the substitutions occur in introns and six in exons, but only two result in amino acid substitutions. One amino acid substitution occurs at position 60 (aspartic acid in Bry vs. histidine in the SL8-18S sequence) and lies in a putative cleaved prosequence region of the protein. The second amino acid substitution occurs at position 386 (lysine in Bry vs. isoleucine in the SL8-18S sequence) and lies in the putative mature region of the polygalacturonase (fig. 6).

The putative propeptide of SL8-18S is expected to shift to a greater pI than that of Bry, based on the inferred amino acid sequences ($pI_{Bry} = 5.17$, $pI_{SL8\text{-}18S} = 5.59$). This putative propeptide, however, lies below the pH range of the IEF gels used and cannot be resolved. In contrast, the pIs of putative mature polygalacturonases, based on the inferred amino acid sequences, result in a lower predicted pI for the SL8-18S sequence than for that of Bry ($pI_{Bry} = 6.74$, $pI_{SL8\text{-}18S} = 6.51$). While the absolute magnitudes of the pIs differ from those estimated on IEF gels (fig. 3; table 2) using pI marker proteins (presumably because of imprecision in estimating pI based on the pI ladder), the predicted difference between the two polygalacturonases (ca. 0.2 pI units) is close to the estimated difference and in the predicted direction (i.e., the pI is lower in SL8-18S than in Bry).

Self-Compatibility and TSPG *Genotype*

One of the parental plants used in this study (Bry) is a self-compatible short-styled plant, while the other is a self-incompatible long-styled plant (SL8-1L). SL8-1L was obtained by selfing another self-compatible short-styled plant (SL8-18S). We do not know whether the self-compatibility of SL8-18S can be transmitted to short-styled progeny through its self-incompatible long-styled progeny. We nevertheless determined whether the short-styled parental plants differed in selfed seed set. Bry sets more seeds after self-pollination than the SL8-18S parental short-styled plant or SB2, the F_1 parent (table 3). Compatible seed set also differs among these plants, with Bry setting ca. 10 more seeds per pollination than either of the other plants (table 3). The ANOVA results based on paired differences between selfed and compatible pollinations, as a further control for daily environmental variation and/or differences in plant vigor, indicated that there is no significant difference among the plants (table 3). The long-styled parental plant (SL8-1L) was thoroughly self-incompatible, as were long-styled F_2 progeny of SB2 and long-styled selfed progeny of Bry. Long-styled progeny were not included in the analyses described below because they are uninformative (i.e., they do not possess the polygalacturonase in their styles).

To explore the potential effects of allelic variation at the *TSPG* locus on self-compatibility, we self-pollinated F_2 progeny of all three *TSPG* genotypes and counted the number of seeds set. We report the results of two replicate experiments, both of which show comparable results (table 4). In the first experiment, a one-way ANOVA of plant means revealed marginally significant differences in seed set after selfing of

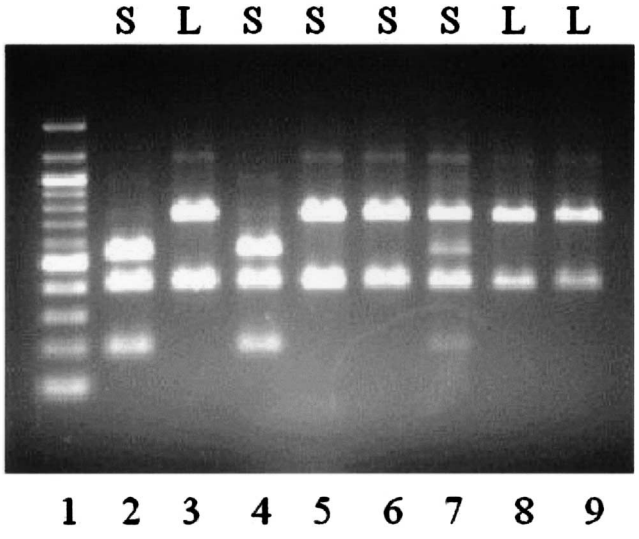

Fig. 4 Segregation of a cleaved amplified polymorphism in F_2 progeny: agarose gel of *ScaI*-digested amplicons of the *TSPG* locus. Lane 1, 100-bp ladder; lane 2, Bry (parental plant); lanes 3–9, F_2 progeny. Lanes 2, 4, $TSPG^B/TSPG^B$; lanes 3, 5, 6, 8, 9, $TSPG^S/TSPG^S$; lane 7, $TSPG^S/TSPG^B$.

Table 2

Correspondence between the Isoelectric Focusing (IEF) Profile (Isoelectric Points [pI] of Each Protein Band) of the Style Polygalacturonase and the Genotype at the *TSPG* Locus Based on a Cleaved Amplified Polymorphism

Plants	*TSPG* genotype	*N*	IEF phenotype (pI)
Bry (parent)	$TSPG^B/TSPG^B$	1	6.5, 6.3
SL8-18S (parent)	$TSPG^S/TSPG^S$	1	6.3, 6.0
SL8-1L (parent)	$TSPG^S/TSPG^S$	1	No bands
SB2 (F_1 plant)	$TSPG^S/TSPG^B$	1	6.5, 6.3, 6.0
F_2 short-styled	$TSPG^B/TSPG^B$	10	6.5, 6.3
F_2 short-styled	$TSPG^S/TSPG^B$	11	6.5, 6.3, 6.0
F_2 short-styled	$TSPG^S/TSPG^S$	12	6.30, 6.0
F_2 long-styled	$TSPG^S/TSPG^S$	3	No bands
F_2 long-styled	$TSPG^S/TSPG^B$	2	No bands

Note. N = number of plants.

F_2 progeny as a function of their *TSPG* genotype. While there were no significant differences in seed set after compatible cross pollinations (the control), an analysis of the mean paired differences in seed set (selfed minus compatible) was statistically significant. $TSPG^S/TSPG^S$ genotypes showed the largest selfed seed set (and the smallest difference between selfed and compatible seed sets), followed by the $TSPG^S/TSPG^B$ and then the $TSPG^B/TSPG^B$ genotype, which had the smallest selfed seed set and the largest difference between selfed and compatible seed set (table 4). Results for the second experiment were comparable. Mean selfed seed set and the difference in seed set were both statistically significant and showed the same ranking of genotypic means (table 4).

Discussion

Despite more than a century of investigations of the genetics and evolution of distyly (Barrett 1992), there have been few attempts at uncovering its molecular basis (Golynskaya et al. 1976; Shivanna et al. 1981; Wong et al. 1994; Athanasiou and Shore 1997; Athanasiou et al. 2003; Khosravi et al. 2003; Miljuš-Đukic et al. 2004; Tamari and Shore 2004; Yasui et al. 2004; Manfield et al. 2005). Ongoing studies of *Turnera* species have led to the discovery of candidate proteins, including both a short-style-specific polygalacturonase (Athanasiou et al. 2003) and a short-style-specific α-dioxygenase (Khosravi et al. 2004). The role that these proteins play in distyly and the associated self-incompatibility, if any, is unknown. At present, we do not have a transformation system for *Turnera* spp., and so antisense strategies to explore the function of these genes cannot be undertaken. Here we take an indirect approach to find evidence for or against the involvement of the polygalacturonase in self-incompatibility. We first detect allelic variation at the locus encoding the polygalacturonase (*TSPG*) and then determine whether that variation influences the degree of self-compatibility.

The occurrence of the polygalacturonase in only one morph (the short-styled morph) and its localization to the style transmitting tissue would seem to support a role in the self-incompatibility system of short-styled plants (Athanasiou et al. 2003). This also raises the possibility that self-incompatibility of long-styled plants might involve a different mechanism. Interestingly, sites of pollen tube inhibition and the phenotype of selfed pollen tubes differ between the morphs, further supporting this possibility (Tamari et al. 2001).

Linkage Relationships

We confirmed the linkage relationships among the *distyly* locus, the *TSPG* locus, and the isozyme loci (*Pgd-c* and *Aco-1*) that were first reported in Athanasiou et al. (2003). We felt that it was important to verify the map because Athanasiou et al. (2003) constructed a map by introgressing genes from *Turnera krapovickasii* into *Turnera subulata*, and their data showed highly aberrant morph ratios. We obtained the same map order for the loci as reported previously (fig. 2). Our estimates of recombination frequencies between the *distyly* locus and the flanking isozyme loci (*Pgd-c* and *Aco-1*) were comparable to those obtained in a large F_2 family of *T. subulata* (Athanasiou and Shore 1997). Our estimate of the recombination frequency between the *TSPG* locus and the *distyly* locus is greater (0.110 ± 0.028 vs. 0.047 ± 0.016) than that obtained by Athanasiou et al. (2003). We used the maximum likelihood method of Allard (1956) to determine whether these estimates are statistically different. The pooled data from both studies yielded a recombination frequency of 0.074 ± 0.015 between the *distyly* locus and the *TSPG* locus. A test of heterogeneity revealed that there is a statistically significant difference between recombination frequencies ($\chi^2 = 4.11$, df = 1, $P < 0.043$). A more detailed study using a reciprocal backcross has revealed greater recombination rates when the female parent is heterozygous (Tamari et al. 2005; J. D. J. Labonne and J. S. Shore, unpublished data).

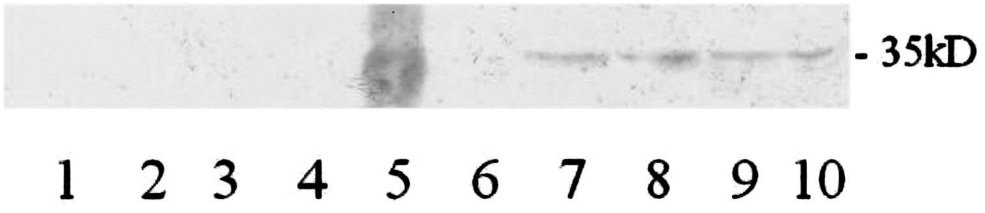

Fig. 5 SDS-PAGE immunoblot to detect the style polygalacturonase in styles of F_2 progeny. Lane 1, long-styled $TSPG^S/TSPG^S$; lanes 2–4, long-styled $TSPG^S/TSPG^B$; lane 5, protein ladder (not well resolved); lane 6, long-styled $TSPG^S/TSPG^S$; lane 7, short-styled $TSPG^B/TSPG^B$; lane 8, short-styled $TSPG^S/TSPG^B$; lane 9, short-styled $TSPG^S/TSPG^S$; lane 10: short-styled $TSPG^B/TSPG^B$. The molecular mass of the polygalacturonase is indicated.

```
BRY   MKTGGFNKKSLTLMILIALCLWCSRFETCQAREGKYWTQSEAGPATSALVKKENGYGGND 60
SL8   MKTGGFNKKSLTLMILIALCLWCSRFETCQAREGKYWTQSEAGPATSALVKKENGYGGNH 60
                                                                  *

BRY   GRRLSKFMGDMWNGEAQATQQYGKPSSFNVLDYGAKGDGHTDDTKAWTDAGKVARSKILV 120
SL8   GRRLSKFMGDMWNGEAQATQQYGKPSSFNVLDYGAKGDGHTDDTKAWTDAGKVARSKILV 120

BRY   PAGSVFFVGPVSFLGSGCGANIELQLDGTILAPVSSKIWGSGLLQWIEFKELNGFTIQGK 180
SL8   PAGSVFFVGPVSFLGSGCGANIELQLDGTILAPVSSKIWGSGLLQWIEFKELNGFTIQGK 180

BRY   GTIDGRGSVWWNDLPSYGSEWESEVGNQLDGKMPSTKPTALRFYGSTGVTVTGITIQNSP 240
SL8   GTIDGRGSVWWNDLPSYGSEWESEVGNQLDGKMPSTKPTALRFYGSTGVTVTGITIQNSP 240

BRY   QTHLKFDSCTGVQVSDFTTSSPGNSPNTDGIHLQNSQNVLIYSSDLACGDDCISIQTGCS 300
SL8   QTHLKFDSCTGVQVSDFTTSSPGNSPNTDGIHLQNSQNVLIYSSDLACGDDCISIQTGCS 300

BRY   NVFIHNVNCGPGHGISIGGLGKDNTKACVSNVTVRDVSMHNTLTGVRIKTWQGGSGSVQG 360
SL8   NVFIHNVNCGPGHGISIGGLGKDNTKACVSNVTVRDVSMHNTLTGVRIKTWQGGSGSVQG 360

BRY   ITFSNVQVSGVGTPIMIDQFYCDGSKCKNDSSAVALTGINYLGIKGTYTEKPIHFACSDS 420
SL8   ITFSNVQVSGVGTPIMIDQFYCDGSICKNDSSAVALTGINYLGIKGTYTEKPIHFACSDS 420
                           *

BRY   MPCVGVSLSTIELTASEEDSPPLCWNAYGTVKTTTLPPVDCLRTGKSRYPTEC 473
SL8   MPCVGVSLSTIELTASEEDSPPLCWNAYGTVKTTTLPPVDCLRTGKSRYPTEC 473
```

Fig. 6 Amino acid sequence alignment of the style polygalacturonase of Bry and SL8-18S (*SL8*). The predicted signal peptide is indicated by a line above the sequences. The shaded sequence is a putative cleaved prosequence, while the unshaded region corresponds to the predicted mature polygalacturonase. The two amino acid substitutions are indicated in bold print and marked by asterisks.

Cause of pI Variation

Athanasiou and Shore (1997) reported what they believed to be a short-styled plant (SL8-18S) of *T. subulata* that did not possess a short-style-specific protein, on the basis of IEF gel electrophoresis and total protein staining. In later work using SDS-PAGE and antibodies made against the short-style-specific polygalacturonase, it was shown that the styles of that plant did indeed possess the polygalacturonase (Khosravi et al. 2003; Tamari and Shore 2004).

Here we show that the plant SL8-18S possesses the polygalacturonase and that it has a reduced pI compared with other plants of *T. subulata* and *Turnera scabra* previously investigated (Athanasiou et al. 2003; Tamari and Shore 2004). Interestingly, pI profiles of short-styled plants show two bands when stained using antibodies against the style polygalacturonase on nondenaturing IEF gels. We do not know why the profiles possess two bands, but we speculate that differential posttranslational modification (e.g., phosphorylation) of the polygalacturonase might be responsible. The two protein bands do not appear to differ in molecular mass based on investigations using two-dimensional electrophoresis and immunoblotting (Khosravi 2004). By comparing inferred amino acid sequences of the polygalacturonase from both plants (Bry and SL8-18S), we show that a single amino acid substitution is apparently responsible for the difference and reduction in pI (fig. 6). While a second amino acid substitution also occurs, it is located in a region of the protein that falls within a putative prosequence that is cleaved from mature polygalacturonase. Khosravi (2004), using N terminal sequencing and mass spectrometry, has identified the location of the N terminal of the mature polygalacturonase (fig. 6) and, through two-dimensional electrophoresis and amino acid sequencing, regions of a cleaved prosequence. Prosequences are known to be cleaved from two polygalacturonases that have been investigated in other species (DellaPenna and Bennett 1988; Dal Degan et al. 2001). The function of these prosequences is unknown.

Table 3

Mean Seed Set (SD) after Self-Pollination and Compatible Pollination of Parental (Bry, SL8-18S) and F_1 (SB2) Plants

			Mean seed set		
Maternal Plant	*TSPG* genotype	*N*	Self-pollination	Compatible pollination	Mean difference
Bry	$TSPG^B/TSPG^B$	12	16.8^A (7.8)	30.0^A (5.3)	−13.3 (9.3)
SL8-18S	$TSPG^S/TSPG^S$	12	7.3^B (4.3)	21.3^B (5.8)	−13.9 (6.1)
SB2	$TSPG^S/TSPG^B$	12	7.8^B (5.5)	20.0^B (10.5)	−12.3 (11.1)

Note. The means of the paired differences between self-pollination and compatible pollination were also analyzed. Means sharing the same superscript are not significantly different (Scheffé's test). N = number of flowers pollinated. One-way ANOVA results: self-pollination, $F_{2,33} = 9.27$, $P < 0.001$; compatible pollination, $F_{2,33} = 6.66$, $P < 0.004$; mean difference, $F_{2,33} = 0.10$, ns.

Table 4

Mean Seed Set per Pollination (SD) after Selfing and Crossing of F_2 Progeny for Each of Three Genotypes of the *TSPG* Locus

Genotype	*N*	Selfed	Compatible	Difference
Experiment 1:[a]				
$TSPG^S/TSPG^S$	10	7.1 (6.5)^A	15.3 (6.5)^A	−8.1 (4.9)^A
$TSPG^S/TSPG^B$	10	6.6 (3.5)^A	20.0 (5.0)^A	−13.4 (5.9)^{AB}
$TSPG^B/TSPG^B$	10	2.5 (1.7)^A	18.9 (6.0)^A	−16.4 (5.8)^B
Experiment 2:[b]				
$TSPG^S/TSPG^S$	10	9.7 (5.4)^A	14.0 (4.2)^A	−4.3 (4.2)^A
$TSPG^S/TSPG^B$	21	6.9 (4.8)^{AB}	18.4 (5.6)^A	−11.4 (7.4)^B
$TSPG^B/TSPG^B$	20	3.6 (3.7)^B	16.7 (5.8)^A	−13.1 (6.4)^B

Note. The paired differences between self-pollination and compatible pollination were also analyzed. Means sharing the same superscript do not differ significantly (Scheffé's test). Both replicates of the experiments are analyzed. N = number of plants.

[a] Results of one-way ANOVA based on mean seed set of each plant after replicate pollinations: selfed, $F_{2,27} = 3.29$, $P = 0.052$; compatible, $F_{2,27} = 1.78$, ns; difference, $F_{2,27} = 5.68$, $P < 0.01$.

[b] Results of one-way ANOVA based on mean seed set of each plant after replicate pollinations: selfed, $F_{2,48} = 6.48$, $P < 0.01$; compatible, $F_{2,48} = 2.18$, ns; difference, $F_{2,48} = 6.24$, $P < 0.01$.

Role of the Style Polygalacturonase

We have demonstrated the occurrence of two alleles of the *TSPG* locus and have exploited this polymorphism in a cross involving a self-compatible short-styled plant and a self-incompatible long-styled plant (derived from a different self-compatible short-styled lineage). The crosses performed allow us to ask whether or not the *TSPG* gene contributes to self-incompatibility/self-compatibility of the F_2 progeny. The results of two replicate experiments revealed small but significant differences in the numbers of seeds set after self-pollination as a function of the *TSPG* genotype (table 4). These results suggest that the style polygalacturonase may play some role in the self-incompatibility system of short-styled plants. Oddly, F_2 progeny bearing the genotype of the more highly self-compatible parental plant (Bry, $TSPG^B/TSPG^B$) exhibited a smaller selfed seed set than progeny bearing the genotype of the parental plant showing a lower degree of self-compatibility (SL18-18S, $TSPG^S/TSPG^S$), while heterozygotes were intermediate. We can think of no easy explanation for this occurrence, which warrants further investigation.

It is possible that genes linked to *TSPG* could be responsible for the observed association between the degree of self-compatibility and *TSPG* genotype, rather than the *TSPG* gene itself. To increase the power of our conclusions, a considerably greater number of progeny would have to be analyzed, coupled with an increased number of mapped molecular markers so that interval mapping can be used to rule out the possibility that linked genes, rather than the *TSPG* locus, are responsible for self-compatibility. A more detailed genetic analysis of self-compatibility of these plants would be required to fully elucidate the role of the style polygalacturonase in this system and/or the causes of self-compatibility. More direct methods, including the use of mutants and/or RNA interference to knock out expression of *TSPG*, may aid in clarifying the role of the style polygalacturonase. The role of a recently discovered short-style-specific α-dioxygenase also requires investigation (Khosravi et al. 2004).

Is TSPG *Regulated by the Distyly Locus?*

Athanasiou et al. (2003) suggested that the style polygalacturonase was upregulated by the *S* allele of *distyly*. To test this hypothesis, we assayed both long- and short-styled plants that exhibited recombination between the *distyly* and *TSPG* loci. We confirmed, using SDS-PAGE and immunoblotting, that long-styled plants do not express the style polygalacturonase even if the allele of *TSPG* they possess was originally derived from the chromosome bearing the *S* allele of *distyly*. Similarly, short-styled plants do express the style polygalacturonase even if homozygous for the *TPSG* allele originally derived from the chromosome bearing the *s* allele of distyly (fig. 5; table 2). Our results support the view of Athanasiou et al. (2003) that the dominant *S* allele of the *distyly* locus must upregulate the style polygalacturonase gene.

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