Distribution of style and pollen polygalacturonases among distylous and homostylous *Turnera* and *Piriqueta* spp. (Turneraceae)

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We explore the distribution of a style and pollen polygalacturonase in a number of distylous and homostylous species of *Turnera*, and two species of *Piriqueta* (Turneraceae). We show, using immunoblotting with antibodies made against these proteins, that the style polygalacturonase is specific to styles of short-styled plants of all the six distylous species of *Turnera* we have investigated. Styles of a somatic homostylous mutant derived from a short-styled plant do not possess the style polygalacturonase. Distylous *P. caroliniana* did not appear to possess this protein. We show that the pollen polygalacturonase, while associated with the short-styled morph in three species, is polymorphic among short-styled plants of *T. krapovickasii*, and absent from *T. joelii*,

T. grandiflora and *P. caroliniana*. These data support a role for the style polygalacturonase in distyly, possibly in the incompatibility system, but cast doubt on any role for the pollen polygalacturonase. In concert with the predictions for the mode of origin, and the response of styles of homostylous species to pollen from long- and short-styled plants, we find that none of the homostylous species possess the style polygalacturonase. The pollen polygalacturonase does occur in some homostylous species, but not in others. It is not clear that the pollen polygalacturonase, however, provides a marker for the mode of origin of homostyly.

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Introduction

While distyly has provided a useful model system in ecological genetics, a clear understanding of the genes/proteins involved and the mechanisms of incompatibility have yet to be determined. There have been no convincing demonstrations of the occurrence of self-incompatibility proteins in any heterostylous species, although some efforts have been made in this direction (Golynskaya *et al*, 1976; Shivanna *et al*, 1981; Wong *et al*, 1994; Athanasiou and Shore, 1997). *Turnera* spp provide a useful system for identifying genes and proteins involved in distyly and self-incompatibility, given the relative ease and speed with which plants may be crossed and brought to flowering, and the increasing amount of work on distyly in this genus (Shore and Barrett, 1985; Barrett and Shore, 1987; Athanasiou and Shore, 1997; Tamari *et al*, 2001; Athanasiou *et al*, 2003). Distyly in *Turnera scabra* and *T. subulata* is determined by a single diallelic locus, where short-styled plants are heterozygous, *Ss*, and long-styled plants are homozygous, *ss* (Shore and Barrett, 1985). Pollen tube studies reveal that there is an asymmetry in the sites of self-incompatibility among the morphs. Self pollen tubes of short-styled plants germinate and penetrate the stigma, rarely enter the style and do not possess callose plugs, while self pollen tubes of long-styled plants tend to

penetrate further into the style, and do possess callose plugs (Tamari *et al*, 2001). Tamari *et al* (2001) suggested that this asymmetry could indicate that the morphs possess different self-incompatibility mechanisms.

Athanasiou and Shore (1997) and Athanasiou *et al* (2003) provided evidence that proteins specific to the styles and pollen of short-styled plants of distylous *Turnera scabra* and *T. subulata* might be involved in distyly, possibly in the incompatibility system. Athanasiou *et al* (2003) showed that both proteins are polygalacturonases and that the style polygalacturonase is localized to the style-transmitting and stigmatic tissue. Furthermore, they showed that the style polygalacturonase was linked to, and approximately 4.6 cM distal to, the distyly locus. The map location of the pollen polygalacturonase is unknown, and Athanasiou *et al* (2003) suggest that it might belong to a small multigene family of pollen polygalacturonases.

In this paper, we explore in more detail the findings of Athanasiou and Shore (1997) and Athanasiou *et al* (2003), by using immunoblotting with polyclonal antibodies made against the short-specific pollen and style polygalacturonases. If these polygalacturonases are involved in distyly, then we would expect them to occur in short-styled plants of all distylous species of *Turnera*, and more widely within the Turneraceae, since it would seem unlikely that distyly and its incompatibility system would have evolved more than once within the genus and/or family. We survey additional species in the genus *Turnera* and extend the analysis to a *Piriqueta* species, a member of another large distylous genus in the Turneraceae. We also investigate the presence of these polygalacturonases in a self-compatible long-homostyle

somatic mutant that arose on a branch of a short-styled plant. We would expect the mutant to lack the style polygalacturonase, but to possess the pollen polygalacturonase.

While distyly is widespread within the Turneraceae, self-compatible homostylous species also occur (Barrett and Shore, 1987). Homostylous species within the series *Turnera* (= *Canaligerae* Urb.), appear to be long homostyles (having long styles and stamens). The homostylous *Turnera* species investigated are self-compatible, and exhibit inheritance patterns and incompatibility responses consistent with a recombinant origin of homostyly (Shore and Barrett, 1985; Barrett and Shore, 1987; Tamari *et al.*, 2001), as occurs in *Primula* (Ernst, 1955; Dowrick, 1956; Richards, 1997). If these self-compatible species, within the Turneraceae, are long homostyles that have arisen through recombination within the distyly locus, we would expect their styles to lack the short-specific style polygalacturonase, but their pollen should possess the short-specific pollen polygalacturonase. We test this hypothesis by investigating the distribution of the polygalacturonases in six homostylous species.

Materials and methods

We investigated the presence/absence of the polygalacturonases in the styles and pollen of 19 populations in 12 species of *Turnera*, and two species of *Piriqueta*. We used SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting to study the presence/absence of the style polygalacturonase. Isoelectric focusing (IEF) gel electrophoresis and immunoblotting were used to investigate the morph-specific pollen polygalacturonase. All procedures were carried out at room temperature, unless otherwise indicated.

Style polygalacturonase

Crude extracts of styles were subjected to SDS-PAGE (discontinuous gel system, stacking $C=5\%$, resolving $C=10\%$, following Hames and Rickwood (1987) with slight modifications). Three styles were ground on ice for each plant, in 20 μ l of phosphate-buffered saline (PBS, 0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4 and 0.024% KH_2PO_4 ; pH=7.4). Extracts were transferred to a microfuge tube containing 5 μ l of the loading buffer (54% glycerol (v/v), 8% SDS (w/v), 25% β -mercaptoethanol (v/v), 0.024% bromophenol blue (w/v)). Samples were vortexed briefly, heated in boiling water for 3 min, and centrifuged at $13\,000 \times g$ for 5 min. The supernatant (approx. 20 μ l of each sample), ladder (precision or kaleidoscope markers, BIO-RAD) and the style fusion protein (as a positive control) were loaded into the gels. As another positive control, on each gel, we ran extracts of styles and pollen of the short-styled plant BRY (*T. subulata* from Arco Verde, Brazil), from which the style and pollen polygalacturonase genes were sequenced. Production of fusion proteins and antibodies is detailed elsewhere (Athanasίου *et al.*, 2003).

Proteins were stacked at 50 V and separated at 140 V. The proteins were electrophoretically transferred, in Towbin-buffered saline (25 mM Tris, 192 mM glycine, 20% methanol, pH=8.3 using a mini-Trans-Blot cell, BIO-RAD), to 0.2 μ m Immuno-blot polyvinylidene difluoride (PVDF) membranes (BIO-RAD), according to the manufacturer's instructions. The transfer was done

overnight at 30 V followed by 100 V for 1 h, at 4°C. Immunoblotting was then carried out as described below.

Pollen polygalacturonase

IEF and immunoblotting were undertaken to investigate the pollen polygalacturonase. IEF Ready Precast Gels (Amersham-Pharmacia Biotech AB, Ampholine™ PAG plate, pH 5.5–8.5, $T=4\%$, $C=3\%$) were prefocused at 6 W for 1 h at 10–12°C, using 0.4 M HEPES and 1 M NaOH as the anodal and cathodal buffers, respectively. All the five anthers from each flower were extracted on ice, in 30 μ l of extraction buffer (0.05 M $\text{NaH}_2\text{C}_6\text{H}_5\text{O}_2$ titrated with an equal volume of 0.05 M Na_2HPO_4). Samples were applied using precut wicks of miracloth (CALBIOCHEM). Electrophoretic separation was conducted at 6 W for 1.5 h. Protein transfer to PVDF membranes was performed electrophoretically in 0.7% acetic acid overnight at 30 V. Immunoblotting was then carried out (see below).

Immunoblotting

Immunoblotting was performed following Riggs and Hasenkampf (1991), with a minor modification. Membranes were blocked in Tris-buffered saline (TBST containing 0.05% Tween 20, pH=7.5, 0.5 h), followed by incubation with a 1:2000 dilution of the 1° antibody (2 h) in TBST. The 1° antibodies were raised against both the style and pollen polygalacturonases, as described in Athanasίου *et al.* (2003). After three 10 min washes with TBST, the membranes were incubated with a 1:10 000 dilution of the 2° antibody (monoclonal goat anti-rabbit antibody conjugated to alkaline phosphatase, Sigma) in TBST (1 h). Three more washes with TBST were performed before the membranes were incubated in substrate solution 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (NBP-BCIP, Sigma) at 37°C in the dark. All immunoblotting procedures, with the exception of the substrate colorimetric reaction, were carried out with gentle agitation.

Results

The results of immunoblotting to detect the presence or absence of the style polygalacturonase are unambiguous, showing a complete association between possession of the protein by all short-styled plants of the *Turnera* species investigated, and its absence in all long-styled plants (Table 1, Figure 1). Immunoblotting revealed a protein band of approximately 35 kDa, unique to short-styled plants. Note that a 120 kDa protein that is neither morph- nor tissue-specific appears using one of two sources of antibody (Figure 1), and provides a convenient internal loading marker (Athanasίου *et al.*, 2003). Combined with the data of Athanasίου and Shore (1997), the results hold for the six species and 12 populations we have investigated. A single short-styled plant of *T. weddelliana* (Series *Salicifoliae* Urb.) possessed a protein band of approximately 37 kDa. Interestingly, for distylous *Piriqueta caroliniana*, neither short- nor long-styled plants showed the presence of the style polygalacturonase (Table 1, Figure 1b).

We carried out a parallel investigation for the pollen polygalacturonase of pI 6.8 identified in Athanasίου and Shore (1997) and Athanasίου *et al.* (2003). The pI 6.8

Table 1 Distribution of the style polygalacturonase (SP) in distylous species using SDS-PAGE immunoblotting

Species	Chromosome number	Short		Long	
		#with SP	N	#with SP	N
<i>T. concinna</i> (Conc)	2x = 10	6	6	0	6
<i>T. grandiflora</i> (Gran)	2x = 10	16	16	0	17
<i>T. joelii</i> (Joli)	2x = 10	17	17	0	15
<i>T. krapovickasii</i> (Krp)	2x = 10	23	23	0	22
<i>T. scabra</i> (Cosr)	2x = 10	8	8	0	8
<i>T. scabra</i> (Mrgd)	2x = 10	5	5	0	5
<i>T. scabra</i> (Nica)	2x = 10	5	5	0	5
<i>T. scabra</i> (Dor4)	4x = 20	9	9	0	9
<i>T. scabra</i> (Dor7)	4x = 20	6	6	0	6
<i>T. subulata</i> (Subu) ^a	2x = 10	15	15	0	16
<i>T. subulata</i> (SL8)	2x = 10	5	5	0	5
<i>T. subulata</i> (E)	4x = 20	5	5	0	5
<i>P. caroliniana</i> (Pird)	2x = 14	0	15	0	15

^aThese are selfed progeny of BRY (see Athanasiou and Shore, 1997). Species (code), chromosome number, number of plants with the style polygalacturonase and sample size (N) are provided for short- and long-styled plants.

Table 2 Distribution of the pl 6.8 pollen polygalacturonase (PP) in distylous species using IEF-PAGE immunoblotting

Species	Chromosome number	Short		Long	
		#with PP	N	#with PP	N
<i>T. concinna</i> (Conc)	2x = 10	5	5	0	5
<i>T. grandiflora</i> (Gran)	2x = 10	0	22	0	21
<i>T. joelii</i> (Joli)	2x = 10	0	16	0	16
<i>T. krapovickasii</i> (Krp)	2x = 10	7	16	0	16
<i>T. scabra</i> (Cosr)	2x = 10	5	5	0	5
<i>T. scabra</i> (Mrgd)	2x = 10	3	6	0	4
<i>T. scabra</i> (Nica)	2x = 10	5	6	0	6
<i>T. scabra</i> (Dor4)	4x = 20	5	5	0	5
<i>T. scabra</i> (Dor7)	4x = 20	5	5	0	6
<i>T. subulata</i> (Subu) ^a	2x = 10	30	30	1	26
<i>T. subulata</i> (SL8)	2x = 10	5	5	0	5
<i>T. subulata</i> (E)	4x = 20	5	5	0	5
<i>P. caroliniana</i> (Pird)	2x = 14	0	23	0	26

^aThese are selfed progeny of BRY (see Athanasiou and Shore, 1997). Species (code), chromosome number, number of plants with the pollen polygalacturonase and sample size (N) are provided for short- and long-styled plants.

Figure 1 SDS-PAGE immunoblots of style extracts. (a) A 35 kDa polygalacturonase occurs in styles of short-styled plants (S) of five *Turnera* spp. The polygalacturonase does not occur in long-styled plants (L). Lanes 1, 2: *T. concinna*; lanes 3, 4: *T. grandiflora*; lanes 5, 6: *T. joelii*; lanes 7, 8: *T. krapovickasii*; lanes 9, 10: *T. subulata*. (b) A 120 kDa protein also appears in immunoblots using one source of antibody providing an internal marker of protein loading levels. Lane 1: short-styled plant of *T. scabra* (Nica) possesses the 35 kDa polygalacturonase; lane 2: long-styled plant of *T. scabra* (Nica) does not have the protein; lanes 3, 4: the protein does not occur in styles of short- or long-styled plants of *P. caroliniana*.

polygalacturonase does not occur in long-styled plants (with a single exception where a band is present at pl 6.8 for a long-styled plant of *T. subulata*), and occurs in all short-styled plants of *T. concinna* and *T. subulata*, and most short-styled plants of *T. scabra* (Table 2). The pl 6.8 polygalacturonase does not occur in short- or long-styled plants of *T. grandiflora*, *T. joelii*, or *P. caroliniana*, nor do

these species show any evidence of a morph-specific pollen polygalacturonase at any other pl (Figure 2a). There is a polymorphism for the presence/absence of the pl 6.8 polygalacturonase among short-styled plants of *T. krapovickasii*, as well as some variation in staining intensity (Table 2, Figure 2b).

In August 1998, we discovered a self-compatible somatic mutant homostyle (having long styles and long stamens) on an otherwise self-incompatible short-styled plant. The inheritance of this mutation will be described elsewhere (F Tamari and JS Shore, unpublished results). The mutant shoot arose on a hybrid we had made between two distylous diploid species, *T. subulata* and *T. krapovickasii*. We have made numerous replicate cuttings of the mutant (homostyle) and nonmutant (short-styled) shoots, and have continued to propagate these in the glasshouse. We ran extracts of styles of the non-mutant short-styled plant and the mutant homostyle on SDS-PAGE, and used immunoblotting to detect the style protein (Figure 3a). Styles of the short-styled plant possess the 35 kDa polygalacturonase, but styles of the mutant homostyle do not. Pollen of both the homostyle and short-styled plant both possess the pl 6.8 pollen polygalacturonase.

Homostylous species

Self-compatible long-homostyled species of *Turnera* and one of *Piriqueta* were investigated for the presence of both polygalacturonases (Table 3). None of these six homostylous species possess the style polygalacturonase (Table 3, Figure 3b). The pl 6.8 pollen polygalacturonase occurs in the pollen of some homostylous species, but not in others (Table 3). For example, the polygalacturonase was detected in all the four plants of *T. velutina*, but not in *T. aurelii* (Figure 3c). *T. orientalis* has a pollen polygalacturonase that appears to be slightly less than pl 6.8 (Figure 3c), and it is not clear if this is the same polygalacturonase that occurs in short-styled plants. Numerous other polygalacturonase bands appear in the

Figure 2 IEF-PAGE immunoblots of pollen extracts. (a) Presence of the pI 6.8 polygalacturonase (see arrow) in the pollen of short-styled plants (S) of *T. subulata* and its absence from long-styled plants (L) and from both morphs of three other species. Lanes 1, 2 and 9: *T. subulata* (Subu); lanes 3–8: *T. joelii*; lane 10: *P. caroliniana*; lanes 11, 12: *T. grandiflora*. For the *Turnera* species, a number of pollen polygalacturonases are common to both morphs. *P. caroliniana* shows only a single band. (b) The pI 6.8 polygalacturonase (arrow) is polymorphic in short-styled plants (S) of *T. krapovickasii* (Krho) and absent from longs (L). Lanes 1, 2: *T. subulata* (Subu) as a control; lanes 3–10: *T. krapovickasii*.

homostylous species, although homostylous *P. cistoides* shows only a single band.

Discussion

Our results confirm the specificity of the 35 kDa polygalacturonase to styles of short-styled plants of all the six species of *Turnera* we have been able to sample. Recent immunocytochemistry analyses show that the polygalacturonase localizes in the style-transmitting tissue (Athanasίου *et al*, 2003; Khosravi *et al*, 2003). All of these species are in series *Turnera* (= *Canaligeræ* Urb.) of the genus, and have a base chromosome number of $x=5$, while most other series and genera have a base number of $x=7$ (Barrett and Shore, 1987; Fernandez, 1987). Outside this series of the genus, we have only been able to examine one short-styled plant of *T. weddelliana* (series *Salicifoliae* Urb.) using immunoblotting, and it possessed a protein band having a molecular mass of approximately 37 kDa. Significantly, a mutant homostyle does

Figure 3 Immunoblots for a somatic mutant homostyle and homostylous species. (a) SDS-PAGE immunoblots of style extracts showing the presence of the 35 kDa polygalacturonase for styles of short-styled plants (S), but not for the somatic mutant homostyle (H). Lane 1: *T. subulata* × *krapovickasii* somatic mutant homostyle (Mhomo H); lane 2: *T. subulata* × *krapovickasii* nonmutant (Mhomo S); lane 3: *T. subulata* (Subu) as a control. (b) SDS-PAGE immunoblots of style extracts for homostylous species. The 35 kDa polygalacturonase is present in styles of the control short-styled plant (S), but not in the long-styled (L) or any of the homostylous plants (H). The nonspecific 120 kDa band is present in all styles. Lanes 1, 2: *T. subulata* (Subu) as a control; lanes 3–5: *T. ulmifolia* (Ulm3); lanes 6, 7: *T. aurelii*. (c) IEF-PAGE immunoblots of pollen extracts for three homostylous species. The pI 6.8 polygalacturonase (arrow) occurs in the control lanes of short-styled *T. subulata* (S) and for homostylous plants (H) of *T. velutina* and possibly for *T. orientalis*, although in the latter species the pI of the band is somewhat less than 6.8. Lanes 1, 6, 7, 10, 11: *T. subulata* (Subu) as a control; lanes 2, 3: *T. orientalis*; lanes 4, 5: *T. aurelii*; lanes 8, 9: *T. velutina*.

not possess the style polygalacturonase, while the short-styled plant that gave rise to it does (Figure 3a). These data, coupled with those in Athanasίου and Shore (1997) and Athanasίου *et al* (2003) demonstrating its localization

Table 3 Distribution of the style (SP) and pollen pI 6.8 (PP) polygalacturonase in homostylous species using SDS-PAGE immunoblotting

Species	Chromosome number	Style		Pollen	
		#with SP	N	#with PP	N
<i>T. orientalis</i> (Orie)	2n = 30	0	5	5 ^a	5
<i>T. ulmifolia</i> (Ulm3)	2n = 30	0	16	4	16
<i>T. ulmifolia</i> (Ulm33)	2n = 30	0	15	15 ^a	15
<i>T. velutina</i> (Velu)	2n = 30	0	4	4	4
<i>T. aurelii</i> (Ar1)	2n = 40	0	15	3	11
<i>T. aurelii</i> (Ar2)	2n = 40	0	15	5	15
<i>T. cuneiformis</i> (Cune)	2n = 40	0	13	5	15
<i>P. cistoides</i> (Pirh)	2n = 14	0	20	0	8

^apI of protein slightly less than 6.8.

Species (code), chromosome number, number of plants with the style polygalacturonase and sample size (N) are provided.

in the style-transmitting tissue, provide strong evidence that the style polygalacturonase is involved in the distylous polymorphism, although its role in the breeding system is unknown.

While no phylogenetic analysis of breeding system variation within the Turneraceae has been undertaken, given the widespread occurrence of distyly within the family, it seems likely that distyly has had a single origin. If so, we might expect morph-specific proteins involved in distyly and the associated incompatibility system, to appear in all distylous species in the family. For the only species outside the genus *Turnera* that we have investigated, plants of distylous *Piriqueta caroliniana* do not possess the style polygalacturonase, based upon our immunoblotting experiments. Three possible explanations for this finding are as follows: (1) the polyclonal antibodies to the style polygalacturonase were made against a portion of a style polygalacturonase fusion protein obtained from *T. subulata* (Athanasίου *et al*, 2003). It is possible that a short-specific style polygalacturonase does occur in *P. caroliniana*, but that it has diverged sufficiently so that the necessary epitopes for antibody detection are absent. (2) The style polygalacturonase may not be necessary for the functioning of distyly, but perhaps acts as an accessory protein involved in some aspect of the pollination process of short-styled plants of *Turnera* spp. (3) Some aspect of the distylous breeding system has arisen independently in *Turnera* and *Piriqueta* (eg the self-incompatibility system). A wider analysis of species within the Turneraceae, using Northern blots and/or rtPCR to detect style polygalacturonase mRNA, might allow us to distinguish among some of these hypotheses.

Investigation of the pollen polygalacturonase casts doubt on its involvement in distyly. We show that the pI 6.8 polygalacturonase detected by Athanasίου and Shore (1997) and Athanasίου *et al* (2003) is not present in a number of distylous species. This suggests that the polygalacturonase might have been linked to and is in linkage disequilibrium with the *S* allele of distyly, at least in *T. concinna*, *T. subulata* and *T. scabra*. The absence of the pI 6.8 polygalacturonase from *T. joelii*, *T. grandiflora* and some short-styled plants of *T. krapovickasii* cast doubt on its involvement in distyly, despite the initial association reported by Athanasίου and Shore (1997).

The distribution of the pollen polygalacturonase varies within and among the homostylous species investigated. Furthermore, it is no longer clear that the pollen polygalacturonase provides a marker for exploring the origins of homostyly, as it does not appear to be a component of distyly (the putative ancestral breeding system), as we note above. All plants of allohexaploid *T. velutina* possess the pollen polygalacturonase, while none of the plants of allooctaploid *T. aurelii* do. Given that the homostyles are allohexaploids or allooctaploids (Fernandez, 1987; Fernandez and Arbo, 2000), the occurrence of the pollen polygalacturonase will depend upon the progenitor species that have given rise to them, and whether the polygalacturonase occurred in pollen of those species.

In marked contrast, none of the six homostylous species we studied possessed the style polygalacturonase based upon immunoblotting of style extracts. Recent immunocytochemical analyses support these results (Khosravi *et al*, 2003). This finding is consistent with the hypothesis that the homostyles have arisen via recombination within a distyly supergene, as in *Primula* spp (Ernst, 1955; Dowrick, 1956), and that their styles are similar to those of long-styled plants. In *Turnera*, styles of the homostylous species usually inhibit pollen from long-styled plants, but allow growth of pollen tubes from short-styled plants through their styles (Barrett and Shore, 1987; Tamari *et al*, 2001). We do not know whether the homostylous species of *Turnera* have arisen independently, and phylogenetic studies to explore this question are currently underway (S Truyens, MM Arbo and JS Shore, unpublished results). While hybrids among hexaploid *T. ulmifolia*, *T. orientalis* and *T. velutina* are completely sterile, suggesting independent origins (Barrett and Shore, 1987), cytogenetic analyses indicate that *T. orientalis* shares three genomes in common with octaploid *T. cuneiformis* and *T. aurelii* (Fernandez and Arbo, 2000). Thus, it is not clear whether the absence of the style polygalacturonase from the homostylous species represents five independent events or just one. Phylogenetic studies of the Turneraceae will aid in addressing this question.

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