Morph-Specific Proteins in Pollen and Styles of Distylous Turnera (Turneraceae)

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ABSTRACT

We used nondenaturing isoelectric focusing (IEF) in a survey of plants from 11 populations to identify style and pollen proteins unique to the short-styled morph of *Turnera scabra*, *T. subulata* and *T. krapovickasii*. Three protein bands [approximately isoelectric points (pIs) 6.1, 6.3 and 6.5] were found only in styles and stigmas of short-styled plants while two bands (approximately pIs 6.7 and 6.8, M_r 56 and 59 kD) occur only in pollen of short-styled plants. Some of these bands appear very late in development, within 24 hr before flowering. Two isozyme loci were mapped to an 8.7 cM region spanning the distyly locus. Using these isozyme markers we identified progeny exhibiting recombination adjacent to the distyly locus. No recombinants between the distyly locus and the locus or loci controlling the presence of the short-styled morph-specific proteins were obtained. This suggests that the loci encoding these proteins are either extremely tightly linked to the distyly locus and in complete disequilibrium with the *S* allele or exhibit morph-limited expression. Crosses to a plant showing an unusual style protein phenotype demonstrated that an additional unlinked locus is required for full expression of the style proteins. The function of the morph-specific proteins is unknown.

DISTYLY is a genetic polymorphism in which the reciprocal heights of anthers and stigmas in flowers of two alternative mating types (style morphs) occurs. A self- and intramorph diallelic incompatibility system is usually associated with the variation in reproductive organ lengths. Distyly has long been used as a model system in genetics and evolutionary biology (BARRETT 1992; LEWIS and JONES 1992; ORNDUFF 1992), in part due to its apparently simple genetic basis and the ease with which mating types can be recognized in the field.

Distyly occurs in ~25 flowering plant families and it is clear from its taxonomic distribution that it has arisen independently a number of times (GANDERS 1979; CHARLESWORTH 1982; BARRETT 1992; LLOYD and WEBB 1992). The short-styled morph is commonly determined by the dominant *S* allele and the long-styled morph is homozygous recessive *ss* (ORNDUFF 1979; LEWIS and JONES 1992), but the dominance relationships are reversed in *Hypericum aegypticum* (ORNDUFF 1979) and possibly in the genus Limonium (BAKER 1966). All characters distinguishing the morphs, including style length and its incompatibility response, stamen length, pollen size, production and pollen incompatibility (see GANDERS 1979; DULBERGER 1992; RICHARDS and BARRETT 1992) appear to be determined by two alternative alleles at this single locus (GANDERS 1979; ORNDUFF 1979; LEWIS and JONES 1992).

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Studies of Primula (MATHER and DEWINTON 1941; MATHER 1950; LEWIS 1954; ERNST 1955; DOWRICK 1956; and see review by LEWIS and JONES 1992) and, to a lesser extent, Armeria (BAKER 1966) and Turnera (SHORE and BARRETT 1985; BARRETT and SHORE 1987), however, indicate that distyly may in fact be determined by a supergene; a number of tightly linked genes held in complete disequilibrium with the dominant alleles of at least three loci linked in coupling and inherited *en masse*. Rare recombination within the supergene (LEWIS and JONES 1992) can lead to the formation and spread of unusual and potentially highly self-fertilizing genotypes (CROSBY 1949; DOWRICK 1956; BODMER 1960; CHARLESWORTH and CHARLESWORTH 1979a; PIPER *et al.* 1984; BARRETT and SHORE, 1987; BELAOUSSOFF and SHORE 1995).

Despite considerable successes using distyly as a model system in ecological genetics (*e.g.*, CROSBY 1949; MATHER 1950; BODMER 1960; ORNDUFF 1971; GANDERS 1974, 1975, 1979; CHARLESWORTH and CHARLESWORTH 1979a,b; PIPER *et al.* 1984; CAHALAN and GLIDDON 1985) details of its molecular genetics and mechanism(s) of incompatibility remain largely obscure. In this paper we initiate investigations of the molecular genetics of distyly by identifying proteins specific to the short-styled morph that are potential incompatibility proteins. These investigations should allow us to begin to understand the mechanisms of incompatibility in distylous systems, which are thought to be quite different from homomorphic sporophytic and gametophytic incompatibility systems (CHARLESWORTH 1982; GIBBS 1986;

LLOYD and WEBB 1992) such as those in Brassica (NASRALLAH *et al.* 1987), members of the Solanaceae (ANDERSON *et al.* 1986; MATTON *et al.* 1994) and *Papaver rhoeas* (FOOTE *et al.* 1994). Finally, a knowledge of the detailed genetics might aid in distinguishing among various evolutionary models for the origin of distyly (DARWIN 1877; MATHER and DE WINTON 1941; BAKER 1966; CHARLESWORTH and CHARLESWORTH 1979b; GANDERS 1979; MUENCHOW 1982; LLOYD and WEBB 1992).

Our specific objectives were to (1) genetically map the distyly locus, (2) use biochemical methods to identify and partially characterize proteins that are unique to the style morphs, including studies of their expression and of variants of these proteins, and (3) determine linkage relationships of the distyly locus and loci encoding morph-specific proteins.

MATERIALS AND METHODS

Plant material: Plants used in this study were obtained from seed collections and stem cuttings taken from natural populations and maintained in a greenhouse at York University, North York, Ontario, Canada. All plants are strongly self-incompatible unless indicated otherwise. *Turnera scabra* Millsp. samples were obtained from Costa Rica ($2x = 10$), El Salvador ($2x = 10$), Nicaragua ($2x = 10$), Venezuela ($2x = 10$) and the Dominican Republic (autotetraploid, $4x = 20$). *T. krapovickasii* Arbo (autotetraploid, $4x = 20$) was sampled in Paraguay. One autotetraploid ($4x = 20$, Recife, Brazil) and two diploid ($2x = 10$) *T. subulata* Smith populations, BRY (from Arco Verde, Brazil) and SL (from Sao Luis, Brazil), were sampled. BRY is a highly self-compatible short-styled plant (SHORE and BARRETT 1985, 1986). One short-styled plant from Sao Luis SL8 was also somewhat self-compatible.

Linkage analysis of *distyly* and two isozyme loci: We refer to the Mendelian locus responsible for the style and stamen length polymorphism as *distyly*. To localize *distyly*, we used the plant BRY, which has the genotype $Pgd\text{-}c^F\ S\ Aco\text{-}1^S / Pgd\text{-}c^S\ s\ Aco\text{-}1^F$ (SHORE and BARRETT, 1985, 1987), where *S* and *s* are the two alternative alleles of *distyly* and the superscripts *F* and *S* designate codominant alleles encoding fast and slow migrating forms of the isozymes *Aco-1* (aconitase-1) and *Pgd-c* (cytosolic 6-phosphogluconate dehydrogenase). BRY was self-pollinated and 620 progeny (referred to as an F_2) were grown to flowering in individual pots and scored for style form (long-styled, *ss, vs.* short-styled, *S*−). We used starch gel electrophoretic methods, detailed in SHORE and BARRETT (1987), to determine the genotype of progeny at both isozyme loci. Three plants died prior to flowering and their genotype at *distyly* could not be ascertained.

We tested single-locus segregation ratios against F_2 Mendelian expectations using the G-statistic for goodness of fit (SOKAL and ROHLF 1981). For the codominant isozyme loci, tests were made against 1:2:1 ratios while for the *distyly* locus the progeny were tested against a 3:1 ratio (short-styled: long-styled). Two-locus segregations were tested against 1:2:1:2:4:2:1:2:1 ratios for independent assortment of the codominant isozyme loci and against 3:6:3:1:2:1 ratios for independent assortment of *distyly* and each isozyme locus (ALLARD 1956). Maximum likelihood estimates of the recombination frequency between each pair of loci were obtained following ALLARD (1956).

Weakened self-incompatibility in BRY and its progeny: We determined whether the incompatibility system of BRY was entirely absent or was merely weaker than normal (resulting in cryptic incompatibility, BATEMAN 1956; WELLER and ORNDUFF 1977, 1989; CASPER *et al.* 1988) in a pollen competition experiment and by examining growth rates of pollen tubes after pollination by long- and short-styled donors. For the pollen competition experiment, we obtained 13 short-styled F_2 progeny (used as males and females) of BRY that were homozygous for the $Pgd\text{-}c^F$ allele and 12 long-styled progeny (used as males) that were homozygous for the alternative allele, $Pgd\text{-}c^S$. We also used one short-styled plant homozygous for $Pgd\text{-}c^S$ (used as a female). We performed competitive pollinations following methods of BAKER and SHORE (1995). All three stigmas of short-styled plants (above) were usually pollinated first with pollen from a short-styled plant followed immediately with pollen from one of the long-styled plants (above). Flowers were emasculated the day before flowering to prevent pollen contamination. We collected ripe seeds, germinated them and assayed seedlings for their *Pgd-c* genotype. A total of 282 progeny were assayed.

To explore the possibility of cryptic incompatibility directly in BRY, we pollinated one style of BRY using pollen from one of its short-styled progeny while a second style in the same flower was pollinated with pollen from one of its long-styled progeny. The experiment was repeated for 19 flowers. Styles were collected 2.5 hr after pollination, fixed for a minimum of 24 hr in 3 ethanol:1 glacial acetic acid, cleared at 55° in 8 M NaOH for 3 hr and stained overnight in 1% aniline blue following MARTIN (1959). Pollen tubes were viewed using fluorescence microscopy and we recorded whether the majority of pollen tubes had entered the stigma or had reached the upper, middle or lower third of the style.

Identification of morph-specific proteins: To identify candidate incompatibility proteins, we initially used nondenaturing polyacrylamide gel electrophoresis (PAGE), SDS-PAGE and nondenaturing isoelectric focusing (IEF). As only IEF gels revealed morph-specific proteins, we provide details only for these gels. IEF polyacrylamide gels (Pharmacia Biotech Inc.), with an ampholine concentration of 2.5% and a pH gradient ranging from 5.5 to 8.5, were run on a LKB Multiphor apparatus with 10° circulating water for cooling. Fresh or frozen (−80°) styles, anthers and pollen, of both long- and short-styled plants from single flowers, were ground separately on ice in 0.05 M Na_2HPO_4-HCl buffer (pH 7.0). Tissue extracts were absorbed on wicks of Miracloth (Calbiochem Corp.) or glass fiber and were placed at the anodal end of a prefocused IEF gel. Prefocusing consisted of running the gel for 30 min at 25 W constant power. Samples were loaded and gels were run for another 90 min at 25 W, at which point the wicks were removed. The run then continued for another 30 min at 25 W.

After focusing was complete, gels were fixed in 10% trichloroacetic acid, 3.5% sulphosalacylic acid, 30% methanol, followed by 12% trichloroacetic acid, 30% methanol, for 1 hr each. Final fixation was in 50% methanol, 10% acetic acid, for 1–16 hr. Gels were then washed in water for 2 hr and were silver stained in 0.15% $AgNO_3$, 0.056% formaldehyde for 30 min. To develop the stain, gels were washed in water for 20 sec and placed in 3% Na_2CO_3, 0.056% formaldehyde, 0.4 mg/liter sodium thiosulphate. Fixing of the stain was achieved with 10% acetic acid. All solutions were made fresh or stored for short periods of time at 4°. Fixing and staining was performed at room temperature. Fixed gels were photographed, wrapped and stored at 4° or vacuum dried for future reference. The isoelectric points (pIs) of proteins of interest were determined by comparing their migration position to those of marker proteins run on each gel (pIs ranging from 5 to 10.5; Pharmacia Biotech Inc.).

Organ-specific and temporal expression of proteins: Once morph-specific proteins were identified, we determined their tissue expression. A range of floral and vegetative organs including leaves, bractioles (linear bracts subtending flowers), ovaries, petals, sepals, anthers containing pollen and washed free of pollen (see below), pollen, styles and filaments were each ground and run on IEF as described above. For a particular IEF gel, all organs were taken from the same short-styled plant to minimize variation between individuals. The experiment was repeated 22 times, each time using a different short-styled plant of *T. scabra* from the Dominican Republic (population 7), Costa Rica, Nicaragua or plants from the F_2 family of BRY (*T. subulata*). We also ran stigmas and bisected the styles into upper and lower halves, to further localize the position of the style-specific proteins. These dissections were done for five short-styled plants taken from different species/populations.

We washed pollen from anthers by vortexing anthers (for 30 sec) in 1 ml of water and repeated this procedure four times. We removed anthers and viewed them under a dissecting microscope, to ensure that all pollen had been removed from the anthers.

To investigate the temporal expression of the morph-specific proteins, we ran extracts of anthers and styles dissected from a developmental series of immature flower buds on IEF gels (as above). The age of flower buds was determined by recording the time of flowering of buds from which anthers and styles had been dissected.

Linkage analysis of *distyly* and morph-specific proteins: If the proteins identified here are products of *distyly*, they should co-segregate with the *S* allele. We determined the IEF style and pollen phenotypes of a number of F_2 progeny of BRY (57 long- and 79 short-styled) by running style and anther extracts on IEF gels (see above). We also ensured that we assayed plants that were known to have undergone recombination between the isozyme loci and *distyly*. To obtain plants homozygous for the recombined chromosomes, we constructed F_3 families for 18 of the short-styled F_2 progeny of BRY that showed recombination between the isozyme loci. F_3 families were made by selfing F_2 plants of interest. A minimum of 16 progeny were grown to flowering for each F_3 family and a number were assayed for their isozyme genotypes at *Pgd-c* and *Aco-1*.

Distribution of morph-specific proteins among populations and species of Turnera: To test the hypothesis that the proteins identified are products of *distyly*, we assayed a number of long- and short-styled plants obtained from three closely related species of Turnera (*T. scabra, T. subulata* and *T. krapovickasii*) and 11 populations, including diploid ($2n = 10$) and autotetraploid ($4x = 20$) cytotypes. Styles and anthers were run on IEF gels as described above.

Protein purification: To isolate pollen proteins, extracts of anthers from short-styled plants were run on IEF gels, and sections of the gel were stained to determine the approximate location of the bands of interest. Successive horizontal gel slices were then taken from the remainder of the gel, crushed in 0.75 ml of 0.05 M Tris-HCl (pH 8.0) in 1.5-ml microcentrifuge tubes, and shaken overnight at 4° to allow diffusion of proteins. The diffusate was then spun briefly at 13,000 *g* to remove the gel remnants. The supernatant was dialyzed against 0.05 M Tris-HCl (pH 8.0) using membrane tubing of 12,000–14,000 M_r cutoff (Spectra/Por, Mandel Scientific Company Ltd.) for 8–16 hr. Dialyzed diffusates were concentrated by lyophilization.

Each diffusate was run on an IEF gel to determine the purity of the diffused proteins and their pI. Diffusates containing proteins of interest were then run on SDS-PAGE to determine their approximate molecular weights. SDS-PAGE was performed as described in LAEMMLI (1970) and gels were fixed and subsequently silver-stained (as above).

RESULTS

Weakened/cryptic incompatibility of BRY: The self-compatible short-styled plant BRY has a fully functional incompatibility system in its pollen, but its style either lacks or has a weakened incompatibility system (SHORE and BARRETT 1986). A majority of its short-styled F_2 progeny are also self-compatible but its long-styled progeny are all self-incompatible (SHORE and BARRETT 1986). For our major goal of identifying incompatibility proteins, plants lacking incompatibility are likely to be very illuminating, so it was important to determine whether the stylar incompatibility of BRY was absent or present in a weakened or "cryptic" form.

Results of competitive pollinations using pollen from short-styled plants *vs.* pollen from long-styled plants were unambiguous in showing that short-styled progeny of BRY possess an incompatibility system. All 282 progeny assayed from competitive pollinations were the result of fertilizations by pollen from long-styled plants (Table 1). Furthermore, in 18 of 19 pairs of pollinations of plant BRY, where one style in a flower received pollen from a short- and the other from a long-styled plant, pollen tubes from long-styled plants traversed a statistically greater proportion of the style than tubes from short-styled plants (sign test, $P < 0.0001$; SOKAL and ROHLF 1981). Pollen from long-styled plants generally traversed at least half the length of the style while pollen from short-styled plants had reached at most the upper third of the style. Taken together, these results support the hypothesis that the styles of BRY and its self-compatible short-styled progeny possess an incompatibility system that is present, but weakened.

Linkage analysis of distyly and two isozyme loci: *Distyly, Pgd-c* and *Aco-1* showed no significant deviation from expected single locus segregation ratios (Table 2). All possible pairs of the three polymorphic loci were tested for independent assortment and recombination frequencies were estimated (Table 3). The results indicate that the loci are linked and that *distyly* lies approximately in the middle of an 8.7-cM region spanned by *Pgd-c* and *Aco-1* (Figure 1).

Identification of morph-specific proteins: Style and pollen extracts of both long- and short-styled F_2 progeny of BRY were run on IEF gels. The protein profile of short-styled plants was compared to those of long-styled plants run in adjacent lanes, to search for morph-specific proteins. Styles of short-styled plants possess three unique protein bands having pIs of ~6.5, 6.3 and 6.1, which were not found in long-styled plants (Figure 2). A fourth band of lower pI was apparent on some gels, but we did not obtain sufficient resolution and/or reproducibility of this band to know whether it is also

TABLE 1

Pollen competition in short-styled progeny of BRY using genetically marked pollen donors

Maternal plant			*Pgd-c* genotype of pollen donors		Progeny numbers		
No.	Style morph	*Pgd-c* genotype	Short-styled	Long-styled	*FF*	*FS*	*SS*
1	S	*FF*	*FF*	*SS*	0	3	—
2	S	*FF*	*FF*	*SS*	0	17	—
3	S	*FF*	*FF*	*SS*	0	14	—
4	S	*FF*	*FF*	*SS*	0	15	—
5	S	*FF*	*FF*	*SS*	0	7	—
6	S	*FF*	*FF*	*SS*	0	30	—
7	S	*FF*	*FF*	*SS*	0	16	—
8	S	*FF*	*FF*	*SS*	0	20	—
9	S	*FF*	*FF*	*SS*	0	18	—
10	S	*FF*	*FF*	*SS*	0	9	—
11	S	*FF*	*FF*	*SS*	0	30	—
12	S	*FF*	*FF*	*SS*	0	9	—
13	S	*SS*	*FF*	*SS*	—	0	94

limited to the short-styled morph. Pollen of short-styled plants possesses two unique protein bands having pIs of ~6.7 and 6.8, which do not occur in pollen of long-styled plants (Figure 3). We found no protein bands unique to long-styled plants in styles or pollen.

To explore the generality of these findings, we ran additional extracts of styles and pollen of both long-styled and short-styled plants from 10 distylous populations in three species of Turnera, on IEF gels. The results were uniformly consistent in showing that only short-styled plants possess these unique proteins (Table 4).

In three instances, however, we have seen very weak staining of proteins in long-styled plants that co-migrate with the short-specific pollen proteins. These may represent instances of cross-contamination from adjacent lanes or very weak expression of the proteins in the pollen of long-styled plants.

A more marked exception occurred for short-styled progeny obtained by selfing a self-compatible short-styled plant (SL8) from the Sao Luis population. For the 14 short-styled selfed progeny assayed, the style bands were absent (see below). None of the 10 long-styled selfed progeny assayed had any of these protein bands, as usual.

TABLE 2

F_2 segregation ratios for *distyly*, *Aco-1* and *Pgd-c*

Locus	Phenotype/genotype frequencies	*G*
Distyly[a]	475 short: 142 long	1.32
Aco-1[b]	143 *FF*: 315 *FS*: 159 *SS*	1.12
Pgd-c[b]	152 *FF*: 317 *FS*: 148 *SS*	0.52

F and *S* represent alleles encoding fast and slow migrating forms of each isozyme, respectively.

[a] Tested against a 3:1 ratio using the *G*-statistic.

[b] Tested against a 1:2:1 ratio using the *G*-statistic.

Organ-specific and temporal expression of proteins: If the morph-specific proteins identified in short-styled plants are incompatibility proteins, they should be present only in organs and tissues where incompatibility substances are expected to occur, *i.e.,* in styles, stigmas, pollen and anthers (containing pollen). We tested this by running extracts of a number of floral and vegetative organs on IEF gels. For the 22 replicates, the style proteins appear clearly only in extracts of styles and, likewise, the anther proteins appear only in anther and pollen extracts (Figures 4 and 5, Table 5). On occasion, very weak bands, possibly corresponding to the pollen-specific bands, appear in the filament and ovary lanes. Likewise in some gels there are very weak bands that might correspond to the style-specific proteins in leaf, sepal and ovary tissues. Overall, the style and pollen proteins are intensely stained only in pollen and styles, but we cannot rule out the possibility of very weak expression in some other organs.

In additional assays, we found that mature anthers washed free of pollen did not possess the pollen-specific proteins. Dissections revealed that the style proteins were present in stigmas, as well as in the upper and lower halves of the styles. We attempted to elute proteins from intact styles by bathing styles for 15–120 min in extraction buffer. While some proteins did elute from the styles, the morph-specific proteins did not.

Short-specific proteins in styles revealed an interesting pattern of developmental expression. Approximately 24 hr before flowering the style proteins are absent (Figure 2). The pI 6.3 band is the first to appear in styles. As the styles mature, the pI 6.5 band appears and increases in staining intensity relative to proteins

TABLE 3

Two-locus segregation ratios and recombination frequencies

Phenotype/genotype at first locus	Genotype at second locus			G	r	SE
	FF	*FS*	*SS*			
Distyly[a]		*Pgd-c*				
Short	152	305	18	8382*	0.050	0.009
Long	0	12	130			
Distyly[a]		*Aco-1*				
Short	11	306	158	8423*	0.037	0.007
Long	132	9	1			
Aco-1[b]		*Pgd-c*				
FF	0	22	122			
FS	24	265	27	633*	0.090	0.009
SS	128	30	2			

r, maximum likelihood estimate of the recombination frequency and SE is the standard error of the estimate; *F* and *S*, alleles encoding fast and slow migrating forms of each isozyme, respectively.

* $P < 0.001$.

[a] Tested against a 3:6:3:1:2:1 ratio.

[b] Tested against a 1:2:1:2:4:2:1:2:1 ratio.

of pI 6.3 and pI 6.1. The pI 6.1 protein first appears ~9 hr before flowering. At flowering, the pI 6.5 band is the most intensely staining (Figure 2).

The anther proteins also exhibit an interesting pattern of developmental expression (Figure 3). Early developmental stages exhibit just a single anther band of pI 6.7 and it is present at least 48 hr prior to flowering. Due to small amounts of protein in very early developmental stages, we cannot state with certainty whether it is present in premeiotic buds. It is not until very late stages of development, ~10–18 hr prior to flowering, that the second band of pI 6.8 appears.

Linkage analysis of *distyly* and morph-specific proteins: On the supergene hypothesis for the inheritance of *distyly*, loci encoding incompatibility proteins should be tightly linked to the *distyly* locus. We used two approaches to determine whether the morph-specific proteins we have identified in pollen and styles of short-styled plants are linked to *distyly*. First, because these proteins behave as if they are determined by a dominant allele, we assayed a number of long-styled F_2 progeny for their IEF style and anther profiles. Any recombination event will be readily observed in long-styled progeny as they should then possess the style- or pollen-specific proteins characteristic of the short-styled morph. None of the long-styled progeny assayed (including 13 plants whose allozyme genotypes indicated that recombination had occurred in this region) had the short-specific style ($N = 57$ plants) or anther ($N = 53$ plants) proteins. The recombination frequency considering only long-styled progeny is given by $r = 1 - (\text{no. of nonrecombinants/total no. longs assayed})^{0.5}$. Using these data alone and assuming that the next long-styled plant assayed would have been a recombinant, the locus encoding the style proteins is at most 0.87 cM from *distyly* (or 0.93 cM for the anther proteins).

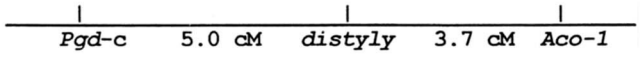

FIGURE 1.—A genetic map of the *Pgd-c*, *distyly* and *Aco-1* loci showing map distances in centimorgans (cM).

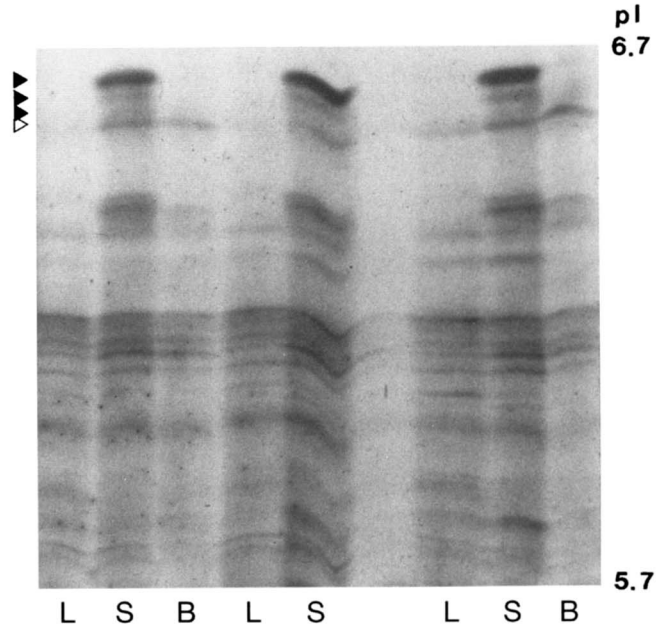

FIGURE 2.—Silver-stained isoelectric focusing gel of extracts of styles from three long-styled plants (lanes marked L), three short-styled plants (lanes marked S) and from flower buds of two short-styled plants sampled 1 day before flowering (lanes marked B). Proteins unique to styles of short-styled plants at flowering are indicated by three solid arrows (the open arrow points to a band present in both morphs). All plants were from the F_2 progeny of BRY. The range of isoelectric points (pI) is provided.

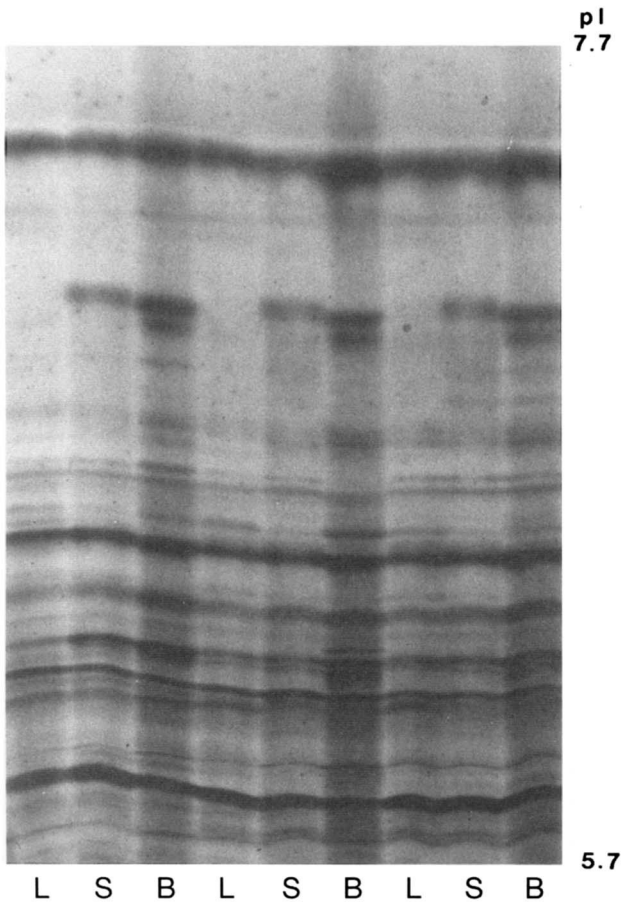

FIGURE 3.—Silver-stained isoelectric focusing gel of extracts of anthers from three long-styled plants (lanes marked L), three short-styled plants (lanes marked S) and from flower buds of three short-styled plants sampled 1 day before flowering (lanes marked B). Proteins unique to pollen of short-styled plants are indicated by the solid arrow. All plants were from the F_2 progeny of BRY. The range of pIs is provided.

The second approach was to identify and assay using IEF, F_2 and F_3 progeny of BRY that we determined (from their allozyme genotypes) had undergone recombination within the 8.7 cM region containing *distyly*. We assayed a number of short-styled F_2 progeny (54 of which had undergone recombination within the 8.7 cM region around *distyly*) and all of them possessed the characteristic style (N = 79 plants) and pollen (N = 67 plants) proteins of the short-styled morph. We also progeny tested 18 of the short-styled recombinants by selfing them, to obtain plants homozygous for the recombined chromosomes (this assumes that the chromosomes have not undergone a second round of recombination). We then assayed on IEF gels one or two progeny that were homozygous for the recombined chromosomes. In no instance was there evidence for recombination of the loci encoding the morph-specific IEF proteins. Taken together, these results suggest that the loci are considerably closer to *distyly* than 0.93 cM.

There are a number of genetic hypotheses, however, that can account for our results. The loci encoding the style and pollen proteins could be (1) tightly linked to *distyly* and in complete disequilibrium with the *S* allele, (2) direct products of *distyly*, or, alternatively, they could (3) exhibit morph-limited expression and the loci encoding them may reside elsewhere in the genome, being regulated or "switched on" by *distyly* only in short-styled plants.

Expression of morph-specific proteins in crosses between BRY progeny and the anomalous Sao Luis plants: One way to distinguish among the above three hypotheses is to identify short-styled plants that have a variant morph-specific protein phenotype and use these in crosses to determine the inheritance patterns and linkage relationships of the variant and normal protein phenotypes. We did this using a self-compatible short-styled plant from the Sao Luis population of *T. subulata*. This plant, referred to as SL8, and its short-styled selfed progeny all lack the style protein bands (Figure 6). We crossed a long-styled plant (*ss*) from the BRY F_2 family with a short-styled plant from the selfed progeny of SL8. The resulting short-styled progeny will all contain the dominant *S* allele derived from the SL8 plant and the recessive *s* allele from BRY. A number of progeny were then assayed for their style protein patterns using IEF.

All 30 short-styled progeny assayed from the cross of BRY long × SL8 short had the short morph style proteins (Figure 6). Thus the absence of these proteins in SL8 is not solely a result of the *S* allele (or adjacent linked genes) derived from SL8, since plants with this *S* allele and a genetic background containing BRY-derived genes possess the proteins. This result may indicate that the dominant *S* allele from SL8 is able to cause the expression of these style proteins in the presence of the BRY *s* allele or other genes from BRY.

To explore this possibility further, we carried out crosses between three different long-styled plants from the BRY selfed progeny, where each was crossed with one of two short-styled selfed progeny of SL8. We selfed one short-styled progeny from each of these F_1s to generate three F_2 families. A number of short- and long-styled progeny from each cross were assayed to investigate patterns of inheritance of the style protein(s). None of the 18 long-styled progeny assayed on IEF gels possessed the style proteins. The short-styled progeny show a 3:1 ratio of short-styled plants with the style proteins to those without (Table 6). These results suggest that the style proteins are expressed only in the presence of a dominant *S* allele at *distyly* and require the presence of a second unlinked dominant allele for expression. The expression of style protein(s), therefore, appears to be determined by two unlinked loci that exhibit complementary gene action.

Purification of short-specific anther proteins: We used gel diffusion from IEF gels to purify the anther proteins and then ran the proteins on both IEF and

TABLE 4

Distribution of putative incompatibility proteins in 11 populations and three species of Turnera

Species/population	No. and style form of plants			
	Style proteins		Anther proteins	
	Present	Absent	Present	Absent
T. subulata				
Arco Verde, Brazil (2*x*)	79 S	57 L	67 S	53 L
Sao Luis, Brazil (2*x*)	11 S	13 L	11 S	13 L
Sao Luis SL8, Brazil (2*x*)[a]	14 S	10 L	14 S	10 L
Recife, Brazil (4*x*)	10 S	10 L	10 S	10 L
T. scabra				
Dominican Republic 6 (4*x*)	30 S	30 L	30 S	30 L
Dominican Republic 7 (4*x*)	15 S	15 L	15 S	15 L
Costa Rica (2*x*)	10 S	10 L	10 S	10 L
Hill Side, Venezuela (2*x*)	8 S	6 L	8 S	6 L
De Cameron, Venezuela (2*x*)	28 S	28 L	28 S	28 L
El Salvador (2*x*)	10 S	10 L	10 S	10 L
Nicaragua (2*x*)	10 S	10 L	10 S	10 L
T. krapovickasii				
Paraguay (4*x*)	2 S	1 L	2 S	1 L

[a] The short-styled selfed progeny of SL8 are missing the style proteins.

SDS gels. As in native IEF gels, two very similar protein bands occur on SDS-PAGE, of ~56 and 59 kD. This suggests that each of the anther proteins consists of a single subunit.

DISCUSSION

We have discovered proteins specific to the short-styled morph of distylous species including *T. scabra, T. subulata* and *T. krapovickasii.* These proteins are found only in styles and stigmas, while two different protein bands appear only in pollen, although we cannot rule out the possibility that weak expression occurs, on occasion, in other organs. The organ- and morph-specific distribution of these proteins indicates that they might be incompatibility proteins. If so, this would represent the first discovery of both pollen and style incompatibility proteins in any heteromorphic incompatibility system. Identification of both pollen and style incompatibility proteins in homomorphic systems has not been demonstrated convincingly (CHARLESWORTH 1995). BOYES and NASRALLAH (1995) have, however, reported the occurrence of an anther-specific gene linked to the *S2* allele in *Brassica oleracea* but not to other *S* alleles in this homomorphic sporophytic system where two style incompatibility genes have previously been identified (NASRALLAH *et al.* 1987; NASRALLAH and NASRALLAH 1993).

Despite continued interest in the ecology, genetics, biochemistry and physiology of distyly, we have only very limited insight into the mechanisms governing this incompatibility system. Several workers have attempted to elucidate the mechanisms of incompatibility including LEWIS (1943), DULBERGER (1974; 1975a,b; 1987), GOLYNSKAYA *et al.* (1976), GHOSH and SHIVANNA (1980), SHIVANNA *et al.* (1981, 1983), RICHARDS and IBRAHIM (1982), STEVENS and MURRAY (1982), SCHOU (1984), SCHOU and MATTSSON (1985) and MURRAY (1986). This work has been reviewed by DULBERGER (1992). Most recently, WONG *et al.* (1994) have discovered a style-specific protein apparently unique to long-styled plants of *Averrhoa carambola* (Oxalidaceae).

No clear picture of the mechanism(s) of incompatibility has yet emerged for distylous species. In part, this could be a result of the fact that different mechanisms likely occur not only among species from different plant families (DULBERGER 1992), but also because the mechanisms may differ among morphs within a species (LLOYD and WEBB 1992). Our discovery of proteins in short-styled plants alone is consistent with hypotheses that propose that the mechanism of incompatibility differs between the two morphs.

In our studies of Turnera spp. we observe multiple morph-specific protein bands on IEF gels, three in styles and two in pollen. We do not know whether these protein bands represent the products of different loci or whether they are different conformational or posttranslationally modified (perhaps differentially glycosylated) forms of the product of a single gene (one for style and one for pollen proteins). While we are presently uncertain about the genetic control of these multiple bands, their appearance very late in style and pollen development (within 24 hr before flowering) lends further support to their involvement in the incompatibility response (ANDERSON *et al.* 1986; CHARLESWORTH 1995) or in some other aspect of distyly.

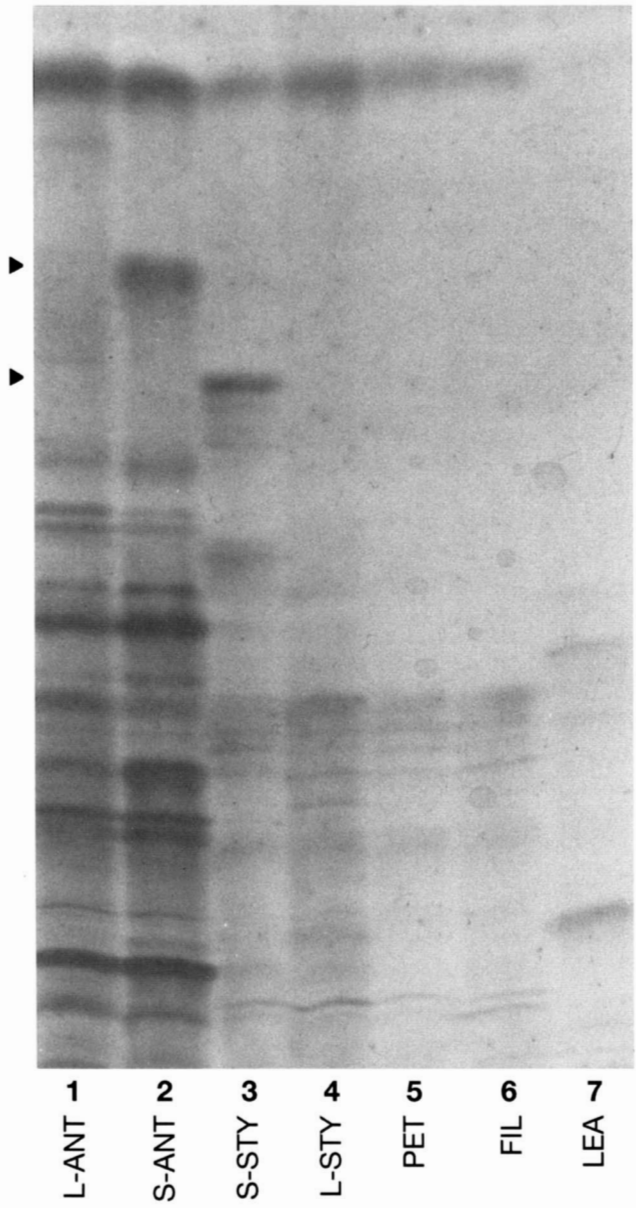

FIGURE 4.—Silver-stained isoelectric focusing gel showing extracts of various organs from plants from the BRY F_2 family. Extracts of anthers (ANT) from a long- and short-styled plant (lanes 1 and 2, respectively); extracts of styles (STY) from a short- and long-styled plant (lanes 3 and 4, respectively); extracts of petals (PET), filaments (FIL) and leaves (LEA) (lanes 5–7, respectively) from a short-styled plant. The arrows indicate the short-specific pollen (lane 2) and style (lane 3) proteins, which stain intensely only in these lanes.

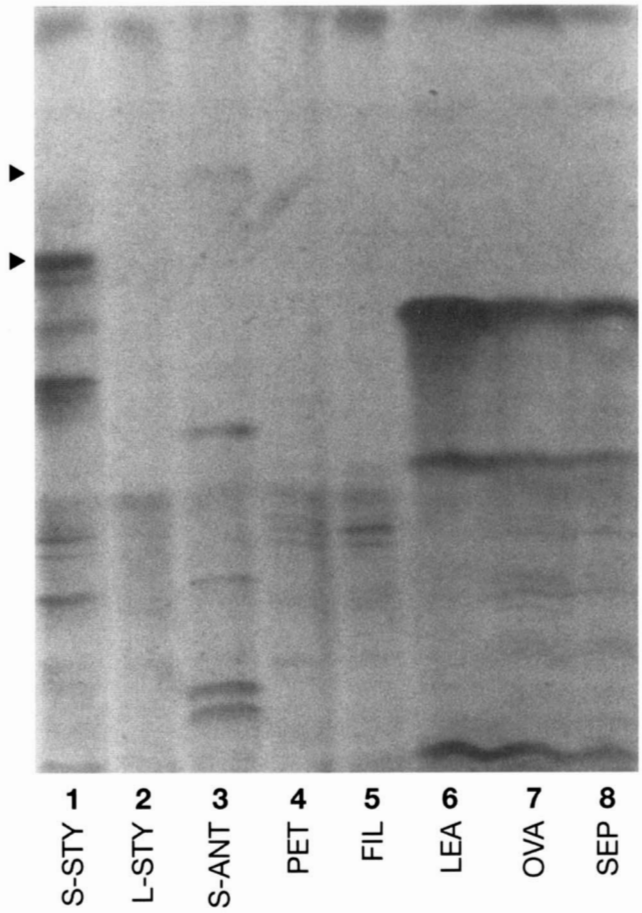

FIGURE 5.—Silver-stained isoelectric focusing gel showing extracts of various organs from plants from the Dominican Republic population 7. Extracts of styles (STY) from a short- and long-styled plant (lanes 1 and 2, respectively); extracts of anthers (ANT) from a short-styled plant (lane 3); extracts of petals (PET), filaments (FIL), leaves (LEA), ovaries (OVA) and sepals (SEP) (lanes 4—8, respectively) from a short-styled plant. The arrows indicate the short-specific style proteins (lane 1) and pollen proteins (lane 3, weakly staining). The short-specific style and anther proteins do not appear to stain in any of the other organs.

The Mendelian locus determining the distylous polymorphism in Turnera apparently controls a number of dimorphic characters (SHORE and BARRETT 1985) and we cannot state with certainty that the proteins we have identified are incompatibility proteins. For example, perhaps the style-specific proteins are involved in development and limit the elongation of the short style. One approach to testing the hypothesis that they are involved in incompatibility is to examine the tissue-specific expression of the proteins using *in situ* assays involving antibodies against the style proteins. Ultimately, cloning the genes encoding the proteins and transformation of incompatibility specificity would be a more powerful test. We have purified the anther proteins, made polyclonal antibodies against them and are using these to screen a pollen cDNA expression library from short-styled plants; we are purifying the style proteins to carry out a similar screen.

An interesting outcome of this study is that we have found no proteins unique to the long-styled morph. There may be unique proteins in styles and pollen of the long-styled morph that we simply have not detected. For example, these proteins might exist but happen to co-migrate with other proteins and hence go unrecognized. We find it interesting, however, that the presence

TABLE 5

Organ-specific distribution of morph-specific proteins

Organ	Style proteins	Pollen proteins
Style	+	−
Upper half of style	+	−
Lower half of style	+	−
Stigma	+	−
Anthers with pollen	−	+
Pollen	−	+
Anther walls	−	−
Filaments	−	−
Ovary	−	−
Petal	−	−
Sepals	−	−
Bractioles	−	−
Seed capsule	−	−
Leaves	−	−

+, Proteins present; −, proteins absent.

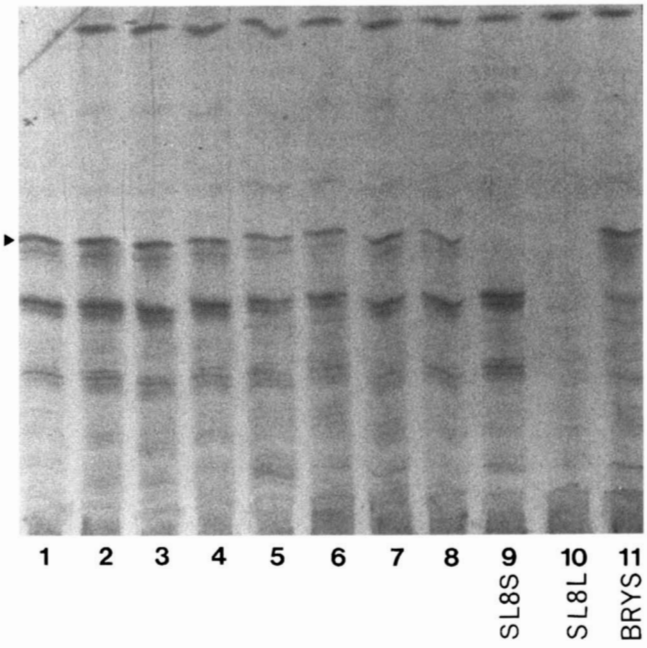

FIGURE 6.—Silver-stained isoelectric focusing gel showing style extracts from parental plants and progeny from the cross of a long-styled plant (*ss*) from the BRY F_2 family (not shown) with a short-styled plant from the variant SL8 selfed progeny (lane 9). Lanes 1–8 are short-styled progeny from this cross and all possess the three style proteins (the most heavily stained band is indicated by the arrow). Lane 9 (SL8S) is from the short-styled parental plant derived from the SL8 selfed progeny and it is missing the style proteins. Lane 10 is a weakly stained extract of a long-styled plant (SL8L) from the selfed progeny of SL8 that does not possess the style proteins. Lane 11 (BRYS) is an extract of a short-styled plant from the BRY F_2 family that has the style proteins.

of proteins in short-styled, but not in long-styled plants, is in concert with the dominance relationships of distyly and with the evolutionary scenario proposed by CHARLESWORTH and CHARLESWORTH (1979b, p. 488). In their scenario, the first step in the evolution of distyly involves a recessive mutation producing a new pollen type that might represent a loss of function mutation and perhaps loss of expression of the pollen protein in the "ancestral" long-styled plants (but this protein is retained in pollen of the "ancestral" short-styled plants). The next step involves a dominant mutation producing a new stigma type, which might represent a gain of function or expression of the unique proteins in styles of short-styled plants.

Not all of our data are consistent with the strict supergene model for the genetic control of distyly. Our results from F_2 families involving the SL8 plants, which have unusual style protein gel phenotypes, suggest that genes elsewhere in the genome (*i.e.*, genes that are not at the distyly locus) are required for the full expression of the style protein(s). Crosses by SHORE and BARRETT (1986) using BRY (another self-compatible plant) showed that genes elsewhere in the genome can also confer self-compatibility in short-styled plants.

A number of hypotheses have been proposed to account for the evolution of distyly. These include all possible scenarios as to whether the morphological polymorphism evolves first followed by the incompatibility system (DARWIN 1877; LLOYD and WEBB 1992), or the reverse scenario, of incompatibility followed by the dimorphism (BAKER 1966; CHARLESWORTH and CHARLESWORTH 1979b; GANDERS 1979). MATHER and de WINTON (1941) suggest that both arose simultaneously. DARWIN (1877), LEWIS (1942, 1943) and DULBERGER (1975a,b, 1992) proposed that incompatibility is a direct consequence of the morphological polymorphism. DARWIN (1877) suggests that self-incompatibility arose almost simultaneously and that incompatibility was an incidental by-product of the adaptation of the different types of pollen to the appropriate style lengths. If the proteins we have discovered are incompatibility proteins, then we can reject the hypothesis that incompatibility is a by-product of the morphological polymorphism or the coadaptation of pollen to different style environments. We believe that a detailed

TABLE 6

Segregation ratios for style proteins in short-styled plants from three F_2 families having SL8 in their parentage

Cross	Style protein		G^a
	Present	Absent	
1	28	6	1.06
2	39	9	1.07
3	26	8	0.04
Total	93	23	1.74

Ratios among crosses are homogeneous, $G = 0.42$.

[a] *G*-statistic for goodness of fit to 3:1 ratio for presence and absence of style proteins.

investigation of the molecular genetics of distyly will allow greater insight into the processes leading to the evolution of this breeding system.

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