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ORIGINAL ARTICLE

Characterization of X-ray-generated floral mutants carrying deletions at the *S*-locus of distylous *Turnera subulata*

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To investigate the genetic architecture of distyly in *Turnera subulata* and test the hypothesis that a supergene determines distyly, we used X-ray mutagenesis to generate floral mutants. Based upon the crossing design, all progeny were expected to be short-styled. Of 3982 progeny screened, 10 long-styled mutants, one long homostyle and one short homostyle were recovered. Assays for molecular markers tightly linked to the *S*-locus showed that the mutants were missing 1–3 markers indicating they are deletion mutants. We investigated the incompatibility phenotype of the mutants and found that both their styles and pollen behaved like those of the long-styled morph. There was a variation in the absolute length of styles, stamens and pollen size of the long-styled mutants. Furthermore, long-styled mutants possessing larger deletions tended to have their anthers and stigmas in closer proximity. We explored the inheritance of the *S*-locus mutations and found that only one of the deletion mutations was transmitted to progeny where we recovered seven such progeny. Remarkably, our data are consistent with the supergene model (*GPA*/*gpa*) of *Primula*. The long homostyle mutant appears to have deletions involving both the *G* and *P* loci. The other mutants appear to have deletions of the entire *S*-locus. The mutants generated will serve as a valuable resource for the molecular dissection of the *S*-locus region, and in the identification of genes determining distyly.
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Introduction

The reciprocal arrangement of reproductive organs and self-incompatibility (SI) system of distylous species appears to be determined by a series of tightly linked genes forming a supergene. This inheritance pattern was first proposed for the genus *Primula*, where there is evidence that three to five, or more, linked genes occur (Ernst, 1955; Dowrick, 1956; Lewis and Jones, 1992; Kurian and Richards, 1997; Barrett and Shore, 2008). There is more limited evidence supporting the occurrence of a supergene in lineages outside the Primulaceae that have independently evolved distyly, including *Fagopyrum* (Woo *et al*., 1999; Matsui *et al*., 2003; Fesenko *et al*., 2006; Wang *et al*., 2005) and *Turnera*, where the inheritance of self-compatible homostyles has been investigated (Shore and Barrett, 1985; Barrett and Shore, 1987; Tamari *et al*., 2001, 2005).

An approach to testing the hypothesis that a supergene determines distyly, is to generate and characterize mutants. Mutational studies have proven useful in the study of homomorphic SI systems (de Nettancourt, 1977; Golz *et al*., 2000) but there appears to have been no systematic investigation of distylous species using this approach. A few mutants have, however, been generated and explored in distylous *Fagopyrum esculentum* (Sharma and Boyes, 1961; Nomura *et al*., 2002). Under the commonly held supergene model of *Primula* spp., three genes tightly linked in coupling, *GPA*/*gpa*, determine the inheritance of distyly. Thus, it should be possible to mutate, or delete the component genes yielding predictable phenotypes, that should appear to be inherited as new alleles of the *S*-locus. Importantly, the generation of deletion mutants will aid efforts to positionally clone the genes determining distyly by delimiting the genomic regions containing the genes.

In this paper, we use X-ray mutagenesis to generate 12 floral mutants of *Turnera subulata*. We provide evidence that the mutants involve deletions of the *S*-locus, or portions thereof. We characterize the floral and incompatibility phenotype of the mutants and attempt to explore their inheritance. We discuss the significance of the mutants to the genetic architecture of distyly and in positional cloning.

Materials and methods

X-ray mutagenesis and mutant screen

We used two plants of diploid *Turnera subulata*, a long-styled plant (S16L, homozygous recessive, *ss*) and F60SS, a short-styled plant homozygous, *SS*, at the *S*-locus.

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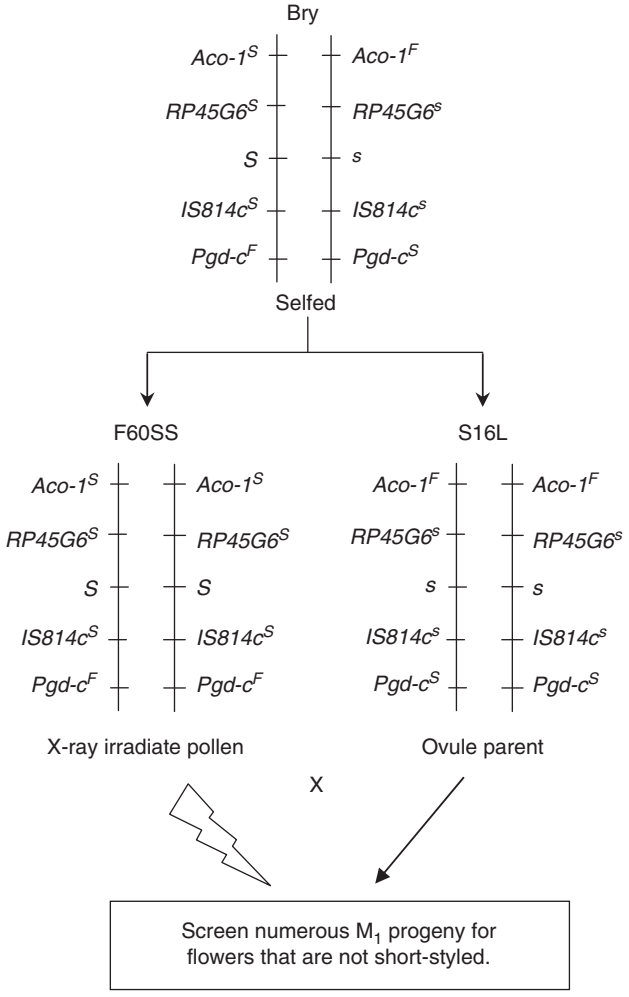

Figure 1 Crossing scheme to produce mutants. Genotypes of parental plants are given for the *S*-locus and four additional linked loci.

Both plants were produced by selfing an unusual self-compatible short-styled parental plant termed Bry. Genotypes of the parental plants at the *S*-locus and four marker loci are provided (Figure 1). Further details of Bry may be found in Athanasiou and Shore (1997) and Labonne *et al*. (2009). We rooted multiple stem cuttings of both plants (S16L and F60SS) to increase the number of flowers available for pollinations and seed production.

On mornings when pollen was to be irradiated, we collected anthers from the short-styled plant F60SS in a Petrie plate and irradiated them with various doses of X-rays using a Willick Xplorer 160 CP 3.2 kW system (Willick Engineering, Santa Fe Springs, CA, USA). Anthers were irradiated using the maximum output of the X-ray machine (160 KVp and 18.9 mA), with varying times of exposure (20–180 s). This yielded doses ranging from ~3.7 to 33.6 Gy. A pilot study was carried out in the year 2001 using a Philips MG-100 X-ray machine (Philips Electronic Instruments) and irradiating anthers with ~45 Gy. We then used the irradiated anthers to pollinate the long-styled plant, S16L, using a pair of forceps to brush 2–3 anthers across the stigmas of each flower. Seeds were collected from S16L when fruits dehisced (~3 weeks following pollination).

Seeds were sown (100 per pot) and ~3 weeks following germination, seedlings were transplanted into individual 15 cm pots. We determined the percent germination for a sample of 62 pots (18.3% ± 0.7). The morph of each plant was recorded at flowering, and short-styled plants (non-mutants) were discarded. All other plants (putative mutants) were retained and genomic DNA was extracted from them using the Mini-CTAB protocol of Labonne *et al*. (2009). Irradiation and mutant screening was carried out in the years 2001 through 2009.

Confirmation of mutant status

To confirm that the putative floral mutants were not pollen or seed contaminants, we used markers known to be linked in coupling with the *S*-allele (Figure 1). These included, initially (2001 pilot study) two isozyme loci, *Aco-1* and *Pgd-c*, known to flank the *S*-locus at distances of 5.7 and 9.1 cM, respectively (Labonne *et al*., 2007). Putative mutants from the later screens were assayed using a dominant inter-simple sequence repeat marker, *IS814c*, and a dominant random amplified microsatellite polymorphism marker, *RP45G6* (Figure 1). These markers lie on opposite sides of the *S*-locus at distances of 1.1 and 3.3 cM, respectively (Labonne *et al*., 2008). Markers *IS814c* and *RP45G6* were assayed following Labonne *et al*. (2008). Mutants are expected to be heterozygous for these markers.

Once it was confirmed that these plants were indeed mutants (and not the result of pollen or seed contamination), we determined whether they possessed a dominant marker, *RP81E18*, known to co-segregate with the *S*-allele (Labonne *et al*., 2008, 2009). We used the end sequences of *RP81E18* to design new primers (RPE18F3: 5′-GGACTGCAGAG-3′ and RPE18R3: 5′-GGGTGGGTG C-3′) to increase the level of amplification of *RP81E18* thereby facilitating scoring of this marker. The PCR cocktail consisted of 1 µl of DNA (~25 ng), 1.0 µl of each primer (10 pmol), 2.75 µl of ddH_2O and 6.25 µl of JumpStart REDTaq Ready mix DNA polymerase (Sigma-Aldrich Inc., Oakville, ON, Canada) in a total volume of 12.0 µl. The PCR machine was programmed for a 2 min initial denaturation followed by 35 cycles of 94 °C for 50 s, 37 °C for 25 s, 72 °C for 85 s and a final extension step of 3 min at 72 °C. Amplification products were run on 7.5% polyacrylamide gels for 65 min and silver-stained following Labonne *et al*. (2008).

Phenotype of mutants

We determined the morph of each mutant. We also measured a number of floral characters for 10 replicate flowers to the nearest 0.1 mm using vernier calipers. We measured both style and stamen lengths from the base of the ovary, and petal length as a measure of flower size. We calculated stigma-anther separation as the difference between style length and stamen length. We measured the length of 20 freshly collected dry pollen grains using a calibrated ocular micrometer on a compound light microscope. The mutants show varying levels of pollen sterility. To avoid measuring the typically smaller sterile grains, we measured only pollen grains that possessed a normal shape. Mutants L1 and SH1 were highly pollen sterile and we were only able to find and measure 6 and 16 pollen grains for each, respectively. We estimated pollen fertility by staining pollen with aniline blue in lactophenol, and scoring stainability for 200 grains from

each of three flowers per plant. We determined seed set per pollination for the mutants by pollinating 10 flowers from each mutant (with the exception of SH1 where only four flowers were pollinated) with compatible pollen from one of the parental plants and counted seed production.

We assessed the incompatibility phenotype of both pollen and styles for each mutant. We pollinated each mutant with pollen from a long- and short-styled plant (S16L and Bry). We also used pollen of the mutants to pollinate a long-styled plant (S16L) as well as a self-incompatible short-styled plant of *T. scabra* (since Bry is self-compatible due to the lack of incompatibility in its style, it was not useful for this purpose). We carried out two replicate pollinations of each type above and collected and fixed the styles (three per flower pollinated) in 3 ethanol: 1 acetic acid 24 h after pollination. We stained the styles in aniline blue and observed pollen tubes using fluorescence microscopy following Tamari *et al*. (2001).

We used analysis of variance (ANOVA) followed by Tukey's studentized range test for multiple comparisons, to determine whether there were significant differences among the mutants for various reproductive characters. We included the parental plants in the analyses when appropriate. In some analyses we controlled for flower size, using petal length as a covariate in analyses of covariance. Both paired and two-sample *t*-tests were carried where indicated. An arcsine transformation was applied to pollen fertility data and a log transformation was applied to seed set data. Analyses were carried out using SAS 9.1 software (SAS Institute Inc. (2004)).

Detection of polygalacturonase and α-dioxygenase

Immunoblotting was used to determine whether the styles of the mutants possessed a polygalacturonase (Athanasiou *et al*., 2003) and an α-dioxygenase (Khosravi *et al*., 2004), both known to be specific to styles of short-styled plants. We collected styles from newly opened flowers of each mutant as well as the parental plants (as controls). We then carried out sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting to detect each of the proteins, following previous protocols (Athanasiou *et al*., 2003; Khosravi *et al*., 2004). Mutant SH1 flowers infrequently as many floral buds abscise before anthesis. Unfortunately, we were unable to obtain freshly collected styles to assay the presence or absence of these proteins for SH1.

Inheritance of mutations

We crossed the mutants as pollen and/or ovule parents to S16L, F60SS and/or Bry, depending on their morph, incompatibility behaviour and degree of pollen or seed sterility. Some of the mutants set no seeds as ovule parents. We recorded the phenotype of the resulting progeny at flowering. For mutants L1 and LH1, which were obtained from the pilot study of 2001, we assayed progeny for both linked isozyme markers, *Aco-1* and *Pgd-c*, following methods of Athanasiou and Shore (1997). For all other progeny, we extracted DNA as above. We then performed PCR to amplify co-dominant marker *P9SP1* from each progeny. This marker has recently been identified in our lab during chromosome walking and it is located at 0.05 cM on the distal side of the *S*-locus (Labonne JDJ and Shore JS, unpublished data). Genotyping progeny with marker *P9SP1* allowed us to determine with high probability whether the mutant allele had been transmitted to progeny. In some cases, further assays with other *S*-linked markers located on opposite sides of the *S*-locus were required to confirm inheritance for long-styled progeny.

For crosses involving mutants for which the *P9SP1* marker has been deleted, we assayed long-styled progeny directly with marker *IS814c* (proximal side of the *S*-locus; Figure 1). For long-styled progeny for which only the *S*-linked marker *IS814c* amplified, we further assayed these plants with a recently identified dominant random amplified microsatellite polymorphism marker (*RP95K7*) located at 11.7 cM on the opposite (distal) side of the *S*-locus (Labonne JDJ and Shore JS, unpublished data).

Mutant L22 is interesting because it is a long-styled plant that possesses marker *RP81E18* that co-segregates with the *S*-allele (Labonne *et al*., 2009). For progeny of the cross L22 × Bry and reciprocal, we assayed short-styled progeny with the co-dominant marker *P9SP1* and long-styled progeny with marker *RP81E18*, to determine whether progeny had inherited the mutation.

Some progeny inherited the mutation of mutant L20. We measured the pollen fertility and pollen size (as above) for all seven progeny that inherited the mutation as well as 27 progeny that did not (as controls). We also used one long- and two short-styled progeny of L20 carrying the deletion and performed reciprocal crosses in an attempt to generate a mutant homozygous for the *S*-locus deletion.

Results

Identification of *S*-locus mutants

Pollinations using irradiated pollen from F60SS (homozygous *SS* at the *S*-locus) crossed onto S16L (*ss*), yielded a total of 3982 progeny. In the absence of mutation at the *S*-locus, all progeny are expected to be short-styled (*Ss*) (Figure 1). Screening resulted in the identification of 12 mutants including one long homostyle (LH1, Figure 2c), one short homostyle (SH1, Figure 2e) and 10 long-styled mutants (L1, L15, L16, L20–L26).

The 12 mutants recovered show varying sizes of deletions as determined by assays of five *S*-linked markers (Figure 3 and 4). Four mutants have a deletion of only one marker (*RP81E18*), three have deletions of two markers (*RP81E18* and *P9SP1*), whereas the remaining mutants have deletions of three or more markers (Figure 3). Interestingly, mutant L22 is the only mutant without a deletion of *RP81E18*, the marker most closely linked to the *S*-locus.

Incompatibility behaviour of mutants

The styles of all mutants inhibited pollen tubes when pollinated by the long-styled parental plant S16L. Pollen germinated penetrated the stigma and grew into the styles, but rarely grew more than half the length of the style. Pollen from the short-styled parent Bry, was compatible on all mutants, with pollen tubes growing to the base of styles (Figure 3). Interestingly, the style of the short homostyle mutant (SH1) had the same incompatibility behaviour as the long-styled mutants.

Figure 2 Photographs showing the styles and stamen positions for parental plants and three mutants. (**a**) Long-styled parent S16L. (**b**) Short-styled parent F60SS. (**c**) Long homostyle mutant LH1. (**d**) Long-styled mutant L22 with a shorter style than parent S16L. (**e**) Short homostyle mutant SH1.

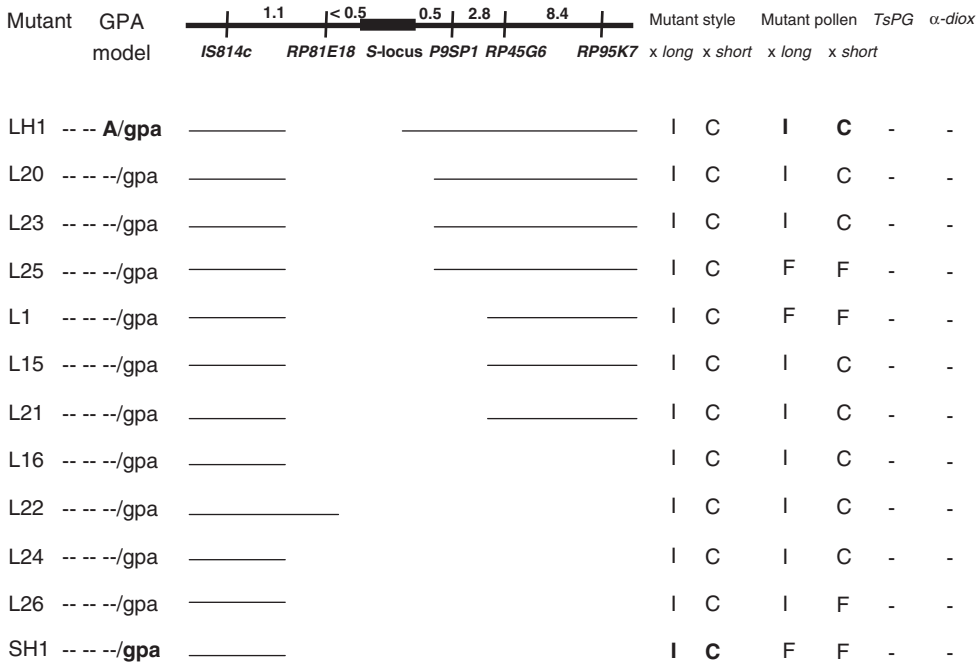

Figure 3 Characterization of 12 *S*-locus mutants. A genetic map of showing the position of five molecular markers relative to the *S*-locus is provided at the top of the figure. For each mutant, a deletion map is also provided. The genotype at the putative *S* supergene (two dotted lines indicate deletion of a gene) as well as the incompatibility phenotype of each mutant are shown. Pollen from S16L (long) and Bry (short) were used to investigate the incompatibility behaviour of mutant styles, whereas the phenotypes of mutant pollen were determined using the styles of S16L and short styles of self-incompatible *T. scabra*. The letter C denotes compatible, with pollen tubes growing to base of style; I—incompatible, with tubes not growing to base of style. F—cannot evaluate compatibility as few or no pollen grains adhere to stigma. *TsPG* represents the results for the style polygalacturonase whereas *α-diox* denotes the α-dioxygenase. The short-style-specific proteins are not expressed in the mutants assayed. Interesting characteristics of some mutants are in bold.

For this mutant, however, pollen of the long-styled parent (S16L) grew as much as ~80% the length of the style.

Pollen from nine mutants (LH1, L15, L16, L20-L23, L24 and L26) was incompatible on the styles of the long-styled parent, S16L and pollen tubes rarely grew more than ~1/3 the length of the style. We could not assess the incompatibility of pollen for mutants L1, L25 and SH1 on stigmas of S16L and short-styled *T. scabra*, nor for L26 on stigmas of the short-styled plant. Pollen of these mutants is highly sterile (Figure 5c) and few or no pollen grains adhered to the stigmas (Figure 3). Pollen of the remaining seven long-styled mutants was found to be fully compatible with short-styled, self-incompatible plants of *T. scabra*. Interestingly, pollen of the long homostyle, LH1, was compatible with short-styled *T. scabra* (Figure 3).

To explore further the pollen compatibility of mutant LH1, we determined seed set following crosses using pollen of LH1 and pollinating the long- and short-styled

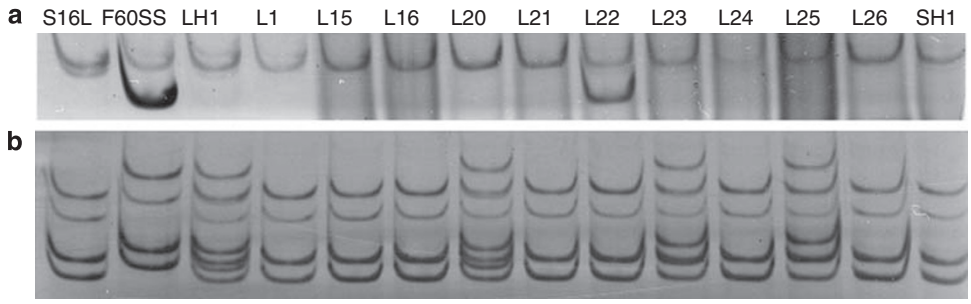

Figure 4 Genotypes of co-segregating marker *RP81E18* and co-dominant marker *P9SP1* for 12 X-ray mutants as well as two controls (S16L and F60SS). Dominant marker *RP81E18* is absent in all but one mutant whereas eight mutants are missing the *S*-linked bands of co-dominant marker *P9SP1*.

parental plants (S16L and Bry). The mean seed set per pollination differed significantly for crosses onto the short- (24.7 ± 2.1, $n = 12$) versus long-styled (4.4 ± 1.5, $n = 9$) parental plants ($t_{19} = 7.3$, $P < 0.001$).

Distribution of polygalacturonase and α-dioxygenase in styles of mutants

Immunoblotting of a short style-specific polygalacturonase and an α-dioxygenase, revealed that none of the mutants expressed either protein in their styles (Figure 3). The short-styled parental plants, Bry and F60SS (controls), expressed both proteins as expected. Mutant SH1 flowers infrequently and, unfortunately, fresh styles could not be included in the assays. Identical results were obtained previously for mutants LH1 and L1 (Khosravi *et al*., 2003, 2004).

Quantification of reproductive characters

We quantified a number of reproductive characters of the mutants and parental plants. The mutants differ significantly in flower size as determined by petal length ($F_{14,135} = 13.1$, $P < 0.001$) with mutants L23 and L1 possessing the smallest, whereas L24 and L26 had the greatest petal length.

We plotted the reproductive character means ranking the long-styled mutants by decreasing stigma-anther separation (Figure 5a). Styles of the mutants are significantly different in length (Figure 5a). This result held when we excluded the short-styled plants and the short homostyle (SH1) mutant from the analysis ($F_{11,108} = 17.2$, $P < 0.001$) and using petal length as a covariate ($F_{11,107} = 22.7$, $P < 0.001$). Comparable analyses revealed significant differences in stamen length among the mutants (Figure 5a).

There is significant variation for stigma–anther separation among the long-styled mutants (ANOVA results excluding the short-styled parental plants; $F_{12,117} = 35.2$, $P < 0.001$). Some mutants, for instance L15, L25 and L23, have their stigmas and anthers well spatially separated in spite of their small flower size (ANCOVA; $F_{10,98} = 17.9$, $P < 0.001$). Long-styled mutants L15, L20, L23 and L25 do not show a significant difference in stigma-anther separation from their long-styled parent, S16L, whereas the remaining mutants have significantly smaller stigma-anther separations.

Mutant SH1 has its stigmas and anthers in very close proximity (Figure 2e) but the stigma–anther separation differs significantly from zero (mean ± s.e. 1.1 ± 0.3 mm; paired *t*-test, $t_9 = 3.8$, $P < 0.005$). The floral phenotype of SH1 is more like that of a short homostyle than a long-styled plant and we have therefore described it as such. The style length of SH1 does not differ significantly from that of its short-styled parent, F60SS, whereas its stamens do not differ from those of the long-styled parent S16L (Figures 2a, 5a).

Mutant LH1 appears to be a 'classic' long homostyle possessing both long styles and long stamens (Figures 2c, 5a) with a stigma–anther separation of 0.1 ± 0.1 mm, not significantly from zero (paired *t*-test, $t_9 = 0.6$, $P > 0.6$). The styles of the LH1 do not differ significantly from those of its long-styled parent, S16L (Figure 5a). The stamens of LH1 (12.3 ± 0.4 mm) are significantly greater than those all other mutants and the long-styled parental plant S16L, but are significantly shorter than those of the short-styled parental plants Bry and F60SS (Figure 5a).

Pollen size showed some interesting differences among the mutants and parental plants. Parental plant F60SS, which is homozygous (*SS*) at the *S*-locus, possessed the largest pollen (Figure 5b) whereas Bry (*Ss*), had the next largest pollen, which did not differ significantly from pollen of mutants L20, or SH1 (Figure 5b). Mutant LH1 had the next largest pollen, which was significantly smaller than that of the parental short-styled plants (F60SS and Bry), but significantly greater than that of the long-styled parent (S16L) and a number of long-styled mutants (Figure 5b). Four of the long-styled mutants had pollen that was not significantly different from that of S16L.

To further explore the large pollen size of mutant L20, we measured the pollen size of progeny from crosses involving this mutant (see Inheritance of mutations). The mean pollen size of short-styled progeny with versus without the mutation did not differ significantly (with the mutation: 81.3 ± 3.3 μm, $n = 2$; without the mutation: 79.0 ± 0.4 μm, $n = 16$, $t_{16} = 1.6$, $P > 0.13$). The mean size of pollen of long-styled progeny with the mutation was 76.8 ± 0.7 μm ($n = 5$, range 74.9–79.1 μm) versus 66.7 ± 0.4 μm ($n = 11$, range 66.1–68.7 μm) and differed significantly ($t_{14} = 13.5$, $P < 0.001$). Long-styled progeny bearing the mutation had a pollen size comparable with that of the L20 mutant and the short-styled parental plants, whereas progeny without the mutation were comparable in size to those of the long-styled parental plant S16L (Figure 5b).

Pollen fertility of all mutants was relatively poor and differed significantly from the parental plants (Figure 5c). Four mutants (LH1, L15, L21 and L22) had pollen fertility

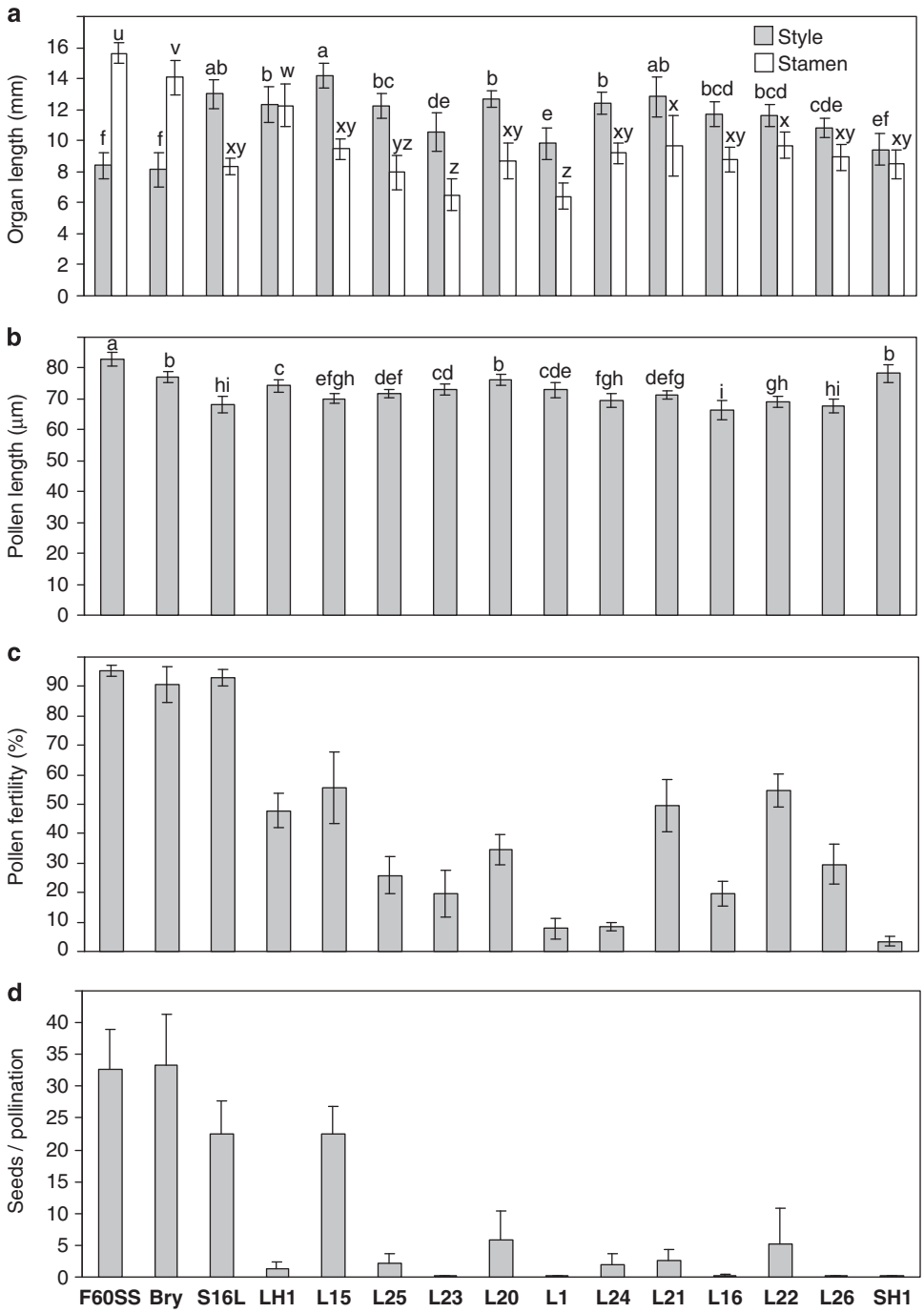

Figure 5 Means (+/−1 s.d.) and ANOVA results for reproductive characters of mutants and parental plants. (**a**) Mean style length and stamen length. Separate 1-way ANOVAs were carried for style ($F_{14,\ 135} = 36.3$, $P < 0.001$) and stamen length ($F_{14,135} = 62.9$, $P < 0.001$). (**b**) Mean length of pollen grains ($F_{14,\ 267} = 92.1$, $P < 0.001$). (**c**) Mean percent pollen fertility ($F_{14,\ 30} = 75.6$, $P < 0.001$). (**d**) Mean seed set ($F_{14,129} = 97.1$, $P < 0.001$). Bars sharing the same letter are not significantly different following ANOVA and Tukey's test.

of ~50% whereas one (SH1) was as low as ~3.5% (Figure 5c). To explore whether deletion of the *S*-locus region alone might be responsible for the reduced pollen fertility, we again exploited progeny from the cross L20 × Bry and the reciprocal. The mean percent pollen fertility of progeny with the mutation was 49.8 ± 3.5% ($n = 7$, range 30.8–58.0%) versus 88.5 ± 2.0% ($n = 27$, range 59.0–98.5%) ($t_{32} = 8.8$, $P < 0.001$).

The mutants showed reduced seed set, with the exception of mutant L15, which did not differ significantly from the long-styled parent, S16L (Figure 5d). Mutants L1, L23, L26 and SH1 set no seeds at all (Figure 5d). The remaining mutants did not have seed set exceeding ~6 seeds per pollination.

Inheritance of mutations

We investigated the inheritance of the *S*-locus mutations using crosses of seven of the deletion mutants and screening a total of 324 progeny. We identified only six

Table 1 Inheritance of mutations identified using *S*-locus linked markers for crosses between mutants and parental plants S16L, F60SS and Bry

Crosses	*Morph*	*Number of progeny*	
		Without deletion	*With deletion*
L20 × Bry	Long	25	5
	Short	15	1
Bry × L20	Long	4	0
	Short	12	0
L20Bry-5 L × F60SS	Short	29	1
F60SS × L20Bry-5 L	Short	24	0
S16 L × LH1	Long	18	0
Bry × L1	Long	5	0
	Short	7	0
L15 × F60SS	Short	3	0
F60SS × L15	Short	62	0
L15 × Bry	Long	30	0
	Short	32	0
Bry × L21	Long	10	0
	Short	14	0
L22 × Bry	Long	17	0
	Short	14	0
Bry × L22	Long	11	0
	Short	18	0
Bry × L23	Long	10	0
	Short	11	0

Maternal plants are listed first in the crosses above.

progeny (five long-styled and one short-styled) that inherited the mutation, as determined by assaying *S*-linked markers (see Materials and methods). All six progeny were derived from mutant L20 (Table 1) and only when it was used as the female parent. Crosses using one of the L20 progeny possessing the deletion (plant L20Bry-5L) as a female parent to F60SS produced one short-styled plant, which inherited the *S*-locus mutation ($n = 30$). No progeny inherited the mutation when L20Bry-5L was the pollen parent ($n = 24$) (Table 1).

In summary, 378 progeny were assayed to study the inheritance of the mutations. No mutations were transmitted to offspring using the mutants as pollen parents ($n = 206$) and only seven (five long- and two short-styled) of 172 progeny inherited the mutation using the mutants as ovule parents. All of these progeny carried the mutation of L20.

Crosses made in an effort to produce a mutant homozygous for the *S*-locus deletion did not yield any such progeny ($n = 154$). Although some progeny carried one copy of the deletion, it was transmitted through the ovules, not the pollen.

Discussion

We have generated 12 floral mutants using X-ray mutagenesis and have shown that they are missing one or more *S*-linked molecular markers indicating they are deletion mutants. We believe this study represents the most extensive series of *S*-locus deletions produced for any heterostylous species. Although X-rays can also produce chromosome rearrangements, our mutant screen was likely biased towards detection of deletions. Five of the mutants generated had relatively large deletions (Figure 3). Using the estimates of 650 kb/cM in *Turnera subulata* (Labonne *et al*., 2008), the size of the deletions in these five mutants is predicted to be greater than 7 mb. Mutants carrying similar-sized deletions have been reported in an extensive mutagenesis study in *Arabidopsis thaliana* (Naito *et al*., 2005).

The genetic architecture of distyly has been explored, especially in *Primula*, by analysing the inheritance of rare recombinants at the *S* supergene (Ernst, 1955; Dowrick, 1956). The production of deletion mutants, however, provides another unexploited means of exploring the genetic architecture of distyly. Under the *Primula S* supergene model, the genotype of the short-styled morph at the *S*-locus is *GPA/gpa* whereas long-styled plants are *gpa/gpa*, where *G/g* determines style length and its incompatibility, *P/p* determines pollen size and its incompatibility and *A/a* determines stamen length (Dowrick, 1956; Lewis and Jones, 1992; Barrett and Shore, 2008). In *Turnera*, genetic studies are consistent with the hypothesis that a supergene comprised of at least two genes, determines distyly (Shore and Barrett, 1985; Tamari *et al*., 2005; Shore *et al*., 2006). In this study, 10 of the X-ray generated floral mutants had the long-styled phenotype indicating that all the *S* components of the putative supergene have been deleted. Long-styled mutant L22 was the only deletion mutant possessing *RP81E18*, a marker, which co-segregates with the *S*-locus in a family of 2013 progeny (Labonne *et al*., 2009). This indicates that, although *RP81E18* is extremely tightly linked, it lies just outside of the *S*-locus, as suggested by Labonne *et al*. (2009).

Under the supergene model, deletion or generation of a non-functional form of the dominant *G*-allele would result in the production of a long homostyle having the genotype $g^{del}PA/gpa$, where g^{del} represents the deleted/mutated allele. Such a plant would possess the style length and style incompatibility of a long-styled plant, but would have all other characteristics of a short-styled plant. The long homostyle (LH1) generated here appears to possess such a mutation, g^{del}. Pollen of LH1, however, is compatible with styles of the short-styled morph, producing considerably greater seed set compared with pollinations of the long-styled morph, with which it is largely incompatible (Figures 3 and 5). Interestingly, this incompatibility phenotype suggests that LH1 has a mutation not only at the *G* locus but also at *P*, which is the locus determining pollen incompatibility behaviour and pollen size, originally postulated for *Primula* (Dowrick, 1956). As the mutation carried by LH1 did not transmit to its progeny, we cannot exclude the possibility that the pollen incompatibility response of LH1 is the result of a mutation elsewhere in the genome.

If both the floral phenotype and incompatibility behaviour of mutant LH1 is the result of a single deletion in the *S*-locus, then this indicates that a *P*-locus may also occur in *Turnera subulata*. The likely gene order would be *GPA* as the deletion would take out two adjacent genes. LH1 has pollen significantly greater in size than that of the long-styled parent, but significantly smaller than that of the short-styled parental plants (Figure 5b). Therefore, we cannot determine with certainty whether separate genes for pollen size and its incompatibility occur at the putative *P* locus in *T. subulata*, as may occur in *Primula Tommasinii* (Kurian and Richards, 1997).

The occurrence of pollen of intermediate size is difficult to rationalize in terms of a single *P*-locus being the sole determinant of pollen size. This finding could

suggest that pollen size is determined by more than one gene. Indeed, five of the long-styled progeny from the cross L20 × Bry, which carry the mutation of L20 have pollen significantly greater in size than the long-styled progeny without the mutation. This strongly supports the notion that additional gene(s) determining pollen size occur and they may reside in the vicinity of the *S*-locus.

Short homostyles can arise via deletions of the *A* component of the *S* supergene or both the A and *P* subunits (Dowrick, 1956). Our SH1 short homostyle, however, appears to have a deletion of the entire *S*-locus region. We believe that SH1 is a long-styled plant with an unusually short style. The five mutants possessing large deletions that include markers *RP45G6* and *RP95K7*, all have their stigmas and anthers in closer proximity than the remaining long-styled mutants (except L21) (Figures 3 and 5a). SH1 may therefore represent the extreme of this situation. Its style incompatibility behaviour is also like that of a long-styled plant. The reduced stigma–anther separation of these five long-styled mutants suggests that gene(s) residing outside the *S*-locus (on the *RP45G6* side) may influence the lengths of styles and stamens. Modifier genes outside of the *S*-locus have been reported to affect style length in *Primula sinensis* and *Fagopyrum esculentum* (Mather, 1950; Matsui *et al*., 2004, 2007).

It has previously been shown that a style polygalacturonase and an α-dioxygenase display short-specific expression patterns and appear to be upregulated by the *S*-allele (Athanasiou *et al*., 2003; Khosravi *et al*., 2004; Tamari and Shore, 2004). Neither gene occurs in the vicinity of *S*-locus. The absence of these proteins from styles of all long-styled mutants and LH1 supports the hypothesis that both genes are regulated by the *S*-allele, possibly the putative *G* locus, or a gene tightly linked to it. This continues to suggest a possible role for the polygalacturonase and α-dioxygenase in distyly.

Inheritance studies yielded only seven progeny possessing an *S*-locus mutation. All progeny were derived from mutant L20, when used as the female parent. Mutant L20 has one of the smallest deletions. Three other mutants (LH1, L23 and L25) possessed deletions of a comparable size (Figure 3), but set little or no seeds when used as female parents (Figure 5) and we could obtain only few, if any, progeny from crosses in this direction. Naito *et al*., (2005) postulated that non-transmission of deletions in *Arabidopsis thaliana* could be due to the knock out of genes required for ovule viability. In our study, the lack of transmission of mutations from the remaining deletion mutants (in the female direction) and the transmission bias against the L20 deletion, could similarly be the result of such genes. It is also possible that non-transmission is caused by the reduced viability of embryos, and/or seeds heterozygous for the deletion.

We did not observe the transmission of any of the mutations through pollen. Mutagenesis is known to lead to poor male transmission (Stadler and Roman, 1948) and can cause male sterility (Nasrallah *et al*., 2000; Koorneef, 2005; Naito *et al*. 2005). Furthermore, Mottinger (1969) noted that X-ray deletions affect the ability of pollen tubes to compete with those that do not possess the deletion. The reduced pollen fertility of all the mutants and the seven progeny (~50% pollen fertility) bearing the mutation of L20 strongly suggests that non-transmission may be a direct result of deletion of the *S*-locus and/or adjacent genes causing pollen sterility.

Naturally occurring and induced *S*-locus mutants have proved to be a valuable resource in studies of homomorphic SI systems (Goring *et al*., 1993; Nasrallah *et al*., 1994; Sassa *et al*., 1997; Golz *et al*., 2000, 2001; Ushijima *et al*., 2004; Sonneveld *et al*., 2005; Hauck *et al*., 2006). In heteromorphic SI systems, the genes determining distyly and tristyly have not yet been discovered (Barrett and Shore, 2008; Labonne *et al*., 2009). To identify the genes controlling distyly, we have initiated chromosome walking in *Turnera* (Labonne *et al*., 2009). Similar approaches are being used in *Primula* and *Fagopyrum* (Li *et al*., 2007; McCubbin, 2008; Yasui *et al*., 2008). Once a bacterial artificial chromosome contig of the *S*-locus region is assembled, the mutants generated will be invaluable in narrowing the specific region of the contig containing the *S*-locus genes of *T. subulata*. Mutant LH1, which we predict to have a deletion breakpoint within the *S*-locus, should aid in identification of the gene determining stamen length since, unlike the other mutants, it has a non-mutant stamen length phenotype.

Conflict of interest

The authors declare no conflict of interest.

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