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Fine Mapping of *ui6.1*, a Gametophytic Factor Controlling Pollen-Side Unilateral Incompatibility in Interspecific Solanum Hybrids

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ABSTRACT

Unilateral incompatibility (UI) is a prezygotic reproductive barrier in plants that prevents fertilization by foreign (interspecific) pollen through the inhibition of pollen tube growth. Incompatibility occurs in one direction only, most often when the female is a self-incompatible species and the male is self-compatible (the "SI × SC rule"). Pistils of the wild tomato relative *Solanum lycopersicoides* (SI) reject pollen of cultivated tomato (*S. lycopersicum*, SC), but accept pollen of *S. pennellii* (SC accession). Expression of pistil-side UI is weakened in *S. lycopersicum* × *S. lycopersicoides* hybrids, as pollen tube rejection occurs lower in the style. Two gametophytic factors are sufficient for pollen compatibility on allotriploid hybrids: *ui1.1* on chromosome 1 (near the *S* locus), and *ui6.1* on chromosome 6. We report herein a fine-scale map of the *ui6.1* region. Recombination around *ui6.1* was suppressed in lines containing a short *S. pennellii* introgression, but less so in lines containing a longer introgression. More recombinants were obtained from female than male meioses. A high-resolution genetic map of this region delineated the location of *ui6.1* to ~0.128 MU, or 160 kb. Identification of the underlying gene should elucidate the mechanism of interspecific pollen rejection and its relationship to self-incompatibility.

FLOWERING plants have evolved several reproductive barriers for preventing illegitimate hybridization with related species. These barriers may be expressed prefertilization and/or postfertilization. Unilateral incompatibility or incongruity (UI) is a prefertilization barrier that occurs when pollen of one species is rejected on pistils of a related species, while no rejection occurs in the reciprocal cross (De Nettancourt 1977). In theory, unilateral incompatibility should reinforce species identity in natural, sympatric populations of related taxa. This barrier also impedes the efforts of plant breeders to transfer traits from wild species into related crop plants. For example, the transfer of cytoplasmic traits from species with maternally inherited chloroplasts and mitochondria may be prevented by unilateral crossing barriers. Nuclear-encoded traits may also be inaccessible if F_1 interspecific hybrids are both male sterile and incompatible as female parents.

In the Solanaceae, unilateral incompatibility is observed in crosses between cultivated tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) and some related wild species. In general, pistils of the cultivated tomato act as a "universal acceptor," in that they fail to recognize and reject pollen of other tomato species. In the reciprocal crosses, pollen of *S. lycopersicum* is rejected on styles of virtually all of the green-fruited species, but not on styles of other red or orange-fruited species (reviewed by Mutschler and Liedl 1994). This pattern is mostly consistent with the "SI × SC" rule, wherein pollen of self-compatible (SC) species (including cultivated tomato) are rejected on pistils of self-incompatible (SI) species, but not in the reverse direction (Lewis and Crowe 1958). Exceptions to the SI × SC rule in the tomato clade include species or populations that have lost self-incompatibility but retain the ability to reject pollen of tomato. This is the case for the facultative outcrossing species *S. chmielewskii*, the autogamous *S. neorickii* (formerly *L. parviflorum*), as well as marginal SC populations of normally SI species such as *S. pennellii* and *S. habrochaites* (formerly *L. hirsutum*). An SC accession of *S. pennellii*, LA0716, is exceptional in having lost the ability to reject self pollen, while retaining the ability to serve as pollen parent on styles of SI accessions of this species (and other SI species, including *S. peruvianum* and *S. lycopersicoides*) (Hardon 1967; Rick 1979; Quiros *et al.* 1986). In this regard, *S. pennellii* LA0716 conforms to the Lewis and Crowe (1958) model in that it behaves like a transitional form lacking SI function in the pistil but not in the pollen.

Unilateral incompatibility may also occur in crosses between populations or races of a single species. In

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S. habrochaites for example, pollen from SC biotypes located at the northern or southern margins of its geographic range is rejected on pistils of the central, SI populations (Martin 1961, 1963). Furthermore, pollen from the northern SC group is rejected by styles of the southern SC populations. Yet pistils of both SC biotypes are able to reject pollen of cultivated tomato. Thus there appear to be at least three distinct unilateral crossing barriers, just within *S. habrochaites*, possibly indicating different pollen tube recognition and rejection systems. The F_1 N × S hybrid is SC, as expected, but SI plants are recovered in the F_2 generation, suggesting that the loss of SI occurred via independent mutations in the north and the south (Rick and Chetelat 1991).

Interspecific F_1 hybrids between SI wild species and SC cultivated tomato are self-incompatible and reject pollen of cultivated tomato, indicating both traits are at least partially dominant (McGuire and Rick 1954; Martin 1963; Hardon 1967). Interestingly, pollen of the F_1 hybrids is incompatible on pistils of the wild species parent (*i.e.*, including other individuals of the same accessions, but with nonmatching S alleles). This observation suggests that there are dominant factors from cultivated tomato that lead to pollen rejection on styles of the wild species, regardless of the pollen genotype. This apparent sporophytic effect contrasts with the purely gametophytic nature of pollen SI specificity in the Solanaceae (De Nettancourt 1977).

Early studies of the inheritance of unilateral incompatibility in tomato suggested the involvement of several genes controlling the pistil response; however, the genetics of pollen responses have received little attention. In F_2 *S. habrochaites* (northern SC accession) × *S. habrochaites* (central SI accession), the rejection of pollen from the SC parent segregated as if controlled by one to two dominant genes from the SI accession (Martin 1964). In crosses of *S. lycopersicum* to both SI and SC accessions of *S. pennellii*, the intra- and interspecific crossing relations were largely consistent with the Lewis and Crowe (1958) model of stepwise mutation at the *S* locus (Hardon 1967); there was also evidence of a second barrier in the SC *S. pennellii* accession. In F_1 and BC_1 hybrids of *S. lycopersicum* × *S. habrochaites,* the segregation of unilateral and self-incompatibilities was consistent with the action of two major genes, with minor polygenes indicated as well (Martin 1967). More recently, several QTL underlying pistil-side unilateral and self-incompatibilities were mapped in BC_1 *S. lycopersicum* × *S. habrochaites* (Bernacchi and Tanksley 1997); the major QTL for both forms of pollen rejection was located at or near the *S* locus on chromosome 1, which controls SI specificity (Tanksley and Loaiza-Figueroa 1985).

There are little data on pollen-side unilateral incompatibility factors in the tomato clade, or any other system. Our previous work identified two to three genetic loci from *S. pennellii* that are required for pollen to overcome incompatibility on pistils of *S. lycopersicum* × *S. lycopersicoides* or *S. lycopersicum* × *S. sitiens* hybrids (Chetelat and Deverna 1991; Pertuze *et al.* 2003). One of these factors mapped to the *S* locus, the other two were on chromosomes 6 and 10. In this system the female tester stocks were either diploid or allotriploid hybrids, the latter containing one genome of the wild, SI parent, plus two genomes of cultivated tomato; both types of hybrids reject pollen of cultivated tomato. The pollen parents were either F_1 *S. lycopersicum* × *S. pennellii* or bridging lines developed by backcrossing the F_1 to cultivated tomato and selecting for the ability to overcome stylar incompatibility. In the progeny, distorted segregation ratios were observed in which the *S. pennellii* alleles were preferentially transmitted, indicating linkage to gametophytic factors required forcompatibility.

This experimental system has several advantages for detecting pollen (gametophytic) unilateral incompatibility genes. First, pollen-expressed factors are readily distinguished from pistil factors because only the former show linkage to *S. pennellii* specific markers. Second, pollen rejection is by unilateral, not self-incompatibility, since both species contributing to the pollen genotype, *S. lycopersicum* and *S. pennellii*, are SC. Finally, as we describe herein, the rejection of tomato pollen by pistils of the interspecific hybrids is weakened by the decreasing dosage of the *S. lycopersicoides* genome, which reduces the number of pollen factors required for compatibility. Thus, the gametophytic factors on chromosomes 1 and 6 (denoted hereinafter *ui1.1* and *ui6.1*), when present in the same pollen, are sufficient for full compatibility on pistils of allotriploid interspecific hybrids, whereas they confer only partial compatibility on diploid hybrids.

Our overall objectives are to identify the genes underlying both the chromosome 1 and chromosome 6 pollen-specific unilateral incompatibility factors from *S. pennellii* and to determine the nature of their interaction. Toward this goal, we report herein the high-resolution genetic and physical mapping of the *ui6.1* region.

MATERIALS AND METHODS

Plant materials: The parental species and accessions used were *S. lycopersicum* cultivars 'Vendor Tm-2[a]' (accession LA2968), 'UC-82B' (LA2801), and 'VF-36' (LA0490), *S. pennellii* (LA0716), and *S. lycopersicoides* (LA1964, LA1991, and LA2951). Seed of these lines was obtained from the C. M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). An allotriploid interspecific hybrid (GH266, Rick *et al.* 1986), containing two genomes of *S. lycopersicum* (cv. UC82B) and one genome of *S. lycopersicoides* (LA1964), was used as a female tester line to detect pollen unilateral incompatibility factors (Figure 1, 'LLS'). For comparison purposes, pollination tests were also carried out with pistils of the 2x F_1 interspecific hybrid (90L4178 = *S. lycopersicum* VF36 × *S. lycopersicoides* LA2951) and pure *S. lycopersicoides* (LA1991). Our previous studies (Chetelat and Deverna 1991) indicated that two pollen factors from *S. pennellii*, located on

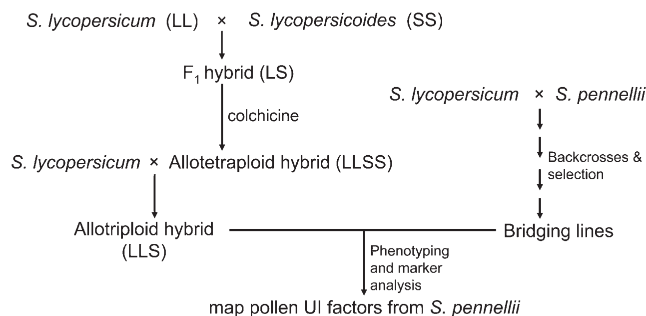

Figure 1.—Crossing scheme used to develop *S. lycopersicum* × *S. lycopersicoides* interspecific hybrids and bridging lines containing gametophytic unilateral incompatibility factors from *S. pennellii*. L, genome of *S. lycopersicum*; S, genome of *S. lycopersicoides*. Pollen of *S. lycopersicum* is rejected on pistils of *S. lycopersicoides* and its interspecific hybrids with cultivated tomato, whereas "bridging lines" containing specific gametophytic factors from *S. pennellii* are compatible.

chromosomes 1 and 6, were required and sufficient for overcoming incompatibility on styles of the allotriploid hybrid. The chromosome 1 factor, *ui1.1*, mapped close to the *S* locus controlling self-incompatibility. The chromosome 6 factor, *ui6.1*, was located on the short arm of chromosome 6. The phenotypes of recombinants around *ui6.1* were determined by test pollinations onto the allotriploid hybrid (Figure 1).

To fine map *ui6.1*, two F_2 populations were developed from *S. pennellii*-derived bridging lines, obtained after several backcrosses from the wild species to *S. lycopersicum* (cv. Vendor or UC-82B) (Chetelat and Deverna 1991, Figure 1). Initial mapping of *ui6.1* was based on a mapping population (denoted "F_2-a") of 1167 plants derived from BC_7F_2 bridging lines heterozygous at both the *ui1.1* and *ui6.1* loci. To simplify phenotyping at *ui6.1*, a second population ("F_2-b") of 1920 plants was obtained from F_2-a individuals that were homozygous for the *S. pennellii* allele at *ui1.1* and heterozygous at *ui6.1*.

Severe recombination suppression was observed in these two mapping populations. To increase recombination rates, we created a heterozygous substitution line (SL-6), carrying a nearly intact (~80 cM, Van Wordragen *et al.* 1996) *S. pennellii* chromosome 6 in the background of *S. lycopersicum* (Figure 6). The heterozygous SL-6 was then crossed as female (BC-♀) or male (BC-♂) to a bridging line, which was homozygous for the *S. pennellii* allele at *ui1.1* and homozygous for the *S. lycopersicum* allele at *ui6.1*. This provided independent estimates of recombination frequency in female and male gametes.

Pollinations and pollen tube growth studies: All crosses were performed in the greenhouse using standard pollination techniques. The compatibility or incompatibility of crosses was determined by visualizing pollen tubes within pistils of the allotriploid hybrid 24 hr after pollination. The staining method was according to Martin (1959), except that pistils were fixed in ethanol: glacial acetic acid (3:1) solution (instead of FAA). After rinsing three times in water, pistils were transferred to 8 N NaOH for 8 hr to clear and soften the tissue. After triple rinsing in water, the softened pistils were soaked in aniline blue solution (0.1% aniline blue in 0.1 N K_3PO_4) for 8–24 hr. Observation and measurements of pollen tubes were carried out under UV light fluorescence microscopy using a Zeiss Axioskop. Pollinations were judged incompatible when the growth of all pollen tubes was arrested in the upper half of the style. In contrast, compatible pollinations resulted in at least some pollen tubes reaching the ovaries. For each cross, the length of the longest pollen tubes was measured and expressed as a percentage of the style length (from stigma surface to base of style). At least three pistils were examined per cross, from which the average maximum pollen tube length was calculated.

For imaging, pistils were fixed in 3:1 ethanol: glacial acetic acid for 24 hr or more. Following a rinse with deionized water, the pistils were soaked overnight in 10 N NaOH, rinsed three times with deionized water, and immersed in a 0.005 g/ml solution of Aniline Blue Fluorochrome (Biosupplies Australia) in 0.1 M K_2HPO_4 buffer, pH 8.5. Pistils were stained overnight in the dark at room temperature. Stained pistils were mounted on glass slides in 50% glycerol, covered with a glass cover slip, and flattened with gentle pressure on the cover slip. Observation and photography of pollen tubes were carried out using a Leica DM5500 B fluorescence microscope with a DAPI filter. Images were captured with a Hamamatsu digital camera and IPLab software (BD Biosciences). Individual micrographs were assembled into whole pistil montages using Adobe Photoshop CS2.

DNA isolations: A small scale DNA extraction method was adopted to isolate DNA from the mapping populations using 96-well microtiter plates. Seeds were treated with 50% household bleach for 15 min, rinsed thoroughly under running tap water, and germinated on blotter paper (Anchor Paper) in acrylic sandwich boxes (10.8 × 10.8 × 3.4 cm, Hoffman Manufacturing) for 7–10 days. Seedlings with fully expanded cotyledons were transplanted to soil in plastic seedling trays and grown in the greenhouse. After 2 or 3 weeks, single expanding leaflets of ~1 cm^2 in area were collected from each plant into 96-well microtiter plates. After adding 400 μl of extraction buffer (200 mm Tris–HCl pH 7.5, 250 mm NaCl, 25 mm EDTA, 0.5% SDS and ~5% sodium metabisulfite) and two levitation stir balls (3.97 mm, cat. no. VP725E, V&P Scientific), leaf tissues were ground on a paint shaker (Skandex SO-10m, Fluid Management Europe) for 8 min and then incubated at 65° for 1 hr. After cooling, 150 μl of chilled 5 m KAc buffer was added and centrifuged at 4000 RPM (3000 × *g*) for 15 min. The supernatant (200 μl) was transferred to a new 96-well microtiter plate. DNA was precipitated by adding 100 μl 100% isopropanol, followed by centrifugation at 4000 RPM for 10 min. The supernatant was poured out and pellets were washed once using 200 μl of 70% ethanol and allowed to dry at room temperature. The pellets were resuspended in 200 μl distilled H_2O. Each PCR reaction used 2 μl (100–200 ng) of resuspended DNA in a total volume of 20 μl.

CAPS markers: Initial mapping of *ui6.1* relied on previously mapped CAPS markers obtained from the Solanaceae Genomics Network (SGN, http://solgenomics.net). Fine-scale mapping was based on markers developed from the sequences of BACs located in the candidate region, also downloaded from SGN. PCR primers of predicted amplification size ranging from 700 to 1000 bp were picked out using the software Primer3. PCR conditions were 94° for 2 min, followed by 35 cycles of 94° for 30 sec, 55° for 30 sec, and 72° for 2 min, with a final extension of 5 min at 72°. Amplification products were separately digested with eight 4-bp recognizing restriction enzymes (*Alu*I, *Hae*III, *Hin*fI, *Mbo*I, *Mse*I, *Msp*I, *Rsa*I, and *Taq*I) to identify polymorphisms. A total of 142 markers were designed for the candidate region, of which 18 polymorphic markers were used in the final linkage analysis (supporting information, Table S1). All CAPS markers were resolved on 2% agarose gels.

Isolation and analysis of recombinants: Marker analysis of the bridging lines indicated the size of the *ui6.1* introgression from *S. pennellii* was ~32 cM on the EXPEN 2000 reference map of tomato (data not shown). To identify the approximate location of *ui6.1*, two markers, SSR47 and T834, located at positions 6.5 and 32 cM on chromosome 6, were selected to genotype the first mapping population, F_2-a. Recombinants

TABLE 1

Pollen tube growth following compatible and incompatible pollinations of *Solanum lycopersicoides* and interspecific Solanum hybrids

Pistil genotype	Pollen genotype	Length of longest tube (% of style)	Location of rejection	No. tubes in ovaries
S. lycopersicoides LA2951	*S. lycopersicoides* LA2951 (self)	50.2 ± 2.76	Style	0
	S. lycopersicoides LA2951 (sib)	100	N/A	>50
	S. pennellii LA0716	100	N/A	4.9 ± 2.91
	S. lycopersicum cv. VF-36	5.6 ± 1.88	Stigma	0
	S. lycopersicum (*ui1.1*)	5.3 ± 0.67	Stigma	0
	S. lycopersicum (*ui6.1*)	4.7 ± 1.52	Stigma	0
	S. lycopersicum (*ui1.1* + *ui6.1*)	10.9 ± 0.96	Stigma/style	0
S. lycopersicum × *S. lycopersicoides* (diploid hybrid 90L4178)	*S. lycopersicoides* LA2951	100	N/A	>50
	S. pennellii LA0716	100	N/A	>50
	S. lycopersicum cv. VF-36	16.6 ± 1.86	Style	0
	S. lycopersicum (*ui1.1*)	13.7 ± 0.71	Style	0
	S. lycopersicum (*ui6.1*)	15.3 ± 3.14	Style	0
	S. lycopersicum (*ui1.1* + *ui6.1*)	42.3 ± 14.5	Style	0
S. lycopersicum × *S. lycopersicoides* (allotriploid hybrid GH266)	*S. lycopersicoides* LA2951	100	N/A	>50
	S. pennellii LA0716	100	N/A	>50
	S. lycopersicum cv. VF-36	47.8 ± 4.93	Style	0
	S. lycopersicum (*ui1.1*)	45.9 ± 6.40	Style	0
	S. lycopersicum (*ui6.1*)	53.0 ± 11.4	Style	0
	S. lycopersicum (*ui1.1* + *ui6.1*)	100	N/A	>50

Pollen compatibility is indicated by the length of the longest pollen tube expressed as a percentage of the total length of the style and stigma (a value of 100% indicates a compatible cross). Values are the averages (±SE) of at least 3 replicate pistils for each cross. On the female side, the genotypes were *S. lycopersicoides*, and diploid and allotriploid F_1 *S. lycopersicum* × *S. lycopersicoides* hybrids. On the male side, crosses were made with pollen from *S. lycopersicoides*, *S. lycopersicum*, *S. pennellii*, and bridging lines containing the *ui1.1* and/or *ui6.1* factors from *S. pennellii* introgressed into cultivated tomato. These genotypes are described in MATERIALS AND METHODS.

identified with these two markers were then subjected to compatibility evaluation by measuring pollen tube growth on pistils of the allotriploid hybrid. The first round of screening indicated that *ui6.1* is closely linked but distal to marker SSR47. Therefore, a CAPS marker nearer the end of chromosome 6, T1928, was used in combination with T834 to screen all subsequent mapping populations. Relatively high recombination frequencies were observed near *ui6.1* in two populations (BC-♀ and BC-♂) derived from a *S. pennellii* substitution line. We focused on the recombinants identified among 1824 individuals of BC-♀. Hundreds of publically available markers, as well as markers developed from sequenced BACs and BAC ends, were used to conduct the linkage analysis. On the basis of the mapping results from this phase, flanking markers 73H07-3 and *Mi* were chosen to analyze the recombinants between T1928 and T834 from the other populations, *i.e.*, the F_2-a, F_2-b, BC-♂, and the remainder of the BC-♀. The additional recombinants were then tested for compatibility on the allotriploid.

Linkage analysis was based on the recombinants isolated from all mapping populations. Map distances were expressed in units of recombination frequency, *i.e.*, (RF = no. of recombinants/total no. of gametes) × 100%, where the no. of gametes = (no. of BC progeny), or (2 × no. of F_2 progeny).

RESULTS

Pollen tube growth in pistils of *S. lycopersicoides* and F_1 hybrids with tomato: Like other SI wild tomato relatives, pistils of *S. lycopersicoides* reject pollen of cultivated tomato (*S. lycopersicum*, SC) (Table 1). No pollen rejection occurs in the other direction, consistent with the SI × SC rule. Rejection of tomato pollen occurs early during growth on the *S. lycopersicoides* pistil, with pollen tubes reaching no further than the stigma or uppermost portion of the style (<5% of style length). In contrast, self pollen tubes are arrested midway down the *S. lycopersicoides* style (∼50% of style length). Diploid and allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrids also reject pollen of cultivated tomato, but the rejection occurs lower in the style: ∼15% of style length for the 2x hybrid, and ∼50% for the 3x hybrid. Thus, the interspecific hybrids have weakened or delayed incompatibility responses compared to *S. lycopersicoides*; rejection of tomato pollen tubes on the allotriploid occurs in about the same position as self pollen tubes on *S. lycopersicoides*. Pollen of *S. pennellii* (SC accession LA0716) is compatible on styles of *S. lycopersicoides* and the diploid or allotriploid hybrids.

Two pollen factors, *ui1.1* and *ui6.1*, are required to overcome stylar UI: We previously showed that two *S. pennellii* gametophytic factors, *ui1.1* and *ui6.1*, are required for pollen to overcome the stylar incompatibility barrier of diploid and allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrids (Chetelat and DeVerna 1991).

These two factors, when bred into *S. lycopersicum*, confer full compatibility on pistils of the allotriploid (Table 1). On pistils of the diploid hybrid or *S. lycopersicoides*, pollen containing both *ui1.1* and *ui6.1* penetrate further into the style than pollen lacking either factor, yet do not reach the ovaries.

To elucidate the inheritance of pollen compatibility in this system, we genotyped BC_7F_2 progeny of bridging lines heterozygous for the *S. pennellii* alleles at both loci (the F_2-a population), using markers SSR47 and T834 on the short arm of chromosome 6 and two *S*-locus flanking markers, TG184 and SSR98, on chromosome 1. Plants heterozygous or homozygous for the *S. pennellii* alleles at only *ui1.1*, or only *ui6.1*, or both loci, in the genetic background of *S. lycopersicum*, were selected from this population. Compatibility of pollen from the various genotypes was tested on pistils of the allotriploid hybrid. The distribution of maximum pollen tube lengths was bimodal, with no intermediate plants; thus pollen compatibility can be treated as a qualitative trait in this system (Figure 2).

Pollen of *S. lycopersicum* cv. VF-36 and bridging containing a single *S. pennellii* factor (*ui1.1* or *ui6.1*) were incompatible on pistils of the allotriploid, as the longest pollen tubes grew to only half the length of the style after 24 hr (Figure 3). In contrast, both *S. pennellii* and the *ui1.1* + *ui6.1* bridging line showed a compatible phenotype, with pollen tubes reaching the ovaries. These results confirmed that either *S. pennellii* factor alone is insufficient to overcome incompatibility, and that both are required to elicit a compatible reaction.

The pollen-side factors *ui1.1* and *ui6.1* do not confer UI or SI in the pistil: Importantly, the two gametophytic factors from *S. pennellii* do not confer a stylar UI or SI response. Bridging lines homozygous or heterozygous for the *S. pennellii* introgressions containing *ui1.1* and *ui6.1* set fruit and seeds following self-pollination (data not shown), indicating they are SC. Furthermore, the bridging lines set fruit and seed following pollinations with cultivated tomato, providing strong evidence that pistil-side and pollen-side UI responses are controlled by different sets of genes.

Map location of *ui6.1*: To determine the approximate genetic location of *ui6.1*, 1167 F_2-a plants were genotyped with markers SSR47 and T834 on the short arm of chromosome 6. Recombinant plants were genotyped with two additional markers in this interval, T892 and T507, and two *S*-locus markers, TG184 and SSR98, on chromosome 1. Compatibility tests of the recombinants were carried out on pistils of the allotriploid. A total of 55 recombinants were identified between SSR47 and T834, of which 47 were tested for compatibility (Figure 4). Thirteen recombinants lacking the *S. pennellii* allele at *ui1.1* showed an incompatible phenotype, regardless of their genotype at *ui6.1*. The average "maximum" pollen tube length in these plants was 48.2% of style length. The recombinants heterozygous or homozygous

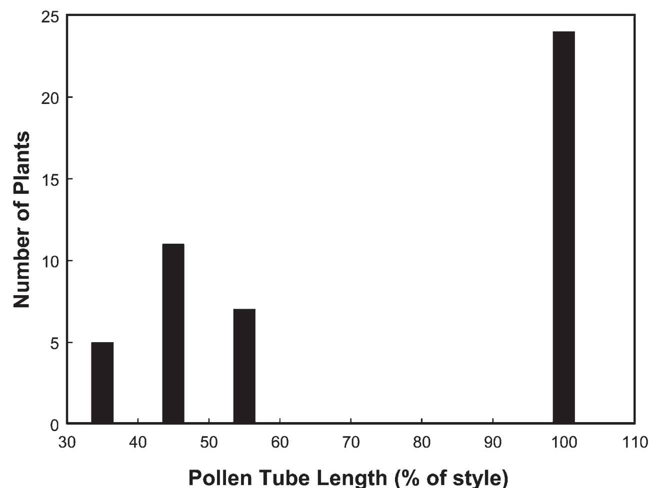

FIGURE 2.—Histogram of pollen tube lengths following pollinations of allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrids (♀) with bridging lines segregating for two pollen compatibility factors from *S. pennellii*. Pollen tube lengths are measured from the position where the majority of tubes are arrested, excluding outlier tubes that may grow a little further (see Figure 3). Measurements were taken 24 hr after pollination.

for the *S. pennellii* allele at *ui1.1* segregated for compatibility, depending on their genotype for *ui6.1*. The 10 recombinants lacking the *S. pennellii* allele of SSR47 were incompatible, with an average pollen tube length of 47.4%, whereas the remaining 24 recombinants (*i.e.*, those that were at least heterozygous) were all compatible on the pistils of the allotriploid.

Some F_2-a recombinants did not provide information on the map location of *ui6.1*. All 13 plants lacking the *S. pennellii* allele at *ui1.1* locus were incompatible, as expected, so they were uninformative regarding *ui6.1*. Recombinants that carried a parental (nonrecombinant) *S. pennellii* segment were also uninformative. These were self-pollinated and plants that carried only the recombinant segment were tested for compatibility. The results showed that *ui6.1* is closely linked to marker SSR47 (Figure 5).

To avoid the problem of segregation for *ui1.1*, a second population (F_2-b), homozygous for the *S. pennellii* allele at *ui1.1* and segregating at *ui6.1*, was analyzed. This population of 1920 plants was screened with a distal marker, T1928 and T834; recombinants were then genotyped with SSR47 and T892 (Figure 5). A total of 86 recombinants were identified, of which 31 had crossovers between T1928 and T892. Only the latter were tested for compatibility phenotype on the allotriploid, since the F_2-a population indicated a location for *ui6.1* closer to SSR47. Of the 31 tested recombinants, only 13 were informative regarding the position of *ui6.1*; the other 18 recombinants also carried a parental *S. pennellii* segment, and thus required an additional generation to obtain an informative phenotype. The results indicated *ui6.1* lies between T1928 and T892 (Figure 5).

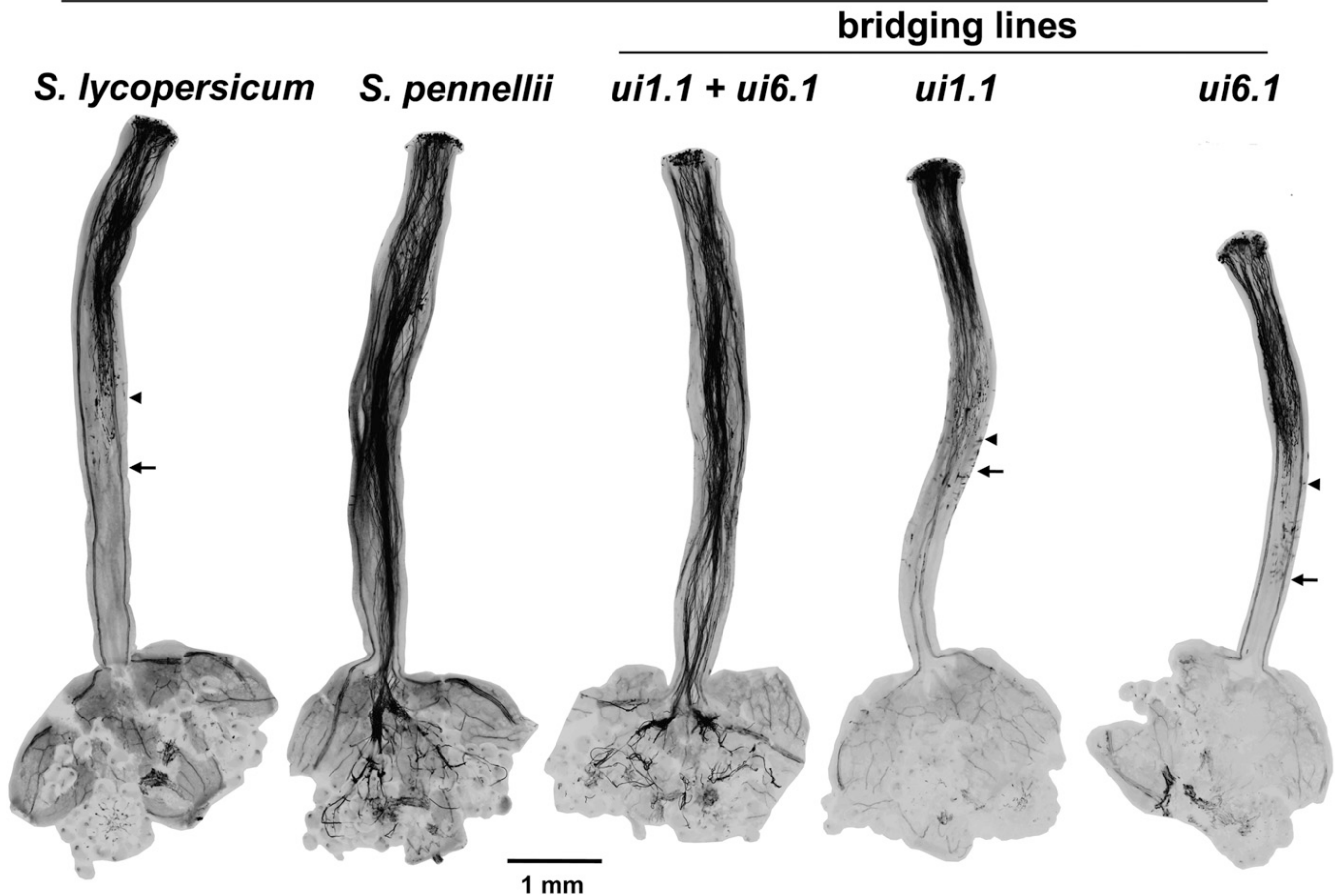

Figure 3.—Pollen tube growth on pistils of allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrids pollinated by different species or genotypes. Pistils were fixed 24 hr after pollination and stained with aniline blue to visualize pollen tubes. (Note: the color of images was inverted, thus pollen tubes appear darkly stained). For the incompatible crosses, arrowheads indicate the zone of the style where growth of most tubes stopped, and arrows indicate the longest pollen tube. The bridging line genotypes contained one or both pollen compatibility factors (*ui1.1*, *ui6.1*) from *S. pennellii* introgressed into the background of *S. lycopersicum*.

Recombination near *ui6.1* is suppressed: Recombination in the *ui6.1* region was strongly suppressed in the two F_2 populations derived from the bridging lines (Figure 6). The total genetic distances observed in the two populations were 2.35 and 2.32 MU, between T834 and either SSR47 or T1928, respectively. These values are less than one-tenth of the recombination frequencies reported for the same markers on the reference map, EXPEN 2000, based on an interspecific F_2 *S. lycopersicum* × *S. pennellii* cross (http://solgenomics.net; Fulton *et al.* 2002). This degree of recombination suppression could impede the fine-scale mapping and cloning of *ui6.1*, so we designed a mapping strategy to elevate crossover frequency in this region. Our previous studies showed that recombination frequency is higher in lines with long introgressed segments or whole chromosome substitutions, relative to short introgressions (Canady *et al.* 2006).

We therefore took advantage of a partial substitution line, SL-6, which contains a long *S. pennellii* introgression spanning most of chromosome 6 (Figure 6). A line heterozygous for SL-6 was crossed as female (BC-♀) or male (BC-♂) to a line homozygous for the *ui1.1* region from *S. pennellii.* All plants in these two populations were screened with the flanking markers T1928 and T834 to identify recombinants, which were genotyped with T892 and SSR47. In 2946 BC-♀ and 672 BC-♂ plants, 294 and 29 recombinants, respectively, were found. The BC-♀ data were derived from two independent reciprocal crosses in which a homozygous SL-6 was crossed as male or female to cultivar M-82 to create the heterozygous stock; since no differences were seen between the reciprocal crosses (data not shown), the mapping data were pooled. The genetic distance between T1928 and T834 in the BC-♀ population was 9.98 MU, or over fourfold higher than observed in F_2-a and F_2-b (Figure 5). Recombination frequency was significantly lower in BC-♂, but the total distance of 4.32 MU was still nearly twice that of the F_2 populations. These results confirmed that longer alien introgressions recombine

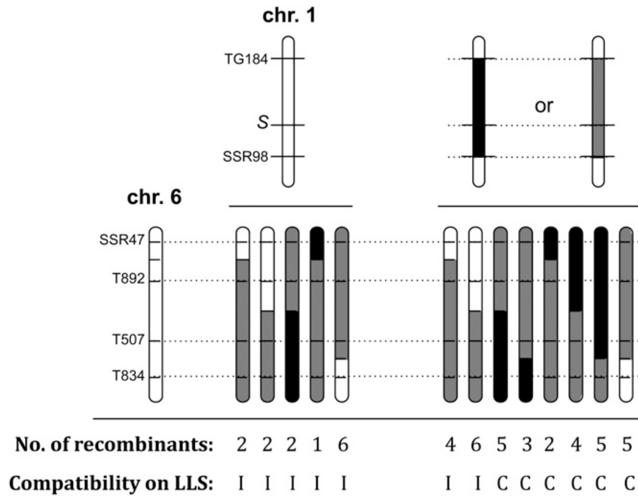

Figure 4.—Genotypes of recombinants recovered in the *ui6.1* region in F_2 progeny of bridging lines containing both the chromosome 1 and 6 factors. The compatibility or incompatibility of recombinants was determined by pollinations onto pistils of the allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrid. Open bars, homozygous for the *S. lycopersicum* alleles; gray, heterozygous; and black, homozygous for the *S. pennellii* alleles. In the absence of the *S. pennellii* chromosome 1 factor, all plants elicit an incompatible reaction, regardless of their genotype at *ui6.1*. Recombinants that were heterozygous for the parental *S. pennellii* introgression were progeny tested to determine the phenotype of *ui6.1*.

more readily than short ones. Furthermore, recombination in female gametes of SL-6 heterozygotes (BC-♀) was over twice that observed in male gametes (BC-♂).

Heterogeneity for recombination rates along the chromosome: We also observed that recombination rates were not uniform along the chromosome. For example, the genetic distance between T1928 and T892 in the BC-♀ population was 4.60 MU, more than six times the distance in BC-♂ (Figure 5). In contrast, the relative increase in recombination between T892 and T834 was only ∼1.5-fold. Fortuitously, *ui6.1* was located in a region of relatively high crossover frequency (Figure 7). When additional markers were placed onto a genetic map derived from selective genotyping of the BC-♀ population, a region of elevated recombination was observed between markers U218000 and *Mi*. The distance between these two markers on our map was 2.13 MU, more than seven times the value on the reference map (Figure 7).

Fine-scale mapping of *ui6.1*: Several hundred plants with crossovers between T834 and T1928 or SSR47 were isolated from the four mapping populations. We initially focused on the recombinants isolated from the partial BC-♀, which produced the highest recombination frequency. The *ui6.1* phenotypes of these were determined by test pollinations on the allotriploid. On the basis of the mapping results, we used two closely flanking markers to further characterize the recombinants isolated from the other populations. These recombinants were selectively phenotyped, focusing on those plants most likely to further resolve the position of *ui6.1*.

Our initial results showed *ui6.1* is located between markers U218000 and *Mi* (Figure 7), based on 39 recombinants in this interval from the partial BC-♀

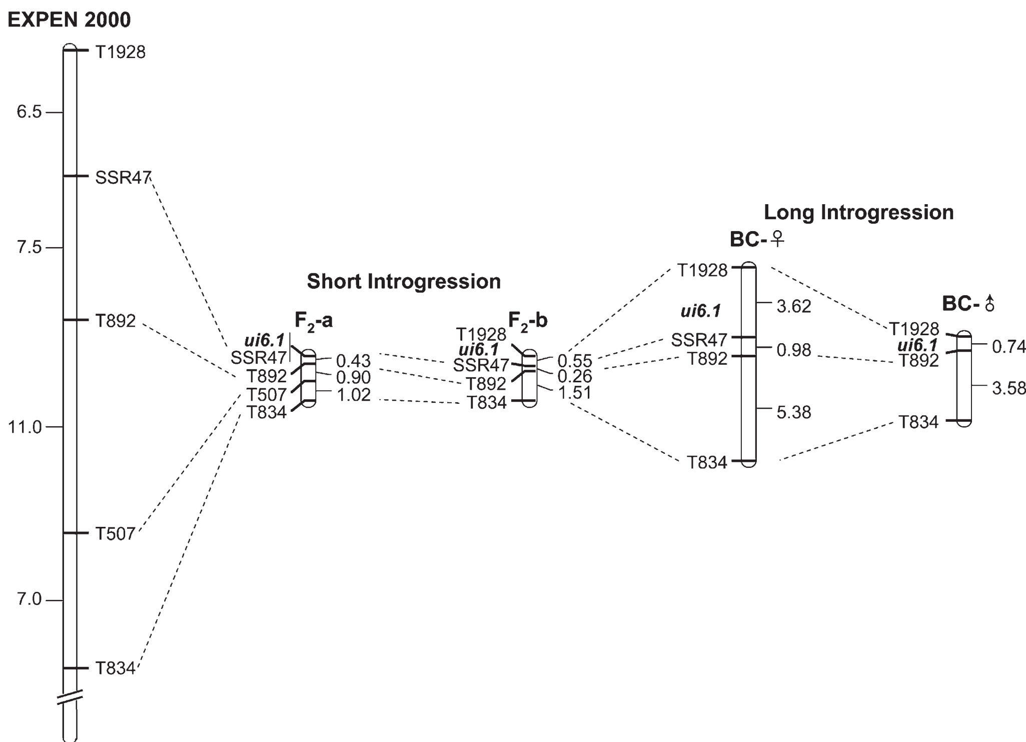

Figure 5.—Comparison of recombination frequencies near *ui6.1* in different mapping populations. The F_2 maps are based on recombination within a relatively short (32 cM) *S. pennellii* segment, whereas the BC maps are derived from a longer segment (∼80 cM) in a substitution line. The reference map is the tomato EXPEN 2000 map (http://solgenomics.net). Genetic distances are in units of percentage of recombination, or, on the EXPEN 2000 map, in centimorgans. On the F_2-b and BC maps, *ui6.1* is shown at its approximate location because not all plants in these populations were phenotyped.

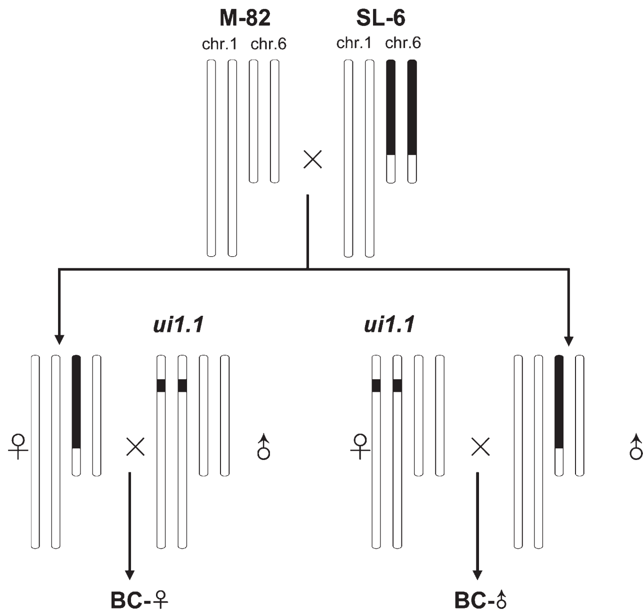

Figure 6.—Crossing scheme used to optimize the rate of recombination around *ui6.1*. A substitution line containing a nearly intact *S. pennellii* chromosome 6 (Van Wordragen *et al.* 1996) was crossed to tomato cv. M-82. The resulting hybrid, heterozygous for the *pennellii* chromosome, was backcrossed as male (BC-♂) or female (BC-♀) parent to a line homozygous for the *S*-locus region from *S. pennellii*. Recombinants were identified by marker analysis in the two BC populations and then tested for compatibility of pollen on pistils of the allotriploid hybrid.

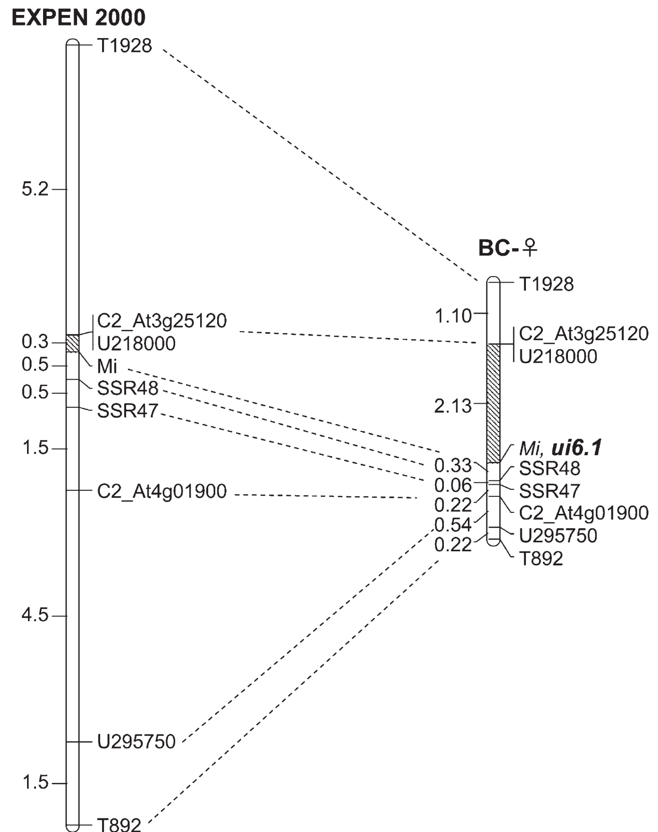

Figure 7.—Genetic map of the *ui6.1* region on the short arm of chromosome 6. Results are based on analysis of progeny from the substitution line, used as female parent (BC-♀), crossed to a line homozygous for the *S. pennellii* factor on chromosome 1. Markers are from the SGN database (http://solgenomics.net). The shaded region of the chromosomes indicates a zone of elevated recombination in the progeny of SL-6 relative to the EXPEN 2000 reference map.

population. To further resolve the location of *ui6.1*, hundreds of markers based on the sequences of overlapping BACs were developed. Of these, 18 polymorphic CAPS markers, developed from the sequences of two overlapping BACs, C06HBa0250I21 and C06HBa0073H07, and the end sequences of LE_H Ba00181H03 (Figure 8, Table S1), were used. A fine-scale map of the *ui6.1* region was constructed from these recombinants and included six flanking markers (73H07-3, 73H07-11, 73H07-13, 73H07-15, *Mi*, and 181H03-2), with one crossover on each side (recombinant 44-32 and 50-58) of *ui6.1* (Figure 8).

The closely linked markers 73H07-3 and *Mi* were then used to genotype the recombinants from the other populations. Two more recombinants, 19-89 from F_2-b and 71-61 from BC-♀, were found in this region (Figure 8). The compatibility of these recombinants was evaluated on the allotriploid (recombinant 19-89 was tested using a homozygous F_3 progeny). Linkage analysis using all the recombinants indicated that the interval spanning *ui6.1* comprised ~0.128 MU. An estimate for the corresponding physical distance was obtained from the prepublication draft genome sequence of tomato, made available by the International Tomato Genome Sequencing Consortium at http://solgenomics.net. This showed that the flanking markers 73H07-11 and *Mi* are separated by ~160 kb.

DISCUSSION

Relationship between unilateral and self-incompatibility: A basic question about the nature of prezygotic interspecific reproductive barriers in plants is their relationship to self-incompatibility. As described by Lewis and Crowe (1958), unilateral crossing barriers often follow the SI × SC rule, wherein pollen of a self-compatible species is rejected on styles of a related self-incompatible species, but no rejection occurs in the reciprocal cross. The general validity of this rule in many plant families, including the Solanaceae, suggests that mutation at the *S* locus is an important element of interspecific barriers. This hypothesis is supported by experiments in Nicotiana in which ectopic expression of *S*-RNase's from an SI species (*Nicotiana alata*) in the background of an SC species (*N. tabacum*) is sufficient to confer on the pistil the ability to reject pollen from several SC species (Murfett *et al.* 1996). However the same experiments also demonstrated pollen rejection pathways that did not depend on *S*-RNase expression. Thus, on the pistil side, the *S* locus is strongly implicated

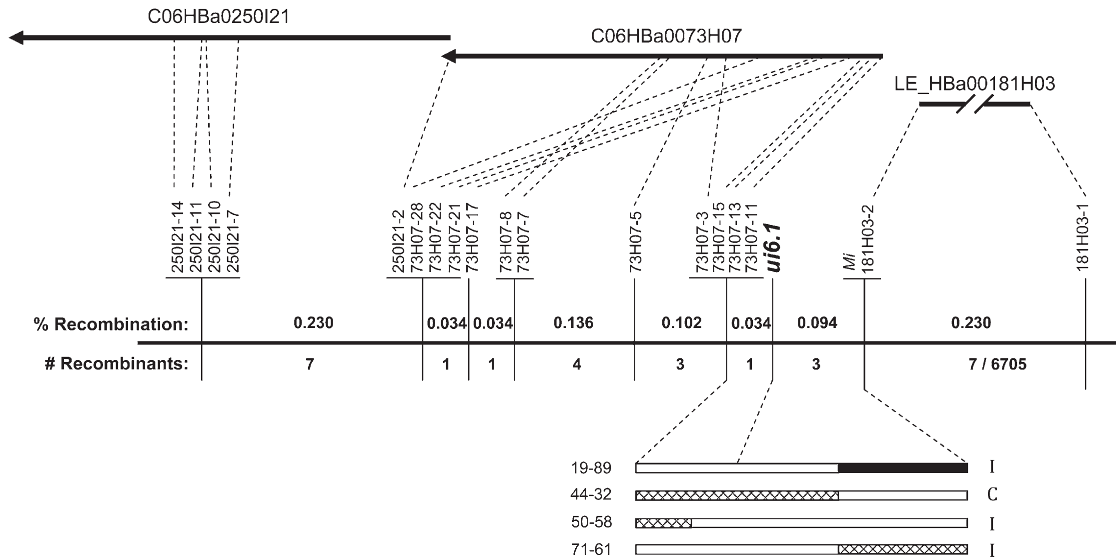

Figure 8.—Fine-scale genetic and physical map of the *ui6.1* region and phenotypes of recombinants with crossovers in the flanking intervals. The position of three BACs is shown relative to a fine-scale map of the region based on the analysis of recombinants. For each marker interval, the percentage of recombination and the number of recombinants obtained from all mapping populations are shown. Markers were derived from the *S. lycopersicum* BAC sequences. The genotypes of four recombinants (solid, homozygous *S. pennellii*; hatched, heterozygous; and open, homozygous *S. lycopersicum*) and their pollen phenotypes on pistils of the allotriploid (I, incompatible; C, compatible) are shown.

in interspecific pollen rejection, but other genetic factors are also involved. In crosses between *S. lycopersicum* (SC) and *S. habrochaites* (SI), several independent QTL were detected, of which the *S*-locus region had the largest phenotypic effect (Bernacchi and Tanksley 1997).

On the other hand, interspecific pollen tube rejection differs from SI in timing and location. Pollen tubes of cultivated tomato are rejected in the upper portion of the *S. pennellii* style (Hardon 1967; Liedl *et al.* 1996), similar to our results with *S. lycopersicoides* pistils (the "early" rejection). In contrast, "self" pollen tubes penetrate further into the style. This early rejection of interspecific pollen tubes superficially resembles sporophytic SI, wherein self pollen is recognized and rejected on the stigma. Further evidence for a sporophytic component of interspecific incompatibility comes from the crossing relationships of F_1 hybrids between cultivated tomato and SI wild relatives: backcrosses to the SI species are only possible when the F_1's are used as female parent (McGuire and Rick 1954; Hardon 1967). In other words, if the compatibility of pollen were purely determined by gametophytic factors, a significant fraction of pollen from the F_1 hybrid should be compatible on pistils of the SI species. Thus, expression of interspecific incompatibility is genetically complex and may involve more than one mechanism for recognizing and rejecting foreign pollen.

In the present study, we report the fine mapping of *ui6.1*, a pollen compatibility factor on chromosome 6 that interacts with *ui1.1*, located at or near the *S* locus on chromosome 1 of tomato. We show that only pollen bearing the *S. pennellii* alleles of both *ui1.1* and *ui6.1* are compatible on styles of *S. lycopersicum* × *S. lycopersicoides* hybrids. These results strongly implicate the *S* locus in pollen-side unilateral incompatibility. They also provide the first genetic evidence (to our knowledge) that factors other than the product of the *S* locus are required for pollen function in self- or interspecific incompatibility.

Patterns of segregation distortion in various interspecific crosses of tomato support these conclusions. They provide further evidence that pollen bearing the cultivated tomato alleles at *ui1.1* and *ui6.1* are selectively eliminated on styles of interspecific hybrids, but only when the wild parent is SI. For example, a pseudo-F_2 *S. lycopersicum* × *S. chilense* (SI) population, obtained by crossing two independent F_1's (to avoid self-incompatibility) exhibited a deficiency of plants homozygous for the *S. lycopersicum* alleles of markers near *ui1.1* and *ui6.1* (Graham 2005). The likely explanation is that pollen lacking either factor from *S. chilense* are eliminated on styles of the F_1 hybrid. In contrast, interspecific F_2's between tomato and *S. chmielewskii* or *S. pennellii* LA0716, both of which are self-compatible yet reject tomato pollen, showed normal Mendelian segregation for markers near *ui1.1* and *ui6.1* (Paterson *et al.* 1991; http://solgenomics.net). Mendelian segregation is also observed in interspecific F_2 populations when both parents are SI, consistent with the hypothesis that the SI species each contain functional alleles at *ui1.1* and *ui6.1* (Pertuze *et al.* 2002; Albrecht and Chetelat 2009). These results suggest the existence of an *S*-RNase dependent pathway for interspecific pollen rejection in

pistils of the SI species, as well as an *S*-RNase independent mechanism in the SI and some SC species.

Thus, both the genetic evidence and the developmental timing of interspecific pollen rejection support the existence of at least two pathways. The first (early) mechanism rejects tomato pollen tubes in the stigma and upper styles on pistils of SI and some SC species; pollen containing the *ui1.1* and *ui6.1* factors does not overcome this barrier. The second mechanism causes pollen tube rejection in the mid position of styles from interspecific SC × SI hybrids, and is overcome by pollen containing *ui1.1* and *ui6.1*. We found evidence of overlap between these two pathways: pollen of bridging lines containing both *ui1.1* and *ui6.1* grow farther into pistils of *S. lycopersicoides* than does pollen of tomato lines lacking either or both factors (10 *vs.* 5% of style length). Further research is clearly needed to understand the genetic and physiological basis of these interspecific pollen rejection pathways.

Relevance to other Solanaceae: We believe our experimental system provides a useful model for studying unilateral incompatibility in other Solanaceae. On the pistil side, the diploid and allotriploid *S. lycopersicum* × *S. lycopersicoides* hybrids are SI and reject pollen of cultivated tomato, indicating these traits are effectively dominant, as they are in other Solanaceae hybrids. For example, diploid F_1 hybrids between cultivated tomato and SI accessions of *S. habrochaites*, *S. pennellii*, *S. peruvianum*, or *S. chilense* are all SI and reject pollen of cultivated tomato (McGuire and Rick 1954; Martin 1961, 1963; Hardon 1967). Allotriploids composed of two genomes from tomato and one from potato (*S. tuberosum*, SI) also reject pollen of *S. lycopersicum* and accept pollen of *S. pennellii* LA0716 (Schoenmaker *et al.* 1993), *i.e.*, the same crossing relationships we report herein. In other solanaceous genera, including Nicotiana (Murfett *et al.* 1996) and Capsicum (Pickersgill 1997), self- and unilateral incompatibility are also dominant in interspecific SC × SI hybrids.

Furthermore, if the underlying pathways for pollen tube rejection by unilateral incompatibility are conserved, then the results we present herein should be relevant to other systems. For example, allotriploid *S. lycopersicum* × *S. sitiens* hybrids also reject pollen of cultivated tomato, and bridging lines containing *ui1.1* and *ui6.1* from *S. pennellii* are compatible (Pertuze *et al.* 2003). In instances where unilateral incompatibility results from the breakdown of self-incompatibility, there are likely to be common mechanisms. Self-incompatibility systems are strongly conserved within plant families, and related families often have the same system. For example, all SI members of the Solanaceae employ the *S*-RNase based gametophytic system, under the control of a single *S* locus determining specificity (De Nettancourt 1977, 1997). While the *S*-RNases are highly divergent, each allele (S_1, S_2, etc.) is strongly conserved, even across plant families (Igic and Kohn 2001).

A weakened UI response in the allotriploid hybrid simplifies the genetics: The ability to reject pollen of cultivated tomato is effectively dominant in pistils of *S. lycopersicum* × *S. lycopersicoides* hybrids. However, expression of the pistil response in the hybrids is weaker than in the wild parent, in two ways. First, pistils of the 3x hybrid arrest pollen tubes of cultivated tomato near the middle of the style, and lack the early (*i.e.*, stigmatic) pollen tube rejection phenotype of *S. lycopersicoides* pistils, suggesting the latter barrier is additive or recessive. Second, bridging lines containing both the *ui1.1* and *ui6.1* genes from *S. pennellii* were fully compatible (*i.e.*, many pollen tubes in the ovaries) with pistils of the allotriploid hybrid, but incompatible with the diploid hybrid or *S. lycopersicoides*. We hypothesize that additional genetic factors from *S. pennellii* contribute to compatibility with these two genotypes; a locus on chromosome 10 was identified in an earlier study (Chetelat and DeVerna 1991). Thus, the use of allotriploid hybrids as tester stocks provides a simpler genetic system in which to isolate pollen-side unilateral incompatibility factors. This method may provide a useful route for genetic analysis of other interspecific incompatibility systems.

The causes of the weakened expression in the allotriploid are unknown, but presumably result from the reduced gene dosage of the SI parent (*i.e.*, one genome of *S. lycopersicoides*, *vs.* two genomes of the SC parent, *S. lycopersicum*). We hypothesize that the different pathways for pollen tube rejection are differentially affected by gene dosage in the pistil. The action of a factor at or near the *S* locus in pollen implies that the *S*-RNase dependent pathway is fully dominant in pistils of the allotriploid hybrid. However, the early rejection pathway may be additive or recessive in hybrids. There are many examples of gene-specific dosage effects in aneuploids and triploids. Genes that are dominant over wild type in normal (2x) heterozygotes may show reduced expression in trisomics containing two doses of the wild-type allele (Khush and Rick 1968). Some protein coding loci are expressed at levels proportional to their gene dosage, while other loci maintain expression at the diploid level via dosage compensation (Smith and Conklin 1975; Birchler 1979; Tanksley 1979). In allotriploid hybrid fish, gene expression is reduced to the diploid level through allele-specific gene silencing (Pala *et al.* 2008). Finally, trisomic dosage may alter gene expression through genic interactions, for example if modifier loci are more sensitive to dosage (Gill 1974).

Toward map-based cloning of *ui6.1*: To decipher the molecular pathways of interspecific pollen tube rejection, it is necessary to clone the underlying genes. Toward this goal, we mapped the *ui6.1* gene to an interval of ∼0.128 MU or 160 kb on the short arm of chromosome 6 of tomato. The efficiency of linkage mapping was limited initially by low recombination

compared to the reference map of tomato. However, the degree of recombination suppression varied along the chromosome, and crossover frequencies close to *ui6.1* were higher than normal. The causes of recombination suppression (or enhancement) in this region of the genome are unknown, but may reflect the degree of divergence (structural or sequence) between the *S. lycopersicum* and *S. pennellii* chromosomes. The two species show colinearity of chromosome 6S, with no rearrangements (Peters *et al.* 2009). In *S. peruvianum*, a large paracentric inversion on chromosome 6S was responsible for recombination suppression around the *Mi* gene (for nematode resistance) in crosses to tomato (Seah *et al.* 2004). The *Mi* locus is close to *ui6.1*, but we found no evidence for an inversion in *S. pennellii*. While a few BAC-derived markers were inverted in our genetic map of the *ui6.1* region compared to their order on the tomato BAC sequence, they were in a region of relatively high recombination. The discrepancy suggests a rearrangement may have occurred in this particular BAC. Furthermore, FISH mapping of many other BACs on chromosome 6 showed an area (position 0–5 cM) where the linear order of markers predicted from the genetic map (EXPEN 2000) is inverted relative to their actual physical arrangement along the chromosome (Peters *et al.* 2009), pointing to a likely error in the linkage map.

We obtained much higher recombination frequencies starting with a nearly intact *S. pennellii* chromosome 6, compared to the shorter introgressed segments present in the bridging lines. This agrees with our previous observations of a positive correlation between crossover frequency and the length of alien chromosome segments (Canady *et al.* 2006). Higher recombination rates were seen in female gametes than in male gametes, as has been reported before (Rick 1969; De Vicente and Tanksley 1991).

Thus, despite an overall suppression of recombination in this region, the efficiency of mapping could be maximized by choice of starting materials and the direction of the cross. In this way, the chromosomal position of *ui6.1* was refined from a relatively imprecise interval, encompassing the entire short arm of chromosome 6, to only 0.128 MU (160 kb). We anticipate that isolation of the *ui6.1* gene will shed light on the mechanism of pollen tube rejection in unilateral incompatibility, and its relationship to self-incompatibility. From a practical standpoint, a more complete understanding of the genetic factors underlying interspecific crossing barriers may eventually enable wider crosses. For example, overcoming the rejection of *S. lycopersicum* pollen on pistils of related SI species might facilitate the development of cytoplasmic male sterility in tomato.

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