The Role of a Pollen-Expressed Cullin1 Protein in Gametophytic Self-Incompatibility in *Solanum*

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ABSTRACT We previously isolated a pollen factor, *ui6.1*, which encodes a Cullin1 protein (CUL1) that functions in unilateral interspecific incompatibility (UI) in *Solanum*. Here we show that CUL1 is also required for pollen function in self-incompatibility (SI). We used RNA interference (RNAi) to reduce CUL1 expression in pollen of *Solanum arcanum*, a wild SI tomato relative. Hemizygous T_0 plants showed little or no transmission of the transfer DNA (T-DNA) through pollen when crossed onto nontransgenic SI plants, indicating that CUL1-deficient pollen are selectively eliminated. When crossed onto a related self-compatible (SC) accession lacking active S-RNase, pollen transmission of the T-DNA followed Mendelian ratios. These results provide further evidence for functional overlap between SI and UI on the pollen side and suggest that CUL1 mutations will reinforce SI-to-SC transitions in natural populations only if preceded by loss of pistil S-RNase expression.

SELF-INCOMPATIBILITY (SI) is a widespread genetic mechanism in hermaphroditic plants that allows for the recognition and rejection of closely related pollen to prevent inbreeding. The breakdown of SI to self-compatibility (SC) through mutation occurs frequently (Igic *et al.* 2008), presumably driven by reproductive assurance under conditions where pollen from compatible mates is limiting. Pollen from SC species or populations is typically rejected on pistils of related SI species or populations, while, in the reciprocal crosses (SC pollinated by SI), no pollen rejection occurs. This pattern of unilateral incompatibility (UI) is known as the "SI × SC rule" (Lewis and Crowe 1958). While the mechanisms underlying SI have been the subject of much investigation, pollen rejection by UI is less well understood.

The cultivated tomato (*Solanum lycopersicum*) and related wild *Solanum* species provide a powerful system with which to study these reproductive barriers. They exhibit a wide range of mating systems, including SI-enforced obligate outcrossing, SC with facultative outcrossing, and SC with high levels of inbreeding (Rick 1988). Self-compatible biotypes or accessions of mostly SI species provide a source of natural variation for studying SI-related factors.

Self-incompatibility in *Solanum* and other Solanaceae is the S-RNase based, gametophytic type, in which S-specificity is determined by S-RNases in the pistil (McClure *et al.* 1989) and S-locus F-box proteins (SLFs) in pollen (Sijacic *et al.* 2004). F-box proteins, together with Skp1 and Cullin1 proteins, are components of Skp, Cullin, Fbox type (SCF) ubiquitin E3 ligases that mark proteins for degradation by the 26S proteasome (Zheng *et al.* 2002; Moon *et al.* 2004). The ubiquitin–proteasome pathway is thought to regulate pollen-side SI responses in the Solanaceae (Zhang *et al.* 2009). In the "collaborative non-self-recognition" model (Kubo *et al.* 2010), the S-locus encodes multiple SLF proteins that together recognize different suites of S-RNases. In compatible pollinations, SLF/S-RNase interactions lead to protection of pollen tubes against cytotoxic S-RNase, while in incompatible pollinations a failure to recognize "self" S-RNase results in pollen-tube inhibition. The absence of any deletions recovered among pollen-part SC mutants in *Nicotiana alata* is consistent with the presence of an S-RNase inhibitor encoded by the S-locus because pollen lacking SLF expression would be eliminated on pistils expressing S-RNase (Golz *et al.* 2001). In addition, modifier genes, such as the HT-B and 120-kDa proteins in the pistil, are required for SI function but not for specificity (McClure *et al.* 1999; Hancock *et al.* 2005).

The molecular mechanisms of pollen rejection in UI are complex. On the pistil side, S-RNase expression is required

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for pollen rejection in some crosses, while, in other cases, an S-RNase-independent pollen rejection system is evident (Murfett *et al.* 1996; Covey *et al.* 2010). On the pollen side, we showed previously that *ui6.1* encodes a Cullin1 protein that is required for pollen to overcome S-RNase-dependent UI (Li and Chetelat 2010). Furthermore, *ui6.1* (designated herein *CUL1*) interacts genetically with another pollen factor, *ui1.1*, which maps to the S-locus and thus might encode an SLF protein(s) (Li *et al.* 2010). These results suggested that pollen rejection in UI is controlled by a mechanism biochemically related to SI, either as an independent pathway or as a secondary effect of loss of SI factor(s) in the pollen. We therefore examined whether *CUL1* functions directly in SI.

Here we show that in *Solanum arcanum*, an SI wild tomato species, silencing of *CUL1* expression in pollen by RNA interference (RNAi) causes pollen rejection in normally compatible sib crosses, whereas the same pollen retain full compatibility on an SC accession expressing an inactive S-RNase. Our results strongly suggest that *CUL1* functions to protect pollen from S-RNases in SI as well as in UI and provide further evidence of overlap between intra- and interspecific pollen rejection pathways.

Results

We used a loss-of-function approach to test whether SI and UI are functionally linked on the pollen side. Figure 1A shows the strategy for testing the effects of suppressing *ui6.1 CUL1* expression in intraspecific crosses. A similar approach was used to test the function of PhSSK1, another putative pollen SI factor that, like CUL1, is proposed to form part of an SCF complex (Zhao *et al.* 2010). We used the *S. arcanum* accessions LA2163 (SI) and LA2157 (SC), which are similar in most respects, apart from mating system (Rick 1986). These two accessions are cross-compatible in either direction (Rick 1986; Kowyama *et al.* 1994). Importantly, LA2157 expresses a mutant S-RNase that lacks RNase activity (Kowyama *et al.* 1994; Royo *et al.* 1994) and confers self-compatibility (Rivers and Bernatzky 1994).

We transformed a *CUL1* RNAi construct (Supporting Information, File S1, Figure S1, and Table S1) into SI *S. arcanum* LA2163, reasoning that if *CUL1* functions in SI as well as in UI, then suppressing *CUL1* expression would disrupt pollen function in "collaborative non-self-recognition," leading to its rejection by pistils of other LA2163 plants, but not by LA2157 pistils that lack S-RNase. Because plants were grown from a random sample of seed from this obligately outcrossing accession, each independent transgenic plant (designated T_0-1, -2, etc.) and each nontransgenic pistil tester plant (LA2163-1, -2, -3) was expected to carry different *S*-genotypes. This was confirmed by controlled crosses between different LA2163 plants (transgenic or nontransgenic), which were all compatible (Figure S3), as expected. Thus, for *CUL1* RNAi plants with a single T-DNA insertion, the specific expectation is a 1:1 transgene segregation in crosses onto LA2157 pistils and little or no transgene transmission in crosses onto LA2163 (Figure 1A). Plants with two unlinked insertions should show a 3:1 ratio in crosses to LA2157 and little or no transmission in crosses to LA2163.

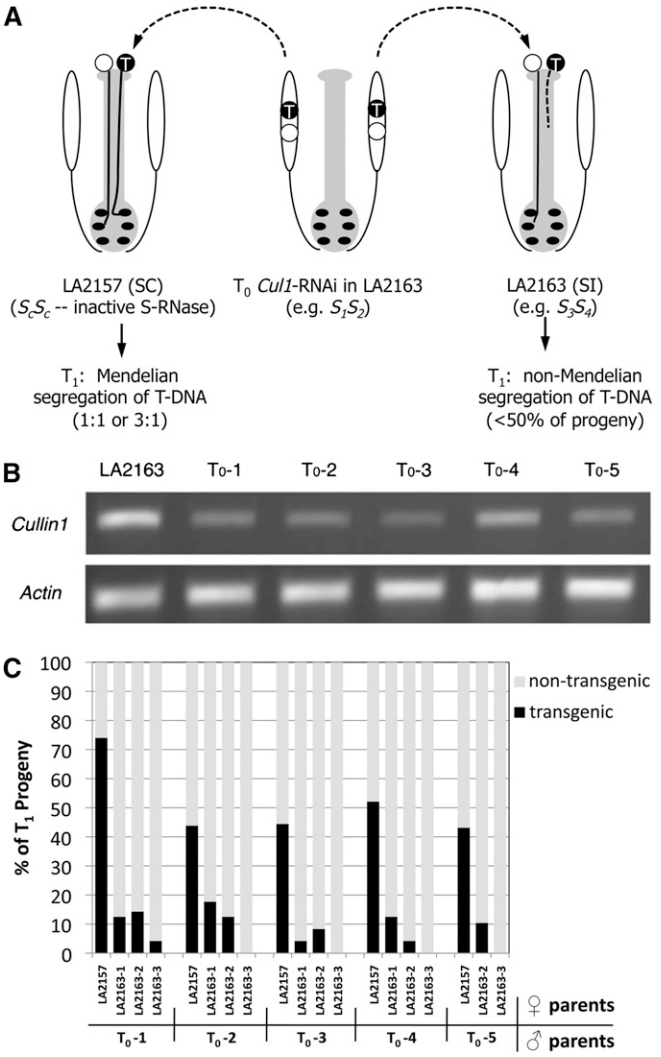

Figure 1 Experimental design and sample results testing CUL1 function in self-incompatibility. (A) Diagram of crosses between transgenic *CUL1* RNAi lines as pollen donors onto self-compatible (LA2157) or self-incompatible (LA2163) accessions of *S. arcanum* with inferred *S*-genotypes of each. (B) RT-PCR analysis of *CUL1* expression in pollen of five independent T_0 *CUL1* RNAi plants (T_0-1 to T_0-5) and nontransgenic control accession LA2163. (C) The frequency of transgenic and nontransgenic T_1 progeny from five independent *CUL1* RNAi transformants (T_0-1, two T-DNA insertions; T_0-2 to T_0-5, one insertion) crossed onto SC LA2157 or three independent SI LA2163 plants. The segregation of the T-DNA in the progeny of pollinations onto LA2157 fits Mendelian ratios, whereas progeny from the crosses onto LA2163 show an extreme deficiency of transgenic plants. These data (and full results in Table S2) indicate that *CUL1*-silenced pollen are rejected on pistils of independent nontransgenic LA2163 plants with different *S*-genotypes.

To avoid cross-silencing of other *Cullin1* gene family members, the RNAi construct was built from the combined 5′ + 3′ untranslated regions (UTRs) of *CUL1* from LA2163 (Figure S1). The UTR sequences showed no homology to other *Cullin1*

genes in the tomato genome. Pollen-specific expression was driven by the LAT52 promoter (Twell *et al.* 1990). Stably transformed plants (T_0) of LA2163 were obtained by *Agrobacterium*-mediated transformation. Figure 1B shows semiquantitative RT-PCR results for five T_0 plants with reduced *CUL1* messenger RNA (mRNA) levels (T_0-1, with two insertions; T_0-2 to T_0-5 with single insertions). Since the T_0 plants are hemizygous, the RNAi construct is present in 50% (single insertion) or 75% (two insertions) of pollen; thus, overall *CUL1* mRNA levels in pollen from T_0 plants was expected to be at least 25–50% of normal.

Observations of pollen tube growth in styles (Figure 2; full results in Figure S2 and Figure S3) showed that T_0 plants behaved like nontransgenic LA2163 SI plants: self-pollinations are incompatible, and sib pollinations onto other LA2163 plants with different *S*-genotypes (transgenic or nontransgenic) are compatible, although some pollen tubes are arrested (Figure 2). These results were expected because hemizygous T_0 plants produce both transgenic and nontransgenic pollen, which should manifest as at least 25–50% compatible pollen in crosses onto other LA2163 plants and 100% incompatible pollen in self-pollinations. The observation of some incompatible pollen tubes in the crosses onto LA2163 (transgenic or nontransgenic) is consistent with this prediction.

Transgene segregation ratios show that CUL1 is required for SI. When the four single-insertion hemizygous transformants (T_0-2 to T_0-5) are crossed onto SC LA2157, segregation of the transgene in the T_1 progeny is consistent with the predicted 1:1 ratio (Figure 1C; full results in Table S2). These results also establish that pollen containing the *CUL1* RNAi construct are viable. In contrast, the *CUL1* RNAi transgene transmits poorly or not at all in crosses onto four different LA2163 tester plants (Figure 1C and Table S2), and the segregation ratios deviate significantly from 1:1 in every case ($P < 0.0001$). A double-insertion transformant (T_0-1) also shows the predicted (3:1) segregation of the *CUL1* RNAi transgene when crossed onto LA2157 but little transmission of the T-DNA in crosses onto other LA2163 plants (Figure 1C and Table S2). Since all five independent T_0 plants, including one with two insertion loci, exhibited highly significant elimination of transgenic pollen on LA2163 pistils, we can rule out insertion-site effects or linkage to other genes under selection.

The best explanation for the transmission ratio differences is that *CUL1* is required for pollen-side SI function. If *CUL1* expression is necessary for pollen resistance to pistil S-RNases, then pollen that harbor the RNAi transgene should be rejected on pistils of any LA2163 plant, regardless of its *S*-genotype, leading to a deficiency of transgene transmission to the T_1 progeny, as was observed. On the other hand, progeny from the crosses onto LA2157, which lacks active S-RNase, should segregate for the transgene in predicted Mendelian ratios, which again is consistent with our results.

Discussion

We previously reported that most accessions of the red-fruited tomato species display *CUL1* mutations and proposed that *CUL1* forms part of a pollen-resistance mechanism for S-RNase-based UI between red-fruited SC species and green-fruited SI species (Li and Chetelat 2010). Here we demonstrate that *CUL1*-deficient pollen are selectively eliminated on pistils following intraspecific crosses in SI *S. arcanum*. In contrast, crosses onto an S-RNase-deficient SC accession do not exhibit pollen elimination. These observations provide direct evidence that *CUL1* function is limited to S-RNase-based pollen rejection mechanisms.

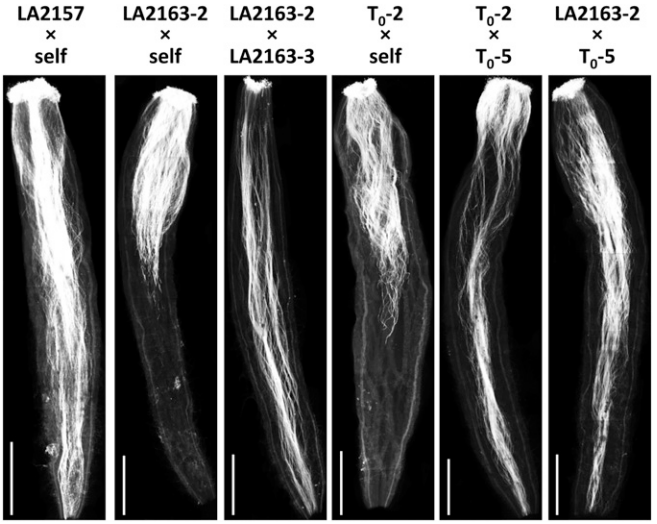

Figure 2 Representative images showing pollen tube growth in pistils of (left to right) LA2157 self-pollinated; nontransgenic LA2163 plants selfed or crossed; T_0 *CUL1* RNAi plants selfed or crossed; and nontransgenic LA2163 pollinated by a T_0 plant. Styles were fixed 24 hr after pollination, and pollen tubes were stained with aniline blue and visualized under UV light. Bar, 1 mm.

Our results also provide an explanation for earlier observations of segregation distortion near *ui6.1* in certain interspecific mapping populations. Specifically, F_2 progeny from hybrids between SC and SI species show preferential transmission of *ui6.1*-linked markers from the SI parent (Graham 2005; Trujillo-Moya *et al.* 2011). In contrast, F_2 progeny from interspecific SC × SC crosses (*i.e.*, lacking functional S-RNase) show normal Mendelian segregation ratios around *ui6.1* (van Heusden *et al.* 1999; Li *et al.* 2010). The simplest interpretation of these results is that *CUL1* activity is necessary for protecting growing pollen tubes against pistil S-RNases in compatible crosses. These findings establish that *CUL1* functions in self- as well as interspecific incompatibility and provide further evidence of overlap, on the pollen side, between these forms of incompatibility.

Our results also suggest that mutations that suppress pollen SI function by blocking CUL1 expression will not be transmitted to the next generation unless preceded by loss of S-RNase expression in the pistil. In our study, CUL1-deficient pollen were selectively eliminated on pistils of other LA2163 plants and were transmitted at a low rate, or not at all, to the next generation. In natural populations, mutations that block CUL1 expression or activity in pollen

are unlikely to become fixed unless expression of S-RNase in the pistil has already been lost. Thus *CUL1* mutations are likely to be secondary mutational events that reinforce a prior loss of pistil-side SI. This prediction is consistent with the presence of a *CUL1* loss-of-function mutant in cultivated tomato and other red- or orange-fruited species (Li and Chetelat 2010), a clade that is entirely SC and lacks both S-RNase and HT expression in the pistil (Kondo *et al.* 2002; Covey *et al.* 2010). Furthermore, to our knowledge, SC tomato species or populations lacking pollen SI function while retaining pistil function have not been reported. In contrast, SC mutations affecting only the pistil-side are known; for example, both *Solanum pennellii* LA0716 and *S. arcanum* LA2157 lack functional S-RNase in the pistil yet produce pollen that functions on pistils of conspecific SI accessions (Hardon 1967; Rick 1986; Royo *et al.* 1994; Covey *et al.* 2010).

Acknowledgments

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Literature Cited

Covey, P., K. Kondo, L. Welch, E. Frank, S. Sianta *et al.*, 2010 Multiple features that distinguish unilateral incongruity and self-incompatibility in the tomato clade. Plant J. 64: 367–378.

Graham, E. B., 2005 Genetic diversity and crossing relationships of *Lycopersicon chilense*. Ph.D. Thesis, University of California, Davis: 1–157.

Hancock, C. N., L. Kent, and B. A. McClure, 2005 The stylar 120kDa glycoprotein is required for *S*-specific pollen rejection in *Nicotiana*. Plant J. 43: 716–723.

Hardon, J. J., 1967 Unilateral incompatibility between *Solanum pennellii* and *Lycopersicon esculentum*. Genetics 57: 795–808.

Golz, J. F., H. Oh, V. Su, M. Kusaba, and E. Newbigin, 2001 Genetic analysis of Nictotiana pollen-part mutants is consistent with the presence of an S-ribonuclease inhibitor at the *S*-locus. Genetics 98: 15372–15376.

Igic, B., R. Lande, and J. R. Kohn, 2008 Loss of self-incompatibility and its evolutionary consequences. Int. J. Plant Sci. 169: 93–104.

Kondo, K., M. Yamamoto, D. P. Matton, T. Sato, M. Hirai *et al.*, 2002 Cultivated tomato has defects in both S-RNase and HT genes required for stylar function of self-incompatibility. Plant J. 29**:** 627–636.

Kowyama, Y., C. Kunz, I. Lewis, E. Newbigin, A. E. Clarke *et al.*, 1994 Self-incompatibility in a *Lycopersicon peruvianum* variant (LA2157) is associated with a lack of style S-RNase activity. Theor. Appl. Genet. 88: 859–864.

Kubo, K., T. Entani, A. Takara, N. Wang, A. M. Fields *et al.*, 2010 Collaborative non-self recognition system in S-RNAse-based self-incompatibility. Science 330: 796–799.

Lewis, D., and L. K. Crowe, 1958 Unilateral interspecific incompatibility in flowering plants. Heredity 12: 233–256.

Li, W., and R. T. Chetelat, 2010 A pollen factor linking inter- and intraspecific pollen rejection. Science 330: 1827–1830.

Li, W., S. Royer, and R. T. Chetelat, 2010 Fine mapping of *ui6.1*, a gametophytic factor controlling pollen-side unilateral incompatibility in interspecific *Solanum* hybrids. Genetics 185: 1069–1080.

McClure, B. A., V. Haring, P. R. Ebert, M. A. Anderson, R. J. Simpson *et al.*, 1989 Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. Nature 342: 955–957.

McClure, B. A., B. Mou, S. Canevascini, and R. Bernatzky, 1999 A small asparagine-rich protein required for *S*-allele-specific pollen rejection in *Nicotiana*. Proc. Natl. Acad. Sci. USA 96: 13548–13553.

Moon, J., G. Parry, and M. Estelle, 2004 The ubiquitin-proteasome pathway and plant development. Plant Cell 16: 3181–3195.

Murfett, J., T. J. Strabala, and D. M. Zurek, B. Mou, B. Beecher *et al.*, 1996 *S* RNase and interspecific pollen rejection in the genus *Nicotiana*: multiple pollen-rejection pathways contribute to unilateral incompatibility between self-incompatible and self-compatible species. Plant Cell 8: 943–958.

Rick, C. M., 1986 Reproductive isolation in the *Lycopersicon peruvianum* complex, pp. 477–496 in *Solanaceae Biology and Systematics*, edited by W. G. D'Arcy. Columbia University Press, New York.

Rick, C. M., 1988 Evolution of mating systems in cultivated plants, pp. 133–147 in *Plant Evolutionary Biology*, edited by L. D. Gottlieb and S. K. Jain. Chapman and Hall, London.

Rivers, B. A., and R. Bernatzky, 1994 Protein expression of a self-compatible allele from *Lycopersicon peruvianum*: introgression and behavior in a self-incompatible background. Sex. Plant Reprod. 7: 357–362.

Royo, J., C. Kunz, Y. Kowyama, M. Anderson, A. E. Clarke *et al.*, 1994 Loss of a histidine residue at the active site of S-locus ribonuclease is associated with self-compatibility in *Lycopersicon peruvianum*. Proc. Natl. Acad. Sci. USA 91: 6511–6514.

Sijacic, P., W. Wang, A. L. Skirpan, Y. Wang, P. E. Dowd *et al.*, 2004 Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature 429: 302–305.

Trujillo-Moya, C., C. Gisbert, S. Vilanova, and F. Nuez, 2011 Localization of QTLs for in vitro plant regeneration in tomato. BMC Plant Biol. 11: 140.

Twell, D., J. Yamaguchi, and S. McCormick, 1990 Pollen-specific gene expression in transgenic plants: coordinate regulation of two different tomato gene promoters during microsporogenesis. Development 109: 705–713.

van Heusden, A. W., M. Koorneef, R. E. Voorrips, W. Brüggemann, G. Pet *et al.*, 1999 Three QTLs from *Lycopersicon peruvianum* confer a high level of resistance to *Clavibacter michiganensis* ssp. *michiganensis*. Theor. Appl. Genet. 99: 1068–1074.

Zhang, Y., Z. Zhao, and Y. Xue, 2009 Roles of proteolysis in plant self-incompatibility. Annu. Rev. Plant Biol. 60: 21–42.

Zhao, L., J. Huang, Z. Zhao, Q. Li, T. L. Sims *et al.*, 2010 The Skp1-like protein SSK1 is required for cross-pollen compatibility in *S*-RNase-based self-incompatibility. Plant J. 62: 52–63.

Zheng, N., B. A. Schulman, L. Song, J. J. Miller, P. D. Jeffrey *et al.*, 2002 Structure of the Cul1-Rbx1-Skp1-F box^{Skp2} SCF ubiquitin ligase complex. Nature 416: 703–709.

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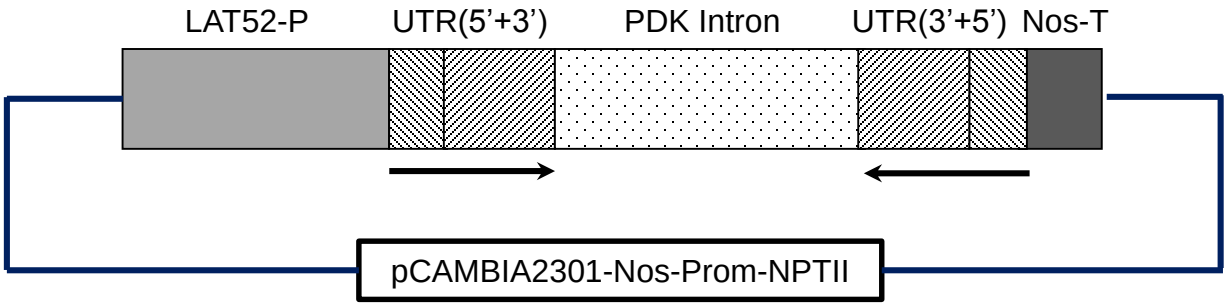

Figure S1 Diagram of the *CUL1* RNAi construct. LAT52-P, pollen specific promoter; UTR (5'+3'), combined UTR regions of *CUL1* gene; Nos-T, nos terminator; pCAMBIA2301-Nos-Prom-NPTII, modified binary vector pCAMBIA2301.

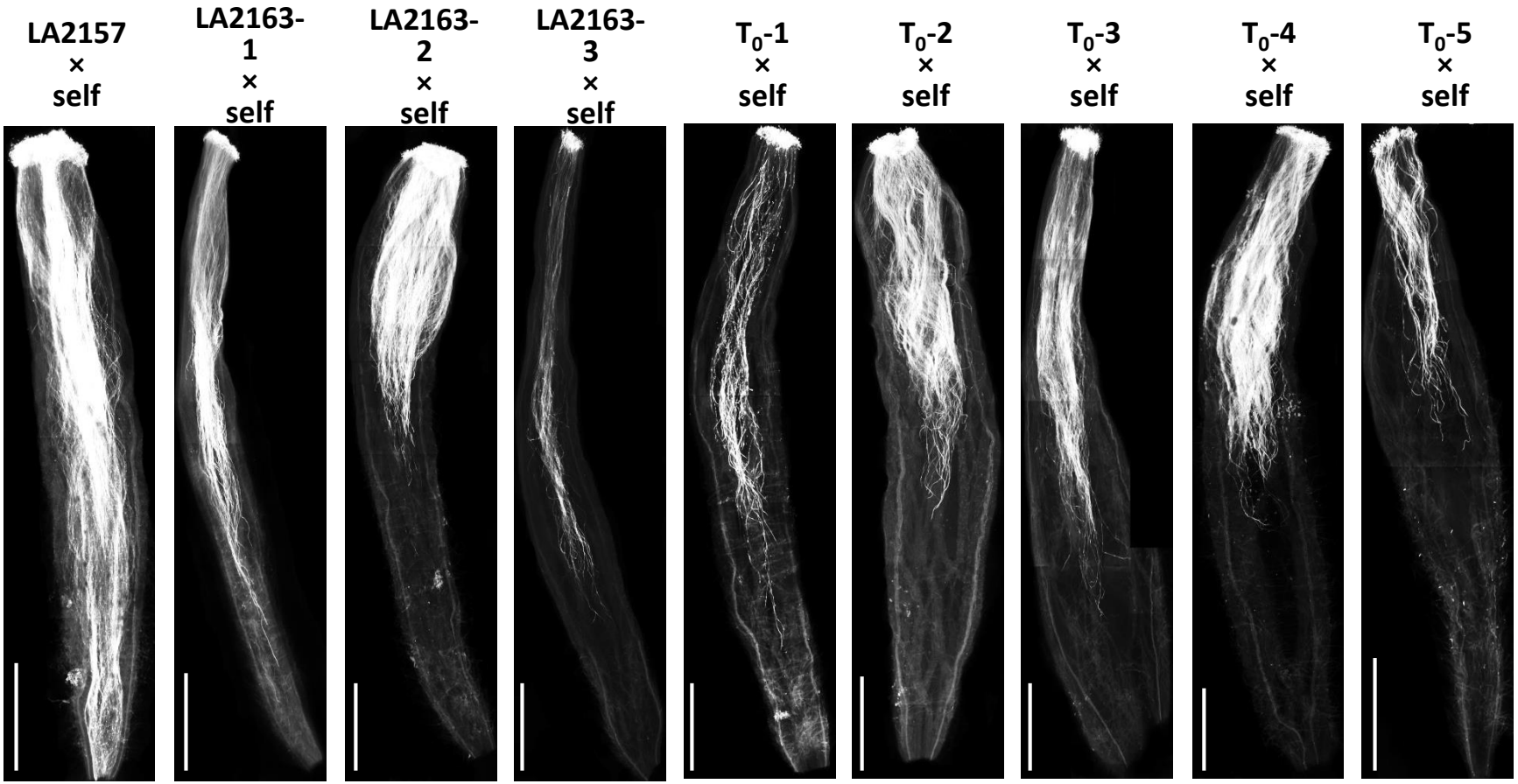

Figure S2 Images of pollen tube growth in pistils after self pollinations. Left to right: LA2157, several independent non-transgenic LA2163 plants, and five independent T_0 *CUL1* RNAi transformants. Styles were fixed 24 hours after pollination and pollen tubes were stained with aniline blue and visualized under UV light. Scale bar is 1 mm.h

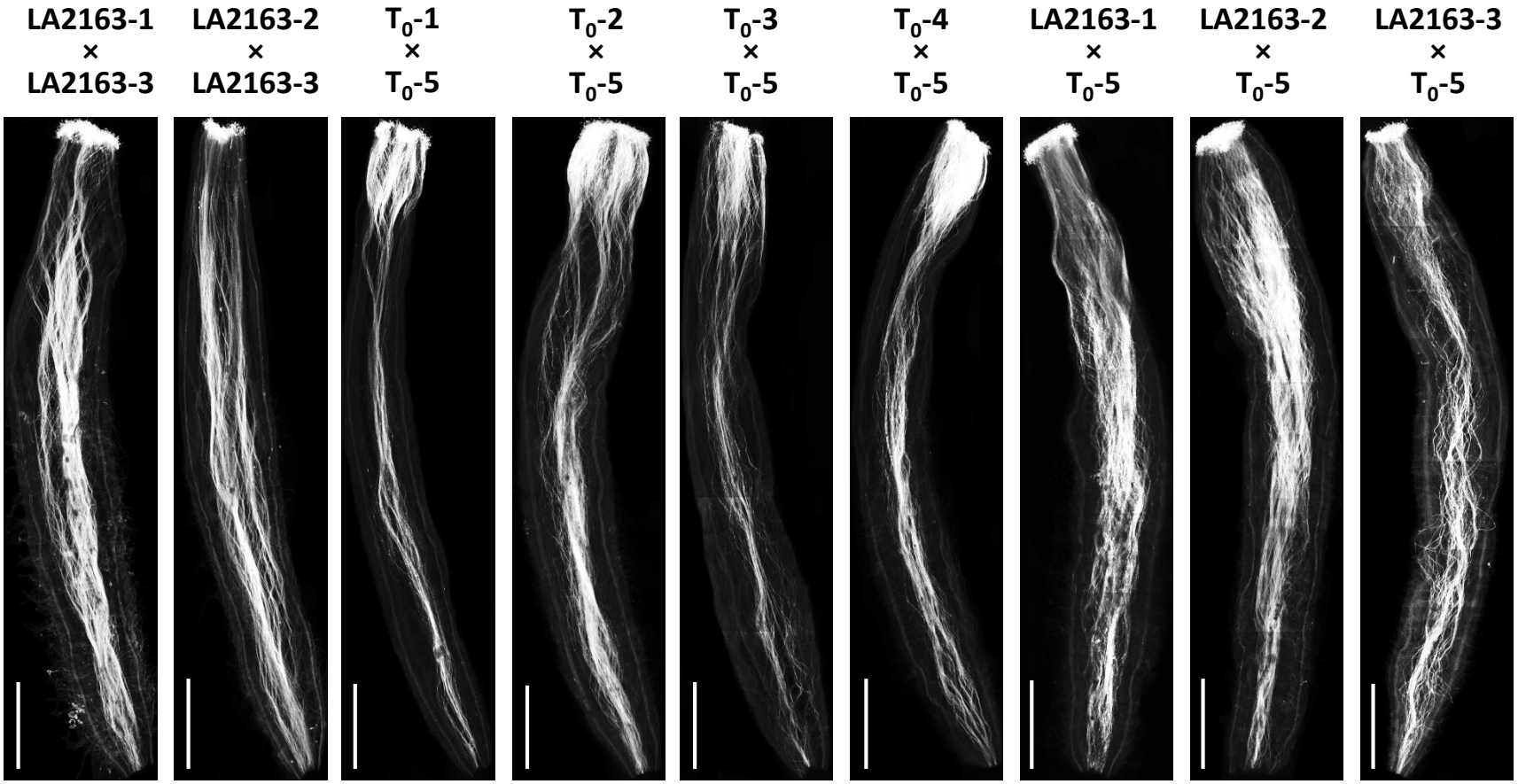

Figure S3 Images of pollen tube growth in pistils after cross pollinations. Left to right: crosses between independent non-transgenic LA2163 plants, crosses between independent T_0 *CUL1* RNAi transformants, and crosses of T_0 plants onto non-transgenic LA2163 pistils. Styles were fixed 24 hours after pollination and pollen tubes were stained with aniline blue and visualized under UV light. Scale bar is 1 mm.

File S1

MATERIALS AND METHODS

Plant materials

Two accessions, LA2157 and LA2163, of the wild tomato species *Solanum arcanum* were used in this study. LA2157 is self-compatible, while LA2163 is self-incompatible, but they are otherwise similar in most respects (Rick 1986). They were collected from nearby sites, separated by ~10 Km, in Cajamarca Department, Peru. Seed of these accessions was obtained from the C.M. Rick Tomato Genetics Resource Center (http://tgrc.ucdavis.edu) where they are maintained by cross pollinating all plants in each generation to maximize heterogeneity. The SI accession LA2163 was transformed with a *CUL1* RNAi construct via Agrobacterium transformation using cotyledonary explants. Selected primary transformants (T_0) were used as pollen donors in crosses onto pistils of non-transgenic LA2163 and LA2157. Plants were drawn at random from the corresponding accessions for the transformations and for the control non-transgenic plants. In the case of LA2163, each primary transformant (T_0-1, -2, etc) or non-transgenic control plant (LA2163-1, -2, -3) was expected to carry different *S*-genotypes (e.g. S_1S_2, S_3S_4, etc), and were cross-compatible in test sib pollinations, as predicted (Figure S3). In the case of LA2157, all plants were SC (Figure 2S) and were therefore expected to carry the same *S*-genotype (i.e. S_cS_c).

Pollen tube growth in styles was visualized by the aniline blue staining technique as described previously (Li et al. 2010).

***CUL1* RNAi construct and plant transformation**

Both conventional digestion-ligation and overlap extension PCR (Ho et al. 1989) were used to synthesize the *CUL1* RNAi construct. For the latter method, the first reaction amplified separate target DNA fragments and a second reaction combined the target fragments in a mixed reaction. Each intermediate primer used for overlap extension PCR had a 5' overhang sequence of ~20 base pairs (bp) that overlapped with the adjacent DNA fragment (Table S1).

To avoid cross-silencing of *Cullin1* genes, 146 and 279 bp 5' and 3' untranslated regions (UTRs) of *CUL1* were combined into a single 425 bp (UTR^{5+3}) sequence and used as the RNAi trigger (Figure S1). The 5' and 3' UTR of *CUL1* from LA2163 were amplified using primers 5UTR-1F/R and 3UTR-1F/R (Table S1) designed from the *SpCul1* sequence (Genbank no. HQ610201). UTR^{5+3} was synthesized by overlap extension PCR using primers 5UTR-1F and 3UTR-1R. The *LAT52* pollen specific promoter was amplified from our previous construct LAT52-SpCUL1 (Li and Chetelat 2010) with primers LAT52-2F/R. The combined LAT52-UTR^{5+3} was obtained by another round of overlap extension PCR using primers LAT52-2F and 3UTR-3R and cloned into pCAMBIA2301. The antisense UTR^{5+3} sequence was amplified with primers 3UTR-2R and 5UTR-2F and ligated to the vector in reverse orientation. The PDK intron was released from pHANNIBAL (Varsha Wesley et al. 2001) and inserted between

the sense and antisense UTR^{5+3} copies. To reduce the frequency of non-transgenic plants recovered from kanamycin selection, the strong plant selection cassette 35S-NPTII in pCAMBIA2301 was replaced with the weak selection cassette Nos-NPTII from pBI121 by PCR with primers Nos-Prom-F and NPTII-R (Table S1). The pCAM2301-Nos-NPT-Cul1-RNAi construct was electroporated into *Agrobactericum tumefaciens* LBA4404 using the Gene Pulser II (Bio-Rad) system and introduced into SI *S. arcanum* LA2163 at the Ralph M. Parsons Plant Transformation Facility at the University of California, Davis.

***CUL1* gene expression and RT-PCR**

CUL1 expression was assayed by RT-PCR. Total RNA was isolated from pollen with TRIzol (Invitrogen). Total RNA (2 mg) was used for cDNA synthesis with SuperScript III (Invitrogen) reverse transcriptase. RT-PCR was performed using the gene specific primer pair Cul-1F/R (Table S1). The PCR conditions for RT-PCR of *CUL1* was 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72°C for 1 min, with a final extension of 5 min at 72 °C. The constitutively expressed *Actin* gene was used as a loading control, and was amplified with the same PCR conditions as *CUL1* except that the cycle number was 25. PCR products were separated in 2% agarose gels.

Transgene segregation tests

To test *CUL1* function in SI, five independent T_0 *CUL1* RNAi transformants were used as pollen parents in crosses onto pistils of SC *S. arcanum* LA2157 and SI *S. arcanum* LA2163 (Table S2). For each T_0 plant, five T_1 progeny arrays -- two from the crosses with LA2157 and three from crosses to three independent non-transgenic LA2163 plants (sibs) -- were genotyped for the presence of the transgene. Data from the two LA2157 progeny arrays of each T_0 plant were pooled since the chi-square tests for heterogeneity were not significant.

A mini-scale DNA extraction method was used to isolate DNA from the T_1 populations as described (Li et al. 2010). The primers 2301-2 and LAT52M-R (Table S1), which spanned the left side of the cloning site where the construct was inserted into the vector, were used to detect the transgene. The presence or absence of the 643 bp amplification product was used to classify the plants as transgenic or non-transgenic, respectively.

Supporting References

Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene 77:51-59.

Varsha Wesley, S. et al. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant Journal 27: 581-590.

Table S1 Primers used to build the RNAi construct and genotype the transgenic lines. Restriction sites are underlined, and sequences that overlap with adjacent primers for overlap extension PCR are underlined with a wavy line.

Primer	Sequence (5'-3')	Restriction Enzyme
5UTR-1F	CACCAAAAAAAAAAATTCCAATTTAACCAAAATGCAACCGAATTCGACG	
5UTR-1R	CGAGGTATGAGATCAGCACCCTCGGAAAATTCTTGTTAACACGAT	
3UTR-1F	TGTTAACAAGAATTTTCCGAGGGTGCTGATCTCATACCTCGG	
3UTR-1R	GTAAATGTTGGCATTACTGCAACCA	
LAT52-2F	TCTATGGAGCTCGGATAAGGGTAGCTCT	*Sac* I
LAT52-2R	TCGAATTCGGTTGCATTTTGGTTAAATTGGAATTTTTTTTTTTGGTG	
3UTR-3R	GTACGGGGTACCGTAAATGTTGGCATTACTGCAACCA	*Kpn* I
3UTR-2R	TTCGCGGATCCGTAAATGTTGGCATTACTGCAACCA	*Bam*H I
5UTR-2F	GTCTATGGGTCACCACCAAAATGCAACCGAATTCGACG	*Bst*E II
Nos-Prom-F	TCTATGCCAACATGGTGGGATCATGAGCGGAGAATTAAGGGA	*Bst*X I
NPTII-R	TTCGCCTCGAGTCAGAAGAACTCGTCAAGAAGGCGA	*Xho* I
Cul-1F	ATCAGGAACGTGAGGGTGAG	
Cul-1R	CAGCAAAACAGCCTTTCACA	
2301-2	CCAGGCTTTACACTTTATGCTTC	
LAT52M-R	TGCTCCTTCTCTTTGTGTGTGT	

Table S2 Segregation through the pollen of the *CUL1* RNAi T-DNA. Five independent transformants (T_0-1 to -5) were crossed as pollen donors onto SC and SI accessions of *S. arcanum* (LA2157 and LA2163, respectively). T_1 progeny arrays from three independent LA2163 plants (LA2163-1, -2, -3 or -4) were analyzed. Chi-square goodness-of-fit statistics (df = 1, with Yates correction factor) were used to test for deviations from the expected 1:1 (single T-DNA insertion) or 3:1 (two insertions) ratios.

Cross (♀ × ♂)	No. Plants + : - TDNA	Total	% Transgenic Progeny	Ratio Tested	χ^2	P value
LA2157 × T_0-1	71 : 25	96	74.0	3:1	0.01	0.92
LA2163-1 × T_0-1	6 : 42	48	12.5	3:1	96.7	<0.0001
LA2163-2 × T_0-1	7 : 41	48	14.3	3:1	90.3	<0.0001
LA2163-3 × T_0-1	2 : 46	48	4.2	3:1	124.8	<0.0001
LA2157 × T_0-2	42 : 54	96	43.8	1:1	1.26	0.26
LA2163-1 × T_0-2	17 : 79	96	17.7	1:1	38.8	<0.0001
LA2163-2 × T_0-2	12 : 84	96	12.5	1:1	52.5	<0.0001
LA2163-3 × T_0-2	0 : 96	96	0	1:1	94.0	<0.0001
LA2157 × T_0-3	64 : 80	144	44.4	1:1	1.56	0.2117
LA2163-1 × T_0-3	2 : 46	48	4.2	1:1	38.5	<0.0001
LA2163-2 × T_0-3	4 : 44	48	8.3	1:1	31.7	<0.0001
LA2163-3 × T_0-3	0 : 48	48	0	1:1	46.0	<0.0001
LA2157 × T_0-4	50 : 46	96	52.1	1:1	0.1	0.7518
LA2163-1 × T_0-4	6 : 42	48	12.5	1:1	25.5	<0.0001
LA2163-2 × T_0-4	2 : 46	48	4.2	1:1	38.5	<0.0001
LA2163-3 × T_0-4	0 : 48	48	0	1:1	46.0	<0.0001
LA2157 × T_0-5	62 : 82	144	43.1	1:1	2.5	0.1138
LA2163-2 × T_0-5	5 : 43	48	10.4	1:1	28.5	<0.0001
LA2163-3 × T_0-5	0 : 48	48	0	1:1	46.0	<0.0001
LA2163-4 × T_0-5	2 : 46	48	4.2	1:1	38.5	<0.0001