The Plant Journal (2002) **29**(5), 627–636

Cultivated tomato has defects in both *S-RNase* and *HT* genes required for stylar function of self-incompatibility

Katsuhiko Kondo[1,†], Mayu Yamamoto[1,†], Daniel P. Matton[2], Takanori Sato[3,‡], Masashi Hirai[3,§], Shigemi Norioka[4], Tsukaho Hattori[5] and Yasuo Kowyama[1,*]

[1]*Faculty of Bioresources, Mie University, Tsu 514-8507, Japan,*

[2]*Institut de Recherche en Biologie Végétale, Département de Sciences Biologiques, Université de Montréal, 4101 rue Sherbrooke est, Montréal, Québec, H1X 2B2, Canada,*

[3]*National Institute of Vegetable and Tea Science, Anou 514-2392, Japan,*

[4]*Institute for Protein Research, Osaka University, Suita 565-0871, Japan, and*

[5]*Center for Molecular Biology and Genetics, Mie University, Tsu 514-8507, Japan*

Received 11 September 2001; revised 22 November 2001; accepted 6 December 2001.

[*]For correspondence (fax +81 59 231 9637; e-mail kouyama@bio.mie-u.ac.jp).

[‡]Present address: Asian Vegetable Research and Development Center, PO Box 42, Tainan 741, ROC, Taiwan.

[§]Present address: Kyoto Prefectural University, Kyoto 619-0244, Japan.

[†]These two authors contributed equally to this work.

Summary

Cultivated tomato (*Lycopersicon esculentum*), a self-compatible species, evolved from self-incompatible (SI) species in the genus *Lycopersicon* following a breakdown of the self-incompatibility system. In order to elucidate the molecular basis of this breakdown in *L. esculentum*, we first analysed the stylar proteins with an in-gel assay for ribonuclease activity and 2D-PAGE. No S-RNase protein or its activity was detected in the style of *L. esculentum*. We then introduced the *S6-RNase* gene from an SI relative, *L. peruvianum*, into *L. esculentum*. However, the styles of transgenic plants expressing S_6-*RNase* at levels comparable to those found in the *L. peruvianum* style were unable to reject self-pollen and *L. peruvianum* pollen in an allele-specific manner. This indicated that defect in the *S-RNase* expression was not the sole reason for the loss of self-incompatibility in tomato. The asparagine-rich HT protein, originally identified from the style of *Nicotiana alata*, is the other stylar factor involved in self-incompatibility reaction. We cloned and sequenced two distinct genes encoding HT-A and HT-B proteins from *L. peruvianum* (*LpHT-A* and *LpHT-B*) and *L. esculentum* (*LeHT-A* and *LeHT-B*). A frame shift mutation in the coding sequence of *LeHT-A* and a stop codon in the ORF of *LeHT-B* were found, and no *LeHT-B* transcript was detected in the style of *L. esculentum*. The results suggest that the breakdown of self-incompatibility in cultivated tomato is associated with loss-of-function mutations in both *S-RNase* and *HT* genes.

Keywords: self-incompatibility, S-RNase, HT-protein, *Lycopersicon*, tomato.

Introduction

Self-incompatibility is a cell-to-cell recognition mechanism that allows a pistil to distinguish self from non-self pollen, and plays a strong role in promoting genetic diversity through outbreeding. In many species of flowering plants, self-incompatibility is genetically controlled by a single multi-allelic locus, called the *S*-locus (de Nettancourt, 1977). In the gametophytic self-incompatibility system of solanaceous plants, pollen-tube growth is retarded when the pollen carries an *S*-allele identical to one of those carried by the pistil. Stylar ribonucleases (S-RNases) are the *S*-allele products in the Solanaceae, Rosaceae and Scrophulariaceae (see reviews by Kao and McCubbin, 1996; Newbigin *et al.*, 1993). S-RNases are abundant proteins in the extracellular matrix of the style, where pollen-tube growth occurs (Anderson *et al.*, 1989). Transgenic studies have shown that a sufficient level of S-RNase expression is necessary for a functional reaction of gametophytic self-incompatibility in *Petunia, Nicotiana*

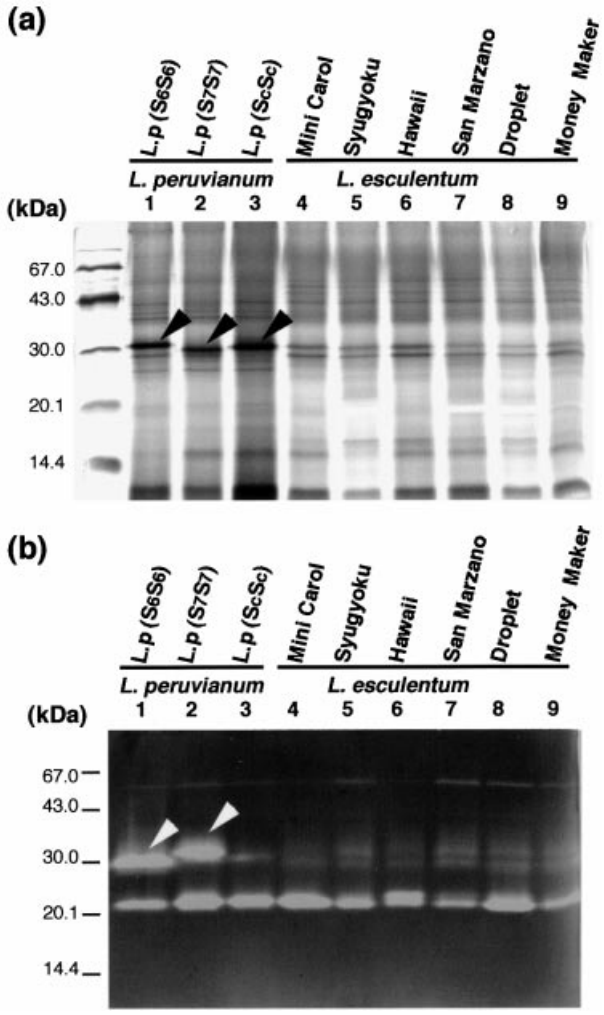

Figure 1. Analyses of S-RNase protein and enzymatic activity in *L. peruvianum* and *L. esculentum.*

(a)SDS–PAGE of stylar proteins extracted from *L. peruvianum* (*L.p.*) and *L. esculentum*. Protein extracts were separated on 15% polyacrylamide and the gel was silver-stained.

(b)In-gel RNase activity staining of SDS–PAGE gel: each lane was loaded with 2.5 μg protein from three *S*-genotypes of *L. peruvianum* and six tomato cultivars. Molecular mass markers loaded on the left lane are indicated in kDa; polypeptide and activity bands corresponding to the S-RNase are shown by arrowheads. A slight difference was found in molecular mass of the RNases between SDS–PAGE (a) and activity-stained (b) gels. This is due to a non-heat treatment of the protein extracts before loading onto the gel in activity staining, while in standard SDS–PAGE, heat-denatured proteins were loaded.

and *Solanum* (Lee *et al.*, 1994; Matton *et al.*, 1997; Murfett *et al.*, 1994).

The genus *Lycopersicon* consists of both self-incompatible (SI) and self-compatible (SC) species. Cultivated tomato, *L. esculentum*, is SC and is generally thought to be derived from an SI species (Miller and Tanksley, 1990; Rick, 1988). Self-compatible plants arise in SI species as a consequence of a breakdown of the self-incompatibility system at some points (de Nettancourt, 1977). Genetic studies suggest that the breakdown of self-incompatibility in *L. esculentum* is due to mutations at some loci, including the *S*-locus (Bernatzky *et al.*, 1995). Some spontaneous SC mutants have been reported in otherwise SI species with defects in the S-RNase. For instance, an SC accession of *L. peruvianum* has an amino acid substitution at one of the essential His residues in the catalytic domain of the S-RNase that leads to a complete loss of enzymatic activity (Kowyama *et al.*, 1994; Royo *et al.*, 1994). Similarly, deletion of an *S-RNase* gene is associated with a loss of stylar SI response in an SC cultivar of *Pyrus serotina* (Sassa *et al.*, 1997).

Related to the breakdown of self-incompatibility within a species is another mechanism of pollen rejection, known as unilateral incompatibility (UI). Unilateral incompatibility is commonly seen when two related plants, one SI and the other SC, are reciprocally crossed. Pollen of an SC species will be rejected when used to pollinate pistils of its SI relative, whereas the reverse pollination is often compatible, i.e. pollen-tube growth is not arrested in the style (Lewis and Crow, 1958). Genetic studies suggest the involvement of multiple loci, including the *S* locus, in the UI reaction of *Lycopersicon* (Chetelat and DeVerna, 1991). The requirement for S-RNases in interspecific pollen rejection has been shown in *Nicotiana* (Murfett *et al.*, 1996). For example, SI *Nicotiana alata* can reject pollen from SC *Nicotiana* species, whereas an SC accession of *N. alata* lacking stylar S-RNase cannot reject pollen of SC *N. plumbaginifolia*. Transgenic studies further show that S-RNase alone is not sufficient for UI reaction in the SC genetic background, indicating both the presence of an S-RNase-dependent mechanism and the involvement of genetic factors other than the S-RNase in controlling interspecific pollination (Murfett *et al.*, 1996). Recently, McClure *et al.* (1999) identified a small asparagine-rich protein, designated HT, that was essential for *S*-allele-specific pollen rejection in the style of *N. alata*. The HT protein is a factor other than S-RNase involved in self-incompatibility response, but it is not known whether HT is also required for the UI response.

The reasons for self-compatibility in cultivated tomato, *L. esculentum*, are still unclear despite the genetic studies mentioned above. In the present study we demonstrate that the style of *L. esculentum* lacks S-RNase activity, and that the expression of a functional S-RNase in transgenic plants is not sufficient to confer *S*-allele-specific or interspecific pollen rejection. Furthermore, from the sequences and expression patterns of *HT* genes in *L. peruvianum* and *L. esculentum*, we suggest that the HT protein, as well as the S-RNase, is one of the missing stylar factors for SI pollen rejection in cultivated tomato.

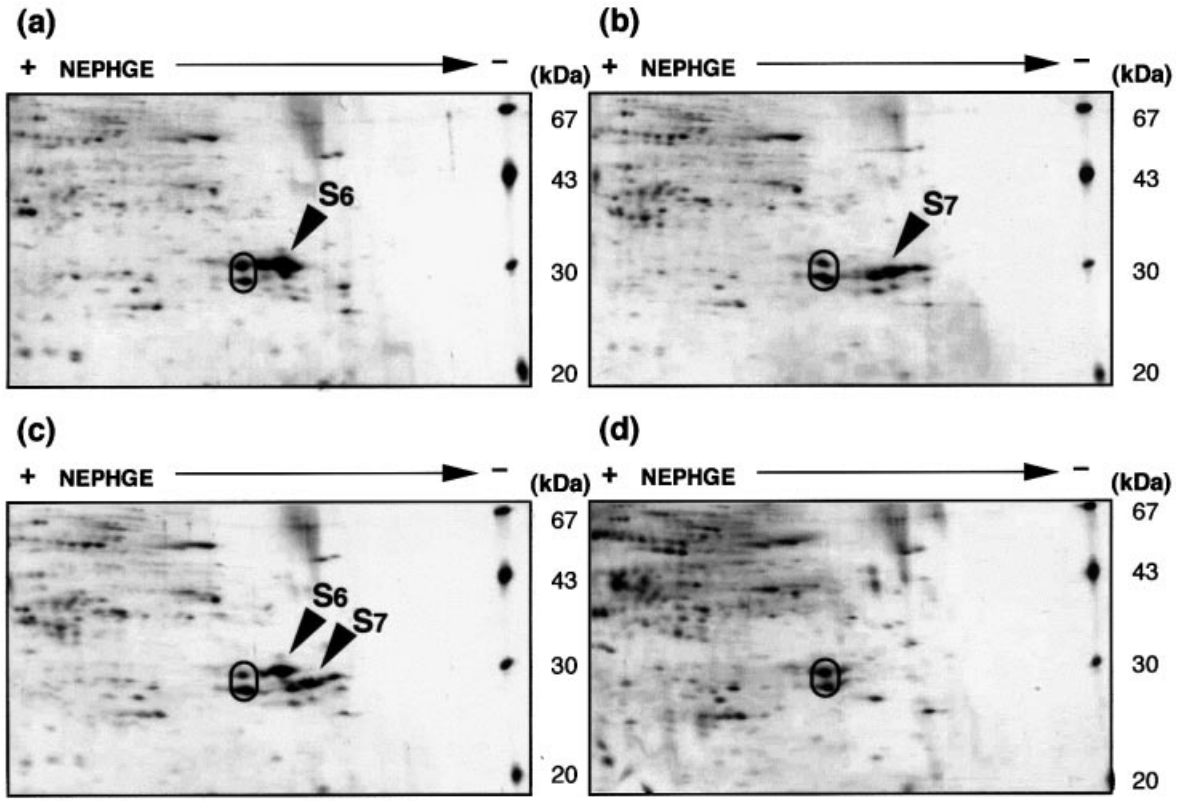

Figure 2. Two-dimensional polyacrylamide gel electrophoresis profiles of stylar extracts.
2D-PAGE profiles of stylar extracts from S_6S_6 homozygote (a); S_7S_7 homozygote (b); and S_6S_7 heterozygote (c) of *L. peruvianum*, and from *L. esculentum,* cv. Alisa Craig (d). Stylar extracts were fractionated by non-equilibrium gel electrophoresis in the first dimension and SDS–PAGE in the second dimension, followed by silver staining. Arrowheads show S-RNases. The 30 kDa doublet spots present in both *L. peruvianum* and *L. esculentum* are enclosed by a circle (see text).

Results

Does cultivated tomato express stylar S-RNases?

The enzymatic activity of S-RNase in the style has been shown to be essential for rejection of self-pollen in solanaceous SI species (Lee *et al.*, 1994; Murfett *et al.*, 1994). To explore the possibility that the lack of S-RNase activity is also the cause of self-compatibility in *L. esculentum*, we performed SDS–PAGE followed by in-gel RNase activity assay on protein extracts from mature styles of *L. esculentum* and SI and SC genotypes of *L. peruvianum*. Under standard SDS–PAGE of stylar extracts, the S-RNases in the SI and SC *L. peruvianum* plants migrated as a band of approximately 30 kDa (Figure 1a, lanes 1–3). The protein bands in the two SI lines (S_6S_6 and S_7S_7) showed a strong RNase activity in the in-gel assay (Figure 1b, lanes 1–2). In contrast, none of the extracts from the SC genotype (*ScSc*) of *L. peruvianum* and the cultivated varieties of *L. esculentum* exhibited such strong RNase-active bands of approximately 30 kDa (Figure 1b, lanes 3–9). In addition to the 30 kDa proteins, high levels of RNase activity were detected around 22 kDa in all the samples examined (Figure 1b) that are estimated to be non-S RNase, corresponding to the RNase LE and RNase LX of tomato (Kock *et al.*, 1995) from sequence analyses of the cDNA clones (K.K. and co-workers, unpublished data). Weak doublets of RNase activity that varied slightly in size among the lanes were observed around 30 kDa in the tomato cultivars. These also were not thought to represent an S-RNase, because similar weak bands were observed in extracts from the SC *L. peruvianum* plant (Figure 1b, lane 3). These bands were seen in styles 4 days before anthesis, when S-RNases are normally not expressed in the SI *L. peruvianum* line (data not shown). Analysis of stylar proteins from *L. esculentum* (cv. Alisa Craig) and three *S*-genotypes of SI *L. peruvianum* by two-dimensional PAGE (2D-PAGE) showed that a 30 kDa doublet was present (Figure 2, circled), indicating they are not S-RNases. The strong RNase-active band of around 30 kDa seen in the in-gel assay of stylar extracts from SI *L. peruvianum* corresponded to abundant 30 kDa protein seen by 2D-PAGE analysis (Figure 2a–c). By contrast, the stylar extract from *L. esculentum* had no major spots typical of S-RNase

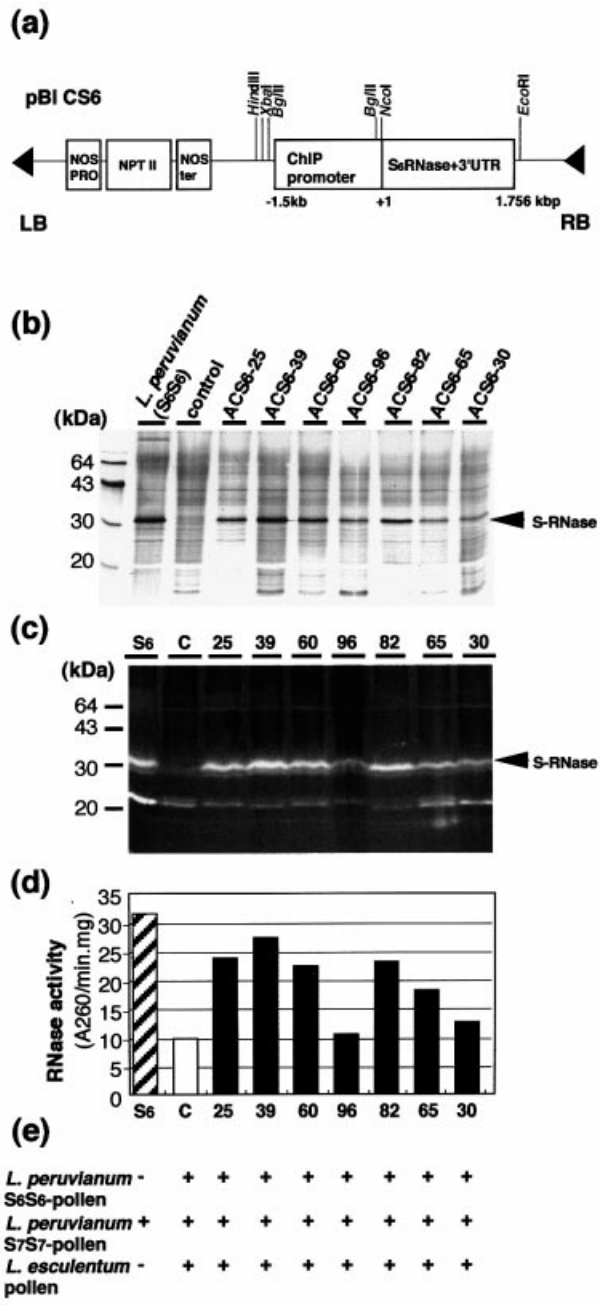

Figure 3. Analysis of *S6-RNase* expressing transgenic plants.
(a) T-DNA construct harbouring the *S6-RNase* gene from *L. peruvianum* used for transformation of *L. esculentum*. The plasmid pBICS6 consists of a tomato chitinase promoter (*Chi*P) and an *L. peruvianum* S_6 RNase genomic fragment, which covers the entire coding exons, an intron and the 3′ untranslated region. Nucleotide position indicated by +1 is the translational initiation site.
(b–e) Detection of S_6-RNase and pollination behaviour in transgenic tomato plants. Stylar extracts from *L. peruvianum* S_6S_6 plant, untransformed tomato cv. Alisa Craig (control), and seven independent transgenic plants (ACS6-25, 39, 60, 96, 82, 65 and 30) were analysed by SDS–PAGE followed by silver staining (b); in-gel RNase activity assay (c); and RNase activity assay of the extracts (d). Pollination behaviour was determined by pollen-tube growth (e). *Lycopersicon peruvianum* S_6S_6, S_6S_7 pollens and *L. esculentum* pollen were used for the pollination. Compatible and incompatible pollinations are indicated by (+) and (–), respectively.

except for the 30 kDa doublet (Figure 2d), suggesting that the styles of *L. esculentum* either lack S-RNase or contain very low levels of this protein.

Production of transgenic tomato plants expressing S-RNase

The results mentioned above suggest the possibility that the loss of S-RNase expression in *L. esculentum* could cause a loss of self-incompatibility during the evolution of this species. To test this possibility, we produced transgenic tomato plants (cv. Alisa Craig) that accumulate the *L. peruvianum* S_6-RNase in their styles. The construct used (pBICS6) is shown in Figure 3(a). A chitinase gene promoter (*Chi*P) that drives a high level of expression specifically in mature styles of solanaceous plants (Harikrishna *et al.*, 1996) was ligated to the genomic sequence of the *S_6-RNase* gene. Plants transformed with the pBICS6 construct efficiently expressed the transgene in the style at both mRNA and protein levels (see below). Analyses of 42 transgenic plants revealed the presence of one to four copies of the transgene and various levels of S_6-RNase mRNA (data not shown). We selected seven representative plants with different levels of transgene expression for further analyses.

S-RNases are not sufficient for self- and S*-allele-specific/interspecific pollen rejection in* L. esculentum

SDS–PAGE and in-gel RNase assays were used to analyse stylar proteins from the seven representative *S_6-RNase* transformants, and revealed that all the transgenic plants expressed a major 30 kDa protein with RNase activity that was absent from an untransformed tomato plant, and co-migrated with the S_6-RNase in an S_6 homozygous *L. peruvianum* plant (Figure 3b,c). The levels of S_6-RNase transcript roughly paralleled with the accumulation of the 30 kDa protein and its associated RNase activity (data not shown). Transgenic S_6-RNase expression was also accompanied by increases in the total RNase activity of the stylar extract (Figure 3d).

The pollination behavior of the transgenic plants was examined to see whether self, *S*-allele-specific or interspecific pollen rejection occurred. Compatibility with self-pollen and pollen with different *S*-genotype was assessed by monitoring pollen-tube growth in the style. The slowed growth characteristic of an incompatible pollen tube was clearly seen in the SI cross (*L. peruvianum* S_6S_6 style with S_6 pollen; Figure 4a), whereas this was not observed when self-pollinated in the styles of a transgenic plant that expressed transgenic *S_6-RNase* at levels comparable to those in an *L. peruvianum* plant (Figure 4b) or pollinated with either S_6 (Figure 4c) or S_7 pollen (Figure 4d). Seeds were set after self-pollination of the transgenic plants.

Figure 3(e) summarizes the results of the pollination test of each transgenic plant. The results indicate that the lack of S-RNase expression is not solely responsible for the lack of capacity for pollen rejection in *L. esculentum*.

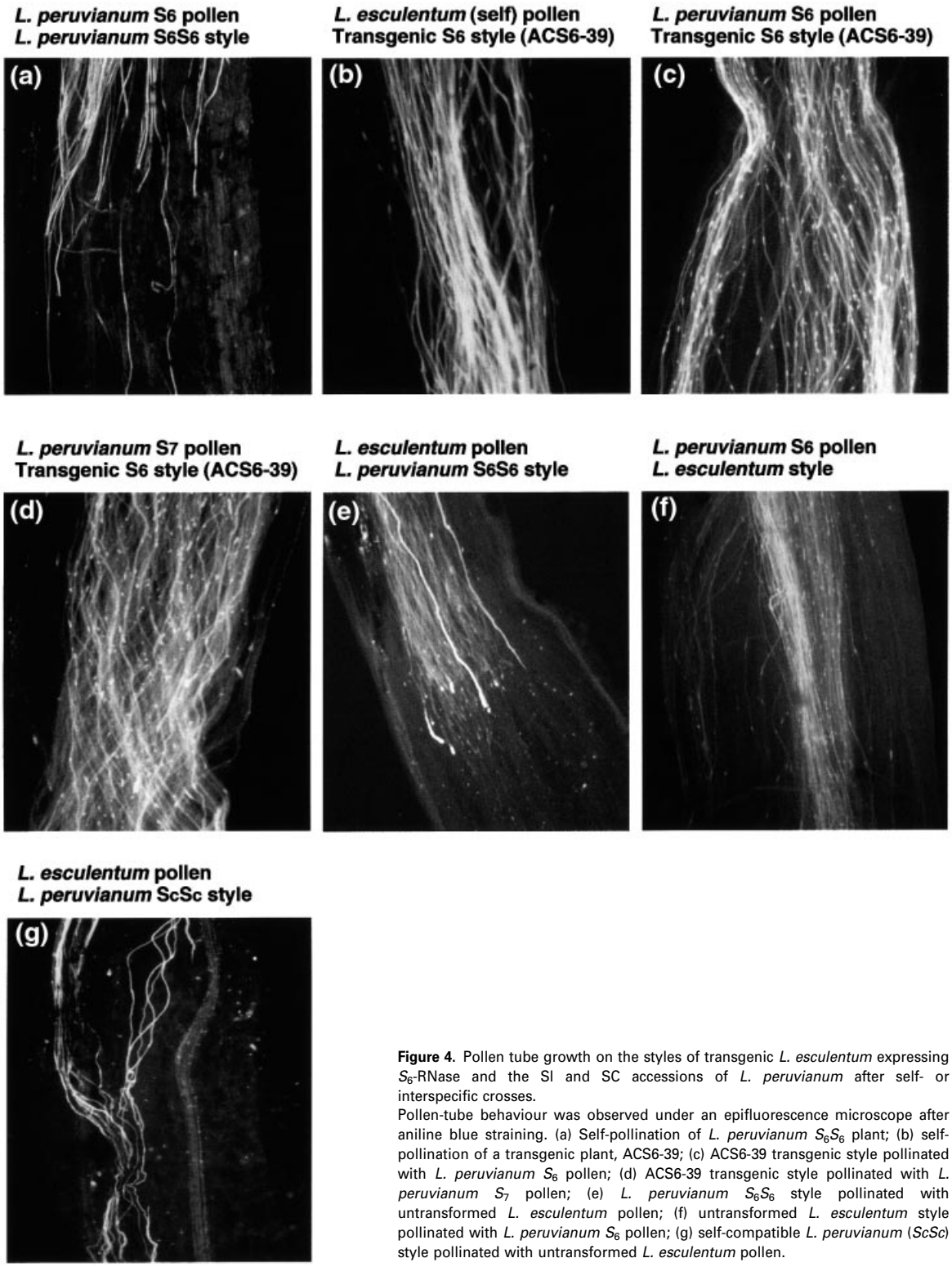

Figure 4. Pollen tube growth on the styles of transgenic *L. esculentum* expressing S_6-RNase and the SI and SC accessions of *L. peruvianum* after self- or interspecific crosses.

Pollen-tube behaviour was observed under an epifluorescence microscope after aniline blue straining. (a) Self-pollination of *L. peruvianum* S_6S_6 plant; (b) self-pollination of a transgenic plant, ACS6-39; (c) ACS6-39 transgenic style pollinated with *L. peruvianum* S_6 pollen; (d) ACS6-39 transgenic style pollinated with *L. peruvianum* S_7 pollen; (e) *L. peruvianum* S_6S_6 style pollinated with untransformed *L. esculentum* pollen; (f) untransformed *L. esculentum* style pollinated with *L. peruvianum* S_6 pollen; (g) self-compatible *L. peruvianum* (*ScSc*) style pollinated with untransformed *L. esculentum* pollen.

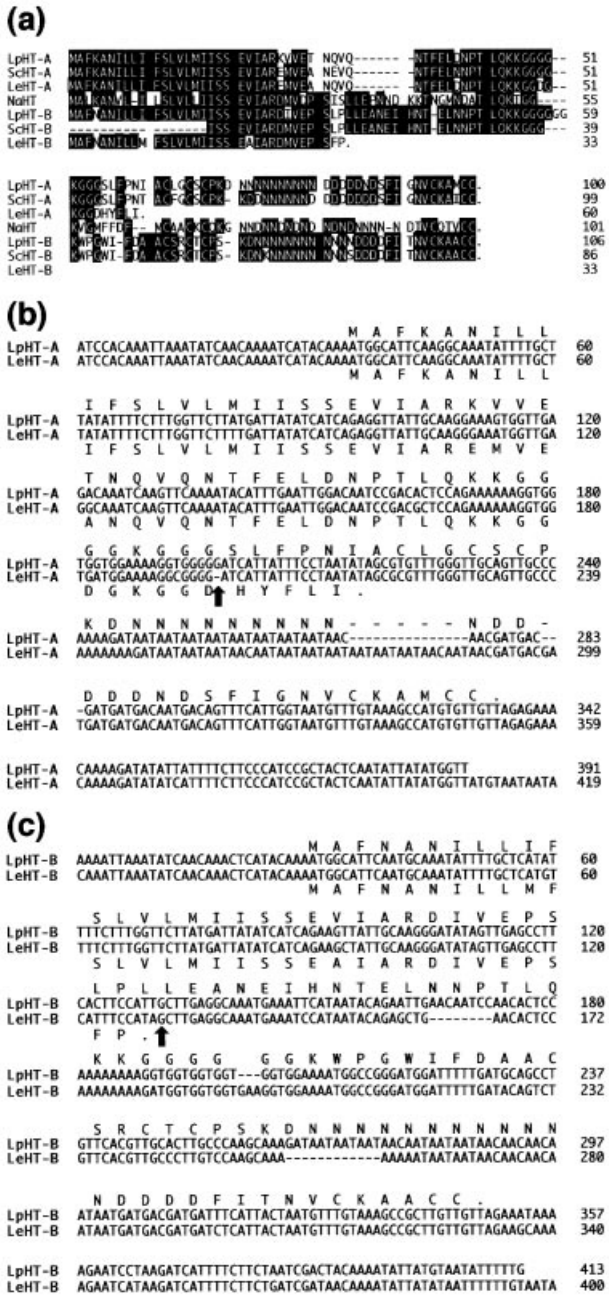

Figure 5. Comparison of the deduced amino acid and nucleotide sequences in the *HT* genes.
(a) Similarity of amino acid sequences in HT-A and HT-B proteins among species. *ScHT-A* and *ScHT-B* are from *Solanum chacoense* (D. Matton, personal communication). *NaHT* is an HT protein reported from *Nicotiana alata* (McClure *et al.*, 1999). Dashes and black background indicate deletions and identity in the sequences, respectively.
(b) Nucleotide/amino acid sequences of *LpHT-A* from *L. peruvianum* (Accession no. AB066582) and *LeHT-A* from *L. esculentum* (Accession no. AB066584).
(c) Nucleotide/amino acid sequences of *LpHT-B* (Accession no. AB066583) from *L. peruvianum* and *LeHT-B* (Accession no. AB066585) from *L. esculentum.*
Arrowheads in (b) and (c) show a single deletion and a stop codon in the ORF, respectively,

In interspecific crosses, the SI style of *L. peruvianum* rejected the pollen of SC *L. esculentum* (Figure 4e), but the reverse cross was compatible (Figure 4f). The SC accession of *L. peruvianum* (ScSc) that lacks S-RNase activity was compatible with *L. esculentum* pollen (Figure 4g), indicating that the UI in the cross between *L. esculentum* and *L. peruvianum* is S-RNase dependent. However, as mentioned above, S-RNase-expressing *L. esculentum* transformants were compatible to *L. peruvianum* pollen, suggesting the UI response involves other factor(s) in addition to the S-RNase.

L. esculentum *has a defect in* HT *genes*

Genetic studies with solanaceous species, including *Lycopersicon*, suggest that, in addition to S-RNase, other genetic factors are required for incompatible pollen rejection in SI (Bernatzky *et al.*, 1995). The only one of these factors characterized so far is a small asparagine-rich protein from *N. alata*, designated HT (McClure *et al.*, 1999). In order to examine if functional HT homologues were present in *Lycopersicon* species, degenerate primers based on the amino acid sequences of HT proteins from *N. alata* (McClure *et al.*, 1999) and *Solanum chacoense* (D. Matton, personal communication) were used for RT–PCR amplifications of stylar total RNA in *L. peruvianum.* The nucleotide sequences of the cloned cDNAs revealed the presence of two distinct HT homologues in *L. peruvianum* that were designated *LpHT-A* and *LpHT-B*. Sequence comparison showed that *LpHT-B* was more similar to the *N. alata* HT protein, with 47% amino acid identity (Figure 5a). Southern analysis with *LpHT-A* and *LpHT-B* probes indicated that the *L. esculentum* genome also contains these genes, and they are located in tandem within 10 kbp of the genomic DNA (Figure 6a). Nucleotide sequences and the deduced amino acid sequences of the tomato homologues (*LeHT-A* and *LeHT-B*) were obtained by PCR amplification of genomic DNA (Figure 5a). Northern blot hybridization revealed that the *LeHT-B* transcript did not accumulate to a high level in stylar RNA (Figure 6b). Furthermore, the nucleotide sequence of the *LeHT-B* gene revealed the presence of a stop codon in the coding sequence (Figure 5c), indicating that *LeHT-B* is a non-functional gene. In contrast, the *LeHT-A* transcript was detected at significant levels in the style (Figure 6b), but its coding region had a single nucleotide deletion that causes frame shift and would result in the production of an aberrant protein (Figure 5b). Therefore both *HT* genes in *L. esculentum* were estimated to be non-functional.

Discussion

In the present study, we have shown that the styles of cultivated tomato, *L. esculentum*, either do not express an

S-RNase, or do so only at a very low level. It is not known whether the defect affecting S-RNase expression in *L. esculentum* is at the transcriptional or translational level, or whether a sequence corresponding to the *S-RNase* gene is deleted in the *L. esculentum* genome. An unsuccessful attempt to amplify the *S-RNase* sequence using primer pairs for conserved regions was made using genomic DNA and cDNA from the different tomato cultivars. This approach had been successfully used to amplify 18 different *S-RNase* alleles from several *Lycopersicon* species (Kondo *et al.*, 2001). This suggests that the S-RNase sequences in the genome of *L. esculentum* have been either deleted or extensively mutated. A similar situation has been reported for *Arabidopsis thaliana*, a self-compatible species (Kusaba *et al.*, 2001). They suggest that self-compatibility in *A. thaliana* is associated with inactivation of genes involved in the self-incompatibility.

As a high level of S-RNase expression in the style has previously been shown to be essential for the gametophytic self-incompatibility response in the Solanaceae, the lack of S-RNase expression may be why tomato styles fail to reject self-pollen. However, expression of a functional S_6-RNase gene in transgenic tomato plants, even at levels comparable to those in SI *L. peruvianum*, did not result in the ability to reject either self-pollen or S_6 pollen from *L. peruvianum*. As the tomato cultivar used in transgenic study is SC, it may be a natural consequence for the styles of the transgenic plants not to reject self-pollen. However, it should be noted that *L. peruvianum* styles reject *L. esculentum* pollen through the UI reaction. Thus the inability of the transgenic plants to reject S_6 pollen from *L. peruvianum* indicates that other stylar component(s) required for *S*-allele-specific pollen rejection are also missing from *L. esculentum*. The result is consistent with previous genetic studies on lines of *L. esculentum* into which chromosomal fragments derived from SI accession of *L. hirsutum* had been introgressed (Bernatzky *et al.*, 1995).

A candidate for the stylar factor missing from *L. esculentum* is the HT protein, which has been shown to be required for *S*-allele-specific pollen rejection in *N. alata* (McClure *et al.*, 1999). We have identified two distinct cDNA clones for HT protein homologues in *L. peruvianum*, *LpHT-A* and *LpHT-B*, and have shown that the latter has a higher level of amino acid identity to the *N. alata* HT protein than the former. Thus the *HT* gene originally reported in *N. alata* corresponds to *HT-B*. Southern

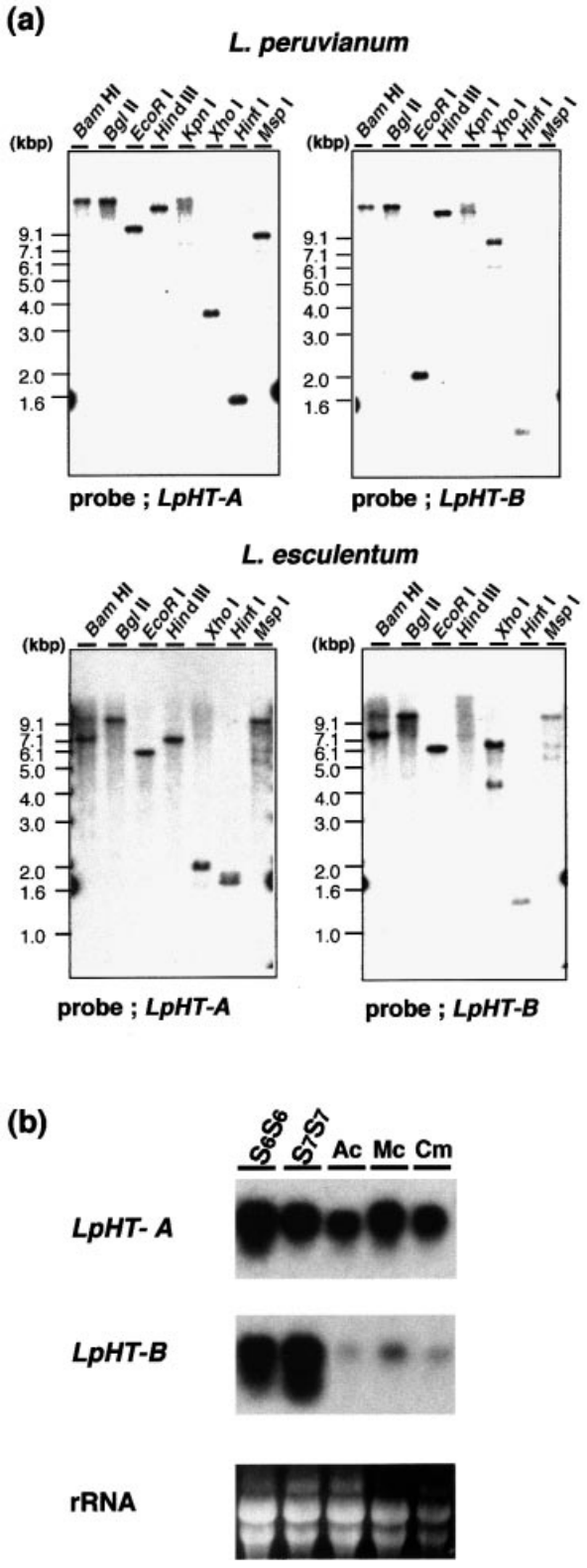

Figure 6. DNA and RNA gel-blot analyses of *HT-A* and *HT-B* genes.
(a) DNA (5 μg) prepared from *L. peruvianum* S_6S_6 homozygote (upper) and *L. esculentum* cv. Alisa Craig (lower) was digested with some restriction enzymes indicated and analysed by DNA gel-blot hybridization using ^{32}P-labeled *LpHT-A* or *LpHT-B* cDNA as a probe.
(b) Total RNA prepared from the styles of *L. peruvianum* (S_6S_6 and *S7S7*) and *L. esculentum* versus Alisa Craig (Ac), Mini Carol (Mc) and Chelsea mini (Cm) was analysed by RNA gel blot probed with ^{32}P-labeled *LpHT-A* or *LpHT-B* cDNA. rRNA is shown as a loading control.

hybridization and PCR analysis with genomic DNA from *L. esculentum* revealed the presence of *LeHT-A* and *LeHT-B* genes, which corresponded to *LpHT-A* and *LpHT-B*, respectively. However, neither of these genes encodes a functional protein. *LeHT-A* was transcribed at a high level, but had a single nucleotide deletion that caused a frame shift in the coding region. *LeHT-B* was not transcribed in the style. Although it is not known if both *HT* gene products are required for pollen rejection in self-incompatibility reactions, the results suggest that *HT-B* is one of the missing stylar factors in *L. esculentum* beside the *S-RNase*. The lack of both S-RNase and HT protein has been also described for *N. plumbaginifolia* (McClure *et al.*, 1999). As the role of HT proteins in the S-RNase mediated self-incompatibility system of *Lycopersicon* is not uncertain, further studies are required through biochemical and molecular analyses of transformed plants.

The failure to confer the capacity for *S*-allele-specific and interspecific pollen rejection has also been reported in transgenic SC *N. plumbaginifolia* with an introduced *S-RNase* gene from *N. alata* (Murfett *et al.*, 1996). The absence of *HT-B* expression is common to both species, *N. plumbaginifolia* and *L. esculentum*, and may be one of the factors still missing in the transgenic plants, as described here. Our study suggests a molecular basis for self-incompatibility breakdown in the cultivated tomato. Nothing is known about whether stylar gene products other than the S-RNase and HT are required for self-incompatibility and are missing in SC species in the genus *Lycopersicon*. Above all, identification of pollen factor(s) involved in the recognition system of gametophytic self-incompatibility remains an important issue. While the molecular identification of such genes is essential for a full understanding of how SC species evolved from SI ancestors, our knowledge of this issue will be advanced by further detailed analyses of transgenic plants with both *S-RNase* and *HT* genes in cultivated tomato.

SI plants usually reject pollen from related SC species, while the reverse pollination is compatible. Such interspecific cross relationships are known as UI. Although a mechanism distinct from that of SI has been proposed for UI in *Lycopersicon* (Hogenboom, 1972), genetic studies indicate the involvement of the *S* locus in UI. Three separate loci, one of which is at or near the *S* locus, were shown to be involved in interspecific pollen rejection in *Lycopersicon* (Chetelat and DeVerna (1991). Murfett *et al.* (1996) demonstrated the presence of both S-RNase-dependent and -independent mechanisms in the UI among various *Nicotiana* species. The SC genotype (*ScSc*) of *L. peruvianum* that lacks S-RNase activity clearly showed a decreased UI response, which implicates the S-RNases in rejection of *L. esculentum* pollen on the style of *L. peruvianum*. In contrast, transgenic tomato styles expressing S_6-RNase showed no UI response towards *L. esculentum* and *L. peruvianum* pollen. This suggests the involvement of a stylar factor(s) other than the S-RNase in the UI response of the *Lycopersicon*. Whether the HT proteins are also involved in interspecific pollen rejection is currently under investigation.

Experimental procedures

Plant materials

Lycopersicon peruvianum plants homozygous for three *S*-alleles, S_6, S_7 and *Sc*, were described in an earlier study (Kowyama *et al.*, 1994). Seeds of seven tomato cultivars were a gift of the National Institute of Vegetable and Tea Science, Japan. One of these cultivars, Alisa Craig, was used for transformation.

Protein electrophoresis and in-gel activity assay

Styles were collected from flowers at anthesis, immediately frozen in liquid nitrogen, and stored at –80°C until needed. For protein extraction, styles were ground into a fine powder in liquid nitrogen and homogenized in extraction buffer [0.1 M Tris–HCl pH 7.8, 10 mM EDTA, 1.5% (w/v) ascorbic acid and 2% (w/v) polyclar AT (polyvinylpyrrolidone) (Nacalai tesque, Kyoto, Japan)]. The extract was separated by the centrifugation at 12 500***g*** for 10 min. The supernatant was recovered and stored at –20°C. Protein concentration was determined with the Coomassie blue protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. SDS–PAGE and in-gel activity assay were performed according to the method described previously (Kowyama *et al.*, 1994). Proteins in the SDS–PAGE gel were detected by silver staining using the Sil-Best Stain for protein/PAGE (Nacalai tesqu, Japan).

Two-dimensional gel electrophoresis

Proteins were extracted with a lysis buffer containing 8 M urea, 2% ampholine (Amersham Pharmacia Biotech, Piscataway, NJ, USA), pH 3.5–10.0, 1% Polyclar-AT and 5% 2-mercaptoethanol, and the supernatant fraction was recovered by centrifugation. Two-dimensional polyacrylamide gel electrophoresis (non-equilibrium gel electrophoresis in the first dimension and SDS–PAGE in the second) was performed as described (Sassa *et al.*, 1993).

Plant transformation

Plasmid pBICS6 was constructed to contain the tomato chitinase promoter (*Chi*P) provided by Gasser (University of California, Davis, USA), and ligated to the genomic DNA fragment of S_6-RNase gene (Figure 3a). The plasmid was electroporated into *Agrobacterium tumefaciens* LBA4404. Tomato plants cv. Alisa Craig were transformed by a leaf disk method (Horsch *et al.*, 1985) using the cotyledons as explants.

Pollination assay

Flowers were emasculated 1 day before anthesis. Pollinations were performed at anthesis using fresh, mature pollen. Pollinated pistils were harvested 48 h after pollination, fixed with 25% acetic acid, treated with heat at 80°C for 20 min, stained with aniline blue

(Kho and Baer, 1968), and observed under an epifluorescence microscope with a green filter. The incompatible reactions were assessed with pollen-tube growth in the style.

PCR amplification and sequencing of cDNA and genomic DNA encoding the HT *genes*

Three degenerate primers were designed based on the amino acid sequences of HT-proteins from *N. alata* (McClure *et al.*, 1999) and *Solanum chacoense* (D. Matton, personal communication). The sequence of the upstream primer [HT-NS2: 5′-ATA TCA TCA GA(G/A) GTT ATT GC(A/T) AGG GA(A/T) GGG A(A/T)A TG-3′] was derived from the signal peptide sequences, and the sequences of the downstream primers [HT-C1: 5′-TCC TTT ATT CAA CCA AT(C/T) TCA TAT TA-3′; HT-C2B: 5′-CAA AAA TAT TAC ATA ATA TTT TGT AGT CG-3′] from the C-terminus of HT proteins. First-strand cDNA was synthesized from 4 μg total RNA prepared from the styles using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech). The PCR amplification was performed with 0.1 μg genomic DNA or the first-strand cDNA corresponding to 4 μg of total RNA as a template in a 50 μl reaction containing 1.25 μM each of upstream and downstream primers, 250 μM dNTPs, 10 mM Tris–HCl pH 8.0, 50 mM KCl, 2.0 mM $MgCl_2$, and 2.0 units of Ex Taq DNA polymerase (Takara, Shiga, Japan), with cycles of 95°C for 1 min, 50°C for 1 min, then 72°C for 1 min. The PCR products corresponding to HT-A and HT-B obtained after the amplification with primer pairs of HT-NS2 and HT-C1 primers, and HT-NS2 and HT-C2B, respectively, were purified by agarose gel electrophoresis, subcloned into pCRII (Invitrogen, Carlsbad, CA, USA) and sequenced. The sequence analysis was performed by using a CEQ dye terminator cycle-sequencing quick-start kit and CEQ 2000 DNA analysis system (Beckman Coulter, Fullerton, CA, USA).

RNA gel-blot analysis

The RNA was prepared from styles by a hot phenol method (Chirgwin *et al.*, 1979). For Northern analysis, 10 μg total RNA were fractionated on a 1.2% agarose gel containing formaldehyde and blotted to a nylon membrane (Bio-Rad, USA). RNA was fixed to the membrane by baking at 80°C for 2 h, prehybridized in a hybridization buffer (50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, 50 mM NaHPO4, 0.5 mg ml^{-1} salmon sperm DNA) at 42°C for 1 h and hybridized in the same buffer containing ^{32}P-labelled probes at 42°C for 16 h. The hybridized filter was washed in 2 × SSC containing 0.1% SDS at room temperature for 20 min and then in 0.1 × SSC/0.1% SDS at 55°C for 20 min, three times. The DNA probe was labeled using a random primer labeling kit according to the manufacturer's instructions (Takara).

DNA-blot analysis

Total DNA was prepared from leaves as described previously (Rogers and Bendich, 1988). After digestion with restriction enzymes, DNA was separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized as described above for RNA gel-blot analysis.

Acknowledgements

We are grateful to Professor A.E. Clarke, University of Melbourne, Australia for providing the *L. peruvianum S*-genotypes, and to Professor C.S. Gasser, University of California at Davis, USA for providing the *Chi*P promoter. We also thank Dr E. Newbigin, University of Melbourne, Australia for his critical reading and revision of the manuscript. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (B, 11238203) and for Scientific Research (B, 10460004) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, to Y.K.

References

Anderson, M.A., McFadden, G.I., Bernatzky, R., Atkinson, A., Orpin, T., Dedman, H., Tregear, G., Fernley, R. and Clarke, A.E. (1989) Sequence variability of three alleles of the self-incompatibility gene of *Nicotiana alata*. *Plant Cell*, **1**, 483–491.

Bernatzky, R., Glaven, R.H. and Rivers, B.A. (1995) S-related protein can be recombined with self-compatibility in interspecific derivatives of *Lycopersicon*. *Biochem. Genet.* **33**, 215–225.

Chetelat, R.T. and DeVerna, J.W. (1991) Expression of unilateral incompatibility in pollen of *Lycopersicon pennellii* is determined by major loci on chromosomes 1, 6 and 10. *Theor. Appl. Genet.* **82**, 704–712.

Chirgwin, J.M., Przybyla, A.E. and MacDonald, R.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*, **18**, 5294–5299.

Harikrishna, K., Jampates-Beale, R., Milligan, S. and Gasser, C. (1996) An endochitinase gene expressed at high levels in the stylar transmitting tissue of tomatoes. *Plant. Mol. Biol.* **30**, 899–911.

Hogenboom, N.G. (1972) Breaking breeding barriers in *Lycopersicon*. 5. The inheritance of the unilateral incompatibility between *L. peruvianum* (L.) Mill. and *L. esculentum* Mill. and the genetics of its breakdown. *Euphytica*, **21**, 405–414.

Horsch, R., Fry, J., Hoffmann, N., Eichholtz, D., Rogers, S. and Fraley, R. (1985) A simple and general method for transferring genes into plants. *Science*, **227**, 1229–1231.

Kao, T.-H. and McCubbin, A.G. (1996) How flowering plants discriminate between self and non-self pollen to prevent inbreeding. *Proc. Natl Acad. Sci. USA*, **93**, 12059–12065.

Kho, Y.O. and Baer, J. (1968) Observing pollen tubes by means of fluorescence. *Euphytica*, **17**, 299–302.

Kock, M., Loffler, A., Abel, S. and Glund, K. (1995) cDNA structure and regulatory properties of a family of starvation-induced ribonucleases from tomato. *Plant Mol. Biol.* **27**, 477–485.

Kondo, K., Yamamoto, M., Itahashi, R., Sato, T., Egashira, H., Hattori, T. and Kowyama, Y. (2001) Insights into the evolution of self-compatibility in *Lycopersicon* from a study of stylar factors. *Plant J.* (in press).

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

Kusaba, M., Dwyer, K., Hendershot, J., Vrebalov, J., Nasrallah, J.B. and Nasrallah, M.E. (2001) Self-incompatibility in the genus *Arabidopsis*. Characterization of the *S* locus in the outcrossing *A. lyrata* and its autogamous relative *A. thaliana*. *Plant Cell*, **13**, 627–643.

Lee, H.-S., Huang, S. and Kao, T.-H. (1994) S proteins control rejection of incompatible pollen in *Petunia inflata*. *Nature*, **367**, 560–563.

Lewis, D. and Crow, L.K. (1958) Unilateral interspecific incompatibility in flowering plants. *Heredity*, **12**, 233–256.

Matton, D.P., Maes, O., Laubin, G., Xike, Q., Bertrand, C., Morse,

D. and Cappadocia, M. (1997) Hypervariable domains of self-incompatibility RNases mediate allele-specific pollen recognition. *Plant Cell*, **9**, 1757–1766.

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

Miller, J.C. and Tanksley, S.D. (1990) RFLP analysis of phylogenetic relationships and genetic variation in the genus *Lycopersicon*. *Theor. Appl. Genet.* **80**, 437–448.

Murfett, J., Atherton, T.L., Mou, B., Gasser, C.S. and McClure, B.A. (1994) S-RNase expressed in transgenic *Nicotiana* causes *S*-allele-specific pollen rejection. *Nature*, **367**, 563–566.

Murfett, J., Strabale, T.J., Zurek, D.M., Mou, B., Beecher, B. and McClure, B.A. (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.

de Nettancourt, D. (1977) *Incompatibility in Angiosperm: Monographs on Theoretical and Applied Genetics*, **Vol. 3**. Heidelberg: Springer-Verlag.

Newbigin, E., Anderson, M.A. and Clarke, A.E. (1993) Gametophytic self-incompatibility systems. *Plant Cell*, **5**, 1315–1324.

Rick, C.M. (1988) *Evolution of Mating Systems in Cultivated Plants*. London: Chapman & Hall.

Rogers, S.O. and Bendich, A.J. (1988) Extraction of DNA from plant tissues. In (Gelvin, S.B. and Schilperoort, R.A., eds). *Plant Molecular Biology Manual*. Dordrecht: Kluwer.

Royo, J., Kunz, C., Kowyama, Y., Anderson, M.A., Clarke, A.E. and Newbigin, E. (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.

Sassa, H., Hirano, H. and Ikehashi, H. (1993) Identification and characterization of stylar glycoproteins associated with self-incompatibility genes of Japanese pear, *Pyrus serotina* Rehd. *Mol. Gen. Genet.* **241**, 17–25.

Sassa, H., Hirano, H., Nishio, H. and Koba, T. (1997) Style-specific self-compatible mutation caused by deletion of the *S-RNase* gene in Japanese pear (*Pyrus serotina*). *Plant J.* **12**, 223–227.

EMBL database accession numbers: AB066582for *LpHT-A*; AB066583for *LpHT-B*; AB066584for *LeHT-A*; AB066585for *LeHT-B*.