Full Length Research Paper

The days anthesis in relation to the floral shapes, pollen size, and S-RNase gene identifying in self-incompatibility of wild species

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Flowering time of tomato (*Solanum.chilense*) which is a self-incompatibility wild species flowers was determined using a series of landmark days. We established a correlation between the floral shapes and the days before anthesis. The model of tomato flower development schedule was then used to analyze the size of bud, anther, style, and the pollen scanning electron microscope (SEM) in the days before anthesis. Two new S-RNase genes of the *Solanum .chilense* which was the stylar SI genes were cloned. The structure of the two S-RNase was also analyzed. Finally, the different topology of the phylogenetic trees produced using available S-RNase sequences suggests that the S-RNase have different evolutionary histories.

Key words: *Solanum.chilense*, flower development, self-incompatibility (SI), the pollen SEM

INTRODUCTION

Research in plant developmental biology has progressed very rapidly in the past decade due to the application of molecular biology techniques, the isolation and characterization of various developmental mutants, and the cloning of genes specifically involved in different stages of plant development. It has been established that floral organ development is controlled by the differential expression of tissue- and organ-specific genes (Meyerowitz, 1998; Theissen et al., 2000). Such genes may be involved in organ/tissue differentiation by triggering the regulatory cascade of key genes controlling the identity of floral organs, that is, the "ABC" model of floral development (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994; Ng and Yanofsky, 2000). The ontogeny of tomato (*Lycopersicon esculentum* cv. sweet cherry) flowers was subdivided into 20 stages using a series of landmark events (Vladimir Brukhin et al., 2003). However, these models are not suitable to the research of pollination biology. Self-incompatibility (SI) is a genetic mechanism found in many flowering plants that acts to inbreeding (De Nettancourt, 2001). In the best- reduce the negative fitness effects associated with understood examples, SI is controlled by a single genetic S locus with many S alleles: styles reject pollen grains when both express the same S specificity. So, the problem that whether the self pollen is rejected by itself style is the key problem .The better method to answer this problem is the pollination biology. In this paper, we established a correlation between the floral shapes and the days before anthesis. And the model of tomato flower development schedule was then used to analyse the size of bud, anther, style, and the pollen EMS in the days before anthesis, the most important is that we know which day of the days before anthesis is the day of the viable pollen.

Using our model of tomato flower development schedule on tomato flower development, we have been able to identify critical stages at which major pollens is the viable pollen. We also show that the flower development schedule can be used to analyse self-incompatibility of wild species pattern of pollination in tomato flowers. And we cloned two new the S-RNase genes illustrated self-incompatibility (SI).

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Table 1. Tert butyl alcohol (TBA) , ddH$_2$O and ethanol concentration gradations.

Gradations	Name and ratio (%)			
	TBA	ddH$_2$O	Ethanol	TBA:ddH$_2$O:ethanol (final concentration)
1	10	40	50	10:40:50
2	20	30	50	20:30:50
3	35	15	50	35:15:50
4	50	0	50	50:0:50
5	75	0	25	75:0:25
6	100	0	0	100: 0:0
7	100	0	0	100:0: 0

MATERIALS AND METHODS

Materials

Seeds of self-incompatibility (SI) S. chilense (LA0130) and self-compatibility (SC)S. habrochaites (LA0094) were obtained from the Charles Rick Tomato Genetics Resource Center at the University of California Davis http://tgrc.ucdavis.edu/index.aspx and were germinated in peat pellets and seedlings with three to four leaves were transferred into 15 cm plastic pots containing the perlite :peat (1:1). The plants were grown in greenhouse under a thermoperiod of 26/20°C (day/night). Plants were supplied every week with a commercial fertilizer.

Methods

Flower development schedule define

Inflorescence and flower sampling were performed on 2-month-old plants after the production of three inflorescences. Then flower development schedule was defined.

Scanning electron microscopy

Samples were fixed in 69% acetone/29% ddH$_2$O/2% glutaraldehyde (100%) for 24 h at 4°C , washed, dehydrated with 30, 50 and 70% ethanol for 15 to 20 min separately and samples were then dried as following tert butyl alcohol(TBA) , ddH$_2$O, and ethanol concentration grads (Table1). Every grad needs 15 to 20 min and sputtered with gold. Specimens were examined by SEM (NOVA NanoSEM 230) at an accelerating voltage of 5.00 kV, magnification of 1000 x, electron microscope detector of LVD. Images were saved as digital files.

Pollen vitality, pollinations and pistil staining

The pollen vitality was tested by the acetocarmine method. Flower buds were emasculated one day prior to anthesis (Brukhin et al., 2003) and pollinated with self pollen. Pollinated pistils were collected after at 6, 24, 30, 32 and 48 h unless otherwise noted, and fixed, cleared and stained with aniline blue fluorochrome (ABF, 4'4-[carbonyl bis (benzene 4,1-diyl) bis (imino)] bis benzene-sulphonic acid from Biosupplies, Victoria, Australia) in 0.1M K3PO4 as a stain for callose in pollen tube walls (Kearns and Inouye, 1993). Slide-mounted pistils were examined using a BX51 microscope (Leica Microsystems, Wetzlar, Germany) running IPLab Version 4 Software (BD Biosciences, Rockville, MD) coupled with a Hamamatsu C4742-95 camera (Bridgewater, NJ). Images were captured at 100 × magnification using a DAPI emission filter.

Primer design and PCR

PCR amplification of *S-RNase* genes was performed as described by Kondo et al. (2002). Two sets of degenerate outer and inner primers were used to amplify S-RNase sequences: outer C2 primer LP-5A:AA(T/C)TT(T/C)AA(C/G/A)AT(T/C/A)CA(T/C)GG, inner C2 primer LP-5B: AT(T/C/A)CA(T/C)GG(C/G)(G/C)T(T/A/G)TGGCC(A/T/G)GAT, outer C5 primer LP-3A: GGT(T/C)(G/T/A)A(A/G)ACATAT(G/A/T)CC, and inner C5 primer LP-3B: ATAT(G/A/T)CC(T/A)A(T/C)(T/C)TC(A/C)(G/T/A)(T/G/A)(T/A)AGTT CAA. Outer primer (LA-5A, LA-3A; 20 cycles: 95°C, 30 sec; 50°C, 60 sec; 72°C, 60 s) PCR products were diluted (1:20) and amplified using the inner set (that is, LA-5B, LA-3B; 20 cycles: 95°C, 30 s; 50°C, 60 s; 72°C, 60 s).

Cloning and DNA sequencing

PCR products were separated in 1% agarose gels, and cloned into pMD18-T vector system (TaKaRa) for sequencing. S-RNase genes identified in the present study are registered in GenBank database as ZS4 (JN226563), ZS5 (JN226564). All evolutionary analyses and alignments were performed using DNAMAN and ClustalW.

RESULTS AND DISCUSSION

Defining flowering time

S. chilense belongs to the *Solanaceae* family. It is a plant featuring a sympodial growth habit of the main shoot and axillary branches; after producing eight to ten leaves, the vegetative meristem transformed into the inflorescence meristem. The next vegetative apex was formed by the lateral bud in the axil of the leaf closest to the apical meristem. Later, the growth of this meristem pushed the inflorescence meristem to the side. The inflorescence meristem ultimately appeared as a lateral monochasial inflorescence, which is a dichotomously branched scorpioid uniparous cyme of six to ten flowers on each branch (Figure 1). Flowers contained five sepals, alternating with five petals, five anti-sepalous stamens forming a cone around the style (lateral cohesion of anthers by the interweaving of hairs) and two fused

Figure 1. Solanum chilense (LA0130) and Inflorescence.

carpels forming a style. The flower was partly gamosepalous. In mature tomato flowers, stamens were shorter than the style, and the anthers formed a tube around the style.

S. chilense (LA0130), the days before anthesis and the days after anthesis is presented in Figure 2. The developmental process has been subdivided into several days (the days before anthesis and the days after anthesis). The following sections detail the comparative development of flower in relation to the days before anthesis and the days after anthesis (Figure 2).

The size of floral organ at the days before anthesis

The very early stages of flower development (ranging from 200 to 1,000 mm in length) have already been described (Sawhney and Greyson, 1972; Chandra and Sawhney, 1984; Rasmussen and Green, 1993) and the size of floral organs summarized as 20 stages (Vladimir et al., 2003). The data reported here that showed in relation to the days before anthesis and the bud forms, the bud size, the anther size, the style length (Table 2).

Comparative the days before anthesis in relation to the bud size, the anther size, the style length showing that the bud size and the anther size continued to grow not to stop from the 7 days before anthesis to the 0 day before anthesis.

Pollen SEM

The size of pollen increased from the 7 days before anthesis to the 3 days before anthesis, and then decreased until the 0 days before anthesis (Figures 3 and 4). The dehydration of pollen will happen at the 3 days before anthesis. Tomato pollen development is presented in Figure 5. The developmental process was subdivided into these stages, as follows: stage 1 pollen mother cell, stage 2 tetrad, stage 3 free microspores, stage 4 uninucleate microspores, stage 5 immature pollen grain, stage 6 mature pollen grain. The dehydration process of pollen happened from the stage 5 to the stage 6. So the 3 days before anthesis, the dehydration process of pollen happened from the stage 5 to the stage 6.The pollen have became the mature pollen before the 2 days before anthesis in *Solanum chilense (*LA0130).

The pollen vitality of the days after anthesis

Testing techniques of plant pollen viability were summarized as follows: staining method, germination method and pollination in field method, etc. But the staining method was the convenient and efficient method. In this paper, the pollen vitality was tested by the acetocarmine method. The pollen exists the vitality from the 0 days after anthesis to the 6 days after anthesis. Pollination can
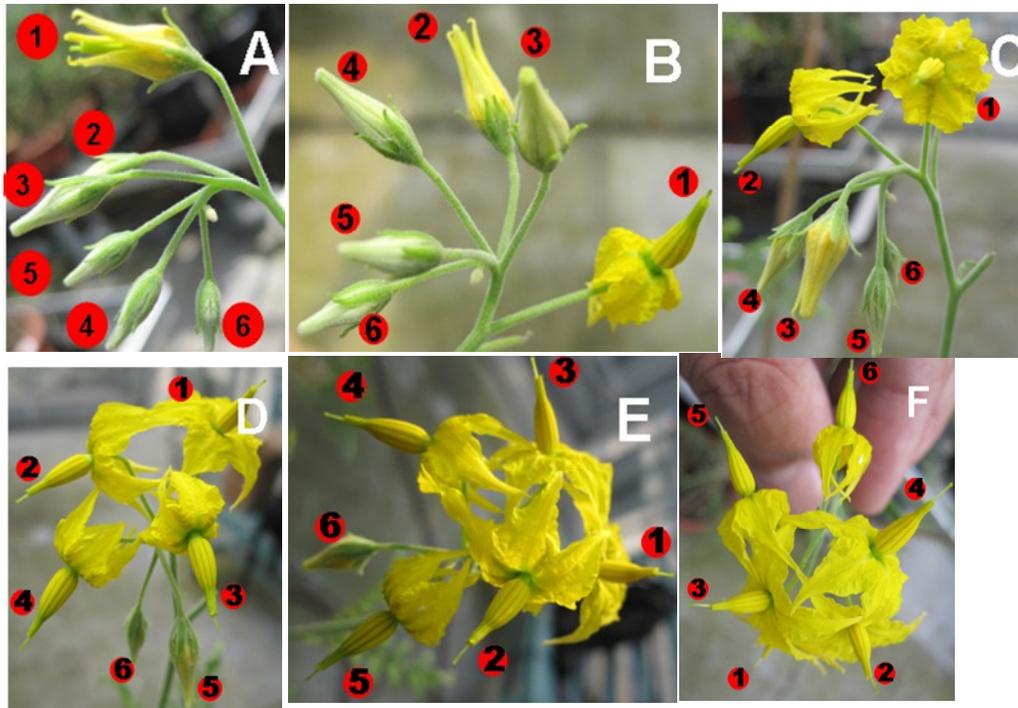
Figure 2. *Solanum chilense* (LA0130) Inflorescence. A—No flower flowering time : 2011-05-30 (am 10:00**;** B— **1** The day of flowering day (0day) time : 2011-05-31 (am 9:00) ; C— **2** The day of flowering day (0day) time : 2011-06-01 (am 9:00) **;** D— **3** **4** The day of flowering day (0day) time : 2011-06-02 (am 9:00) **;** E— **5** The day of flowering day (0day) time : 2011-06-03 (am 9:00) **;** F— **6** The day of flowering day (0day)   time : 2011-06-04 (am 9:00) **;** Drawing a conclusion from the Figure 2 : The floral shapes of the days before anthesis in B: **1** the floral shapes of the 0 day before anthesis , **2** the floral shapes of the 1 day before anthesis, **3** **4** the floral shapes of the 2 days before anthesis , **5** the floral shapes of the 3 days before anthesis , **6** the floral shapes of the 4 days before anthesis ; The days after anthesis in F: **1** The 4 days after anthesis, **2** The 3 days after anthesis, **3** **4** The 2 days after anthesis, **5** The 1 day after anthesis, **6** The 0 day after anthesis.

be done in these days.

Pollination with self pollen

Pollen tube growth in self pollinations was quantified, as shown in Figures 7 and 8. After 48 h, pollen tubes reached the ovary in self pollinations of a SC accession LA0094 and didn't reach the ovary in self pollination of a SI accession LA0130 as predicted. In the SI accession LA0130, the pollen tube almost stopped growing from 6 to 24 h, started growing after 24 h, but don't reached the ovary. In the SC accession LA0094, the pollen tube constantly grew, reached the ovary and fruiting finally.

Identify S-RNase genes of SI accession LA0130

PCR and sequence analysis were used to detect the

identity of S-RNase genes. Degenerate PCR primers were designed using sequences encoding conserved regions C2 and C5 (Ioerger et al., 1991) and used to identify the S-RNase genes. S-RNase genes were identified by the presence of additional conserved regions C3 and C4 and by the presence of an intron characteristic of all solanaceous S-RNase genes (Igic and Kohn, 2001) (Figure 9).

Sequences in the tree and their accession numbers are Lycium cestroides (HM195097.1), Lycium cooperi (HM195096.1), Lycium pallidum (HM195095.1), Petunia hybrida S3(U07363.1), Petunia integrifolia S13 (AF301174.1),Solanum peruvianum SP17(HM357228.1), Solanum carolinense N-SC(L40551.1), Solanum chilense S11(EF680110.1), Solanum chacoense S-arg (DQ00-7316.1), Lycopersicon hirsutum LhgSRN-2(AB072479.1), Solanum chmielewskii LcwSRN-1(AB072477.1), Solanum neorickii LpfSRN-2(AB072476.1), L.peruvianum (X76065.1), Physalis crassifolia S9(L46661.1), Physalis

Table 2. The size of floral organ at the days before anthesis.

	Bud form bud anther style the days before anthesis					
	Length (mm)	width (mm)	Length (mm)	width (mm)	length (mm)	day
A			10.17	2.79	13.00	0
B	12.76	4.89				2
C	11.48	4.05				3
D	10.09	3.41				4
E	8.22	3.04				5
F	6.83	2.48				6
G	6.04	2.45				7
H	5.6	2.28				Buddies
I	4.81	2.25				Buddies

A— , B— , C— , D— , E— , F— , G— , H— , I—
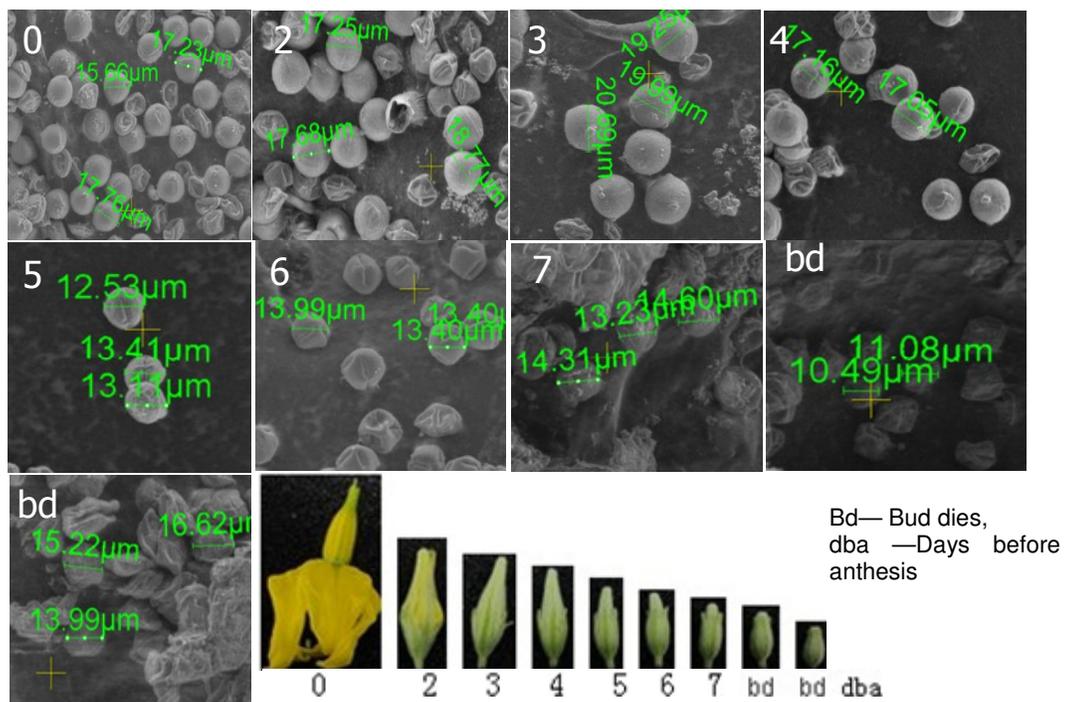
Figure 3. EMS of Pollen in relation to the pollen form of the days before anthesis (dba).

cinerascens PCIN4(AF058933.1), Physalis longifolia S5(AF374420.1), Witheringia solanacea S23(AY454121.1), Witheringia maculate S6(AF102073.1), Nicotiana tabacum relic (AJ011532.1), Nicotiana sylvestris (AJ002296.1), Nicotiana alata (GQ375153.1), Iochroma loxense S2(EU382087.1), Iochroma gesnerioides S2(EU382083.1), Iochroma cyaneum S1(EU382081.1), Iochroma australe S3(EU382080.1), Vassobia breviflora S2(EU382085.1), Vassobia breviflora S1(EU382084.1), Dunalia brachyacantha S1(EU382077.1), Eriolarynx lorentzii S3(EU382076.1), Eriolarynxm lorentzii S2(EU382075.1), Eriolarynx lorentzii S1(EU382074.1), Solanum habrochaites S-hab-1 (GU361144.1).

A Neighbor-joining tree was constructed using the PHYLIP program and is shown in Figure 10. The Neighbor-joining tree grouped the ZS4, ZS5, Solanum chmielewskii LcwSRN-1, Solanum chilense S11 in the same cluster, in agreement with the ZS4 and ZS5 are of the S-RNase family.

In the past study, the ontogeny of tomato (Lycopersicon esculentum cv. sweet cherry) flowers was subdivided into
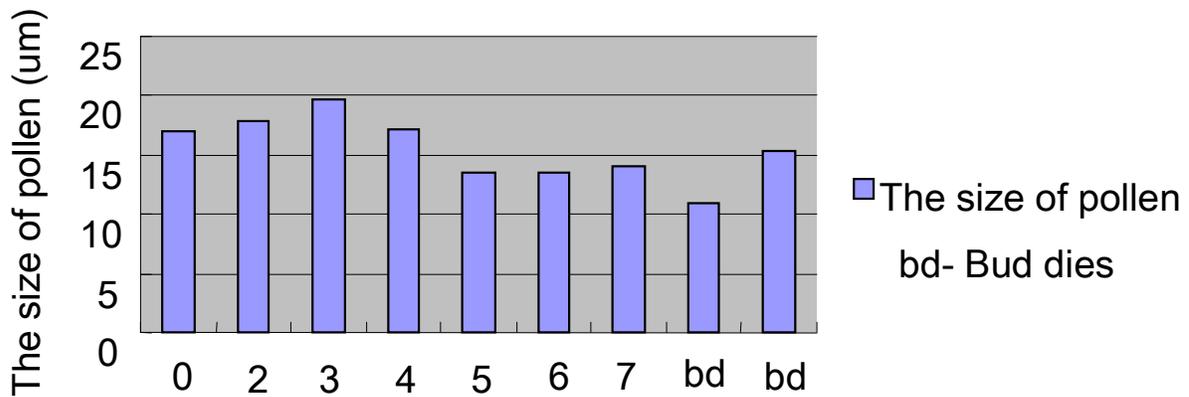
Figure 4. The size of Pollen in relation to the days before anthesis (dya).
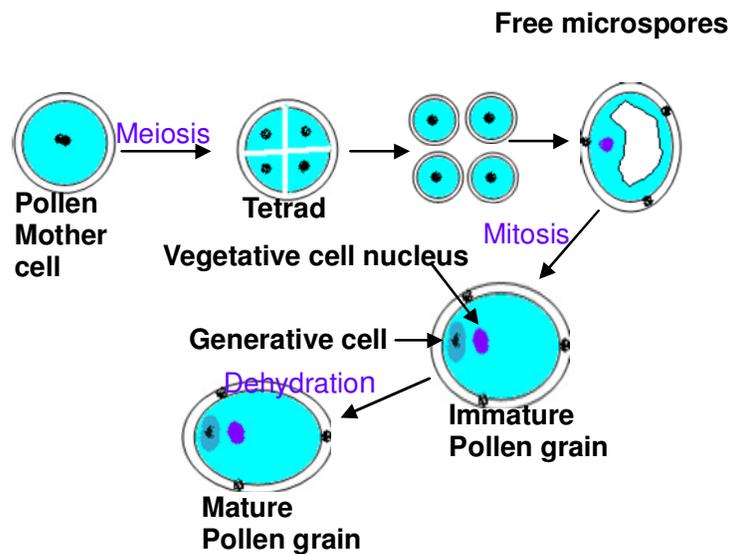
Figure 5. The developmental process of tomato pollen.
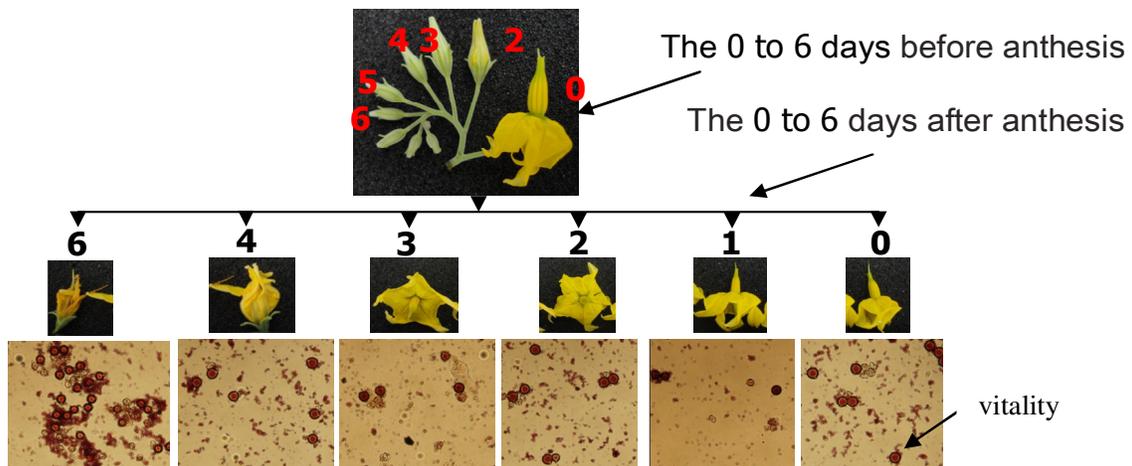
Figure 6. The pollen vitality of the days after anthesis
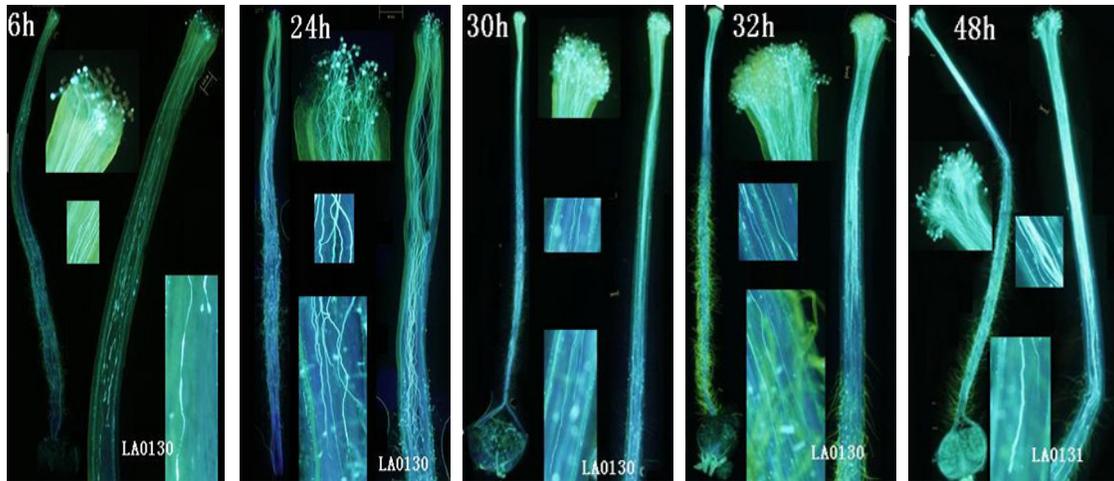
a
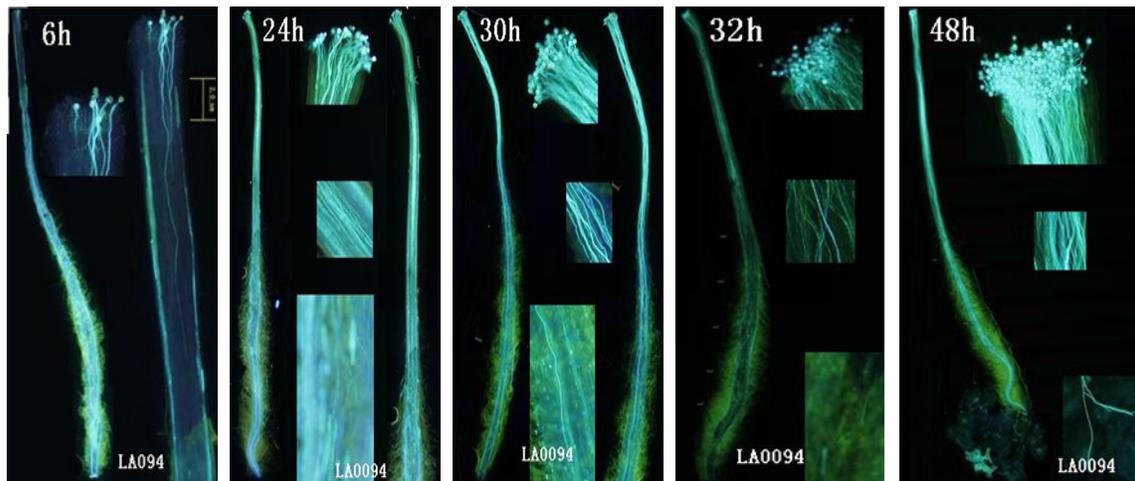
b

Figure 7. SI and SC pollen tube growth in self pollinations.

20 stages using a series of landmark events (Vladimir et al., 2003). The 20 stages were used to study the ontogeny of tomato, which was not suitable for use in the studying of pollination biology. In this study, we detailed the comparative development of flower in relation to the days before anthesis and the days after anthesis and established the model of tomato flower development schedule, the correlation between the floral shapes and the days before anthesis, the days after anthesis. And then the model of tomato flower development schedule was used to analyse the size of bud, anther, style, and the pollen SEM in the days before anthesis. Also, we established the correlation between the pollen vitality and the days after anthesis and used to study the pollination biology of the self-incompatibility of wild species. And we knew which day was the best day for the pollination. In addition, this paper describes the identifying of the S-RNase genes of the *Solanum.chilense* (LA0130) which will be useful to us in future study.

In conclusion, we have constructed a detailed reference model of the correlation between the floral shapes and the days before anthesis and the pollen vitality of the days after anthesis for the later study of the pollination and especially for self-incompatibility of wild species pollination.
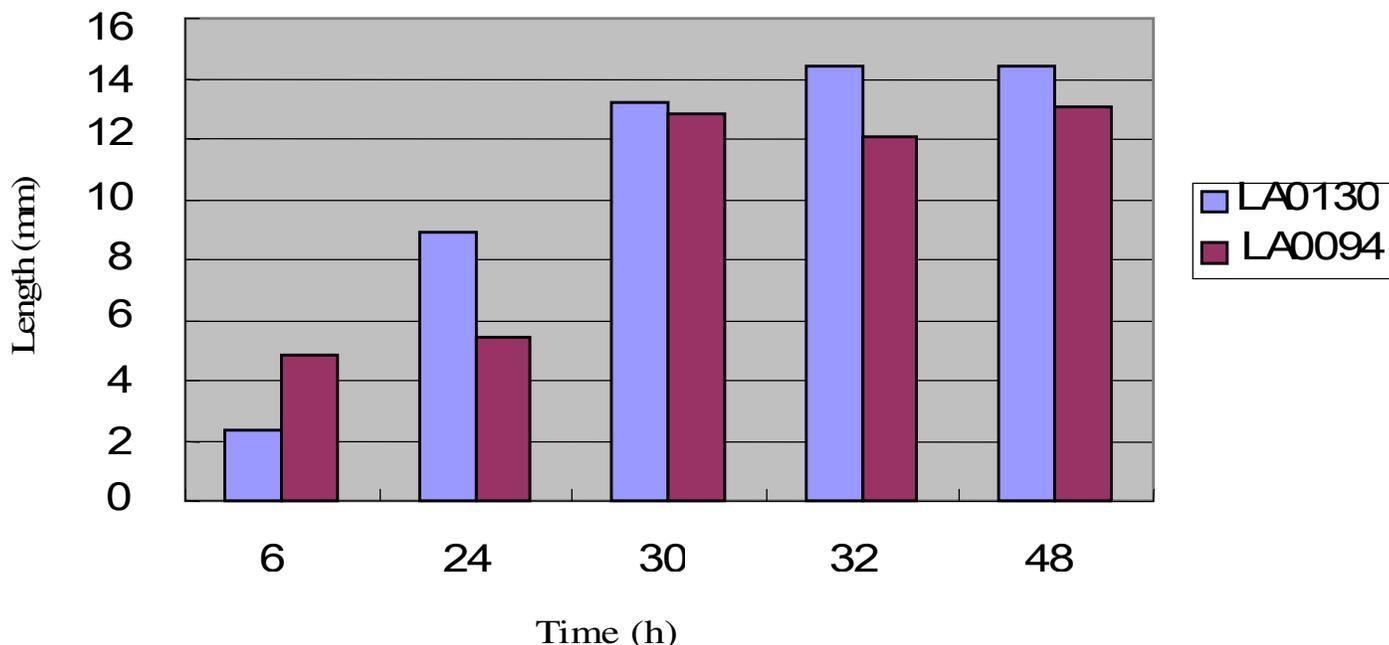
Figure 8. SI and SC pollen tube length in self pollinations.
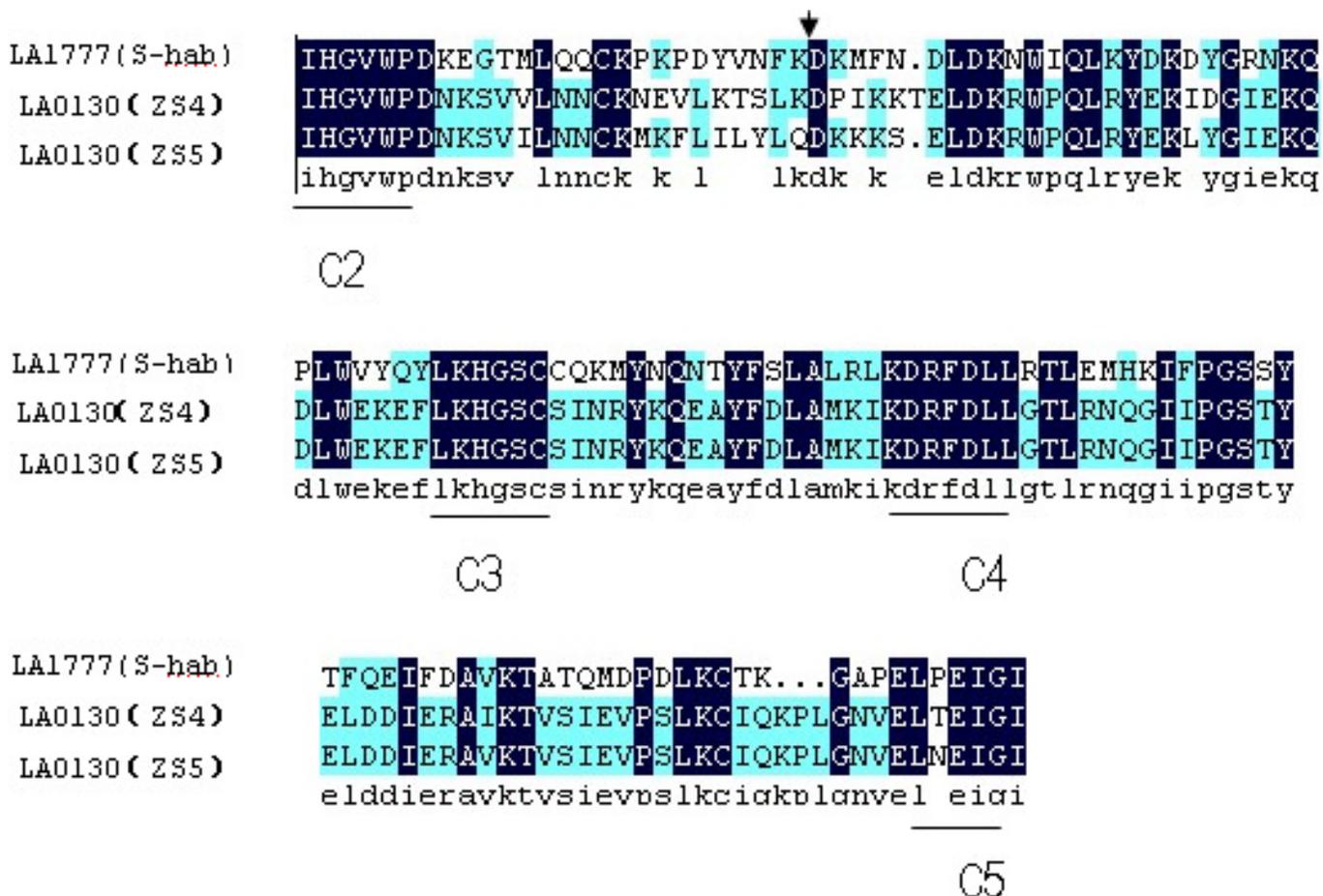
Figure 9. Amino acid alignment of predicted S-RNases from SI LA0130.
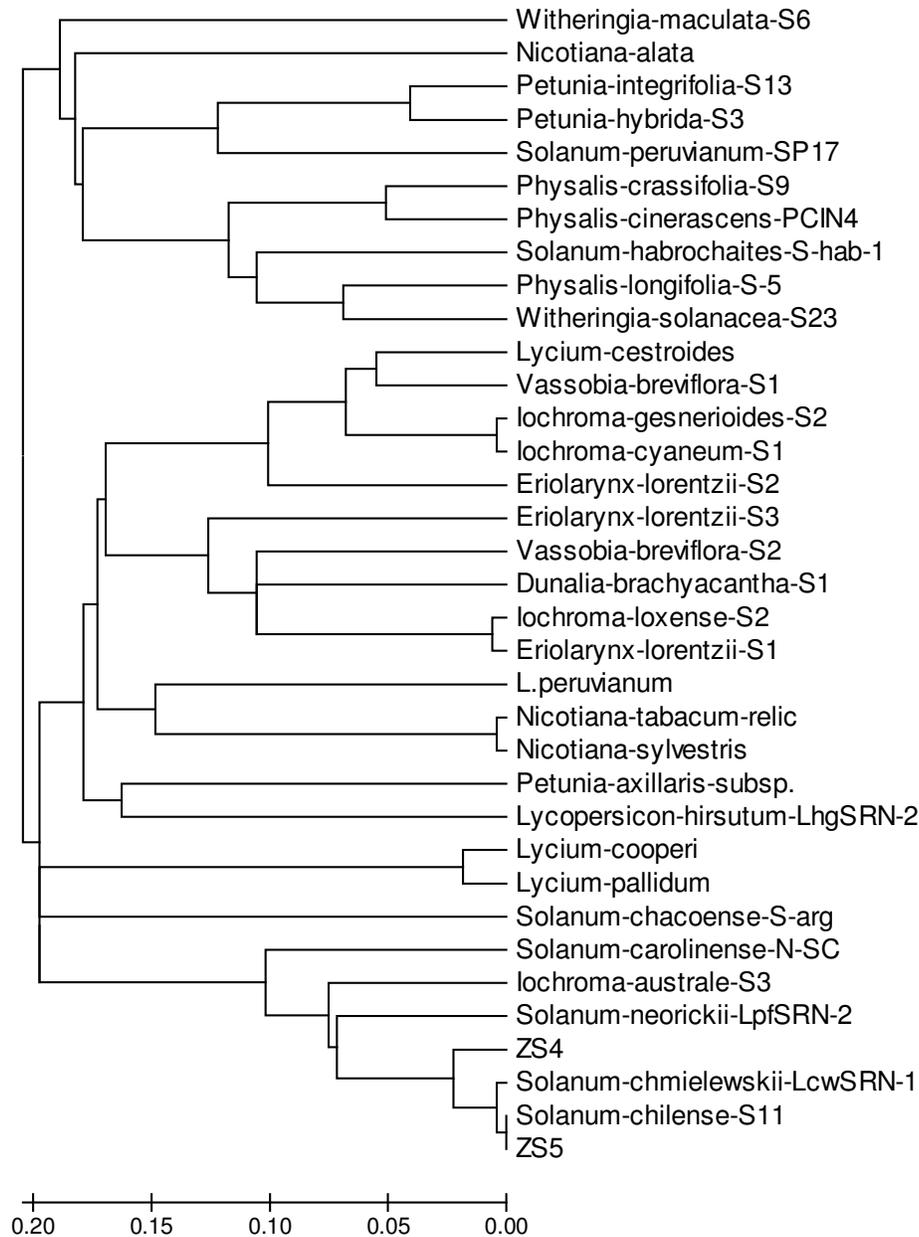
Figure10. Neighbor-joining tree constructed with the S-RNase gene DNA sequences using the PHYLIP program.

Sequences in the tree and their accession numbers are Lycium cestroides (HM195097.1), Lycium cooperi (HM195096.1), Lycium pallidum (HM195095.1), Petunia hybrida S3(U07363.1), Petunia integrifolia S13 (AF301174.1),Solanum peruvianum SP17(HM357228.1), Solanum carolinense N-SC(L40551.1), Solanum chilense S11(EF680110.1), Solanum chacoense S-arg (DQ007316.1), Lycopersicon hirsutum LhgSRN-2(AB072479.1), Solanum chmielewskii LcwSRN-1(AB072477.1), Solanum neorickii LpfSRN-2(AB072476.1), L.peruvianum (X76065.1), Physalis crassifolia S9(L46661.1), Physalis cinerascens PCIN4(AF058933.1), Physalis longifolia S5(AF374420.1), Witheringia solanacea S23(AY454121.1), Witheringia maculate S6(AF102073.1), Nicotiana tabacum relic (AJ011532.1), Nicotiana sylvestris (AJ002296.1), Nicotiana alata (GQ375153.1), Iochroma loxense S2(EU382087.1), Iochroma gesnerioides S2(EU382083.1), Iochroma cyaneum S1(EU382081.1), Iochroma australe S3(EU382080.1), Vassobia breviflora S2(EU382085.1), Vassobia breviflora S1(EU382084.1), Dunalia brachyacantha S1(EU382077.1), Eriolarynx lorentzii S3(EU382076.1), Eriolarynxm lorentzii S2(EU382075.1), Eriolarynx lorentzii S1(EU382074.1),Solanum habrochaites S-hab-1 (GU361144.1).

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