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## Identification and characterization of TcCRP1, a pollen tube attractant from *Torenia concolor*

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- **Background and Aims** During sexual reproduction in higher angiosperms, the pollen tubes are directed to the ovules in the pistil to deliver sperm cells. This pollen tube attraction is highly species specific, and a group of small secreted proteins, TfCRPs, are necessary for this process in *Torenia fournieri*.
- **Methods** A candidate pollen tube attractant protein in *Torenia concolor*, a related species of *T. fournieri*, was isolated and the attractant abilities between them were compared.
- **Key Results** *TcCRP1*, an orthologous gene of *TfCRP1* from *T. concolor*, is expressed predominantly in the synergid cell. The gene product attracted pollen tubes in a concentration-dependent manner, but attracted fewer pollen tubes from the other species.
- **Conclusions** The results indicated that this class of CRP proteins is a common pollen tube attractant in *Torenia* species. The sequence diversity of these proteins is important for species-specific pollen tube attraction.

**Key words:** *Torenia fournieri*, *T. concolor*, sexual reproduction, TcCRP1, fertilization, pollen tube guidance, synergid cell, defensin, cysteine-rich polypeptide, CRP, speciation.

### INTRODUCTION

Higher angiosperms have evolved a unique fertilization system. Unlike gymnosperms, the sperm cells of angiosperms do not have the ability to move long distances. Instead, the pollen tube carries the sperm cells to the female embryo sac for fertilization. When pollen grains are deposited on the female stigma, they hydrate and pollen tubes grow to penetrate into the female tissue. Pollen tubes continue to grow inside the style and, once they reach to the ovary, they change their growth direction toward the ovule. The pollen tube enters into the embryo sac from the micropyle of the ovule, and bursts to release sperm cells. The growth direction of pollen tubes must be tightly and precisely regulated throughout this long journey (Higashiyama, 2010).

After the finding of the pollen tube, attempts have been made to identify the chemoattractants that determine pollen tube growth orientation in the female pistil. In classic studies, calcium ions were first thought to be a candidate for the attraction molecule, because a calcium concentration gradient could be formed to attract the pollen tube through the female tissue (Mascarenhas and Machlis, 1962). However, as external calcium ions are required for pollen tube tip growth, and the growth stimulation and the change in growth orientation of pollen tubes were not distinguished in this system, whether attractant molecules really exist was not determined (Higashiyama and Hamamura, 2008).

Based on the genetic analysis of *Arabidopsis thaliana*, several mutants defective in the tube attraction were identified. In an analysis of plants lacking embryo sacs, pollen tubes were

found to be able to grow inside the ovary but could not direct themselves toward the ovule (Hulskamp *et al.*, 1995; Ray *et al.*, 1997). Pollen tubes could reach to the funiculus of the ovule, but could not find the micropyle and failed to fertilize in *magatama* mutants defective in the development of female gametophytic cells, suggesting the involvement of the embryo sac for guidance (Shimizu and Okada, 2000).

The importance of the embryo sac for pollen tube attraction has also been shown during the *in vitro* fertilization of *Torenia fournieri* (Higashiyama *et al.*, 1998). When synergid cells were ablated by UV laser, pollen tubes were not attracted to the ovule, suggesting that synergid cells secrete attractants (Higashiyama *et al.*, 2001; Higashiyama, 2002). The necessity of synergid cells for attraction was also shown by the identification of an Arabidopsis transcription factor, MYB98, which is predominantly expressed in synergid cells and whose mutation caused a defect in micropylar pollen tube attraction (Kasahara *et al.*, 2005). The synergid cell is also required for pollen tube rupture and sperm cell release into the embryo sac (Huck *et al.*, 2003; Rotman *et al.*, 2003; Escobar-Restrepo *et al.*, 2007).

A key feature of pollen tube attraction is that it is species-specific. When ovules from two related species are placed on a medium close enough to cause interference of signals from each ovule, pollen tubes are attracted to the ovules from the same species. Adding excess calcium ions to the medium does not perturb the attraction, suggesting that the attractant is not a simple universal molecule such as calcium, but rather a more complex molecule that can vary even in closely related species (Higashiyama *et al.*, 2006).

Recently, a family of secreted proteins that have the ability to attract pollen tubes was discovered (Okuda *et al.*, 2009). Using an expressed sequence tag analysis of cDNAs from the isolated synergid cells of *T. fournieri*, it was found that genes encoding cysteine-rich polypeptides (CRPs) are abundantly expressed in the synergid cells. Among them, TfCRP1 (LURE1) and TfCRP3 (LURE2) proteins attracted pollen tubes *in vitro*. Down-regulation of *TfCRP1* and *TfCRP3* caused less attraction of the pollen tubes to the ovule *in vitro*. Based on these results, it was concluded that TfCRP1 and TfCRP3 are pollen tube attractants responsible for micropylar attraction in *Torenia* (Okuda *et al.*, 2009; Goto *et al.*, 2011).

These pollen tube attractants possess six cysteine residues, a cysteine-stabilized  $\alpha$ -helix  $\beta$ -sheet (CS $\alpha\beta$ ) motif and a  $\gamma$ -core motif, which are typical molecular features of antimicrobial defensin proteins (Cornet *et al.*, 1995; Thomma *et al.*, 2002; Yount and Yeaman, 2004; Yount *et al.*, 2007). Another defensin-like protein from maize, ZmES4, is involved in pollen tube rupture, suggesting the importance of this family of protein not only for defence response but also for reproduction (Amien *et al.*, 2010). Rapid sequence divergence is also a feature of defensin proteins (Maxwell *et al.*, 2003; Patil *et al.*, 2004), thus these pollen tube attractants were thought to be good candidates for species-specific pollen tube guidance.

In this study an orthologous gene of *TfCRP1* was isolated from *Torenia concolor* and named *TcCRP1*. *TcCRP1* was expressed exclusively in the synergid cells. Purified *TcCRP1* protein attracted *T. concolor* pollen tubes *in vitro*. It attracted *T. fournieri* pollen tubes less frequently, suggesting that the diversification of CRP1 proteins among species may contribute to species-specific pollen tube attraction.

## MATERIALS AND METHODS

### *Plant materials, crosses and pollen tube observations*

*Torenia fournieri* 'Blue and White' and *T. concolor* were grown in a plant growth room or in a chamber under the ambient conditions. Because both *T. fournieri* and *T. concolor* are self-fertilizing and do not auto-pollinate, emasculation was not needed before the crossing experiment. Fully opened flowers were hand-pollinated with pollen grains from the same species or other species. The pistils were collected 24 h later and fixed in the fixation solution (ethanol:acetic acid = 9:1) overnight. Subsequently they were rehydrated with 90% ethanol and 70% ethanol, respectively, for 20 min. After rehydration, they were kept in 1 N NaOH solution overnight, then stained with 1% aniline blue solution dissolved in 0.1 M K<sub>3</sub>PO<sub>4</sub> (pH 12.4) for >1 h. They were mounted in 50% glycerol and observed under an IX71 fluorescence microscope (Olympus, Tokyo, Japan).

For the pollen tube elongation experiment, hand-pollinated pistils were cut at the junction between the style and ovary and placed on the pollen tube growth medium. Pollen tubes emerged from the cut end of the style approx. 8 h after pollination. The pistils were dissected 24 h after pollination to pick up the ovules and to monitor fertilization *in vivo*. Pistils were mounted on 0.12 M sorbitol and observed under a

BX51 microscopy (Olympus) to determine whether the pollen tube entered into the embryo sac.

### *Isolation of synergid cells and egg cells*

Flowers 2 d after maturation were used as material to isolate synergid cells. Pistils were dissected and carpel walls were removed to take ovaries. The ovaries were sunk in the 300  $\mu$ L of enzyme solution [1% (w/v) cellulase (Worthington), 0.3% macerozyme RS (Yakult), 0.05% pectinase Y-23 (Yakult), 5 mM CaNO<sub>3</sub>, 0.4 M mannitol] for 1 h at 28 °C. Synergid and egg cells separated from ovule tissues were distinguished by their morphology, captured in the pico pipette (Altair, Japan), pooled separately by cell type, and washed with 100  $\mu$ L of wash solution (CaNO<sub>3</sub>, 0.4 M mannitol) three times. Cells were kept frozen until being used.

### *RNA isolation, the cloning of *TcCRP1* and RT-PCR*

The mRNA from isolated synergid cells and egg cells was extracted using RNAqueous-Micro (Ambion, Austin, TX, USA) with Plant RNA Isolation Aid (Ambion). The RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) was used for the remaining tissues. cDNA was synthesized using SuperScript<sup>®</sup> reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The expression of cell-type specific marker genes in these mRNAs (Kawano *et al.*, 2011) was confirmed to clarify that no contamination of other cell types in the pool of synergid cell mRNA and egg cell mRNA. *TcCRP1* was first isolated from ovule cDNA by PCR using gene-specific degenerate primers (Supplementary Data Table S1, available online). Full-length cDNA was isolated by 5'- and 3'-RACE-PCR (GENE RACER Kit; Invitrogen) and the sequence was confirmed. The intron sequence was determined from the genomic sequence. Thirty-five-cycles of RT-PCR were conducted using cDNA from sporophytic tissues as templates, and 50 cycles of RT-PCR were performed using cDNA from synergid cells and egg cells as templates. The full-length *TcCRP1* sequence including the intron was deposited to the GenBank under the Accession No. AB594190.

### *Alignment and phylogenetic analyses of *TfCRPs* and *TcCRP1**

Signal sequences were predicted using SignalP 3.0 software (Bendtsen *et al.*, 2004). Mature CRP proteins were aligned using the ClustalX version 2.0 algorithms (Larkin *et al.*, 2007) and modified manually. A phylogenetic tree was generated using the neighbor-joining method (Saitou and Nei, 1987) in Clustal X.

### *Construction*

His-tagged TfCRP1 was constructed as previously reported (Okuda *et al.*, 2009). *TcCRP1* cDNA corresponding to its mature peptide encoding region was amplified using the following primer set and subcloned into the pBluescriptII SK (+) vector (Toyobo, Osaka, Japan):

forward primer: 5'-CACCGGATCCGGTCAAATTCCACCT  
GAG-3'

reverse primer: 5'-CTCGAGTTATTTCTTTTCTTTTCA  
GAAC-3'

The underlines represent the *Bam*HI recognition sequence in the forward primer and the *Xho*I recognition sequence in the reverse primer, respectively. After subcloning, The *Bam*HI and *Xho*I recognition sequences were cleaved using *Bam*HI and *Xho*I restriction enzymes and then ligated into the pET28a vector in *Escherichia coli* BL21 cells to generate His-tagged TcCRP1.

#### Protein purification and refolding

One and a half litres of BL21 *E. coli* culture cells expressing His-tagged TfCRP1 or TcCRP1 proteins were collected 24 h after the induction of protein expression. Protein purification and refolding was done as described previously (Okuda *et al.*, 2009). Refolding reagents were removed from the protein solution [50 mM Tris-HCl (pH 8.0) buffer] by dialysis.

#### Pollen tube attraction assay

Purified proteins were mixed with Alexa488-conjugated dextran or Alexa546-conjugated dextran and vortexed with 5 % (w/v) gelatin to generate gelatin beads containing these proteins. Pollen tube culture and the procedure of the *in vitro* pollen tube attraction assay were as described previously (Okuda *et al.*, 2009).

#### Estimation of the $K_a/K_s$ rate

The nucleotide sequences encoding the signal peptide and mature polypeptide in TfCRP1 and TcCRP1 were subjected to counting the ratio of nonsynonymous substitutions/ nonsynonymous sites ( $K_a$ ) and synonymous substitutions/

synonymous sites ( $K_s$ ) using DnaSP version 5.0 (Librado and Rozas, 2009).

## RESULTS

### Flower structure and pollen tube elongation in the style of *Torenia* species

Initially pollen tube elongation in the style and attraction to the ovules were compared in *Torenia* species to investigate pollen tube guidance. *Torenia concolor* was used as a partner for the crossing experiment, because it has a similar floral structure to *T. fournieri* (Fig. 1A, B). Similar to *T. fournieri*, the embryo sac of *T. concolor* was protruded from the ovule (Fig. 1C, D), and the synergid cells, the egg cell and a part of the central cell could be easily observed under the microscope.

It has been previously observed that pollen tubes grow through the style, enter the ovary and fertilize the ovule within 24 h after intra- and interspecific pollination in *T. fournieri* and *T. concolor* (Higashiyama *et al.*, 2006). However, the time needed for pollen tubes to grow through the style was not clear. Some secreted molecules, such as chemocyanins, function in chemotropic attraction of pollen tubes elongating in the style of lilies (Kim *et al.*, 2003). Self- and interspecies crossing experiments between *T. fournieri* and *T. concolor* were performed because it was necessary to adjust the culture periods for the *in vitro* pollen tube attraction assay and we wanted to clarify whether pollen tubes could grow through the style of the other species at the same time. After hand pollination, the pistils were cut at the junction of the style and the ovary, and the styles were placed in a pollen tube germination medium to observe pollen tube emergence from the cut end of the style (Fig. 1E–H). When the stigma of *T. fournieri* was pollinated with *T. fournieri* pollen grains, pollen tubes did not emerge from the cut end of the style at the time point of 6 h after pollination, but instead

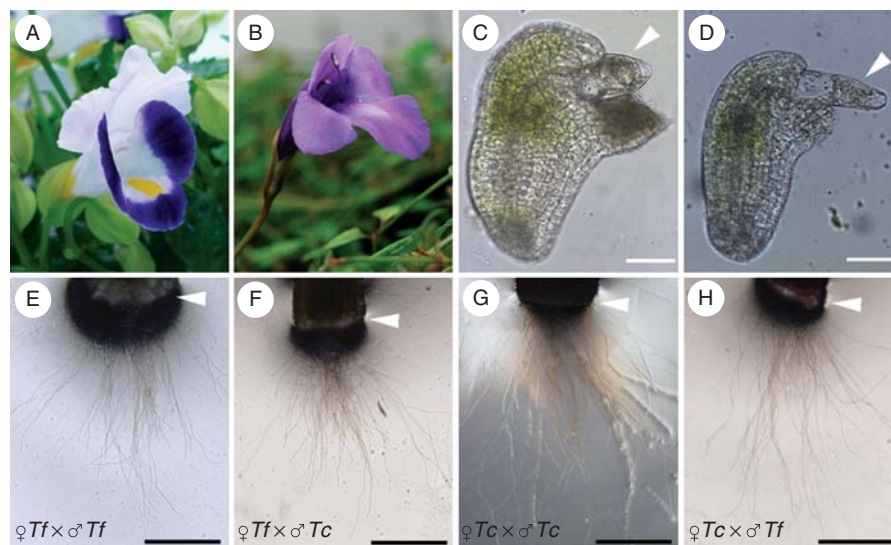


FIG. 1. Flower morphology and pollen tube attraction in *Torenia* species: (A, B) flowers of (A) *T. fournieri* and (B) *T. concolor*; (C, D) protruding embryo sac of (C) *T. fournieri* and (D) *T. concolor*; (E–H) pollen tubes emerging from the cut end of the style (arrowheads). The cross combinations (female  $\times$  male) are indicated: Tf = *Torenia fournieri*; Tc = *T. concolor*. Scale bars: (C, D) = 50  $\mu$ m; (E–H) = 200  $\mu$ m.



emerged from the other end of the style 8 h after pollination, and the tubes continued growing on the medium (Fig. 1E). When the style of *T. concolor* was pollinated with *T. fournieri* pollen grains, the pollen tubes emerged from the cut end of the style 8 h after pollination, the same timing as the self-pollination of *T. fournieri* (Fig. 1F). Similarly, both *T. concolor* and *T. fournieri* pollen tubes emerged from the cut end of *T. concolor* style 8 h after pollination (Fig. 1G, H). These results suggest that the elongation of the pollen tube was not affected by the plant species, and that the speed of growth of the pollen tubes and the timing to enter the ovary were the same in *T. fournieri* and *T. concolor*.

*Interspecies cross between T. fournieri and T. concolor revealed less pollen tube attraction to ovules from different species*

It has previously been observed that reduced penetration of pollen tubes into the embryo sac occurs during cross pollination (Higashiyama *et al.*, 2006). When *T. fournieri* pistils were self-pollinated, all the ovules accepted pollen tubes, while only 4.5 % of the ovules accepted pollen tubes when

*T. concolor* pollen grains were used. When *T. concolor* pistils were self-pollinated, 80.3 % of the ovules accepted pollen tubes, while only 18.9 % of the ovules accepted pollen tubes when *T. fournieri* pollen grains were used (Higashiyama *et al.*, 2006). To determine how these pollen tubes behaved *in vivo* and when defects in tube guidance were occurred, pollen tube elongation was observed *in vivo* in the ovary (Fig. 2). Stigmas of *T. fournieri* and *T. concolor* were pollinated with either self- or cross-pollen grains and observed 24 h after pollination. As the result, pollen tubes grew normally in the style in all the self- and cross-pollination experiments. During self-crossing of *T. fournieri*, the pollen tubes changed their growth orientation toward the ovules upon arriving at the top part of the ovary (Fig. 2A, E). All of the pollen tubes that came close to the ovule were trapped at the entrance of the protruding embryo sac, suggesting that they entered and fertilized the ovule (Fig. 2I). When *T. concolor* pollen grains were crossed with the *T. fournieri* stigma, the pollen tubes grew normally and entered the ovary (Fig. 2B). However, most of the pollen tubes did not change their direction to the ovule (Fig. 2F), and only a few

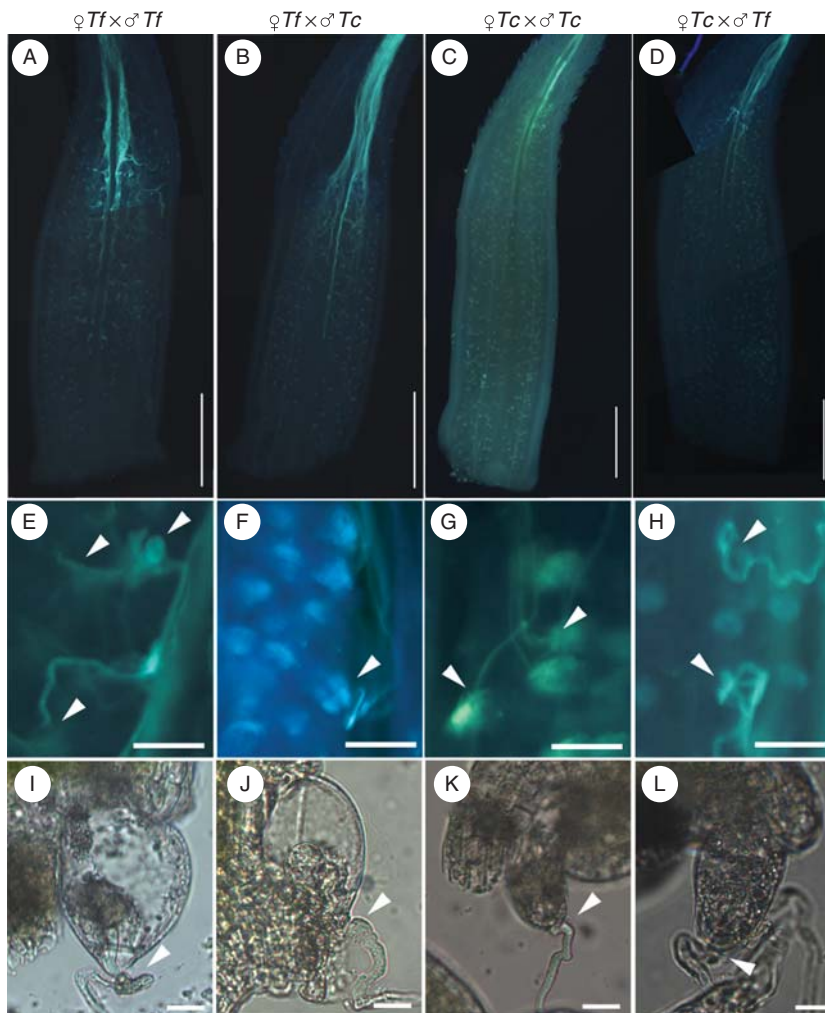


FIG. 2. *In vivo* pollen tube growth and attraction to the ovule: aniline blue staining of (A–D) pollen tubes in the ovary and (E–H) pollen tubes attracted to the ovules (arrowheads). (I–L) DIC images of ovules attracting pollen tubes. The cross combinations (female × male) are indicated above each column of images: *Tf* = *Torenia fournieri*; *Tc* = *T. concolor*. Scale bars: (A–D) = 1 mm; (E–H) = 100 μm; (I–L) = 20 μm.

ovules with pollen tubes at the micropylar end were observed (Fig. 2J), suggesting that they did not precisely attract the pollen tubes. In the cross of *T. fournieri* pollen grains with *T. concolor*, fewer pollen tubes were directed toward the ovule compared with the *T. concolor* self-cross (Fig. 2K, L). These results suggest that short-range pollen tube guidance, from the ovary to the ovule, is important for precise fertilization among *Torenia* species.

#### Isolation of *TcCRP1*, an orthologue of *TfCRP1*, and its synergid-specific expression

*In vivo* crossing experiments suggested that short-range guidance is critical for species-specific pollen tube attraction in *T. fournieri* and *T. concolor*. Previously *TfCRP1* (LURE1) and *TfCRP3* (LURE2) proteins had been isolated as pollen tube guidance molecules secreted from the synergid cells (Okuda *et al.*, 2009). Because *TfCRP1* and *TfCRP3* function as relatively short-range guidance cues within a 200  $\mu$ m diameter, they were considered as good candidates for species-specific pollen tube guidance molecules. To investigate whether pollen tube attraction in *T. concolor* is regulated by these proteins or not, the orthologous genes of *TfCRP1* and *TfCRP3* were isolated from the cDNA of *T. concolor* ovules. Using gene-specific and degenerated primers, genes with similarity to *TfCRP1* were amplified; however, it was not possible to amplify that of *TfCRP3*. RACE-PCR and PCR with genomic DNA revealed total sequence similarity and gene structure between *TfCRP1* and the amplified fragment, therefore it was named *TcCRP1* (Supplementary Data Fig. S1, available online). The predicted *TcCRP1* protein had a signal peptide at its N-terminus, and the mature *TcCRP1* protein had high sequence similarity with *TfCRP1*, rather than *TfCRP3* (Fig. 3A). A phylogenetic analysis of group 1 DEF/L CRPs (Okuda *et al.*, 2009) and *TcCRP1* showed that *TcCRP1* was closest to *TfCRP1* within the group (Fig. 3B). All cysteine residues, necessary for intramolecular disulfide-bond formation and tertiary structure of CRP molecules (Thomma *et al.*, 2002), are conserved among *TfCRP1* and *TcCRP1*, although eight of 62 amino acid residues in the mature protein were different between them (Fig. 3A). RT-PCR analysis revealed that *TcCRP1* was expressed in the ovule-containing tissues and strongly expressed in synergid cells but not egg cells (Fig. 3C). These results suggest that *TcCRP1* has similar molecular features to *TfCRP1* so we thought it was a good pollen tube attractant candidate in *T. concolor*.

#### Concentration-dependent pollen tube attraction activity of *TcCRP1* against the *T. concolor* pollen tube

Next tests were done to find out if *TcCRP1* could attract *T. concolor* pollen tubes. Recombinant His-tagged *TcCRP1* protein was expressed in *E. coli* and purified in an Ni-column. After refolding, the protein was embedded in the gelatin beads and placed in front of pollen tubes grown through the style. Generally, pollen tubes on solid agarose medium grow slightly wavy. In fact, when the beads that contain buffer only were used, most of the pollen tubes did not change their direction of growth, but 11.1% of the

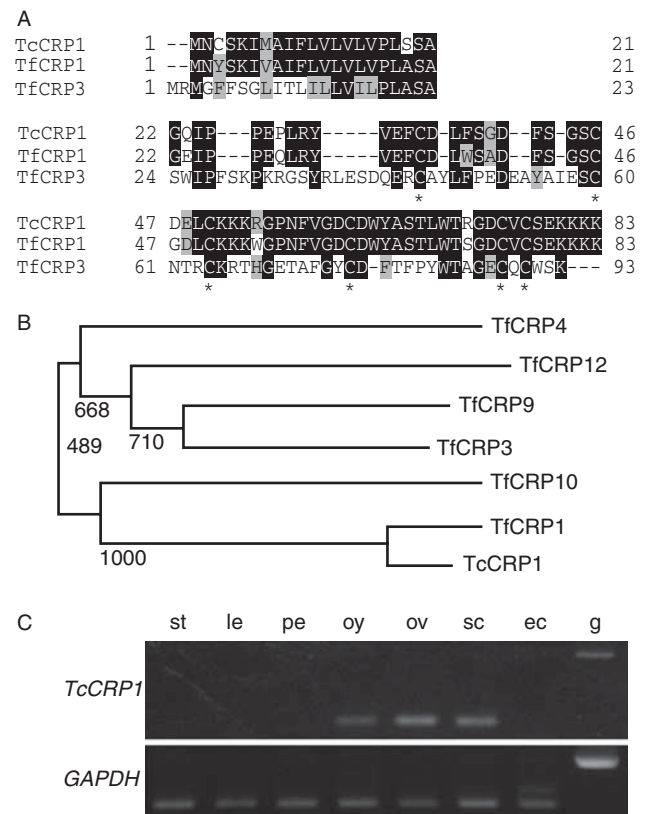


FIG. 3. Amino acid sequence of *TcCRP1* and *TcCRP1* expression. (A) Multiple alignments of *TcCRP1*, *TfCRP1* and *TfCRP3* proteins. Sequences in the top row represent signal sequences, and those in the middle and bottom rows represent mature proteins. Conserved amino acids in all three sequences are shown in black boxes, and in two of the three are shaded. Conserved cysteine residues have asterisks. (B) Phylogenetic analysis of group 1 (LURE-like) CRPs. Bootstrap values are shown on each branch. (C) RT-PCR of *TcCRP1*. *GAPDH* served as a control. Abbreviations: st, style; le, leaf; pe, petal; oy, ovary; ov, ovules; sc, synergid cells; ec, egg cells; g, genomic DNA.

pollen tubes did due to this random waving growth (Fig. 4A, B). When the beads that contained 40 nM of *TcCRP1* protein were used, 55.1% of the pollen tubes turned to the beads (Fig. 3C, D). This pollen tube attraction by *TcCRP1* decreased when a lower protein concentration was tested, suggesting that the attraction was concentration-dependent (Fig. 3E). When 40  $\mu$ M of *TcCRP1* protein was tested, pollen tube attraction decreased to 36.4%, suggesting that a high concentration of attractant molecules affects recognition of the attractant concentration gradient from the signal source. These results suggest that *TcCRP1* functions as a pollen tube attractant in *T. concolor* in a concentration-dependent manner.

#### Species-specific pollen tube attraction by CRPs

Defensin-like CRP proteins often have divergent amino acid sequences between orthologues (Thomma *et al.*, 2002). Because eight amino acid substitutions occur between *TfCRP1* and *TcCRP1* (Fig. 3A), and fewer pollen tubes were attracted to the embryo sac from different species (Fig. 2), these different amino acids may contribute to pollen tube

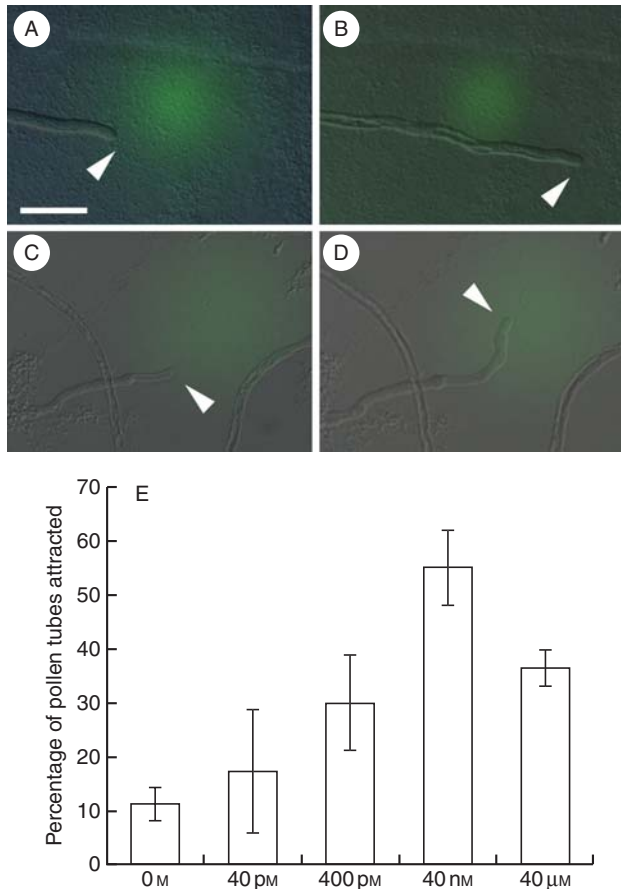


FIG. 4. Pollen tube attraction by TcCRP1: (A, B) *T. concolor* pollen tube just after (A) and 20 min after (B) applying assay buffer only (green); (C, D) *T. concolor* pollen tube just after (C) and 20 min after (D) applying TcCRP1 protein (green). Arrowheads indicate the pollen tube tip. (E) Concentration-dependent pollen tube attraction by TcCRP1. Data are means  $\pm$  s.d. ( $n > 3$  with  $> 10$  pollen tubes per replicate). Scale bar = 50  $\mu$ m.

attraction by the same species. Next tests were done to find out if these orthologous CRP1s could attract pollen tubes from different species (Fig. 5). The attraction activity of TfCRP1 and TcCRP1 were compared against *T. fournieri* and *T. concolor* pollen tubes. When beads containing 40 nM of TfCRP1 protein, which is the most efficient concentration for pollen tube attraction against *T. fournieri* pollen tubes *in vitro* (Okuda *et al.*, 2009) were applied, 56.3% of the pollen tubes were attracted to the protein beads in *T. fournieri* pollen tubes (Fig. 5C). In contrast, when 40 nM of TcCRP1, which is the most effective concentration for pollen tube attraction against *T. concolor*, were added (Fig. 4), only 38.7% of *T. fournieri* pollen tubes were attracted (Fig. 5A–C) and the remaining 61.3% of the pollen tubes continued growing straight. Although it was not significantly different (Fischer's exact test,  $P = 0.12$ ), the attraction of *T. fournieri* pollen tubes by TfCRP1 was 1.45-fold higher than that of TcCRP1. When 40 nM TfCRP1 was applied to *T. concolor* pollen tubes, 33.3% were attracted and the remaining 66.7% ignored the protein-containing beads and kept growing straight. The attraction rate of *T. concolor* pollen tubes by TcCRP1 was 1.65-fold higher than that of TfCRP1, and this

attraction was significantly higher than that of TfCRP1 (Fig. 5D–F) (Fischer's exact test,  $P < 0.05$ ). These results suggest that CRP1s most effectively attracted pollen tubes from the same species.

#### CRP1 may be diverse under the positive selection

In some classes of CRP proteins, typically defensin-like proteins, the amino acid sequences of the orthologous proteins are less conserved between species (Maxwell *et al.*, 2003; Patil *et al.*, 2004). This rapid evolution is thought to contribute to their defensive functions against diverse pathogens. Because pollen tubes from different species were less attracted to the CRP1 protein compared with its own pollen tubes (Fig. 5), we thought that CRP1 might selectively change the amino acid sequence. To demonstrate this point, the rate of nonsynonymous substitutions versus synonymous substitutions ( $K_a/K_s$ ) was calculated in the CRP proteins. If  $K_a/K_s$  is smaller than 1, most of the nucleotide substitutions are silent and do not affect amino acid substitution. If  $K_a/K_s$  is larger than 1, the protein might change the sequence selectively (Fiebig *et al.*, 2004).

Because the signal peptide was cleaved out and the mature polypeptide functions in pollen tube attraction, the  $K_a/K_s$  ratios were calculated separately for the signal peptide and the mature protein region. The  $K_a/K_s$  ratio in the signal peptide region was smaller than 1 (0.801) and that of the mature peptide region was larger than 1 (1.153), suggesting that the mature CRP1 protein may change to alter the amino acid sequence selectively.

## DISCUSSION

### *Micropylar pollen tube guidance may be a key step for species recognition in Torenia*

Multiple steps are believed to maintain successful fertilization within the species and prevent undesired crosses with different species (Johnson and Preuss, 2002; Shimizu, 2002; Swanson *et al.*, 2004; Chapman and Goring, 2010). The first reproductive barrier to prevent undesired crosses is pollen–stigma recognition. When distantly related plant species are cross-pollinated, pollen grains placed on the stigma do not adhere to the stigma surface (Zinkl *et al.*, 1999). In some combinations, although pollen grains attach to the stigma, they fail to hydrate and pollen tubes do not germinate. Several prevention steps have been observed when crossing more related species, including the arrest of pollen tube growth in the style, a lack of funiculus guidance, and a lack of micropylar guidance (Shimizu, 2002; Kikuchi *et al.*, 2007).

When *T. fournieri* and *T. concolor* were crossed, pollen tubes grew normally through the style (Figs 1E–H and 2A–D), indicating that the mechanism to support pollen tube growth through the style is maintained between these species. In contrast, fewer ovules guided the pollen tubes from crossing partner species at least to the filiform apparatus of the protruding embryo sac (Fig. 2), suggesting this rather short-range guidance is a key step for species recognition in *Torenia*.



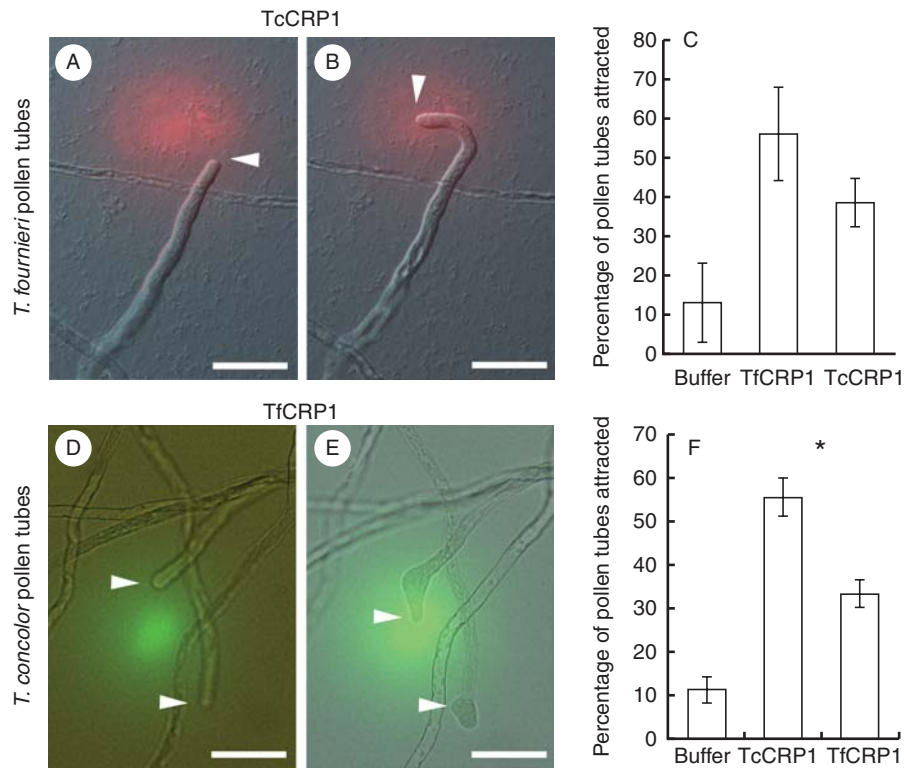


FIG. 5. Interspecies pollen tube attraction by CRP1: (A, B) *T. fournieri* pollen tube (A) just after and (B) 20 min after applying the TcCRP1 protein (red); (C) comparison of the *T. fournieri* pollen tube attraction rate using buffer, 40 nM of TfCRP1 and 40 nM of TcCRP1; (D, E) *T. concolor* pollen tube (D) just after and (E) 20 min after applying the TfCRP1 protein (green); (F) comparison of the *T. concolor* pollen tube attraction rate using buffer, 40 nM of TcCRP1 and 40 nM of TfCRP1. (C, F) Data are means  $\pm$  s.d. ( $n > 3$  with  $> 10$  pollen tubes per replicate). The asterisk in (F) indicates a significant difference between TcCRP1 and TfCRP1 (Fisher's exact test,  $P < 0.05$ ). Scale bars = 50  $\mu$ m.

#### Identification of *TcCRP1* as a pollen tube attractant in *T. concolor*

A CRP1-like gene, *TcCRP1*, was found in the *T. concolor* ovule cDNA based on nucleotide sequence similarity (Fig. 3). The entire nucleotide sequence similarity, including the intron, synergid cell-abundant expression, and protein sequence similarity suggested that TcCRP1 is related to TfCRP1. Indeed, the recombinant TcCRP1 protein attracted *T. concolor* pollen tubes *in vitro*, suggesting that TcCRP1 is a TfCRP1 orthologue and a pollen tube attractant in *T. concolor* (Fig. 4). TcCRP1 attracted pollen tubes in a concentration-dependent manner, and it attracted pollen tubes most effectively at the concentration of 40 nM, which is similar to the attraction by TfCRP1 (Okuda *et al.*, 2009). These results suggest conservation of CRP1-like molecules as a micropylar pollen tube attractant within *Torenia*.

#### Functional similarities and differences in CRP1 and CRP3

Although TcCRP1 showed strong attractant activity to *T. concolor* pollen tubes, the attraction activity against *T. fournieri* pollen tubes was weak (Fig. 5). When 40 nM TcCRP1 was used in the assay, the attraction rate was 55.1% and 30.3% for *T. concolor* pollen tubes and *T. fournieri* pollen tubes, respectively (Fig. 5). This same tendency was seen when TfCRP1 was applied to *T. concolor*

pollen tubes. Because eight of 62 amino acid residues were different between mature TfCRP1 and TcCRP1, key residues may influence the attractant activities in these amino acids.

A mutational analysis of radish defensin Rs-AFP2 has been performed to identify the antifungal activity site (De Samblanx *et al.*, 1997). Two adjacent sites affecting its activity were found: one was located between the second and third  $\beta$ -strand, and the other was in the loop region connecting the first  $\beta$ -strand and the  $\alpha$ -helix. The loop region sequence of some  $\beta$ -defensins is positively selected (Gao and Zhu, 2010). Most of the amino acid substitutions between TfCRP1 and TcCRP1 are located in similar positions, suggesting the importance of these regions for defensin-like protein function.

In contrast, although entire sequence similarity between TfCRP1 and TfCRP3 is low (35% identical), both proteins show almost the same activities (about 55% attraction) for *T. fournieri* pollen tubes (Okuda *et al.*, 2009). Two possibilities have been proposed for the function of CRP-related pollen tube attraction. First, although their sequences are diverse, both TfCRP1 and TfCRP3 possess a CS $\alpha\beta$  motif and a  $\gamma$ -core motif, typical molecular features of antimicrobial defensin proteins (Cornet *et al.*, 1995; Thomma *et al.*, 2002; Yount and Yeaman, 2004; Yount *et al.*, 2007). These factors may result in a similarity of their tertiary structures and in the interaction with the pollen tubes. Second, because TfCRP1 and TfCRP3 act solely in pollen tube attraction (Okuda

*et al.*, 2009), they may be received by different machinery, possibly by different receptors on the pollen tube surface. Future investigations on the key amino acid residues that yield species-specificity, as well as the identification of their tertiary structures and receptors, will shed light on how these small secreted polypeptides function in pollen tube attraction.

#### Rapid evolution and diversification of reproductive proteins

Many genes that mediate in sexual reproduction, such as the sperm–egg interaction in sea urchin and the pollen–stigma interaction in *Arabidopsis thaliana*, have diverged rapidly, which is often a result of adaptive evolution (Swanson and Vacquier, 2002). Although when these *Torenia* species diverged from a common ancestor is unclear, the sequence diversities of the CRP genes expressed in the synergid cells suggests their importance in pollen tube attraction and they may be a driving force in speciation.

Although CRP genes evolve rapidly, entire regions in a gene do not change in the same manner. The nucleotide sequence encoding a signal peptide is more conserved than the sequence encoding a mature peptide, and the sequences encoding mature peptides are often under positive selection (Morrison *et al.*, 2003; Semple *et al.*, 2006). A comparison of the Ka/Ks value between nucleotide sequences encoding signal sequences and mature proteins in TfCRP1 and TcCRP1 revealed that the protein-encoding region was less conserved between them. This result supports the assumption that the LURE-class of pollen tube attractants (CRP1 and CRP3) has diversity in their sequences.

#### Diversification of micropylar pollen tube attractants in a wide range of plant species

Several mutants defective in micropylar pollen tube guidance have been reported in *A. thaliana*. A mutation in the *MAGATAMA3* gene, which encodes a yeast Sen1-like RNA helicase, causes pollen tube misguidance near the micropyle (Shimizu *et al.*, 2008). *CENTRAL CELL GUIDANCE1*, a transcription regulator expressed in the central cell, and *MYB98*, a transcription factor expressed in synergid cells, also affect micropylar guidance (Kasahara *et al.*, 2005; Chen *et al.*, 2007). These genes are thought to function as the upstream regulator for producing attractant molecules or regulating guidance signalling, so their precise contribution to guidance is still unclear (Higashiyama, 2010). GABA, a candidate guidance molecule that is produced in the sporophytic cells of the pistil, can create a concentration gradient through the pistil (Palanivelu *et al.*, 2003). However, as adding excess external GABA does not affect pollen tube guidance by the ovule, it may be a general stimulation factor for tube attraction functioning together with other attractants (Higashiyama *et al.*, 2006).

The maize gene *ZmEA1* is expressed predominantly in the egg apparatus (egg and synergid cells) (Marton *et al.*, 2005). Knockdown of *ZmEA1* causes defects in pollen tube penetration into the intercellular space of micropylar nucellus cells. Recently, an N-terminal cleaved, predicted mature *ZmEA1* protein of 49 amino acids was shown to attract maize pollen tubes *in vitro* (Dresselhaus and Marton, 2009).

As *ZmEA1* possesses only two cysteine residues, its secondary structure is not related to CRP proteins such as TfCRP1 and TfCRP3. Whether these different classes of small secreted proteins can function as attractants in both species, or whether they are species-specific attractants is unknown. Because *ZmEA1*-like genes are limited to the Poaceae species (Marton *et al.*, 2005), it may be a monocotyledon-specific attractant. Further discovery of pollen tube attractants, particularly in dicotyledons including *A. thaliana*, will reveal the evolution, conservation and diversification of pollen tube attractants in plants.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxford-journals.org](http://www.aob.oxford-journals.org) and consist of the following. Table S1: list of primers used in this study. Fig. S1: sequence comparison of the whole genomic region of *TcCRP1* and *TfCRP1*.

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