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Molecular Characterization of a Reproductive Organ-Specific cDNA Clone, ISP11 from *Ipomoea trifida*

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Summary

A cDNA clone, ISP11, that exhibits a specific expression in the stigma and pollen has been isolated from a cDNA library constructed for poly(A)⁺ RNA from mature stigma of *Ipomoea trifida*. The cDNA with a length of 1037 nucleotides excluding poly(A) tail codes for a predicted polypeptide containing 201 amino acid residues and with a molecular mass of 22.8 kilodaltons. The hydropathy profile suggests that the polypeptide is a water-soluble protein without a signal sequence on the amino terminus. A computer search of sequence database failed to reveal any significant homologies with known molecules. RNA gel blot analysis showed that gene expression of ISP11 was developmentally regulated along with the progress in flower bud growth. Southern hybridizations of genomic DNA to ISP11 probe indicated that the *ISP11* gene is present as a single copy in the haploid genome and is not linked to the self-incompatibility locus. These facts suggest that the ISP11 protein plays a general role in pollen germination and/or the resulting process of fertilization, rather than in self-recognition associated with the self-incompatibility of *I. trifida*.

Key Words : *Ipomoea trifida*, cDNA clone, stigma, pollen, organ-specific expression.

Introduction

Some species of the genus *Ipomoea* (Convolvulaceae) including the sweet potato exhibit a sporophytic type of self-incompatibility (SI), by which pollen germination is completely inhibited on the stigma surface after self-pollination. Genetic analysis of the diploid *I. trifida*, a wild relative closely related to sweet potato, showed that the SI is controlled by a single locus (S-locus) with multiple alleles (Kowyama *et al.*, 1980), and that there is a linear dominance hierarchy among the 28 S-alleles identified from some natural populations in Central America (Kowyama *et al.*, 1994a).

As the SI reaction of *Ipomoea* occurs between pollen and papilla cells on the stigma surface soon after self-pollination, *i.e.* after about 5-10 min, it is suggested that S-locus gene products had already been accumulated in both pollen and papilla cells at the time of anthesis. In order to analyse the molecular mechanism underlying self-recognition associated with SI reaction and resulting failure of pollen germination, it is essential to identify the gene products that show preferential expression in the pollen and/or stigma. In this report, we present data on one of the cDNA clones obtained from differential screening of the stigma cDNA library of *Ipomoea*. The cDNA (ISP11) codes for a predicted polypeptide containing 201 amino acid residues, and the *ISP11* gene is specifically expressed in the stigma and pollen, and developmentally regulated along with the progress in flower bud growth.

Materials and Methods

Three homozygotes for S-alleles, S³S³, S⁴S⁴ and S²²S²² were derived from cross-pollinated progenies in our S-genotype stocks. F₁ population segregating for S-genotypes was obtained from a cross between two heterozygotes, S³S⁴ and S³S²² which were derived from crosses between the homozygotes. Total RNA and poly(A)⁺ RNA of the homozygotes were isolated from mature stigmas and pollen one day before anthesis, and also from leaves and petioles, as described previously (Kowyama *et al.* 1995a). A cDNA library was constructed from 2 µg poly(A)⁺ RNA of S⁴-homozygous stigma using the phage vector λgt11 with a library size of approximately 2.5 × 10⁵ phages. Differential screening of organ-specific cDNA clones in the stigma cDNA library was performed using plaque-lifted replica filters: the filters were hybridized to cDNA probes that were prepared from the poly(A)⁺ RNAs isolated from stigma, leaves and petioles of S⁴-homozygote and from stigma of S³-homozygote. Twenty phage clones screened from the library were further classified into several groups by dot-blot cross hybridization. PCR-amplified insert DNAs from randomly selected clones were used as probes for the dot-blot hybridization. A representative clone (ISP11) from one of the groups was sub-

The nucleotide sequence data of ISP11 have been deposited in the GenBank database under the accession number U29432.

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cloned into a plasmid vector, Bluescript, and sequenced by the dideoxy-termination method (Sambrook *et al.* 1989). For RNA gel blot analysis, total RNA was extracted from petals, stems and roots in addition to the RNA described above, and also from stigmas and anthers at different stages of flower bud development (Kowyama *et al.* 1995a). Twenty micrograms of total RNA was subjected to electrophoresis in 1.5 % agarose formaldehyde gel and transferred to a membrane filter. The filters were hybridized to the ISP11 cDNA labeled with α -[32 P] dCTP using random oligonucleotide priming, washed twice for 20 min at 60 °C in buffer containing 0.1x SSC and 0.1x SDS, and exposed to X-ray film with an intensifying screen at -80 °C. For DNA gel blot analysis, genomic DNA was extracted from the leaves of the parent plants and F₁ individuals as described previously (Kowyama *et al.* 1995b). Ten micrograms of the DNA was digested with appropriate restriction endonuclease, electrophoresed on 0.6 % agarose gel and transferred to a membrane filter. Hybridization and washings were performed in the same way as for the RNA gel blots.

Results and Discussion

By differential screening of about 10⁴ phages from the S⁴-homozygous stigma cDNA library, we obtained 20 clones that hybridized intensely to the homologous probe (S⁴-stigma cDNA) and weakly to the S³-stigma cDNA probe, but did not hybridize to leaf and petiole cDNA probes. Dot-blot cross hybridization of the 20 clones revealed two major classes, group A and B which contain 9 and 6 homologous clones, respectively, and 5 minor classes independent of each other. A single phage clone from group B, which had the longest insert of cDNA and designated as ISP11, was further characterized in the present study.

RNA gel blot analysis using the insert cDNA of ISP11 as a probe showed that transcripts of about 1.1

kb were preferentially expressed in the mature stigma and pollen, whereas no detectable signals were observed in petal and vegetative organs such as leaf, petiole, stem and root (Fig. 1). The amount of transcripts in the stigma was significantly larger, about ten times the amount in the pollen. Northern blot of RNAs extracted from flower buds at different stages of growth also indicated that the expression of the ISP11 coding gene was developmentally regulated, with a maximum accumulation in mature stigma and anther, as shown in Fig. 2 where a 2-fold amount of total RNA (40 μ g) was loaded in each lane of anther compared with the stigma. These experimental results indicate that expression of the *ISP11* gene is both spatially and temporally regulated in the plant.

The insert size of ISP11 cDNA was 1037 bp excluding the poly(A) tail (Fig. 3A), in accordance with the transcript size of about 1.1 kb estimated from the RNA gel blot. An open reading frame encoded a 201-amino acid polypeptide with a molecular mass of 22.8 kilodaltons. A polyadenylation consensus motif AATAAA was identified at the distance of 91 nucleotides upstream from the poly(A) site (underlined in Fig. 3A). Although in most of the plant mRNA this motif was detected at about 30 bases upstream from the polyadenylation site (Joshi, 1987), longer distances of 50-100 bases have also been reported for some plant genes (Lycett *et al.* 1984, Sachs *et al.* 1986). Hydropathy plot of the deduced amino acid sequence (Fig. 3B) revealed the presence of two major hydrophilic regions around residues 86 and 115, and the absence of a hydrophobic region at the amino terminus, suggesting that the *ISP11* gene

Fig. 1. Northern hybridization to demonstrate the reproductive organ-specific expression of *ISP11* gene. Twenty micrograms of total RNA isolated from different organs of the S⁴-homozygote were hybridized to ³²P-labeled cDNA of ISP11.

Fig. 2. Northern hybridization to demonstrate the developmentally regulated expression of *ISP11* gene. Total RNA of stigma and anther was separately isolated from the S⁴-homozygous flower buds with different sizes; 5-7 mm (stage 1), 7-9 mm (stage 2), 9-11 mm (stage 3), 11-15 mm (stage 4) and 15-20 mm (stage 5) in length. Stage 1 corresponds to the meiosis-tetrad stage in pollen development, and stage 5 occurs one day before anthesis (Kowyama *et al.* 1995a). Twenty and 40 μ g of total RNA from stigma and anther, respectively, were hybridized to ³²P-labeled cDNA of ISP11.

(A)

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1   GGGGTTTGGGCCTTAAAAAGAATGTTCCCATGGCAACTGCTTTATACAAACTAGTTGCA
                                     M A T A L Y K L V A      10
60  GAGCGTGGACCATGGAGTTCTTTGTCTAGATGGGCATTAGAGTCGTATTGAAGGGAGAT
    E R G P W S S L S R W A L E S Y L K G D      30
120 GTTGGGAAAGCTTTTCTCCTGTACTCTAGGATGGCAGAGTTGGGATATGAAGTCGCACAA
    V G K A F L L Y S R M A E L G Y E V A Q      50
180 AGTAATGCAGCATGGATTCTTGACAAATATGGACCGAGGAGTATGTGCATGGGAGGATCT
    S N A A W I L D K Y G P R S M C M G G S      70
240 GGAATTTGCACAGATGCAGAGAGACATCAGCGCTCCCATGCATTGTGGTGGCCAAGTTCT
    G I C T D A E R H Q R S H A L W W P S S      90
300 GAGCAGGGTAACGAACATGCTGCCCTGCTCATTGGAGATGCATACTACTATGGTCGGGGT
    E Q G N E H A A L L I G D A Y Y Y G R G      110
360 ACTGAACGAGATTATAAGCCTCTGCGGCACGATTATCACTACTCTCTTTGTTATAAGAT G
    T E R D Y K P L R H D Y H Y S L C Y K M      130
420 GGCCTAAAAGCAAGGTACTTGGGCAGGTAGAGATCAAGTTTCAATGGAGATGTTTTC
    G L K G K V L G Q V E I K F S M E M F S      150
480 ATGAAATCCTTAGAGAAAGACCACATCATGTGCCCTCCATGTGCTCAACTATTCTCGGAG
    M K S L E K D H I M C P P C A Q L F S E      170
540 TTGAAGGCCAATGGGGAACATATGGGCTCTGTCATCATCTGGAAAATTAGCCATGATGGGA
    L K A N G E L W A L S S S G K L A M M G      190
600 AGACCAAACCGTGGAGGATGTTATTTAGGCCATAGATGATGAGAAGAAATTAGTGAAAT
    R P K P W R M L F R P                        201
660 TCAGCGTGATTGGAGGTAATCTCAAGGAATCCTATAAGAGCTTCATCATAACATGTTCATG
720 TTGATAGTAATGGTGATGACAACCTTTGTGACTTGGGCTCTCGAGTATGAGAAAAGTAAAG
780 AGGAGATTACTGAGCCACTCACCTATCTGGAGCTTCTTCTTAATATGACCAAGGATATGG
840 ATGATCACCATGCCAACTGAAGCCATGAATATGAAGTCATAATAATTTAATAATTATAA
900 TAATGATAATAATGATGATGATGATGAGGGAAGAGTGTGTTGTGTAATAATAAACTGCCC
960 ATCTGTTTAGTTTACGCGTGGTATTATATGTATGTTGTTGTTGAATATATCAATAATA
1020 AGGTTTTTAATTTATTTTAAAAA

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(B)

Fig. 3. (A) cDNA sequence and deduced amino acid sequence of ISP11 clone, and (B) hydropathy plot of the amino acid sequence. The hydropathy was averaged over a window of 5 amino acid residues using the algorithm of Kyte and Doolittle (1982).

codes for a water-soluble protein without an amino-terminal signal peptide. A computer search of GenBank database (NCBI, USA) using both the nucleotide and deduced amino acid sequence of ISP11 failed to reveal any significant homologies with known proteins.

Southern hybridization of genomic DNA was carried out to determine whether the *ISP11* gene was present in the genome as a member of multigene family or as a single copy gene. Based on our estimation of the nuclear DNA content (1.18 pg/2C) of the diploid *I. trifida* by flow cytometry (unpublished data), the amount of DNA corresponding to a single copy of ISP11 cDNA (1.04 kb) was estimated at 0.97 pg per μ g of genomic DNA. Signal intensity of a single band detected by Southern hyb-

ridization of the *Eco*RV-digested genomic DNA was found to be equivalent to 2 copies per nucleus (Fig. 4), indicating that the *ISP11* gene is present as a single copy in the haploid genome of *I. trifida*.

To determine the presence of a genetic linkage between the *ISP11* gene and the *S*-locus, restriction fragment length polymorphism (RFLP) analysis was carried out in an F_1 population segregating for *S*-alleles. *Dra*I-digested genomic DNAs from parental homozygotes and F_1 individuals were hybridized to ISP11 cDNA. Hybridizing fragments of about 2.9 and 3.8 kb were detected in the S^3S^3 and S^4S^4 homozygotes, and a 3.8 kb fragment in the $S^{22}S^{22}$ homozygote (data not shown). In addition, a weak common band of about 2.4 kb was found in all the

plants, suggesting that there exists an additional gene with a low sequence homology in the genome of *I. trifida*. None of these fragments, however, co-segregated with *S*-alleles in the F_1 progeny (Fig. 5). Segregation ratio for the hybridizing pattern in the progeny, [four plants with a 3.8 kb fragment]:[seven plants with 2.9 and 3.8 kb fragments]:[five plants with a 2.9 kb fragment] fitted well to the 1:2:1 ratio expected for two alleles of a single-locus gene ($\chi^2=0.16$, $d_f=2$, $p>0.9$) and the hybridizing patterns segregated independently of *S*-genotypes ($\chi^2=1.6$, $d_f=7$, $p>0.99$). This finding indicates that the *ISP11* gene is not linked to the *S*-locus, and is presumably located in another chromosome.

In *Brassica* which shows a sporophytic type of SI, *S*-locus-specific glycoprotein (SLG) and receptor kinase (SRK) have been identified from the stigma as molecules associated with the SI reaction (for review, Nasrallah *et al.* 1995). In solanaceous plants such as *Nicotiana alata*, *Lycopersicon peruvianum* and *Petunia inflata* which display

a gametophytic type of SI, on the other hand, *S*-locus ribonuclease (*S*-RNase) detected in the style has been shown to be involved in the SI system (for review, Newbigin *et al.* 1993). Recent studies have provided some evidence that the ribonuclease activity of *S*-RNase is essential for the expression of SI in solanaceous plants (Kowyama *et al.*, 1994b, Lee, *et al.*, 1994, Murfett *et al.*, 1994, Royo *et al.*, 1994). All these molecules, SLG, SRK and *S*-RNase, are predominantly expressed in the pistill, while no *S*-locus gene products exclusively expressed in the pollen or anther have yet been identified in any of these self-incompatible plants. In *I. trifida* that shows genetically the same SI system as *Brassica* species, expression of *SRK*-like genes with sequence similarities to the *Brassica SRKs* was found in the stigma and pollen as well as in the vegetative organs, and it was observed that these genes were not linked to the *S*-locus (Kowyama, *et al.*, 1995b). This fact suggests that a unique reaction mechanism different from that of *Brassica* is operating in the SI system of *I. trifida*. We have no information yet about the function of the *ISP11* protein in the reproductive organs of *I. trifida*. The Northern and RFLP analyses, however, suggest that the *ISP11* protein is not involved in the *S*-allele-specific self-recognition between the pollen and stigma cells, but probably plays a general role in pollen germination and/or pollen-tube growth following pollination.

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Fig. 4. Southern hybridization to indicate the copy number of *ISP11* gene in the nucleus of diploid *I. trifida*. Ten micrograms of *EcoRV*-digested genomic DNA and *ISP11* plasmid DNAs corresponding to a single copy (9.7 pg), 2, 4, and 6 copies were hybridized to 32 P-labeled cDNA of *ISP11*.

Fig. 5. RFLP analysis of an F_1 population segregating for *S*-alleles. *Dra I*-digested genomic DNAs from F_1 individuals were hybridized to 32 P-labeled cDNA of *ISP11*. The *S*-genotypes of the plants are indicated above the lanes.

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