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A pistil-specific gene of *Solanum tuberosum*
is predominantly expressed in the stylar cortex

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Abstract In a program aimed at studying genes expressed in pistils, the cDNA clone STS15 was isolated from a cDNA library of pollinated pistils of *Solanum tuberosum* and was found to be expressed only in pistils. During development of the pistil, the accumulation of STS15 transcripts, which are 0.7 kb long, reached a maximum just before anthesis and declined in fully open flowers. Southern blot analysis revealed that *sts15* was present as a small gene family in dihaploid potato. In situ hybridization experiments indicated that STS15 was strongly expressed in the cortex of the style and at a low level in the stigma. No hybridization signal was observed in the transmitting tissue. The temporal and spatial expression patterns of STS15 indicate that the gene products of the *sts15* gene might be involved in the function of the stylar cortex or in making the pistil competent for pollination.

Key words Pistil-specific gene · Stylar cortex · STS15 cDNA · *Solanum tuberosum*

Introduction

In flowering plants, the pollen, i.e. the male gametophyte, and the pistil, which contains the female gametophyte (Esau 1977), come into close contact at the onset of pollination. During this interaction, the genetic background of both tissues determines whether or not successful fertilization will take place. Although rejection of the pollen, or self-incompatibility, has been extensively investigated at the molecular level (Sims 1993), relatively few data have been obtained that shed light on the molecular basis of a successful pollen-pistil interaction. Such an interaction is characterized by germination and undisturbed growth of the pollen tubes that ultimately leads to fertilization of the egg cell in the embryo sac (Lord and Sanders 1992).

The process of pollination starts when mature pollen, developed in the pollen sacs of the anther (Mascarenhas 1990), is shed and transferred to the mature stigma of a receptive pistil. After landing, recognition processes initiate the cascade of events that lead to fertilization (Mascarenhas 1990). This recognition implies that pistils are able to discriminate between the different types of pollen they receive and to determine whether they will accept or reject the pollen (Knox 1984). According to the species, pollen acceptance or rejection may take place at two distinct moments after pollination, either when the pollen has-landed on the stigma or when the pollen tubes are in the pistil. After the pollen has been accepted on the stigma, it germinates and produces a pollen tube. This pollen tube penetrates the stigmatic tissue (Van Went and Willems 1984) and grows into the intercellular matrix of the stylar transmitting tissue. In some species, the choice between acceptance and rejection is delayed until the pollen tube has travelled one third of the length of the style (gametophytic self-incompatibility, such as in the Solanaceae). After the pollen or the pollen tubes are accepted, successful growth depends on the qualitative and quantitative composition of the nutrients in the stylar transmitting tissue. The intercellular matrix of the transmitting tissue is filled with proteinaceous substances and low-molecular-weight compounds like glucose and galactose (Herrero and Dickinson 1979; Sedgley et al. 1985; Konar and Linskens 1966). Its mass is influenced before and during pollination by temperature and other environmental factors (Van Herpen 1984). Compounds in the tissue may affect the growth of the pollen tubes (Herrero and Arbelo 1989; Kroh et al. 1971; Ichimura and Yamamoto 1992), because substances from the intercellular matrix are taken up by the pollen tubes (Kroh et al. 1970; Labarca and Loewus 1973). In the So-
To understand more about the genes involved in the acceptance or rejection of the pollen and pollen tubes in the different tissues, several pistil genes have been isolated and are being investigated. These genes, predominantly expressed in the pistil, can be divided into two major groups (Gasser and Robinson-Beers 1993). The first group contains the S genes involved in self-incompatibility; these are all expressed in the pistil and thoroughly described (Sims 1993; Newbigin et al. 1993; Nasrallah and Nasrallah 1993). The second group comprises pistil genes that are not involved in self-incompatibility. This group includes genes expressed in the stylar transmitting tissue of tomato (Gasser et al. 1989; Budelier et al. 1990) and tobacco (Ori et al. 1990). Several genes in this group encode proline-rich proteins. Some of them are extensin-like (Goldman et al. 1992; Chen et al. 1992; Baldwin et al. 1992), whereas others are characterized only by a high proline content (Chen et al. 1993; Cheung et al. 1993). Other pistil-specific genes are a proteinase inhibitor precursor of Nicotiana alata (Atkinson et al. 1993) and STIG1, a tobacco gene that is specifically expressed in the stigmatic secretory zone (Goldman et al. 1994). None of the genes described so far have been identified as specific for compatible pollination; however, some extensin-like genes have a modulated expression upon pollination (Goldman et al. 1992; Wang et al. 1993). Also, nothing is known about the effect of the pollen gene products on the expression of these pistil-specific genes.

The aim of our research was to isolate and characterize genes expressed in pollinated pistils and establish their functional role during pollen tube-pistil interaction. To achieve these goals, we differentially screened a cDNA library of pollinated pistils of Solanum tuberosum and isolated several pistil-specific cDNAs. The temporal and tissue-specific expression and localization of the transcripts of one of these genes and its putative function are described.

Materials and methods

Plant material

Plants of self-incompatible dihaploid (2n=2x=24) Solanum tuberosum clones heterozygous with respect to the S-alleles (S-S and S-S) were supplied by Professor E. Jacobsen (Wageningen Agricultural University, The Netherlands). Cuttings were grown in climate chambers at 20°C under a light/dark regime of 16/8 h.

Pistils (without the ovary) were collected from flowers at anthesis. Mature pollen was collected at anthesis and dried at room temperature for 48 h in open petri dishes (Van Herpen 1984). Pollinations were carried out just before anthesis, and pistils were collected after 24 h. All tissues were frozen in liquid nitrogen and stored at −80°C.

Development of potato flowers was divided into four stages according to Kaufmann et al. (1991). Stage 1 is characterized by a closed green bud (3–4 mm). Stage 2 is a flower bud (5–6 mm), still closed, in which the anthers are turning yellow. Stage 3 is a partly opened flower bud (8–9 mm) in which the petals and anthers are colored. Stage 4 corresponds to flowers at anthesis (>10 mm).

RNA and DNA extraction

Total RNA was isolated using the method of Frankis and Mascarenhas (1980) with slight modifications according to Goldberg et al. (1981). Plant tissue frozen in liquid nitrogen was homogenized in 4 ml RNA extraction buffer [100 mM TRIS- HCl pH 8.0, 50 mM EDTA, 1% SDS, 0.1 mM NaCl, 50 mM β-mercaptoethanol and 1% Tri-isopropylphenylphosphate sodium salt (Kodak)] and an equal volume of phenol, and incubated at 60°C for 10 min. Additional phenol/chloroform/isoamyl alcohol (25:24:1) extractions were performed until no interface remained. RNA was obtained after successive ethanol and 2 M LiCl precipitations.

The same procedure, omitting β-mercaptoethanol, was used to extract high-molecular-weight DNA from young leaves.

cDNA library construction and differential screening

Cross-pollinated (S1S2xS3S4) pistils were harvested 24 h after pollination. Poly(A)+ RNA was isolated using a PolyATtract poly(A)+ mRNA isolation kit (Promega). cDNAs were synthesized from poly(A)+ RNA, using a Uni-ZAP XR cDNA synthesis kit (Stratagene), according to the protocols of the manufacturer. The library was packaged using Gigapack II gold packaging extracts (Stratagene). The cDNA library was differentially screened on nitrocellulose with 32P-labelled single-stranded cDNA probes. The cDNA probes were prepared from either cross-pollinated pistil poly(A)+ RNA or unpollinated pistil poly(A)+ RNA (Sambrook et al. 1989). The ExAssist/SOLR in vivo excision system (Stratagene) was used for automatic excision of the positive cDNA clones from lambda Zap II to yield the Bluescript II SK(+) vector in XL-1 Blue E. coli cells (Stratagene).

The isolated STS15 cDNA clone was not full-sized. Therefore, the 5'-AmpliFinder RACE kit (Clontech) was used to isolate the 5' end of the STS15 cDNA clone. Two nested internal primers were designed (VE5, TCGAAAATCCATTCAAGGCACCCGGAG; VE6, GCCACATGGACTCGCGGAGCAGCTTG). The primer VE5 was used to synthesize cDNA and the primer VE6 was used for PCR amplification of the 5' cDNA end according to the protocols of the manufacturer. The PCR fragments were cloned with the TA Cloning System (Invitrogen) into the pCRRI vector.

DNA manipulations and sequence analysis

Plasmid DNA was prepared as outlined in Sambrook et al. (1989). Radioactively labelled probes were prepared from cDNA fragments in low melting point agarose using the random-primer labelling method (Church and Gilbert 1984). Nucleotide sequence analysis was carried out by the dyeodeoxyribonucleotide chain termination method (Sanger et al. 1977) using the T7 DNA polymerase sequencing system of Pharmacia. Both nucleotide and deduced protein sequences were analyzed using the University of Wisconsin Computer Group programs (Devereux et al. 1984).

Northern blot analysis

Equal amounts of total RNA (10 µg) from different tissues were electrophoretically separated on 1.5% agarose gels and transferred to Hybond-N (Amersham) according to Sambrook et al. (1989). The STS15 cDNA was used as a probe. Hybridization was performed for 20 h at 55°C in 6x SETS (1x SETS is 0.15 M NaCl, 0.02 M TRIS-HCl pH 7.8, 1 mM EDTA), 5x Denhardt's (1x Denhardt's is 0.02% Ficoll, 0.02% PVP and 0.02% BSA), 0.1% SDS and 75 µg/ml denatured herbing sperm DNA. Washing was carried out at 55°C in 1x SSC, 0.1% SDS or at 55°C 4x SSC, 0.1% SDS.
Localization of STS15 spliced expression

in any tissue examined (Fig. 3E–G). The sense STS15 cDNA probe showed no hybridization with any tissue surrounding the plasmid, the cortex of the style (Fig. 3D), or the ovary. The hybridization signal was observed in the transgenic line (Fig. 3C). No hybridization signal was observed in the transgenic line (Fig. 3B). Whether the STS15 mRNA is also present in the epidermis could not be distinguished with this approach (Fig. 3B). However, the hybridization signal was observed with confocal laser-scanning microscopy (CLSM), a very strong signal was detected in the cortex of the style (Fig. 3B). When using an antisense RNA probe of STS15 in the leaves of 3-week-old seedlings, the hybridization signal was localized by in situ hybridization. An overview of the hybridizations is shown in Fig. 3A. Using an antisense RNA probe of STS15 revealed two recombinant clones which proved to be the two clones isolated from the library. Although we expected to identify pollination-induced genes, by this protocol we isolated two pollen-specific clones which were not pollination-induced. One of the clones was identified by in situ hybridization, the other clone was isolated from the library by in situ hybridization. A detailed analysis of the expression patterns revealed that the STS15 gene was expressed in pollen in all tissues during pollination. A detailed analysis of the expression patterns revealed that the STS15 gene was expressed in pollen in all tissues during pollination. A detailed analysis of the expression patterns revealed that the STS15 gene was expressed in pollen in all tissues during pollination.
The ST15 cDNA was produced with ST15 cDNA from an 18-day-old embryo. The ST15 RNA was extracted from four stages: (a) blastula, (b) gastrula, (c) neurula, and (d) larva. The RNA was hybridized to a single mRNA transcript of 0.7

The expression of the ST15 gene during blastula development was assayed by Northern blotting. The ST15 cDNA was probed with ST15 cDNA. The ST15 mRNA was detected at four stages: (a) blastula, (b) gastrula, (c) neurula, and (d) larva. The mRNA was hybridized to a single mRNA transcript of 0.7
The conservation of the sis15 gene in other plant species

Conservation of the sis15 gene and its expression

Conservation in the computer database:

amino acid sequences do not resemble any homology with
some other amino acids. However, amino acids 18, 75, and 106 are highly conserved.

Figure 1: Isoelectric point of the mouse and human isoforms.

Figure 2: Immunocytochemical staining of the sis15 gene

Figure 3: Expression of the sis15 gene in various plant species.

Figure 4: Expression of the sis15 gene in various plant tissues.
Fig. 4 Nucleotide sequence of the STS 15 cDNA and its deduced amino acid sequence. The stop codon is shown in boldface letters and putative N-glycosylation sites are underlined.

Discussion

For a better understanding of the process of pollination, it is important to identify genes specifically expressed in all different tissues of the pistil and establish their functional role during pollination. All the pistil-specific genes previously isolated are expressed in the stigmatic tissue and/or in the transmitting tissue of the pistil (Sims 1993; Gasser and Robinson-Beers 1993; Nasrallah and Nasrallah 1993).

As can be concluded from the northern blot analysis, stsl5 is a gene highly expressed in pistil. Since stsl5 homology at the genomic level was shown in B. oleracea, P. hybrida, and N. tabacum, stsl5 is a member of a small gene family which is conserved during evolution. Conservation at the expression level differs for the tested species, with stronger hybridization signals observed in S. tuberosum pistils compared to potato. STS15 cDNA hybridized with two transcripts of 0.7 kb and 0.75 kb in tobacco. Shorter exposure of the blot revealed that the very strong hybridization signal observed in S. tuberosum pistils consists of transcripts of one length.
species; only potato and tobacco showed expression of the st5 gene or a gene homologous to st5. Despite the high level of conservation at the genomic level, the expression of st5 is not conserved in the Solanaceae. In contrast to the presence of a transcript of 0.7 kb in potato, an additional transcript of 0.75 kb is present in tobacco pistils (Fig. 5C). This may have been caused by the use of two different polyadenylation sites in the tobacco gene.

The st5 gene is the first pistil-specific gene described that is expressed in the cortex of the pistil. The localization of STS15 expression in the stolar cortex (Fig. 3) is, so far, unique among pistil-specific genes. However, localization of expression of STS15 in the pistil epidermis as well cannot be excluded. The other described genes with expression in the cortex are the flower-specific fts gene isolated by Gu et al. (1992) and the ta20 gene described by Koltunow et al. (1990). The fts and ta20 genes are expressed in the cortex of the style, but also in petals, stamens, and other parts of the pistil. Furthermore, in some species the cortex is demarcated from the transmitting tissue by a zone of callose-containing cells around the stolar channel (Knox 1984). In this zone, Wu et al. (1993) showed the accumulation of CEP (Cys-rich extensin-like protein) mRNAs.

The localization of STS15 expression primarily in the stolar cortex combined with its maximum expression just before anthesis suggests that the gene products of st5 do not play a direct role in processes during or after pollination even though the temporal expression pattern is similar to that of the potato S genes (Kaufmann et al. 1991). The deduced protein sequence of the STS15 cDNA has no homology with other sequences in the databases and could not, therefore, elucidate a possible function for st5. The specific expression of STS15 in the parenchymatous tissue of the cortex suggests that these celltypes are highly differentiated and may play a different role than in other organs.

Most of the pistil-specific genes isolated so far are expressed in the tissues that come into close contact with the pollen or pollen tube (stigma and transmitting tissue). However, the mature form and proper functioning of the stigma and style depend on a developmental program that includes all tissues of the pistil. The spatial and temporal patterns of expression of STS15 strongly suggest that the gene is developmentally regulated and may have a specific function in the cortex. To further understand the process of pollination, knowledge both of the tissues in direct contact with the pollen tubes and of the other parts of the pistil is necessary. To this end, greater effort should be made to isolate and characterize genes involved in the development of the pistil cortex and epidermis.

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