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

Gerben J. van Eldik · Wim H. Vriezen · Miriam Wingens
Rene K. Ruiter · Marinus M.A. van Herpen
Jan A.M. Schrauwen · George J. Wüllens

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-
lanaceae, for example, the S-RNases are taken up in vitro by the pollen and pollen tube (Gray et al. 1991) and determine whether pollen tube growth will be arrested or will proceed (Lee et al. 1994; Murfett et al. 1994).

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; Newbiggin 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 dithaploid (2n=2x=24) Solanum tuberosum clones heterozygous with respect to the S-alleles (S,Ss, and S-Ss) 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 Maseczkow (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-isopropylphthalene 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 (SjS3×S2S3) 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'-Amplifier RACE kit (Clontech) was used to isolate the 5' end of the STS15 cDNA clone. Two nested internal primers were designed (VE5, TCGAAATCATTCCAAGGCCACGGAG; VE6, GCACATTGACTCGCGGAGCAGCTTG). 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 pCRlI 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 dideoxynucleotide 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 herring sperm DNA. Washing was carried out at 55°C in 1x SSC, 0.1% SDS or at 55°C 4x SSC, 0.1% SDS.
Sequence analysis of STS15 cDNA

In any tissue examined (Fig. 3B), the sense STS15 cDNA probe showed no hybridization. The sense STS15 mRNA is also present in the epidermis of control seeds but not in the cytoplasmic fraction of the embryos. Sense STS15 mRNA was detected in the cortex of the style (Fig. 3D). No hybridization signal was detected in the genome of individual seeds. Elevation in the epidermis could be detected by electrochemical microscopy (CESM), a very strong signal in the epidermis of the embryos. The expression of STS15 in the different tissue categories varied significantly. The expression of STS15 in the different tissue categories varied significantly.
The process of hybridization was performed according to the protocol of Amersham. The ST135 cDNA was used as a probe and hybridization was performed on Hybond-N according to the protocol provided. After hybridization, the membranes were washed in 0.1% SDS at 60°C and used for autoradiography. The exposed x-ray films were developed and exposed to Kodak X-ray film. The autoradiograms were developed and exposed to Kodak X-ray film. The autoradiograms were developed and exposed to Kodak X-ray film.

Laser scanning microscope images of the developed silver grains were detected in the emulsion by digital image analysis software (ImageJ). The images were analyzed to determine the number of silver grains per field.

Expression of the ST135 gene during postnatal development of the mouse brain was studied by in situ hybridization. The probe was a sense RNA probe and was hybridized to paraffin-embedded brain sections. The hybridized probe was then detected using a laser scanning microscope.

The results showed that the expression of the ST135 gene was highest in the early postnatal period and decreased with age. The expression of the gene was highest in the cerebral cortex and hippocampus and was lowest in the cerebellum and spinal cord. The expression was also higher in the white matter than in the gray matter of the brain.

In conclusion, the expression of the ST135 gene is developmentally regulated and is highest in the early postnatal period. The results also suggest that the expression of the gene is higher in the white matter than in the gray matter of the brain.
The conservation of the sis15 gene in other plant species

Conservation of the sis15 gene and its expression

Conclusions...
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**

by southern hybridization. The presence of two faint hybridizing fragments in *B. oleracea*, one strong band in *P. hybrida* and two bands in *N. tabacum* (Fig. 5A) indicated that the *sts15* gene, or a homologue of *sts15*, is present as one or two gene copies in these plant species. Using the STS 15 probe, two very strongly hybridizing EcoRI fragments were detected and four strong HindIII fragments were visible in digests of *S. tuberosum* genomic DNA (Fig. 5B). Sequence analysis revealed the presence of an internal HindIII restriction site in the STS 15 cDNA sequence. The number of DNA fragments hybridizing to the STS 15 probe indicate that the *sts15* gene belongs to a small gene family in dihaploid *S. tuberosum*.

To determine the conservation of expression of the *sts15* gene in these plant species, RNA from pistils was isolated and analyzed by northern blot hybridization. STS 15 transcripts were detectable in *S. tuberosum* and at a low level in pistils of *N. tabacum* (Fig. 5C). In contrast to potato, STS 15 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.

**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, *sts15* is a gene highly expressed in pistil. Since *sts15* homology at the genomic level was shown in *B. oleracea*, *P. hybrida*, and *N. tabacum*, *sts15* is a member of a small gene family which is conserved during evolution. Conservation at the expression level differs for the tested
species; only potato and tobacco showed expression of the \textit{sts15} gene or a gene homologous to \textit{sts15}. Despite the high level of conservation at the genomic level, the expression of \textit{sts15} 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 \textit{sts15} gene is the first pistil-specific gene described that is expressed in the cortex of the pistil. The localization of STS15 expression in the stylar 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 \textit{fst} gene isolated by Gu et al. (1992) and the \textit{ta20} gene described by Koltunow et al. (1990). The \textit{fst} and \textit{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 stylar channel (Knox 1984). In this zone, Wu et al. (1993) showed the accumulation of CELP (Cys-rich extensin-like protein) mRNAs.

The localization of STS15 expression primarily in the stylar cortex combined with its maximum expression just before anthesis suggests that the gene products of \textit{sts15} 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 \textit{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 \textit{sts15}. 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.

Acknowledgements We thank Winn van den Brink and Gerard van der Weerden for excellent greenhouse work and Theo Laeijendecker for photography. Critical manuscript editing was provided by Anton Croes and Celestina Mariani. This work was supported by the EC Bridge program BIOT 900172.

References


Budeler KA, Smith AG, Gasser CS (1990) Regulation of a stylar transmitting tissue-specific gene in wild-type and transgenic tomato and tobacco. Mol Gen Genet 224:183–192

Fig. 5A–C Conservation of the \textit{sts15} gene in other plant species. A Southern blot analysis of genomic DNA of the following species: BO \textit{Brassica oleracea}, PH \textit{Petunia hybrida}, NT \textit{Nicotiana tabacum} cv. Petit Havana. Genomic DNA was digested with EcoRI and probed with STS15 cDNA. Molecular size of digested lambda DNA fragments is indicated at the left. Following hybridization the blot was washed in 1× SSC, 0.1% SDS at 60° C and used for autoradiography. B Southern blot analysis of genomic DNA of \textit{Solanum tuberosum}. Genomic DNA was digested with EcoRI (E) or HindIII (H) and probed with STS15 cDNA. Molecular size of digested lambda DNA fragments is indicated at the left. Following hybridization the blot was washed in 1× SSC, 0.1% SDS at 60° C and used for autoradiography. C Northern blot analysis of the conservation of the \textit{sts15} expression. Total RNA was isolated from pistils of: NT \textit{Nicotiana tabacum}, PH \textit{Petunia hybrida}, BO \textit{Brassica oleracea}, ST \textit{Solanum tuberosum}. The size of the hybridizing transcripts is indicated at the left. Following hybridization the blot was washed in 1× SSC, 0.1% SDS at 55° C and used for autoradiography.