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Pollination, wounding and jasmonate treatments induce the expression of a developmentally regulated pistil dioxygenase at a distance, in the ovary, in the wild potato *Solanum chacoense* Bitt.

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Abstract

Pollination and fertilization trigger unique developmental programs leading to embryogenesis, ovary maturation and seed set. Pistil tissues are actively involved in pollen tube growth and respond to the presence of the growing pollen tubes by modulating the expression of specific genes. Using subtractive hybridization to isolate genes involved in pollen-pistil interactions and fertilization, we have isolated a pollination- and fertilization-induced dioxygenase which is predominantly expressed in the pistil. *In situ* hybridization analyses revealed that the SPP2 dioxygenase (*Solanum* pollinated pistil) from the self-incompatible wild potato *Solanum chacoense* Bitt. is also developmentally regulated, with mRNA levels gradually regressing from the tip of the style towards the ovary during pistil development. At maturity, the upper limit of SPP2 transcript distribution coincided with the abscission zone of the style and SPP2 dioxygenase expression in ovaries coincided with the fertilization receptivity period of the flower. Pollination, as well as wounding of the style, induced an increase in SPP2 mRNA steady-state levels at a distance, in the ovary. Treatments with stress hormones including methyl jasmonate, jasmonic acid and salicylic acid mimicked the wound response and also induced SPP2 transcripts in the ovary. The SPP2 dioxygenase could be involved in the biosynthesis of deterrent alkaloids in reproductive tissues or in generating chemical signals involved in pollen tube guidance.

Abbreviations: AA, arachidonic acid; ABA, abscisic acid; DPA, days after anthesis; DPP, days after pollination; DPW, days after wounding; JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid.

Introduction

Sexual reproduction in flowering plants involves intimate interactions between the growing pollen tube and the female reproductive structure, the pistil. After landing on the stigma surface where the pollen grains adhere and hydrate, the pollen grains grow a protruding tube which carries the sperm cells through the transmitting tissue of the style towards the ovary. The two sperm nuclei carried at the tip of the growing pollen tube enter the ovules through the micropyle and fuse with the egg and the central cell nuclei, forming the zygote and the endosperm (Russell, 1992).

During pollen tube growth, the pistil plays an active role in pollen tube guidance and nourishment (Cheung, 1996). Furthermore, in self-incompatible species, such as *Solanum chacoense*, the pistil acts as a sieve, blocking pollen tubes carrying an *S*-allele identical to the ones expressed in the style from reaching the ovary (Matton *et al.*, 1998). Although many genes involved in male and female gametophyte development have been characterized in recent years (Raghavan, 1997), less attention has been devoted to

The nucleotide sequence data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number AF104925 (SPP2 dioxygenase cDNA).

the characterization of genes involved in compatible pollen-pistil interactions (Cheung, 1995). Pollination induces multiple responses, the most dramatic being ovule development in orchid flowers (Zhang and O'Neill, 1993). The best characterized responses to pollination are the increase in ACC synthase and ACC oxidase gene expression leading to ethylene production and to perianth senescence (O'Neill, 1997). Other pollination-induced events include cell deterioration and death of transmitting-tissue cells throughout the path of pollen tube growth as well as the deglycosylation of stylar transmitting tissue-specific proteins and the shortening of the poly(A) tails from transmitting-tissue specific mRNAs (Wang *et al.*, 1993, 1996). Differential screening strategies were recently used in potato (van Eldik *et al.*, 1997b) and apple (Dong *et al.*, 1998a) to isolate pollination-inducible genes. Genes involved in basic metabolic functions including the L1 ribosomal protein, histone H2B, translation elongation factors 1α and vacuolar ATPase were found to be up-regulated upon pollination in apple (Dong *et al.*, 1998a). Genes involved in flavonoid biosynthesis were also found to be up-regulated in both apple (Dong *et al.*, 1998a) and potato (van Eldik *et al.*, 1997a, b) upon pollination. Other pistil genes also characterized as being up-regulated after pollination include an *Arabidopsis* E2-related ubiquitin-conjugating enzyme (Watts *et al.*, 1994), two barley calreticulins (Chen *et al.*, 1994), a peroxidase (Chandra-Sekhar and Heij, 1995), a tobacco receptor-like protein kinase (Li and Gray, 1997) and an apple homologue of the DAD1 gene (defender against cell death 1), an inhibitor of programmed cell death in *Caenorhabditis elegans* (Dong *et al.*, 1998b). After successful pollination, fertilization initiates the developmental program leading to ovary maturation and seed set. Apart from knowledge gained from the analysis of embryo-defective mutants (reviewed in Howell, 1998) little progress has been made in the understanding of the early responses that follow zygote formation. *In vitro* fertilization approaches offer great promise in deciphering the early steps in embryogenesis, but production of large number of isolated gametes is limiting, difficult to master, and limited to a few amenable species (Kranz and Dresselhaus, 1996).

In order to characterize early responses upon pollination and fertilization we have begun to isolate pollination- and fertilization-induced genes by differential display analysis, virtual subtraction, and subtractive hybridization approaches in a self-incompatible relative of the potato, *Solanum chacoense*. In this study, we report the isolation of a developmentally regulated pistil dioxygenase which is induced by pollination, wounding, and fertilization in the wild potato species *S. chacoense*.

Materials and methods

Plant material

The diploid ($2n = 2x = 24$) *Solanum chacoense* Bitt. self-incompatible genotypes used include line PI 458314 (which carries the S_{11} and S_{12} self-incompatibility alleles) and line PI 230582 (which carries the S_{13} and S_{14} alleles) which were originally obtained from the Potato Introduction Station (Sturgeon Bay, WI), and generously provided by Prof. Mario Cappadocia (Université de Montréal). Plants were grown in greenhouses with 14 h of light per day.

Library construction and screening

cDNA libraries were made from 5 μg of poly(A)$^+$ mRNA isolated from either compatibly pollinated pistils 48 h after pollination or from ovaries 96 h after pollination in the ZAP express pBK vector following the manufacturer's instructions (Stratagene, La Jolla, CA). Subtracted cDNA libraries were made by suppression subtractive hybridization (PCR-select, Clontech, Palo Alto, CA) with poly(A)$^+$ mRNA from pistils 48 h after pollination or ovaries 96 h after pollination as tester mRNAs and poly(A)$^+$ mRNA from unpollinated pistils as driver mRNAs. The subtracted cDNA pool was PCR-amplified, radiolabeled and used to screen the ZAP-pBK 48 and 96 h post-pollination libraries. Hybridizing phage plaques were cored and, after *in vivo* excision, tested individually by RNA slot blot analysis (Sambrook *et al.*, 1989) to confirm their induction by pollination and fertilization.

Isolation and gel blot analysis of RNA and DNA

Total RNA was isolated as described previously (Jones *et al.*, 1985). Polyadenylated RNA was prepared by Oligo(dT) spin column chromatography as described in the mRNA separator kit protocol (Clontech). RNA concentration was determined by measuring its absorbance at 260 nm and verified (adjusted if necessary) by agarose gel electrophoresis and ethidium bromide staining. To confirm equal loading of total RNA on RNA gel blots, a 1 kb

fragment of the *S. chacoense* 18S RNA was PCR-amplified and used as a probe. The oligonucleotides used (5′-TCGATGGTAGGATAGTGGC-3′ and 5′-GCATAGCTAGTTAGCAGG-3′ were derived from highly conserved regions determined from a ClustalW alignment of 18S RNA sequences from *Solanum avicular*, *S. lycopersicon*, *S. melongena*, *S. petophylum*, and *S. tuberosum*. Genomic DNA isolation was performed via a modified CTAB extraction method (Reiter *et al.*, 1992). DNA gel blot analysis, including restriction, electrophoresis, and capillary transfer to a positively charged nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Baie D'Urfé, Canada) were performed as described in Sambrook *et al.* (1989). Hybridization of the membrane was performed under high-stringency conditions at 65 °C as described previously (Church and Gilbert, 1984) for 16 to 24 h and, after hybridization, the membrane was washed at room temperature, once with 4× SSC/0.1% SDS for 1 h, twice with 1× SSC/0.1% SDS at 50 °C for 30 min and twice with 0.1× SSC/0.1% SDS at 55 °C for 30 min (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA gel blot analyses were performed as described in Sambrook *et al.* (1989), following the formaldehyde denaturing protocol. RNAs were capillary-transferred onto Hybond N+ nylon membranes and cross-linked (120 mJ/cm^2) with a Hoefer UVC 500 UV Crosslinker. Hybridization of the membranes was performed under high-stringency conditions at 45 °C in 50% deionized formamide, 5× Denhardt's solution, 0.5% SDS, 200 μg/ml denatured salmon sperm DNA and 6× SSC for 16 to 24 h. After hybridization, the membranes were washed once at room temperature with 2× SSC/0.1% SDS for 1 h, twice with 1× SSC/0.1% SDS at 50 °C for 30 min and twice with 0.1× SSC/0.1% SDS at 55 °C for 30 min. Probes for both DNA and RNA gel blot analysis were synthesized from random-labeled isolated DNA inserts (Roche Diagnostics, Laval, Canada) with α-^{32}P-dCTP (ICN Biochemicals, Irvine, CA). The membranes were autoradiographed at −85 °C with one intensifying screen on Kodak Biomax MR film (Interscience, Markham, Canada).

In situ *hybridization*

Tissues samples were fixed in FAA (60% ethanol, 5% acetic acid, 5% formalin) at 4 °C overnight for pistils or for 2 days for flower buds and larger ovaries (cut in half). After dehydratation with *tert*-butanol and embedding in paraffin, samples were cut into 10 μm sections and mounted on slides coated with AES (3-aminopropyltriethoxy-silane; Sigma, Oakville, Canada). Tissue sections were deparaffinized, hydrated through decreasing ethanol baths (100%, 95%, 70%, 50%; distilled water twice, 5 min each) and subjected to the following series of treatments: 0.2 M HCl for 20 min at room temperature, 2× SSC for 30 min at 70 °C, 2 μg/ml proteinase K (Roche Diagnostics) for 30 min at 37 °C, 4% paraformaldhehyde for 20 min at room temperature, 10% triethanolamine/0.25% acetic acid anhydride for 10 min at room temperature. After these treatments, tissues were dehydrated through increasing ethanol baths (50%, 70%, 95%, 100%, 5 min each). Slides were then air-dried. Sense and anti-sense labeled riboprobes were synthesized from the SPP2 cDNA clone with digoxigenin-11-UTP (Roche Diagnostics) using the T7 and T3 RNA polymerases (RNA transcription kit, Stratagene, Palo Alto, CA). Slides were pre-hybridized for 1 h at 50 °C in 40% deionized formamide, 10% dextran sulfate, 3 mM NaCl, 10 mM Tris-HCl, 10 mM sodium phosphate pH 6.8, 5 mM EDTA, 1 mg/ml tRNA, 0.01 M DTT, 0.5 mg/ml poly(A)$^+$ (Roche Diagnostics) and 40 U of RNase inhibitor (Promega, Madison, WI). Tissue sections were hybridized in the same solution containing between 0.4 and 0.6 μg/ml digoxigenin-labeled RNA probe overnight at 50 °C. After hybridization, slides were treated in a 60 mg/l RNase A solution for 30 min at 37 °C to digest unhybridized probe and subsequently washed in 2× SSC for 1 h at room temperature, 1× SSC for 1 h at room temperature, 0.1× SSC for 1 h at room temperature. Hybridized probe was detected with anti-digoxigenin antibodies conjugated to alkaline phosphatase (Roche Diagnostics) and visualized by color development using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates for 15 h. Sections were photographed with an Olympus BHT microscope on Kodak Ektachrome 160 T film.

Phytohormone and wounding treatments

Flowers were sprayed with an aqueous solution (in 0.05% Tween) of either 50 μM abscisic acid (ABA), 50 μM jasmonic acid (JA, mixed isomers), 5 mM salicylic acid (SA), a 1 mg/ml emulsion (3.28 mM) of arachidonic acid (AA) or 0.05% Tween as a control once per day on two consecutive days. For volatile treatments, methyl jasmonate (MeJA) or *trans*-2-hexenal were first diluted to 0.1 M in ice-

cold methanol (MeOH) and 100 μl of the compound (equivalent to 10 μl/l of air space) was added to a piece of Whatman paper suspended over the plant in an airtight container as described previously (Bate and Rothstein, 1998). Treatments were repeated on two consecutive days. As a control, 100 μl of MeOH was used in the same way. All phytohormones and elicitors were purchased from Sigma/Aldrich (Oakville, Canada). For wounding, a small forceps was used to slighly crush the upper region of the styles and leaves were crushed perpendicularly to the mid-vein three times. Tubers were sliced and incubated in a moist Petri dish. All wounded tissues were harvested 48 h after the wounding.

Results

Isolation of the SPP2 cDNA

We have used subtractive hybridization (Diatchenko *et al.*, 1996) to characterize genes involved in pollen–pistil interactions and fertilization. Subtracted cDNA libraries were made with mRNA from either pollinated pistils 48 h after pollination or from ovaries 96 h after pollination from which were subtracted mRNAs common to mature unpollinated pistils. The pool of subtracted cDNAs was then PCR-amplified and used to screen 48 h and 96 h post-pollination *Solanum chacoense* pistil cDNA libraries made in the λZap-pBK vector. Positive plaques were purified and plasmids were rescued by *in vivo* excision. cDNA clones were tested individually by RNA slot-blot hybridization to determine if they were truly up-regulated after pollination and/or fertilization events (data not shown). One clone, SPP2 (*Solanum* pollinated pistil), was isolated from both the 48 h and 96 h subtracted pools. The nucleic acid sequence and the deduced amino acid sequence of SPP2 are shown in Figure 1A. The SPP2 clone contains a cDNA of 1210 bp (excluding the poly(A) tail) with a short 5′-untranslated leader of 43 nucleotides (nt) and a 3′ UTR of 140 nt. The size of the SPP2 cDNA corresponds to the size of the mRNA, as determined by RNA gel blot analysis (1250 nt, Figure 4), suggesting that the SPP2 cDNA is full-length or near-full-length. The SPP2 cDNA is predicted to encode a 341 amino acid polypeptide of 37.8 kDa with a pI of 5.6. Hydrophilicity plots suggest a cytoplasmic localization as no hydrophobic segment that could serve as a signal peptide could be identified in the N-terminal region. Intron positions (Figure 1A, arrowheads) were obtained by sequencing the products of PCR reactions amplified from *S. chacoense* genomic DNA using oligonucleotides corresponding to internal sites and to the ends of the SPP2 cDNA.

Gene copy number of SPP2

A Southern blot of *S. chacoense* genomic DNA was probed with the complete SPP2 cDNA insert (Figure 2). Only one hybridizing fragment could be detected from the *Bam*HI (2.1 kb), *Eco*RI (6.6 kb), *Eco*RV (5.5 kb) and *Xba*I (5 kb) digested genomic DNA and two hybridizing fragments, of 6.6 kb and 0.5 kb respectively, were detected from the *Hin*dIII digestion. Since there is one *Hin*dIII restriction site in the cDNA sequence (position 219), this strongly suggests that SPP2 is a single-copy gene in *S. chacoense*. Fragments hybridizing to the same probe were also detected in other solanaceous species (eggplant, tomato, ground cherry and petunia) but not in members of the Brassicaceae like *Arabidopsis thaliana* or *Brassica oleracea* (data not shown).

Sequence analysis

Homology searches in DNA and protein databases revealed striking similarities with a large group of 2-oxoglutarate-dependent (or 2-oxoacid-dependent) dioxygenases in plants. Figure 3 shows an alignment of the five dioxygenases most similar to SPP2. Dioxygenases most similar to SPP2 are from solanaceous plants and include a previously described pistil-expressed dioxygenase from tomato named TPP1 (Milligan and Gasser, 1995) also described as a gibberellin down-regulated partial cDNA clone (GAD2) isolated from a tomato leaf cDNA library (Jacobsen and Olszewski, 1996). Both TPP1 and an eggplant dioxygenase (GenBank accession number X77368) share extensive amino acid sequence identities (94% and 86%, respectively) with the SPP2 dioxygenase from *S. chacoense*. No substrates have been reported for either of these dioxygenases. The most similar dioxygenase of known function (44% amino acid identity, 60% similarity) is the hyoscyamine 6β-hydroxylase (H6H) from *Hyoscyamus niger* involved in tropane alkaloid biosynthesis. H6H catalyzes both the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine and the subsequent epoxidation of 6β-hydroxyhyoscyamine to scopolamine in several solanaceous plants (Hashimoto *et al.*, 1993). Significant amino acid identities ranging from 29% to 34% throughout the entire sequence could also be observed with other plant dioxygenases, including two genes

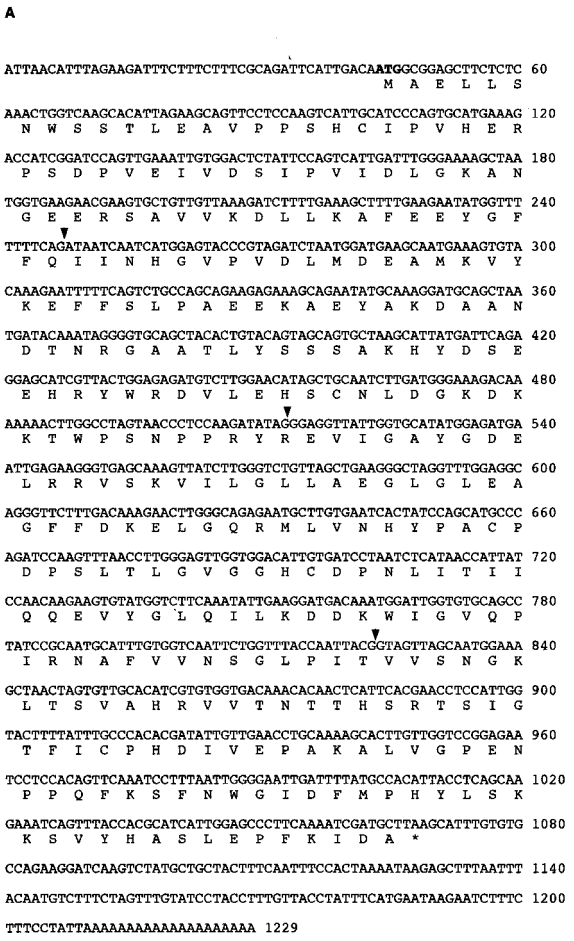


Figure 1. Nucleotide and deduced amino acid sequence of the *S. chacoense* SPP2 dioxygenase cDNA. A. The sequence of the coding strand of the SPP2 cDNA insert is shown together with the deduced amino acid sequence. The start of translation was chosen as the first methionine in the sequence, as it is in good agreement with the usual plant and animal initiation context AACAATGGC (Fütterer and Hohn, 1996). Black triangles indicate the position of introns as determined by PCR amplification of genomic DNA and sequencing. B. Sequence of a 5′ extension showing a potential extended open reading frame. Putative initiation codons are in bold. A potential TATA box element that could explain the selection of an alternative transcriptional start site producing the cDNA shown in A is marked with asterisks.

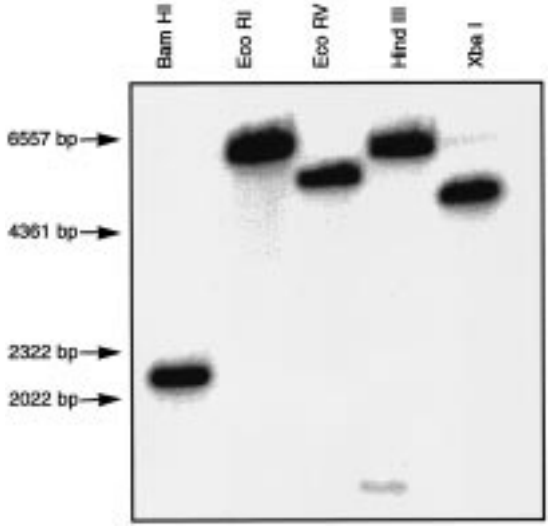


Figure 2. DNA gel blot analysis of the SPP2 gene. Genomic DNA (10 μg) isolated from *S. chacoense* leaves was digested with various restriction enzymes and probed with the 1.2 kb complete SPP2 cDNA insert. Molecular weight markers appear on the left.

from barley, Ids2 and Ids3 (34% amino acid identity), which are expressed under iron deficiency conditions in roots (Nakanishi *et al.*, 1993; Okumura *et al.*, 1994). Other dioxygenases sharing significant amino acid sequence identities include flavonol synthase (FLS, 31%) from *Petunia* (Holton *et al.*, 1993), a putative *Arabidopsis* gibberellin β-hydroxylase (31%, GenBank accession number AC003672), a flavanone-3-hydroxylase or naringenin-3-dioxygenase (FHT, 30%) from *Dianthus caryophyllus* (Britsch *et al.*, 1993), and a leucoanthocyanidin dioxygenase from *Perilla frutescens* (29%, GenBank accession number AB003779). Amino acid residues involved in the iron binding site of dioxygenases (Britsch *et al.*, 1993) are conserved in the above-mentioned dioxygenases (asterisks in Figure 3).

SPP2 dioxygenase mRNA expression in mature tissue

Tissue-specific expression of SPP2 was determined using RNA extracted from different tissues of *S. chacoense*. RNA gel blot analyses indicate that SPP2 is predominantly expressed in pistils, in leaves (Figure 4A) and in tubers (Figure 6A). Highest expression levels are seen in pollinated pistils 48 h after pollination. Upon prolonged exposure, clear hybridization signals can be seen in mature stems and to a lesser extent, in mature petals and roots (Figure 4B). No hybridization signal could be detected in mature anthers, pollen or pollen tubes even after prolonged exposure

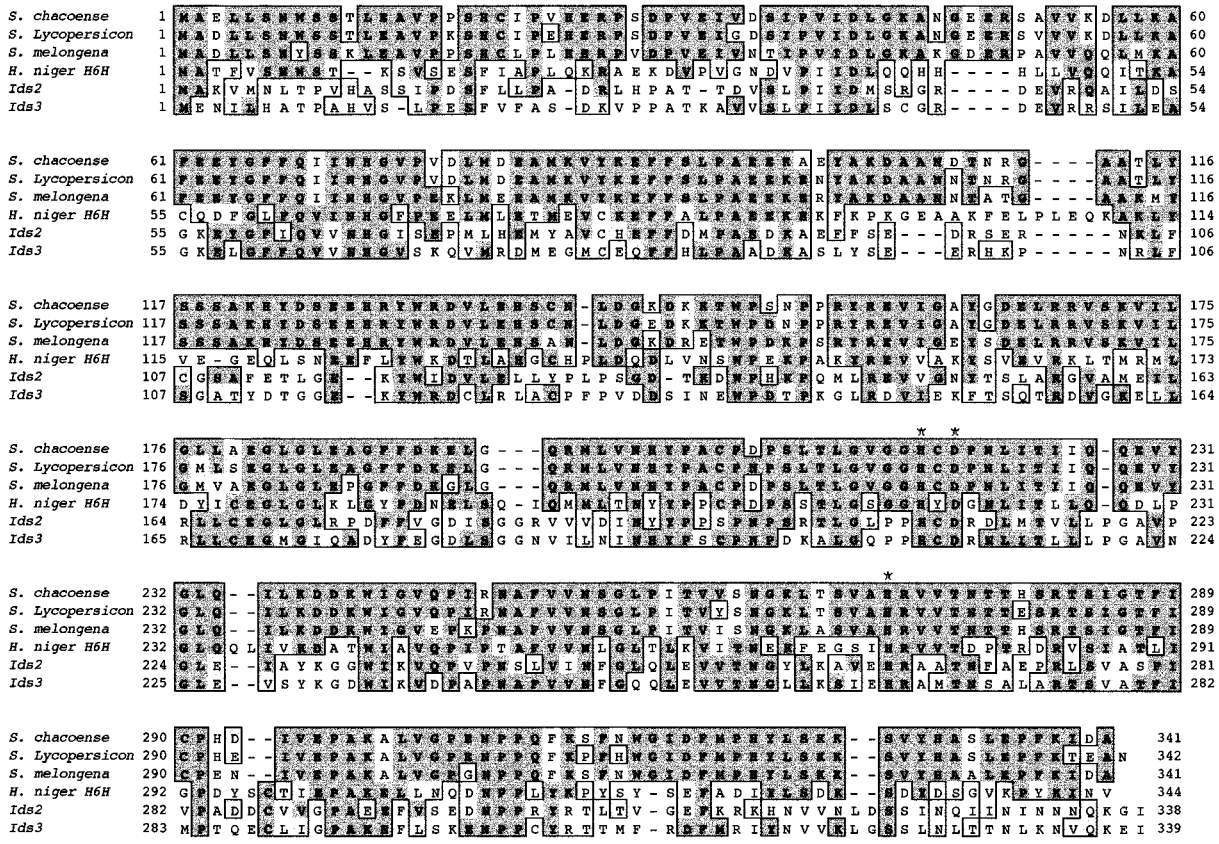


Figure 3. Alignment of the SPP2 deduced protein sequence with the five most closely related 2-oxoglutarate-dependent dioxygenases. The deduced amino acid sequence of SPP2 was aligned with those of the TPP1 clone from tomato (Milligan and Gasser, 1995, GenBank accession number U20596), a dioxygenase cDNA from eggplant (X77368), hyoscyamine 6β-hydroxylase (H6H) from *Hyoscyamus niger* (Matsuda *et al.*, 1991, M62719), and the Ids2 and Ids3 dioxygenases from barley (Okumura *et al.*, 1994, D15051; Nakanishi *et al.*, 1993, D37796). Asterisks represent amino acid residues involved in the iron-binding site of dioxygenases (Britsch *et al.*, 1993).

(Figure 4B). Densitometric scans of multiple film exposures indicate that SPP2 mRNA levels are about 30-fold less abundant in roots, and six-fold less abundant in leaves and in unpollinated pistils than in pollinated pistils 48 h after pollination. Levels observed in leaves and in unpollinated pistils are roughly equal if we take into account the SPP2 doublet seen in unpollinated pistils (see also Figure 6A). Since the SPP2 dioxygenase is a single-copy gene (Figure 2), the presence of two hybridizing fragments on some RNA gel blots was surprising. To determine the relationship between the two mRNAs, PCR reactions were carried out on the 48 h pollinated pistil library using a variety of internal and vector-derived primers. A single fragment of the expected size was amplified using internal primers only and using an internal primer paired with a 3′ primer from the cloning vector, indicating that the longer mRNA did not arise from alternative splicing or from the selection of an alternative polyadenylation site. However, when an internal SPP2 primer was paired with a 5′ primer derived from the cloning vector, two fragments were amplified. One fragment was of the size expected from our SPP2 cDNA clone, while the second contained a 68 bp extension from the original SPP2 cDNA clone. This extension contained an upstream ATG codon in frame with the putative initiation methionine and no stop codons (Figure 1B). However, it is not known if this region is translated in *S. chacoense* since in the corresponding 5′ UTR of the tomato TPP1 cDNA (86% nucleotide sequence identity with the 5′-UTR of SPP2), two in-frame stop codons are present. Furthermore, the translation initiation context of the upstream ATG is not optimal for translation (Fütterer and Hohn, 1996). This longer fragment most probably corresponds to the upper hybridization signal in some RNA gel blots. Thus, either

alternative 5′ start sites are selected or an unspliced intron is located in the 5′ UTR. The selection of alternative 5′ start sites could be explained by the presence of more than one TATA element. Indeed, one such consensus TATA element, which could be used to generate the shorter SPP2 transcript, is found in the 5′ UTR of the longer extension product (Figure 1B).

In order to determine if pollination triggered the accumulation of SPP2 mRNAs or if fertilization was necessary, an incompatible pollination was performed. *S. chacoense* is a gametophytic self-incompatible species that expresses stylar ribonucleases (S-RNases) in the transmitting tissue of the style (Matton *et al.*, 1998). These S-RNases recognize and reject pollen that share the same *S* allele as the ones expressed in the pistil. We observe that mRNA levels in pistil harvested 48 h after pollination with either incompatible pollen (Figure 4D, lane 1) or compatible pollen (Figure 4D, lane 2) were very similar, despite the fact that in incompatible pollinations, the pollen tubes never reach more than two-third of the style length and most are arrested in the top half of the style. In order to determine where in the mature pistils the SPP2 gene was expressed, styles and ovaries were collected separately. No hybridization signal could be detected in the style of mature pistils, even after prolonged exposure (Figure 4D, lane 3). In mature flowers, SPP2 mRNAs are thus mostly confined to the ovary (Figure 4D, lane 4).

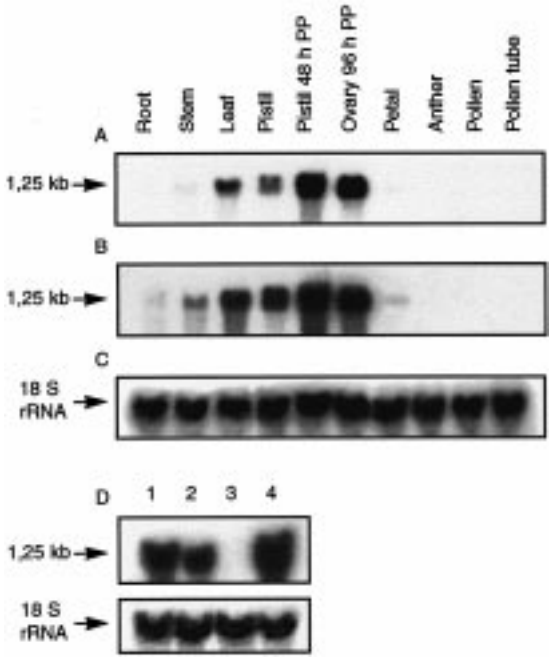


Figure 4. RNA gel blot analysis of SPP2 transcript levels in mature tissues. A. Total RNA from various tisues (10 μg) was probed with the 1.2 kb complete (*Eco*RI/*Xho*I) SPP2 cDNA insert. B. Overexposure of the RNA gel blot in A. C. Same as in A except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*. D. SPP2 mRNA accumulation two days after an incompatible pollination (lane 1, whole pistil), or a compatible pollination (lane 2, whole pistil). Little or no mRNA is found in styles from a 48 h compatible pollination (lane 3) while ovaries from a 48 h compatible pollination contain high levels (lane 4).

Pollen tube growth and wounding of the style trigger SPP2 mRNA expression in the ovary

The increase in SPP2 mRNA abundance seen 48 h post-pollination (Figure 4A) could be due to pollination-dependent or developmentally regulated processes. To distinguish between these two possibilities, an RNA time-course after a compatible pollination (Figure 5A) was compared to mRNA levels in unpollinated pistils collected from the day before anthesis to five days after anthesis (Figure 5B). SPP2 mRNA levels increased gradually from time 0 (unpollinated flowers) to 48 h after pollination in pollinated pistil tissues (Figure 5A) and were quite stable thereafter (96 h ovaries, Figure 4A and B). No increase was seen in unpollinated flowers, from the day before anthesis until 3 DPA (Figure 5B), suggesting that new expression of the SPP2 mRNA in pistil tissues is induced by pollination. The increase was not due to SPP2 mRNA expression from pollen tubes or styles, since in a separate time course experiment, with styles only, no hybridization signal could be detected (data not shown; see also Figure 6). Furthermore, no SPP2 mRNA could be detected in *in vitro* grown pollen tubes (Figure 4A and B). Therefore pollen tube growth in the style triggers an increase in SPP2 mRNA expression in the ovary, at a distance, before fertilization has occurred.

In many species, pollination is known to induce deterioration and death of specific cells or tissues, including the secretory cells in the stigmatic region and the transmitting tissue of the style (Cheung, 1996). To determine if cell death caused by wounding could also trigger SPP2 mRNA accumulation at a distance in the ovary, the styles of young flowers were crushed with tweezers and the styles and ovaries were collected separately 48 h later. Figure 6A shows that wounding of the style also induced SPP2 mRNA accumulation at a distance in the ovary, to the same extent as a 48 h compatible pollination (a six-fold increase). Thus, cellular deterioration and death caused by pollination (either compatible or incompatible) or by wounding, might

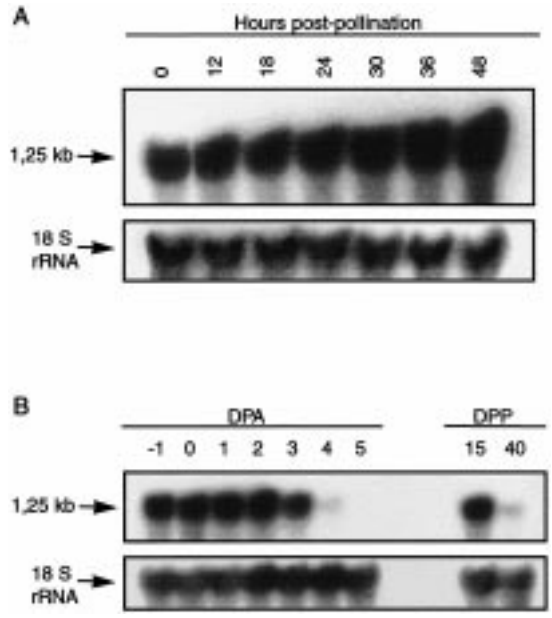


Figure 5. Temporal expression pattern of SPP2 mRNA levels in pollinated and unpollinated pistil tissues. A. Pollination-induced SPP2 mRNA accumulation. SPP2 transcript levels were determined by RNA gel blot analysis following a compatible pollination (pollen from a $S_{11}S_{12}$ genotype, female recipient, $S_{13}S_{14}$ genotype). Ten μg of total RNA from whole pistils (including stigma, style and ovary) collected at various times after pollination was probed with the 1.2 kb complete SPP2 cDNA insert. Lower panel: same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*. B. Developmental expression pattern of SPP2 mRNA levels in unpollinated pistil tissues and in fruits. SPP2 transcript levels were determined by RNA gel blot analysis of unpollinated pistil tissues from the day before anthesis (−1) until five days after anthesis (DPA) and in fruits 15 and 40 days after pollination (DPP). Lower panel: same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*.

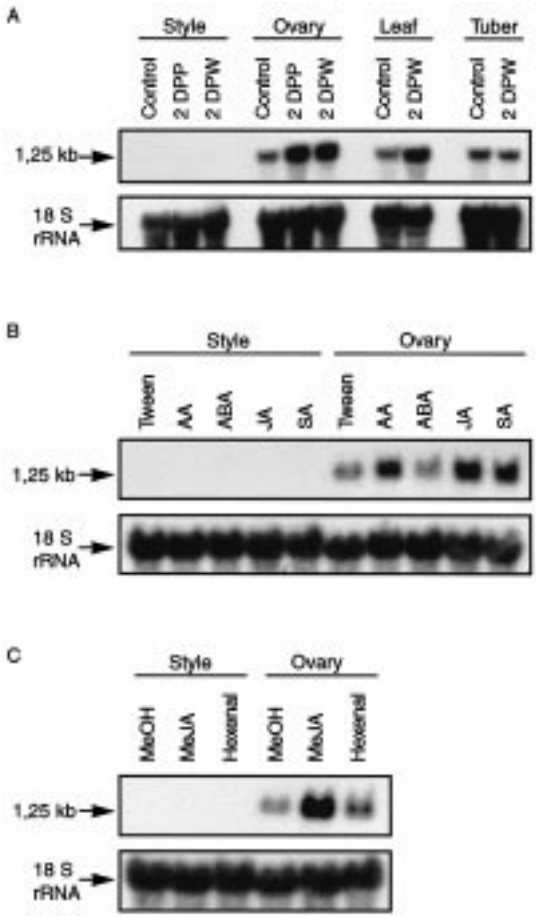


Figure 6. Effect of wounding and stress hormone treatments on SPP2 mRNA levels. A. SPP2 transcript levels were determined by RNA gel blot analysis of tissues 2 days after pollination (DPP) or 2 days after wounding (DPW). Total RNA from various tissues (10 μg) was probed with the 1.2 kb complete SPP2 cDNA insert. Styles were either pollinated or slightly crushed with tweezers and collected two days later. Unpollinated and unwounded styles served as controls. Ovaries from the same pistils were also collected either 2 DPP or 2 DPW. Lower panel: same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*. B. SPP2 transcript levels were determined by RNA gel blot analysis of unpollinated pistil tissues collected 48 h after various hormonal spray treatments (ABA and JA as 50 μM solutions in 0.05% Tween; AA as a 3.28 mM emulsion in 0.05% Tween; SA as a 5 mM solution in 0.05% Tween). Flowers sprayed with a 0.05% Tween solution served as control. Lower panel: same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*. C. SPP2 transcript levels were determined by RNA gel blot analysis of unpollinated pistil tissues collected 48 h after volatile treatments (10 μl of a 0.1 M solution in MeOH of either methyl jasmonate (MeJa) or trans-2-hexenal (Hexenal) per liter of air space). Treatment with 100 μl of MeOH served as control. Lower panel: same as upper panel except that the membrane was stripped and reprobed with an 18S ribosomal cDNA probe from *S. chacoense*.

be the initial trigger that induces SPP2 mRNA accumulation in the ovary. Since wounding of the style induced SPP2 mRNA accumulation in the ovary, we also tested if wounding could also induce SPP2 mRNA accumulation in leaves and tubers where SPP2 expression is also high. Figure 6A shows that SPP2 mRNA levels increase in wounded leaves but not in wounded (sliced) tubers.

Stress hormones are active inducers of SPP2 mRNA expression in the ovary

The nature of the signal that triggers SPP2 mRNA accumulation in the ovary remains to be determined but phytohormones have been implicated in many physiological processes during flowering and fertilization. Since pollen tube growth, which causes progressive

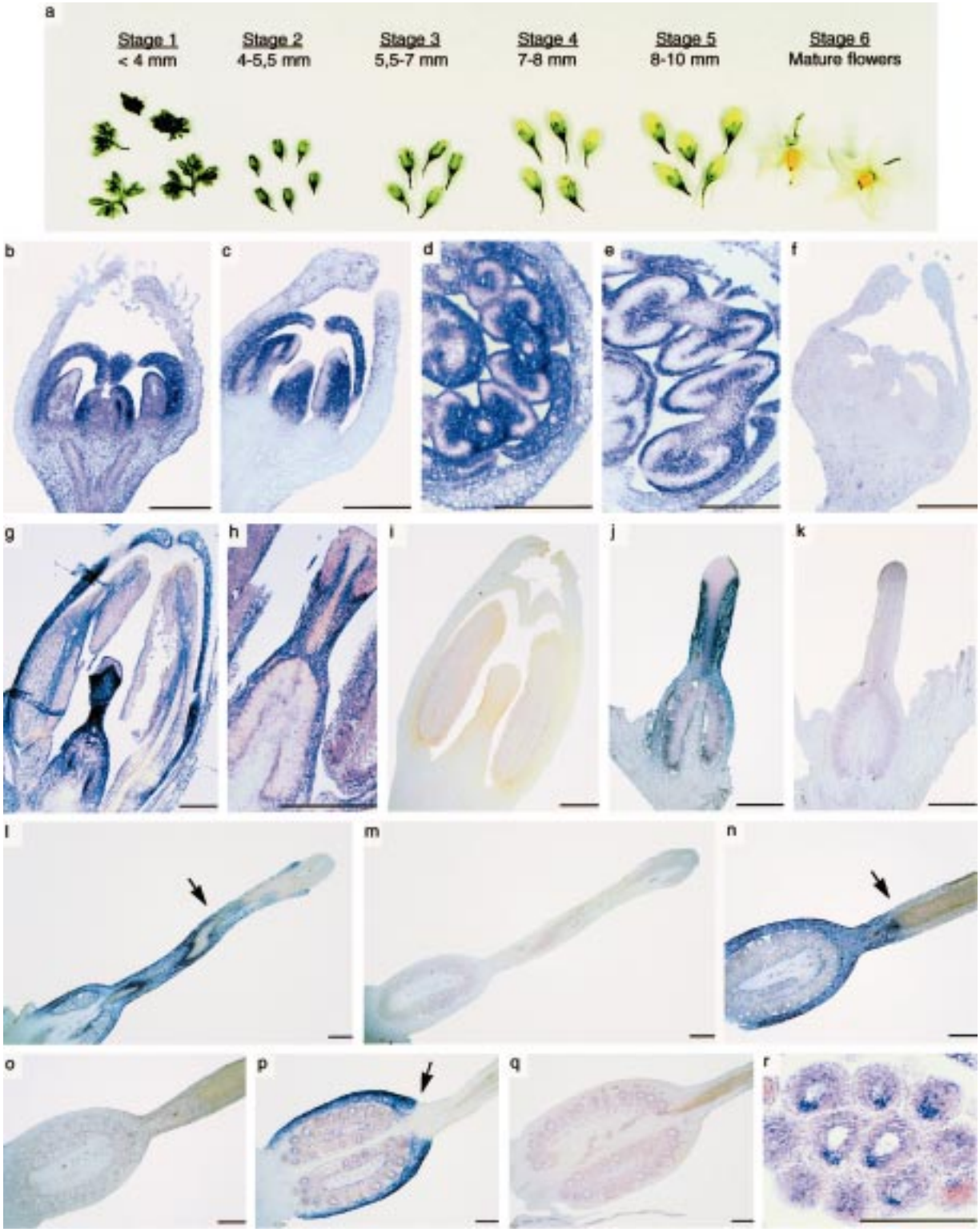
cell death of the style transmitting tissue (Cheung, 1996) and wounding of the style induced an identical increase in SPP2 mRNA at a distance in the ovary (Figure 6A), we tested if SPP2 mRNA expression was under stress hormone control. *S. chacoense* flowers were either sprayed with various phytohormones or chemical elicitors once per day for two consecutive days (Figure 6B) or treated with volatile compounds in an airtight chamber (Figure 6C). Styles and ovaries from 2 DPA flowers (in order to be in the same developmental state as for the 2 DPW and 2 DPP flowers in Figure 6A) were then collected separately. Stress hormones known to be involved in mediating the wound response, namely MeJa and JA, were the most potent inducers, as a six-fold increase in SPP2 mRNA levels, identical to the effect of wounding or pollination, could be observed in ovaries (Figure 6B and C). Arachidonic and salicylic acids, both potent inducers of defense response genes in potato (Matton *et al.*, 1990; Choi *et al.*, 1994; Coquoz *et al.*, 1995), also induced a strong increase in SPP2 mRNA accumulation in the ovary (Figure 6B). *Trans*-2-hexenal, a C_6 volatile produced from damaged or wounded tissue and also derived from the lipoxygenase pathway (Bate and Rothstein, 1998), had little inducing activity. ABA, although thought to be a primary signal in the systemic wound-signaling cascade (Pena-Cortés *et al.*, 1995), had no effect on SPP2 mRNA levels. Other phytohormones also delivered as a spray directly to the flowers (in concentrations ranging from 10 to 100 μM) including ethylene (ethephon), GA_3, kinetin, IAA and NAA had only weak or no inducing activity (data not shown).

SPP2 mRNA expression coincides with fertilization receptivity of the flower

Figure 5B also shows that, if unpollinated, SPP2 mRNA levels in flowers decline sharply after 3 DPA. These older flowers, either 4 DPA (closed petals) or 5 DPA (flowers will abscise if touched), are already showing signs of senescence. Densitometric scans of multiple film exposures indicated that in flowers 3 DPA, when the flowers are still receptive for fertilization, SPP2 mRNA levels were still quite high (50% of the level in -1 to 2 DPA). In flowers 4 and 5 DPA, which are no longer responsive to fertilization (flowers will abscise even if pollinated), SPP2 mRNA levels were at least 20- and 40-fold less, respectively, than levels observed in flowers -1 to 2 DPA. Thus, unless fertilized, SPP2 mRNA levels sharply decline in ovaries (compare flowers 4 and 5 DPA with the corresponding ovaries 96 h after pollination in Figure 4). Similarly, SPP2 mRNA levels remained high in young developing fruits (Figure 5B, 15 DPP) with levels similar to flowers 3 DPA, but declined again (8-fold decrease) in mature fruits (Figure 5B, 40 DPP).

Developmental expression in floral tissues: SPP2 expression regresses from the tip of the style to the ovary during pistil development

In order to further characterize the expression pattern of the SPP2 dioxygenase in floral tissues, *in situ* hybridizations were performed on 10 μm sections of whole flowers or pistils from different developmental stages. Figure 7a shows the six different developmental stages used in our experiment. Flower buds of stage 1 were fixed whole without prior dissection, while for later stages, pistils were dissected out prior to fixation. In stage 1 flower buds, even before carpel fusion, expression of SPP2 mRNA can be detected in the fusing carpels, in petals and in the epidermis of the anthers (Figure 7b). Once the carpels have fused, expression is still detected in the pistil and anther epidermis as well as in immature petals, but no hybridization signal is seen in the developing stigma (Figure 7c). SPP2 mRNAs are strongly expressed in the connective tissue as well as in the epidermis of young anthers (Figure 7d and e). SPP2 mRNAs are also clearly detected in the epidermis and parenchyma cells of the developing ovary around the locules (Figure 7e). In later stages, hybridization signals from the anther tissue are much weaker, while strongest expression is seen in the style and ovary epidermis and parenchymous tissue (Figure 7g, h and j). As for earlier stages, no SPP2 mRNA expression is detected in the stigma region and in the transmitting tract of the style. In stage 4 flowers, two days before anthesis, SPP2 expression has decreased from the tip of the style cortex and is now mostly detected only in the lower two-third of the style (Figure 7l). In stage 5 flowers, one day before anthesis, SPP2 expression has regressed further down the style and can only be detected in the lower third of the style, near the ovary (Figure 7n). In stage 6 flowers, on the day of anthesis, no hybridization signal can be detected in the style. Expression is now seen exclusively in the ovary cortex (epidermis and parenchyma) and very slightly in the region of the placenta nearest to the ovules (Figure 7p). A magnified view of the ovules shows that SPP2 is expressed specifically in the mi-


a
Stage 1
< 4 mm
Stage 2
4-5,5 mm
Stage 3
5,5-7 mm
Stage 4
7-8 mm
Stage 5
8-10 mm
Stage 6
Mature flowers
b
c
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e
f
g
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i
j
k
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n
o
p
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Figure 7. In situ localization of SPP2 transcripts. a. Developmental stages of *S. chacoense* flowers used in this study. Stages 4 and 5 represent flowers 2 days and 1 day before anthesis (flower opening) and stage 6 represents flowers on the day of anthesis. b. Longitudinal section of a very young flower just before carpel fusion. Anti-sense probe. c. Longitudinal section of a very young flower after carpel fusion. Anti-sense probe. d. Transverse section of a stage 1 flower. Anti-sense probe. e. Transverse section of a stage 1 flower. Anti-sense probe. f. Longitudinal section of a stage 1 flower. Sense probe. g. Longitudinal section of a stage 2 flower. Anti-sense probe. h. Longitudinal section of a stage 2 flower showing the complete absence of SPP2 mRNA expression in the transmitting tissue of the style. Anti-sense probe. i. Longitudinal section of a stage 2 flower. Sense probe. j. Longitudinal section of a stage 3 pistil. Anti-sense probe. k. Longitudinal section of a stage 3 pistil. Sense probe. l. Longitudinal section of a stage 4 pistil, two days before anthesis. The arrow represents the area where in most of the stage 4 pistil, the SPP2 mRNA expression has regressed. Anti-sense probe. m. Longitudinal section of a stage 4 pistil, two days before anthesis. Sense probe. n. Longitudinal section of a stage 5 pistil, one day before anthesis. The arrow represents the area, where in most of the stage 5 pistil, the SPP2 mRNA expression has regressed. Anti-sense probe. o. Longitudinal section of a stage 5 pistil, one day before anthesis. Sense probe. p. Longitudinal section of a stage 6 pistil, day of anthesis. The arrow represents the upper limit of SPP2 mRNA expression in mature pistil. Anti-sense probe. q. Longitudinal section of a stage 6 pistil, day of anthesis. Sense probe. r. Magnification of the ovules in a stage 6 pistil showing SPP2 expression in the micropylar region. Anti-sense probe. Digoxigenin labeling is visible as a blueish staining. All hybridizations used 10 μm thick sections and an equal amount of either SPP2 sense or antisense probe. Scale bars represent 100 μm.

cropylar region, where the pollen tube will enter the ovule to deliver its two sperm nuclei and effect double fertilization (Figure 7r).

To confirm that SPP2 mRNAs are both disappearing and regressing from the tip of the style to the ovary during pistil development, an RNA gel blot analysis was conducted on styles and ovaries from three days before anthesis to two days after anthesis. Figure 8 shows that strong SPP2 mRNA expression is detected in samples extracted from ovaries at all developmental stages, while in the style, SPP2 mRNA levels were high until two days prior to anthesis and then decreased to undetectable levels by anthesis (Figure 8, left panels). When upper and lower halves of styles were collected separately two days before anthesis, SPP2 mRNA levels were already much stronger in the lower part, as expected from the corresponding *in situ* hybridization (Figure 7l). Furthermore, one day before anthesis, no SPP2 signal can be detected in the upper part of the style, while only a weak signal is detected in the lower part, thus fully confirming the pattern observed by *in situ* hybridization (Figure 7n).

Fertilization induces a localized accumulation of SPP2 mRNA in the ovary

Pollen tubes reach the embryo sac about 36 h after pollination, and fertilization occurs between 36 and 48 h after pollination in *Solanum* spp. (Clarke, 1940; Williams, 1955; and our unpublished observations). In pollinated pistils, the expression pattern of the SPP2 dioxygenase 48 h post-pollination shows a new area of mRNA expression, mainly in the parenchyma tissue of the interlocular space (Figure 9a and g). This pattern of expression was not seen in unpollinated mature pistils (Figure 7p and Figure 9f). To determine if interlocular space expression is a pollination-dependent event or a fertilization-dependent phenomenon, we also performed incompatible pollinations and collected the pistils 48 h after pollination (Figure 9c and h). No interlocular expression could be detected from incompatible pollinations, suggesting that this spatially restricted expression is strictly fertilization-dependent. In ovaries 96 h after pollination, strong SPP2 mRNA expression is now detected all over the ovary pericarp, as well as in the developing placenta, mainly in the region immediately adjacent to the ovules (Figure 9d). SPP2 mRNA expression can also be detected in the ovule's integument (Figure 9d, inset).

Discussion

Homologous dioxygenases show major differences in expression profiles

While searching for genes involved in pollen-pistil interaction and fertilization by subtractive hybridization, we have isolated a cDNA clone induced by pollination and fertilization that shares strong sequence similarity to 2-oxoacid-dependent dioxygenases found in plants. In a report on the preliminary characterization of genes predominantly expressed in tomato pistil tissues, Milligan and Gasser (1995) cloned a nearly identical gene, most probably the tomato homologue of SPP2, named TPP1 (tomato pistil-predominant). RNA gel blot analysis has shown that TPP1 is expressed in immature anthers to high levels, but is absent in mature anthers as found for SPP2. The TPP1 gene was also expressed in pistils and its amount did not vary significantly from flower buds to mature flowers. However, no TPP1 mRNA expression was detected in tomato roots and fruits, while SPP2 mRNA is expressed in mature roots (Figure 4B), in the pericarp of young developing fruits (4 days after pollination, Figures 4A

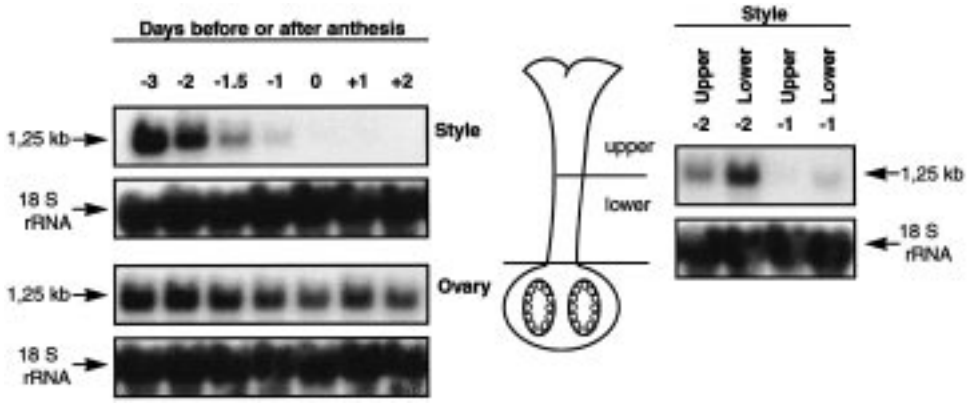

Figure 8. SPP2 mRNAs are regressing from the tip of the style to the ovary during pistil development. Left panel: SPP2 mRNA levels in styles and ovaries of unpollinated pistil tissues were analyzed by RNA gel blot analysis. Ten μg of total RNA from styles and ovaries from pistil three days before anthesis to two days after anthesis were probed with the 1.2 kb SPP2 cDNA insert. Right panel: RNA gel blot analysis of SPP2 mRNA levels in the upper and lower halves of styles from flowers two days (−2) and one day (−1) before anthesis.

and 9d) and in more mature fruits (15 and 40 days after pollination, Figure 5B). Furthermore, TPP1 was expressed as highly in stems as in pistils, while in *S. chacoense*, SPP2 is barely detectable in stems (Figure 4A). Similarly, while TPP1 expression in leaves and stems was equal, there is a 7-fold difference in SPP2 mRNA expression between leaves and stems in *S. chacoense*. Thus, despite the extremely high amino acid sequence identity between these two genes (94%), which suggests that SPP2 is the potato homologue of TPP1, the overall expression profile of the two genes is quite different.

Developmental regulation of SPP2 expression in flowers

RNA gel blot analysis (Figure 5B) showed that SPP2 mRNA accumulation was high and stable during the period when flowers are responsive to pollination and fertilization, from the day before anthesis (if flowers are opened manually) to 3 DPA. In older senescing flowers, 4 and 5 DPA, SPP2 mRNA accumulation declined sharply to barely detectable levels. Although known to induce senescence and flower abscission, ethylene did not induce a decline in SPP2 mRNA levels (data not shown). The decrease in SPP2 mRNA levels seen in unpollinated senescing flowers thus seems to be independent of the action of ethylene.

In situ detection of SPP2 mRNAs during flower development revealed an unusual pattern of expression (Figure 7). In young flowers, from stages 1 to 3, SPP2 mRNAs are clearly expressed in the style and ovary cortex while being totally absent from the transmitting tissue and stigmatic region (Figure 7b, c, g, h and j). From stage 4 onward, the site of SPP2 expression regresses gradually from the tip of the style to the ovary. RNA gel blot analyses of whole and sectioned styles confirmed this pattern (Figure 8). On the day of anthesis, SPP2 mRNAs are strongly expressed only in the ovary cortex (epidermis and parenchyma) with a clear division zone between the style and the ovary (Figure 7p). If fertilized, the pistil will shed its style about three days after pollination, and the upper limit of SPP2 mRNA expression coincides with the abscission zone of the style. This pattern of gradual decline in SPP2 mRNA expression from the tip of the style to the ovary could be involved in the production of a gradient of a secondary metabolite involved in pollen tube growth and guidance. Also in mature flowers, weaker SPP2 expression can also be detected in the placenta, in the region closest to the ovules, and in the micropyle of the ovules (Figure 7r). Accumulation of a specific secondary metabolite in the micropylar region could also serve as an attractant molecule, guiding pollen tube growth towards the ovule.

Pollination, wounding and stress hormone treatments induce SPP2 mRNA expression in the ovary

After pollination SPP2 mRNA levels in the ovary increase gradually from the time of pollination until 48 h post-pollination (Figure 5A). Since pollen tubes only reach the ovary about 36 h after pollination, and since no SPP2 mRNA expression is detected in pollinated or unpollinated mature styles (Figures 6 and 8), pollen or pollen tubes (Figure 4A and D), the increase observed in pistil tissues is a localized response, in the ovary, triggered by the growth of the pollen tube at a distance

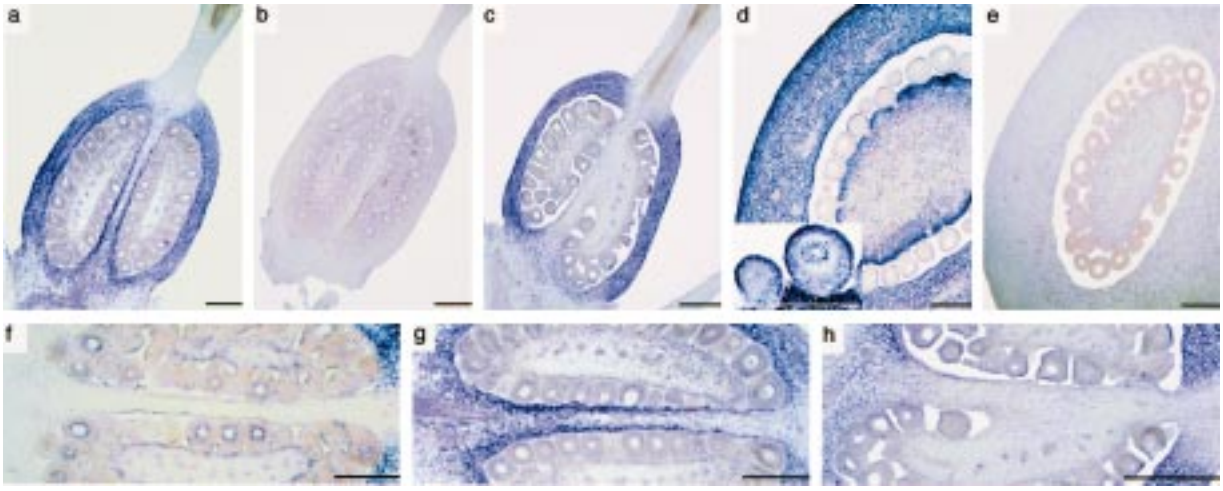

Figure 9. In situ localization of SPP2 transcripts in pistil following compatible and incompatible pollinations. a. Longitudinal section of a 2DPP pistel (compatible pollination) showing interlocular expression of SPP2 transcripts. Anti-sense probe. b. Longitudinal section of a 2DPA pistil. Sense probe. c. Longitudinal section of a 2DPP pistil (incompatible pollination) showing no interlocular expression of SPP2 transcripts. Anti-sense probe. d. Section through an ovary 96 h after pollination. Inset: magnification of the same section. Anti-sense probe. e. Section through an ovary 96 h after pollination. Sense probe. f. Magnification of the interlocular space from unpollinated pistil in Figure 7p. g. Magnification of the interlocular space from a compatible pollination in a. h. Magnification of the interlocular space from an incompatible pollination in c. Digoxigenin labeling is visible as a blueish staining. All hybridizations used 10 μm thick sections and an equal amount of either SPP2 sense or antisense probe. Scale bars represent 100 μm.

in the style. Pollen tube growth in the style is known to cause major cellular deterioration and ultimately death of the transmitting tissue (Cheung, 1996). Wang *et al.* (1996) observed by transmission electron microscopy (TEM) the vacuolation and the progressive deterioration of the transmitting tissue cells as pollen tubes grew in tobacco styles. Pollen tube growth in *S. chacoense* styles also induced a massive disorganization of the transmitting tract cells as observed by light microscopy and TEM (our unpublished observations). This pollination-induced cellular deterioration has previously been postulated to contribute to pollen tube growth by providing more nutrients than normally found in the extracellular matrix of the transmitting tissue of the style, and the release of calcium and other small molecules from the dead cells of the transmitting tissue have also been hypothesized to play a role in pollen tube guidance (Cheung, 1996). If injuries and cell death in the transmitting tissue of the style caused by the growing pollen tubes is the primary event that triggers SPP2 mRNA accumulation at a distance in the ovary, then wounding the style without pollination would also induce SPP2 mRNA accumulation. Indeed, wounding of the style also induced an identical response in SPP2 mRNA levels, at a distance in the ovary (Figure 6A).

To determine if stress hormones or elicitors known to induce wound or defense responses could be mediating the SPP2 mRNA increase in the ovary, flowers were locally sprayed or treated in an airtight chamber with volatile compounds. ABA, JA and MeJA are well known mediators of wound-responsive genes (Hildmann *et al.*, 1992; Wasternack and Parthier, 1997) while jasmonates and SA are involved in mediating defense gene expression (Reymond and Farmer, 1998). Surprisingly, ABA, thought to be a primary signal in the systemic wound-signaling cascade (Pena-Cortés *et al.*, 1995), had no effect on SPP2 mRNA levels (Figure 6B). A recent report suggests that ABA might be more important in the general maintenance of a healthy physiological state that enables the plant to respond to wounding (Birkenmeier and Ryan, 1998). *Trans*-2-hexenal, recently shown in *Arabidopsis* to induce a subset of the wound- and defense-response genes induced by MeJA, albeit with lower efficiency (Bate and Rothstein, 1998), had only a very weak inducing activity on SPP2 mRNA levels (Figure 6C). Of the stress hormones used, MeJA and JA were the most potent inducers (Figure 6B and C), mimicking the effect of pollination or wounding (Figure 6A). AA and SA also strongly enhanced SPP2 mRNA levels (Figure 6B) but were used at much higher concentrations, although these concentrations are routinely used

by other investigators. Although not a substrate for the octadecanoid pathway leading to JA and MeJA, AA is metabolized by a 5-lipoxygenase activity in potato tubers (Bostock *et al.*, 1992). It is not known if metabolites of AA are directly bioactive but, alternatively, AA may act through SA, since AA spray treatments of potato leaves induce the rapid and localized synthesis of SA (Coquoz *et al.*, 1995).

Although an inhibitory effect of SA on JA-regulated gene expression has been previously reported (Doares *et al.*, 1995), this is not always observed. Since AA, SA and jasmonates strongly induced SPP2 mRNA accumulation, this suggests that the SPP2 dioxygenase gene is regulated through both wound-inducible and defense response signaling pathways.

Fertilization induces the accumulation of SPP2 transcripts in a new area of the ovary

In order to test the possibility that fertilization could account for at least part of the increase seen from 36 h after pollination onward, we took advantage of the gametophytic self-incompatibility system in *S. chacoense* and analyzed by *in situ* hybridization the SPP2 mRNA profile following both compatible or incompatible pollinations (Figure 9). A new and highly localized accumulation of SPP2 mRNAs only occurred after fertilization (Figure 9a and g). Thus, following the expression induced by pollination, fertilization also triggers a localized accumulation of SPP2 mRNAs in the interlocular space. Since incompatible pollinations induced similar levels of SPP2 mRNAs in the ovary (Figure 4D), this highly localized accumulation of SPP2 mRNAs immediately after fertilization does not contribute significantly to the overall level of SPP2 mRNAs induced by pollination and wounding. Later during fruit development, SPP2 mRNA accumulation seems to be developmentally regulated (Figure 5B), and can be strongly detected in pericarp of the developing fruit and in the placenta in the region closest to the ovules (Figure 9d) as well as in the ovule's integument (Figure 9d, inset).

Dioxygenases are involved in multiple metabolic pathways and most probably derive from a common ancestral enzyme

Dioxygenases are nonheme iron-containing enzymes important in the biosynthesis of plant signaling compounds such as gibberellins, ethylene, abscisic acid as well as in the biosynthesis of secondary metabolites, mainly alkaloids and flavonoids (Prescott and John, 1996). Two distinct classes of plant dioxygenases are known: the lipoxygenases and the 2-oxoacid-dependent dioxygenases (2-ODD). This latter class of enzymes catalyzes many different chemical reactions including oxidation, hydroxylation, epoxidation and desaturation of various plant compounds. Although amino acid sequence identities of various 2-ODDs with different functions show 27–32% identity, intron positions in many of these are often conserved, suggesting a common origin for this highly diversified class of enzymes (Prescott and John, 1996). The SPP2 dioxygenase shares significant sequence identity with hyoscyamine 6β-hydroxylase (H6H) from *Hyoscyamus niger*, and, to a lesser extent, with Ids2 and Ids3 from barley (Figure 3). Interestingly, all three introns in these enzymes and in SPP2 are located at the same positions (Figure 1A), indicating a possible functional grouping of these enzymes as previously suggested (Prescott and John, 1996). Several alkaloids with structures similar to 1-hyoscyamine are also hydroxylated by H6H. Hyoscyamine, scopolamine and their derivatives are the major tropane alkaloids in the Solanaceae and scopolamine is a medicinally important anticholinergic drug present in several solanaceous plants. These alkaloids are the esters of tropane derivatives and tropic acid, which originate from ornithine and phenylalanine, respectively (Hashimoto and Yamada, 1997). Ornithine decarboxylase expression was recently shown to be transiently increased by pollination in tomato ovaries (Alabadi and Carbonell, 1998).

Many alkaloids are known to have anti-microbial or herbivore-deterrent properties (Kutchan, 1995). The fact that the SPP2 dioxygenase is strongly expressed in flowers, fruits, leaves and tubers might indicate a function in the biosynthesis of an alkaloid with defense properties that would also protect the reproductive organs of the plant as well as the berries that serve to disperse its seeds. Interestingly, the major differences in the expression pattern observed between SPP2 and its tomato homologue, TPP1, might reflect the fact that strong expression of TPP1 in tomato fruits could have been bred out during the selection of the cultivated tomato. This could be tested by comparing the expression profiles of SPP2 homologues in fruits of both cultivated and wild tomato and potato.

Alternatively, the role of SPP2 could be to generate secondary metabolite signaling molecules. Considering the pattern of expression of SPP2 in developing

pistils as well as after fertilization it is tempting to speculate that SPP2 generates such signaling metabolite(s) that could also serve as a cell differentiation marker. In mature ovaries, the upper expression limit coincides with the style abscission zone, consequently SPP2 expression could also act as a cellular delineation marker. Furthermore, the highly localized expression in the mycropyle suggests a possible role in pollen tube attraction and guidance.

Although thousands of alkaloids have been isolated, and many have been found to have medicinal values, their exact role in plants is still mostly unknown (Kutchan, 1995). Recruitment of an initially deterrent alkaloid into a molecule that could also have roles in reproduction cannot be excluded. Another class of secondary metabolites, the flavonoids, which are also the substrates of dioxygenases, are known to have roles in both plant defense, as phytoalexins and UV protectants, and in reproduction (Shirley, 1996). Flavonols are found in the pollen of many higher-plant species and are essential for pollen germination and promote pollen tube growth in some species (Vogt *et al.*, 1994; Ylstra *et al.*, 1994). Chalcone synthase mutants have been reported to be self-sterile in both maize and petunia (Mo *et al.*, 1992; van der Meer *et al.*, 1992; Ylstra *et al.*, 1994). As for the flavonoid secondary metabolites, alkaloids might also have specific functions during plant reproduction.

In order to understand the role of this wound-, pollination-, and fertilization-induced dioxygenase, transgenic plants either overexpressing the SPP2 transcript or expressing anti-sense SPP2 cDNA constructs are currently being produced and will be tested for their pollination behavior and fertility, as well as for their response towards insect herbivores.

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