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## Multiple *S* Gene Family Members Including Natural Antisense Transcripts Are Differentially Expressed during Development of Maize Flowers\*

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Within the large *Brassica S* gene family, *SLG* (*S* locus glycoprotein) and *SRK* (*S* locus receptor kinase) participate to the control of pollen-stigma self-incompatibility. In the self-compatible species maize, *S* gene family members are predominantly expressed in vegetative organs but are also expressed to a lesser extent in the stigma (silk). To determine if the expression of any *S* gene family members correlates with female receptivity, we analyzed their expression in developing maize silks. We show that a large family of maize *S* transcripts is expressed in developing silks. Surprisingly, we isolated a cDNA complementary to a large portion of the antisense strand of the maize receptor kinase *S* domain. Rapid amplification of cDNA ends (RACE)-polymerase chain reaction, RNase protection, and Northern hybridization with single-stranded riboprobes confirmed that natural antisense *S* transcripts exist in leaves and seedling shoots and in all sexual tissues tested except mature pollen. These natural antisense *S* transcripts co-exist with several less abundant sense *S* transcripts. The accumulation of sense and antisense *S* transcripts is differentially regulated during pollen and silk development. Thus, these results support a role for *S* gene family members in sexual tissue development and/or compatible pollination and reveal a new level of complexity in the regulation and function of the *S* gene family in maize.

glycoprotein (*SLG*) genes. *SRK* and *SLG* genes encode, respectively, a receptor-like protein kinase and a secreted glycoprotein that is highly similar to the extracellular domain of *SRK* (reviewed in Refs. 1 and 2). This common domain in *SRK* and *SLG* is called the *S* domain. A number of *S* domain-related sequences, unlinked to the *S* locus, have been found in the *Brassica* genome (3, 4), indicating that *SRK* and *SLG* genes are two members of a large gene family, the *Brassica S* gene family (or *S* multigene family).

Further studies have demonstrated that *S* domain-related sequences are not restricted to *Brassica*. To date, *S* gene family members that encode either secreted glycoproteins (reviewed in Ref. 1) or transmembrane receptor protein kinases (reviewed in Ref. 5) have been identified in monocots (maize (6, 7); rice (8)) as well as in dicots (*Brassica* (reviewed in Ref. 9); *Arabidopsis* (10, 11); carrot (12); *Ipomea* (13)).

Cell-cell signaling between pollen and stigma leading to the self-incompatibility response in *Brassica* has been extensively studied (reviewed in Ref. 9), and the individual biological functions of the various *S* locus genes in self-incompatibility have been elucidated. Gain-of-function experiments have shown that *SRK* alone determines the *S* haplotype specificity of the stigma and that *SLG* acts to promote a full manifestation of the self-incompatibility response (14). The gene encoding the pollen *S* determinant that would provide the ligand for the *SRK* receptor has also been identified; it is unrelated to *SLG* or *SRK* but belongs instead to a family of genes encoding pollen coat proteins. This gene, termed *SCR* (*S* locus cysteine-rich protein) is a single copy, *S* locus-encoded, anther-expressed gene (15–17).

In contrast to *SLG* and *SRK*, the biological functions of the other various *S*-like genes are still largely unknown. Distinct patterns of expression have now been described for different *S* gene family members (reviewed in (1)). Different members are specifically expressed in reproductive tissues in self-incompatible as well as self-compatible species, but others are expressed predominantly in vegetative structures, so that it is obvious that the *S* gene family may have diverse roles in plants.

Among the *S* gene family, genes expressed in reproductive tissues are thought to play essential roles in pollination (reviewed in Ref. 1) and recent data support this hypothesis. In *Brassica*, *S* locus-related glycoprotein 1 (*SLR*<sub>1</sub>), not encoded at the *S* locus and specifically expressed in stigma, is involved in the pollen-stigma adhesion process (18) and interact specifically with new members of pollen coat proteins (19). Such *S* domain-related transcripts have been reported in self-compat-

Flowering plants have evolved self-incompatibility systems that prevent or substantially reduce the level of self-fertilization. In the genus *Brassica*, at least two stigmatically expressed genes at the self-incompatibility locus or *S* locus are required for the recognition and the rejection of self pollen by the stigma: the *S* locus receptor kinase (*SRK*)<sup>1</sup> and the *S* locus

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ001485 and AJ001486.

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<sup>1</sup> The abbreviations used are: *SRK*, *S* locus receptor kinase; *SLG*, *S* locus glycoprotein; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; kb, kilobases(s); nt, nucleotide(s); ORF, open

reading frame; *SLR*, *S* locus-related glycoprotein; *ARK*, *Arabidopsis* receptor kinase.

ible species such as *Arabidopsis* (20) and maize (6, 7). The *S* gene family in maize includes an *S* receptor kinase, *ZmPK<sub>1</sub>* (for *Zea mays* protein kinase), and three additional *S* domain-related genes without the protein kinase domain, *ZmSLRs* (for *Z. mays S* locus related); all are predominantly expressed in vegetative organs. One or more of these *ZmSLR* genes is also expressed to a lesser extent in the maize stigma, the silk (6, 7).

For pollination success, the basic requirements are pollen viability and female receptivity. In maize, although parameters of pollen quality have been extensively described (21), little information is available concerning female receptivity at the molecular level. Female receptivity seems to rely principally on stigma receptivity, which corresponds to a precise developmental window of the silk (22). Here, our objective was to analyze the structure and expression of the *S* gene family in developing maize silks to determine if this gene family was related to the acquisition of female receptivity and further processes implicated in pollination success. We show that a large number of maize *S* transcripts are expressed in developing silks and that some of them are more highly expressed at the maximum of female receptivity. The cloning of the corresponding cDNAs surprisingly led to the isolation of a cDNA complementary to a large portion of the antisense strand of the *S* domain of *ZmPK<sub>1</sub>*, suggesting the presence of natural antisense *S* transcripts. Further experiments were designed to assess antisense *S* RNAs in maize tissues and to clarify the origin of these molecules. These experiments confirmed that several *S* loci encode antisense RNAs. We show that the antisense *S* transcripts co-exist with sense *S* transcripts. The developmental expression patterns of sense and antisense transcripts in male and female tissues show distinct cellular and spatial distribution, demonstrating a high level of complexity in the regulation and function of the *S* gene family in maize.

#### EXPERIMENTAL PROCEDURES

**Plant Material**—Four maize (*Z. mays* L.) inbreds were used in this study: A188, B73, F564, and F546. These belong to different heterotic groups; A188 is from Minnesota 13 complex, B73 is from Reid Yellow Dent, F564 is from European flint, and F546 is derived from lines tracing back to 50% European flint and 50% Reid Yellow Dent (23). The hybrid line DH5xDH7 used for microspore and immature pollen isolation was derived from Chinese stocks (24). Maize plants were grown to flowering in a growth chamber with a 16-h illumination period (700  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) at 24/19 °C (day/night) and 80% relative humidity.

Maize seedlings were germinated in the dark on damp filter paper at 25 °C for 6 days. Shoots and roots were then quickly excised from the grain, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

**Sexual Tissues Staging and Harvest**—Ear development of the A188 maize line was defined relative to female receptivity. This physiological parameter was assessed from seed-set after controlled hand pollination performed in the growth chamber with non-limiting quantities of high quality pollen. High quality pollen corresponds to mature pollen collected at anthesis, whose water content was at least 55% (w/w) of its fresh weight (21). Under these conditions, female receptivity is the major factor involved in seed-set. Ear developmental stage also was estimated using the sensitive morphological index "external silk length" defined by Dupuis and Dumas (22), i.e. the maximum length of the silks protruding from the husks (Fig. 1A). The female receptivity pattern of A188 maize line is shown in Fig. 1B. Accordingly, four main ear developmental stages have been defined: an immature non-receptive stage (before silk emergence from the husks), an immature partially receptive stage (external silk length from 2 to 12 cm), a mature fully receptive stage (external silk length from 12 to 18 cm), and a senescent non-receptive stage (external silk length above 20 cm). Total silks from ears at these different receptivity stages were harvested, immediately frozen in liquid nitrogen, and stored at -80 °C until analysis.

The developmental stages of immature pollen within anthers in the tassel branches were assessed cytologically using topographical staining (25) as previously used (26) to evaluate number of nuclei, state of vacuolar system, and starch storage. Anthers were then quickly dissected from selected tassel fragments, immediately frozen in liquid

nitrogen, and stored at -80 °C until analysis.

Four different stages of immature pollen were isolated from anthers of selected spikelets of DH5xDH7 tassels. Microspores were isolated as described by Gaillard *et al.* (27). Bicellular, early-tricellular, and mid-tricellular pollen were isolated according to Gagliardi *et al.* (28). All these samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

Mature pollen was collected by shaking tassels at anthesis over aluminum foil. Only pollen with the same quality criterion as for pollination (see above) was used. This pollen was immediately frozen in liquid nitrogen and stored at -80 °C until analysis.

**RNA Isolation and Analysis**—RNA was extracted from a range of tissues by various methods according to its final use. When large amounts of poly(A)<sup>+</sup> RNA were required for the analysis of *S* family transcripts from silks at different stages of development or for constructing a silk cDNA library, total RNA from 10–20 g of material for each desired developmental stage was extracted by the large scale method recommended by McCarty (29). Then poly(A)<sup>+</sup> RNAs were selected by two rounds of affinity chromatography on oligo(dT)-cellulose (30). For analyses by ribonuclease protection or Northern blot with riboprobes, total RNA from approximately 1–2 g of sexual or vegetative tissues was isolated using guanidinium hydrochloride-phenol-chloroform extraction according to the procedure of Gurr and McPherson (31).

For Northern blot analysis, either poly(A)<sup>+</sup> RNA (10  $\mu\text{g/lane}$ ) or total RNA (25  $\mu\text{g/lane}$ ) was separated on denaturing 1.5% agarose, 2.2 M formaldehyde gels and stained with ethidium bromide to ensure that equal amounts of RNA had been loaded. The RNA was then capillary-blotted to nylon filters for hybridization (Hybond N, Amersham Pharmacia Biotech). Equal transfer was monitored by visualizing ethidium bromide stained RNA on filters. Double-stranded DNA probes were prepared using a random priming DNA labeling kit (Roche Molecular Biochemicals), and single-stranded RNA probes were transcribed from linearized DNA plasmids using T7 RNA polymerase (Promega). Filters were prehybridized and hybridized at 42 °C for DNA probes and 60 °C for RNA probes in 50% formamide, 6 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, and herring sperm DNA (100  $\mu\text{g ml}^{-1}$ ). Filters were washed under standard conditions for DNA probes (1 $\times$  SSC, 0.1% SDS at 65 °C) and at high stringency for RNA probes (0.1 $\times$  SSC, 1% SDS at 70 °C). Hybridization signals were detected by autoradiography on Kodak X-Omat films at -80 °C. When RNA probes were used, the blots were imaged with a Molecular Dynamics  $\beta$ -PhosphorImager, and the signal intensities were quantified using ImageQuant software package (Molecular Dynamics).

**cDNA Library Construction and Screening**—To construct a mature silk cDNA library, poly(A)<sup>+</sup> RNA (6  $\mu\text{g}$ ) was extracted from total silks of one mature fully receptive ear with 16-cm external silk length (see above), treated by digestion with RNase-free DNase (Roche Molecular Biochemicals) to remove any contaminating DNA, and processed for directional cloning into  $\lambda$  ZapII (Stratagene). After *in vitro* encapsulation, approximately  $3 \times 10^5$  recombinant bacteriophages were amplified to produce the working library. Approximately  $10^6$  recombinant clones were screened with a <sup>32</sup>P-labeled DNA probe derived from the extracellular *S* domain of *ZmPK<sub>1</sub>* (6, 7). Hybridizing clones were plaque-purified, rescued as recombinant phagemids in pBluescript II SK (-) by *in vivo* excision, and sequenced.

**DNA Isolation and Analysis**—Genomic DNA was extracted from the leaves of 10-day-old maize plantlets. Tissue was frozen in liquid nitrogen and ground with a mortar and pestle until a fine powder was obtained. DNA was then extracted using the method of Rogers and Bendich (32). For Southern blot analysis, genomic DNA (10  $\mu\text{g}$ ) was digested with appropriate restriction enzymes and separated on 0.8% agarose gels (30). Fragments were capillary-transferred in 0.4 M NaOH onto Hybond-N<sup>+</sup> (Amersham Pharmacia Biotech) for hybridization. Ethidium bromide staining was used to check that similar amounts of DNA were loaded and that transfer was efficient. Double-stranded DNA probes were prepared by using a random primer DNA labeling kit (Roche Molecular Biochemicals). Filters were prehybridized, hybridized, and washed as for Northern blots with DNA probes.

**Isolation of Partial Genomic Clone of *ZmPK<sub>1</sub>***—Genomic *ZmPK<sub>1</sub>* sequences of inbred line A188 were obtained by PCR amplification with primers designed from the *ZmPK<sub>1</sub>*-B73 gene sequence: S (5'-CATCA-GACGGGACATTC-3') within the coding strand of the *S* domain (nucleotides +149 to +165 from ATG start codon) and K (5'-TTTGAT-CAGTGTCTTGCTTG-3') within the non-coding strand of the kinase domain (nucleotides +2367 to +2347 from ATG start codon). Amplification was carried out with AmpliTaq DNA polymerase according to the instructions of the supplier (Perkin-Elmer) for 30 cycles at 94 °C for 45 s



for denaturation, 55 °C for 1 min for annealing, and 72 °C for 2.5 min for polymerization. The 2.2-kb PCR product obtained was directly cloned in the plasmid vector pCR II (TA-cloning kit, Invitrogen).

**Sequence Analysis**—Sequencing was performed by the dideoxynucleotide chain termination method (33) using the Sequenase system (version 2.0, U. S. Biochemical Corp.) and custom-synthesized oligonucleotides. Sequence data were analyzed with Lasergene sequence analysis software (DNASTAR, London, UK) and compared with EMBL and GenBank® data bases using the BLAST algorithm. The nucleotide sequences reported here have been given the accession numbers AJ001485 and AJ001486.

**Rapid Amplification of cDNA Ends (RACE)-PCR**—RACE-PCR amplification of 3' ends was carried out using mRNA extracted from various tissues and reverse transcribed in first-strand cDNA synthesis primed from an oligo(dT) primer (first strand cDNA synthesis kit, Stratagene). Subsequent PCR amplifications employed an oligo(dT) primer in combination with the AS primer (5'-GTTCTGGTCAGGGTCTG-3') designated from the non-coding strand of *ZmPK<sub>1</sub>* (nucleotides +654 to +637 from ATG start codon). These amplifications led to the specific amplification of a cDNA derived from antisense *S* transcripts. After a first amplification for 30 cycles (1 min at 94 °C, 1 min at 55 °C, 3 min at 72 °C), an aliquot of 1 μl was used for reamplification in the same conditions with AmpliTaq DNA polymerase according to the instructions of the supplier (Perkin-Elmer). The amplification products were shown to be derived from *S* transcripts by Southern blotting and hybridization with the *S* domain probe derived from *ZmPK<sub>1</sub>*.

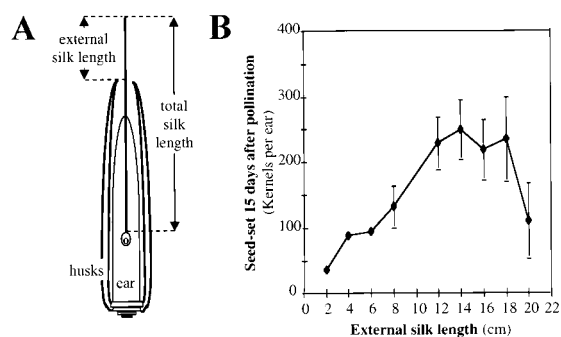
**Ribonuclease Protection**—Linearized plasmids were transcribed *in vitro* with T7 RNA polymerase (Promega) in the presence of [ $\alpha$ -<sup>32</sup>P]UTP, yielding strand-specific probes. The probes were gel-purified, and a 100,000 cpm aliquot was used in hybridizations with 100 μg of total RNA from a range of tissues using the Guardian™ RNase protection assay kit (CLONTECH). After digestion with 300 ng of RNase A and 5 units of RNase T1 (1:100 dilution of the provided RNase mix), the protected fragments were separated on a 5% acrylamide denaturing gel. *In vitro* transcribed perfect RNA™ marker template mix (Novagen) was used as a size standard.

## RESULTS

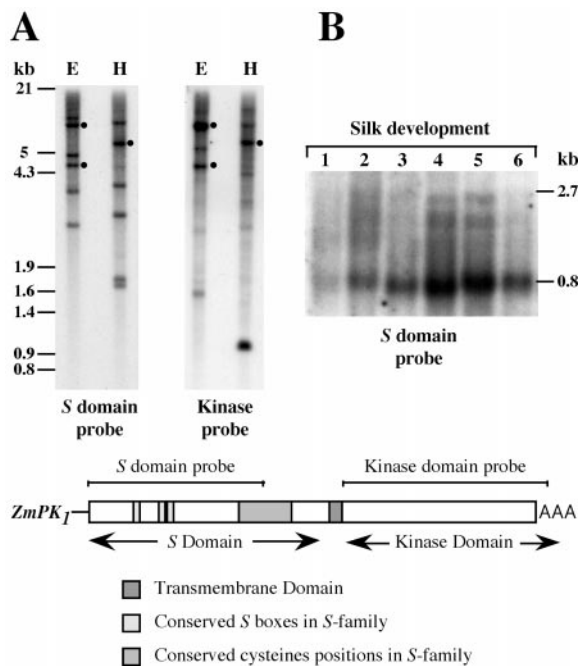
**The *S* Gene Family in the Maize Inbred Line A188**—Previous work devoted to the characterization of the *S* gene family in maize (7) was carried out using the line B73. Because B73 is genetically highly divergent from the inbred line A188 (23), which is commonly used for sexual reproduction studies (34), we first analyzed the structure and expression of *S* gene family members in silks of A188 (Fig. 2).

Genomic Southern blot analysis of A188 reveals a more complex *S* multigene family (Fig. 2A) than previously described in B73 (7). A higher number of restriction fragments hybridize with an *S* domain probe derived from *ZmPK<sub>1</sub>*, suggesting the presence of extra *S*-related genes. For example, three restriction fragments hybridize with both *S* domain and kinase domain probes, indicating that at least one other *S* receptor kinase in addition to *ZmPK<sub>1</sub>* is present in A188.

Northern blot analysis of silk mRNAs was conducted to analyze whether the accumulation of *S* domain-related transcripts during development correlated with the acquisition of female receptivity (see "Experimental Procedures, Sexual Tissues Staging and Harvest" and Fig. 1). Poly(A)<sup>+</sup> RNA was isolated from silks of ears at four developmental stages (immature non-receptive stage, immature partially receptive stage, mature fully receptive stage, and senescent non-receptive stage) and was analyzed by Northern blot using the *S* domain probe derived from *ZmPK<sub>1</sub>*. As shown in Fig. 2B, the probe detected one major *S* domain-related transcript of 0.8 kb at a level varying with silk receptivity. A set of diffuse bands of hybridization ranging in size from 0.8 to 3 kb was detected in partially and fully receptive silks. Only one band of ~0.8 kb was observed in immature and senescent non-receptive stages. Maximal levels of *S* domain-related transcripts were detected in silks of fully receptive ears. Throughout silk development, the levels of *S* domain-related transcripts were very low, and 10 μg of poly(A)<sup>+</sup> RNA was required for their visualization. This



**FIG. 1. Determination of female receptivity.** A, schematic representation of a maize ear. Ear developmental stage is estimated using the sensitive index external silk length defined by Dupuis and Dumas (22), *i.e.* the maximum length of the silks protruding from the husks. O, ovary. B, female receptivity of A188 maize line assessed 15 days after pollination by seed-set relative to ear developmental stage estimated as described in A. Bars represent S.D.



**FIG. 2. Structure and expression of *S* gene family in maize.** A, genomic Southern blot analysis of the *S* gene family in maize inbred A188. Each lane contains 10 μg of maize genomic DNA digested with either *Eco*RI (E) or *Hind*III (H). Identical Southern blots were, respectively, hybridized with *S* domain or kinase domain DNA probes. Dots indicate restriction fragments that hybridize with both probes. Numbers at the left represent molecular length markers in kilobases. B, *S* gene family expression in maize silks at several stage of female receptivity. *S* domain-related transcripts accumulation was analyzed by Northern blot hybridization with *S* domain double-stranded DNA probe. Each lane contained 10 μg of poly(A)<sup>+</sup> RNA isolated from silks at different stages of development: 1, immature non-receptive stage (5-cm long ear with total silk length less than 4 cm); 2–3, immature partially receptive stage (2, silk just emerging from husks; 3, ear with external silk length of 9 cm); 4–5, mature fully receptive stage (4, ears with external silk length of 12 cm; 5, ears with external silk length of 17 cm); and 6, senescent non-receptive stage (ear with external silk length of 21 cm). Numbers at the right represent the size of transcripts in kilobases. The probes used in panel A and B are derived from *ZmPK<sub>1</sub>* cDNA (7) and are shown diagrammatically in the lower part of the figure in relation to *ZmPK<sub>1</sub>-B73* mRNA (accession number X67733).

low abundance was already noted for *ZmPK<sub>1</sub>* and *ZmSLRs* (6, 7). Reprobing of the same blot with a *ZmPK<sub>1</sub>* kinase domain probe (data not shown) indicated that *ZmPK<sub>1</sub>* transcripts correspond to the 2.7-kb band and are faintly expressed in mature silks.

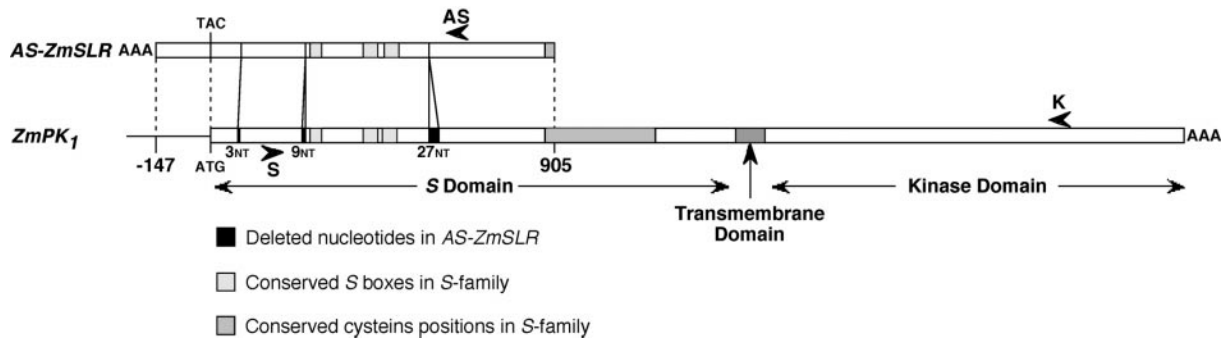


FIG. 3. Schematic representation of sequence homology between silk *AS-ZmSLR* cDNA and *ZmPK<sub>1</sub>* gene. The silk *AS-ZmSLR* cDNA is compared with the *ZmPK<sub>1</sub>* mRNA. Numbering is relative to the ATG translation initiation codon of the *ZmPK<sub>1</sub>* gene (accession number X67733, overlapping region extending from nucleotide 1580 to nucleotide 2631). Homologous regions are boxed and identically shaded; positions of the deleted nucleotides in *AS-ZmSLR* are noted on the *ZmPK<sub>1</sub>* sequence. The positions of the primers S, K, and AS used for partial *ZmPK<sub>1</sub>-A188* genomic clone isolation or RACE-PCR experiments are marked by arrowheads.

**Isolation of a Silk S Domain-related cDNA Clone**—To further characterize maize silk S domain-related RNAs, a cDNA library was generated from RNA of mature silks and screened with the S domain probe derived from *ZmPK<sub>1</sub>*. From 11 cDNAs hybridizing with the probe, the two that gave the strongest signals were further purified and were found to represent the same gene. This silk S-related cDNA clone was not an S receptor kinase because no kinase domain was present in the sequence. This clone (accession number AJ001485) contains a 1029-nucleotide sequence and a poly(A) tail. Computer analysis revealed that this silk cDNA corresponds to a large portion of the antisense strand of S domain of *ZmPK<sub>1</sub>*, the overlapping region extending from 147 nucleotides upstream of the ATG initiation codon to 905 nucleotides downstream.

An alignment of the silk S domain-related cDNA and *ZmPK<sub>1</sub>* is schematically drawn Fig. 3. The nucleotide sequence of the silk S domain-related cDNA has some distinguishing features of the S domain, *i.e.* conserved S boxes and the first two conserved cysteines, but was interrupted by 3-, 12-, and 27-nt deletions (Fig. 3). 42 punctual mutations were also noticed.

To demonstrate that the newly described cDNA was not transcribed from an allele of *ZmPK<sub>1</sub>* in the A188 maize line, the authentic *ZmPK<sub>1</sub>* gene was isolated from A188. Genomic DNA of A188 was PCR-amplified with two specific primers, one (S) within the S domain, the other (K) within the kinase domain (see Fig. 3) to generate a 2.2-kb fragment of the *ZmPK<sub>1</sub>-A188* gene (accession number AJ001486). Cloning and sequencing of this partial genomic clone revealed the presence of all the hallmarks of the *ZmPK<sub>1</sub>* gene: serine/threonine protein kinase signature, transmembrane domain, S domain with conserved S boxes, and the array of 12 conserved cysteines. The nucleotide and amino acid sequences predicted to be encoded by the S domain of *ZmPK<sub>1</sub>-A188* clone were, respectively, 98.7% identical and 99.3% similar to *ZmPK<sub>1</sub>-B73* (data not shown). Moreover, this partial genomic clone shared with *ZmPK<sub>1</sub>-B73* the same 12-nt and 27-nt sequences that were deleted in the silk S domain-related cDNA and 22 point mutations in the overlapping region (data not shown). This strongly indicated that the silk cDNA isolated here did not arise from the transcription of the antisense strand of *ZmPK<sub>1</sub>* but rather from another member of the *ZmSLR* gene family. This silk S domain-related cDNA will be designated *AS-ZmSLR*, for *anti-sense-Z. mays S* locus related, “antisense” referring to a direction of transcription opposite from that encoding the *ZmPK<sub>1</sub>* protein.

**Sequence Analysis of AS-ZmSLR Silk cDNA**—The entire *AS-ZmSLR* silk cDNA sequence is presented in Fig. 4. The *AS-ZmSLR* transcript does not exhibit typical features of eukaryotic mRNAs. The predicted first translation initiation codon in *AS-ZmSLR* cDNA occurs at position 53, but only a

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cacaaacctgaaattgcaaggctgggtcattactaccattgaaactgaccatgaacca 60
tctgaatcgcttcaggctgtacagacgaagatggatcatcagggtcaagagttagccttctc 120
ttgatgcttggccctacgtcggaggccacaagcctggatcatcagcaagctgctcgag 180
tcaagcaccctgtatcagtaagcattcctaactcgtacggttatactggttccgacca 240
tcctgtagatgttctggtcagggtctggccaggtatattgctgagactgggcaagctcg 300
ctgaagcagaagataagttaccaggactacgcgattgggtgtgggactaatctggctc 360
gcagcagctGATGagcTGCCTCGGCAGGAAAGTTCGTTGGGGAATCAAACCTCTGCCAT 420
M S C V G R K V F V G E S K L C H
ACAGTGTACCTCCTGAGTCTCGATGACGAGGTTCCAGTGTTCAGGACCCGAGCAGCG 480
T V L P P E S S M T R F P V F R S R A R
TGGACACCCGGTGAAGTTGTCATCAGCTGCCACACCGGTCGCTGCTGATCgggtg 540
W T P V K L L P S A R H T A A S S .
agCACCATGtTGTATCCTTTTTCAGGGTTAGAGCCGACCTCTGCATGGCAGGGCGG 600
M L S S F C R V R A D L L A W T G R
TCAAGTTTTCGCTCCACACGATGCTTGTGTCGCCGCCCTTTCAGTACCATACTGAG 660
S R F A L H T M V L L A A A F E Y H T E
AATGTGAGGCGATGGGTGTAGacttcatagaagccagagagaatgctcccgctcggatgat 720
N V K A W V .
tgtaaggcgtgattcttaggactcgacttggagagagaaccaggtgtaggatgtct 780
caggatgatcagctcttggaaatagagcgaaggaggagagatggcagcagtggaagg 840
agagctgcaagaggacgagacatcattgctgagatggggtttggtgatgaacgagcag 900
ggccagctactgtatcctggtatgccaggtttctgcttagctgatagcgaaacgc 960
cattactggtcaattcagagtgccatggaggtcaagttgtaagtttaaaaaaaaaa 1020
aaaaaaaa 1029

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FIG. 4. Silk *AS-ZmSLR* cDNA sequence. The nucleotide sequences are in lowercase letters. The sequences of longest predicted ORFs are given in the single-letter amino acid code. Putative translation initiation codons are underlined, and termination codons are dark gray-shaded and indicated within the ORF by dots. The sequence contexts surrounding the putative initiating methionine codon of the two ORFs are light gray-shaded, and they have been compared with the maize translation initiation consensus sequence (35). The predicted initiating methionine codon is underlined. Identity with the maize consensus sequence (G/A C/A C/G AUG G C/A G) is indicated by uppercase letters, with most frequent nucleotides underlined; mismatches are indicated in lowercase.

14-amino acid open reading frame (ORF) follows. Furthermore, 11 translation termination codons are present at frequent intervals in all three reading frames, precluding the existence of an appreciable ORF anywhere within the sequence. However, two putative short ORFs do exist, as shown in Fig. 4. The longest ORF present in the sequence, ORF<sub>1</sub>, would produce a 54-amino acid polypeptide beginning at position 370 and terminating at position 534. The deduced amino acid sequence of ORF<sub>1</sub> corresponds to a hydrophilic molecule with a predicted pI

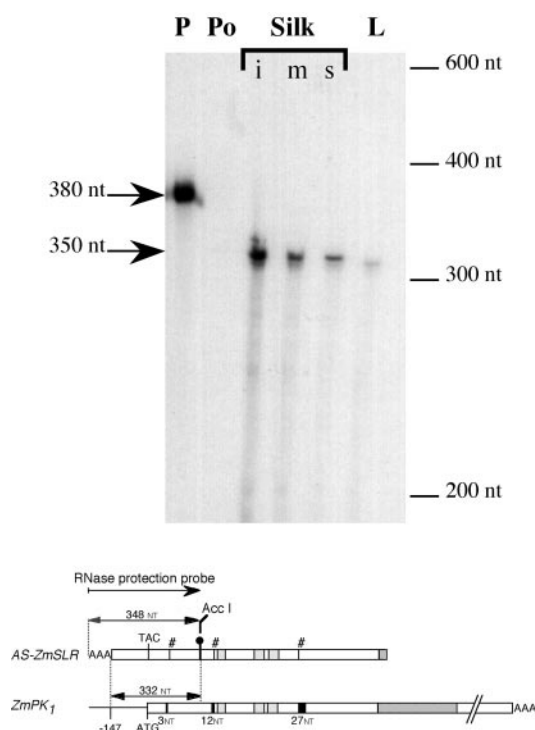
of 11.4 and molecular mass of 6 kDa. ORF<sub>2</sub> begins at position 547 and extends for 44 amino acids before terminating at position 681. Similar to ORF<sub>1</sub>, ORF<sub>2</sub> predicts a small hydrophilic molecule with a pI of 9.2 and molecular mass of 5 kDa. Neither ORF is predicted to encode an N-terminal hydrophobic signal sequence typical of secreted proteins, and a search of the DNA and protein data bases revealed no significant homology between the ORFs and any previously characterized sequence.

To determine if the ORFs of *AS-ZmSLR* could be translated *in vivo*, we compared the sequence context surrounding their putative initiating methionine codon with the AUG context consensus sequence for effective translation initiation in maize (35). As presented Fig. 4, neither of the AUG codons is contained within the ideal context for use as a translation initiator. Nevertheless, the relaxed requirements for AUG context in plants (36) and the fact that the translation initiation of *ZmPK<sub>1</sub>* (A C G AUG c C t) also fails to conform to the consensus sequence context (G/A C/A C/G AUG G C/A G) but retains the ability to initiate translation effectively (37) do not preclude the possibility that the *AS-ZmSLR* ORFs could be translated *in vivo*.

**Antisense *AS-ZmSLR* Transcripts Are Expressed in Both Reproductive and Vegetative Tissues**—To confirm the existence of *AS-ZmSLR* transcripts in maize plants, we performed further experiments with methods giving directional information about *S* transcripts. Because *AS-ZmSLR* appears to encode a polyadenylated RNA, we first performed 3' RACE-PCR using RNA from mature silks, where *AS-ZmSLR* expression was expected, but also seedlings and mature pollen. Polyadenylated RNA samples from those three tissues, treated with DNase I to remove any residual genomic DNA, were reverse-transcribed from their poly(A) tails using an oligo-dT primer and then amplified by 3' RACE PCR using an oligo(dT) primer in combination with the AS primer, indicated in Fig. 3. Such a PCR amplification was expected to generate an 800-base pairs PCR product uniquely from antisense transcripts of the *AS-ZmSLR* gene. The result of this PCR amplification was that the expected 800-base pairs PCR product was amplified from mature silk and to a less extent seedling RNA, although not from mature pollen RNA (results not shown). This indicates that the *AS-ZmSLR* gene is transcribed to generate antisense *S* transcripts in silks and seedlings but not in mature pollen.

To compare the levels of *AS-ZmSLR* antisense transcripts in different plant tissues, RNase protection assays were used. A single-stranded riboprobe that had the same sequence as a part of the sense strand of the *AS-ZmSLR* cDNA, represented in Fig. 5, was generated by *in vitro* transcription, and the results of an RNase protection assay performed with this probe are shown in Fig. 5. The presence of a band at the 350-nt position in any track indicates the existence of an exactly matching, complementary RNA sequence in the RNA sample analyzed. This 350-nt fragment was protected by RNA samples from maize silks over three developmental stages and from leaves but not by RNA from mature pollen. These results confirm the findings of 3' RACE-PCR analysis: that antisense *AS-ZmSLR* transcripts are present in silks and vegetative tissue, but not in pollen. They further indicate that the steady state level of antisense *AS-ZmSLR* transcript is highest in immature silks and declines as the silks mature and then senesce. Antisense *AS-ZmSLR* transcripts appear to be less abundant in leaves, as their level is lower than the lowest one during silk development.

**Sense *S* Transcripts Originating from *AS-ZmSLR* Gene Locus Are Co-expressed with Antisense *S* Transcripts**—To further analyze *S* gene transcription in maize, we try to map the sense

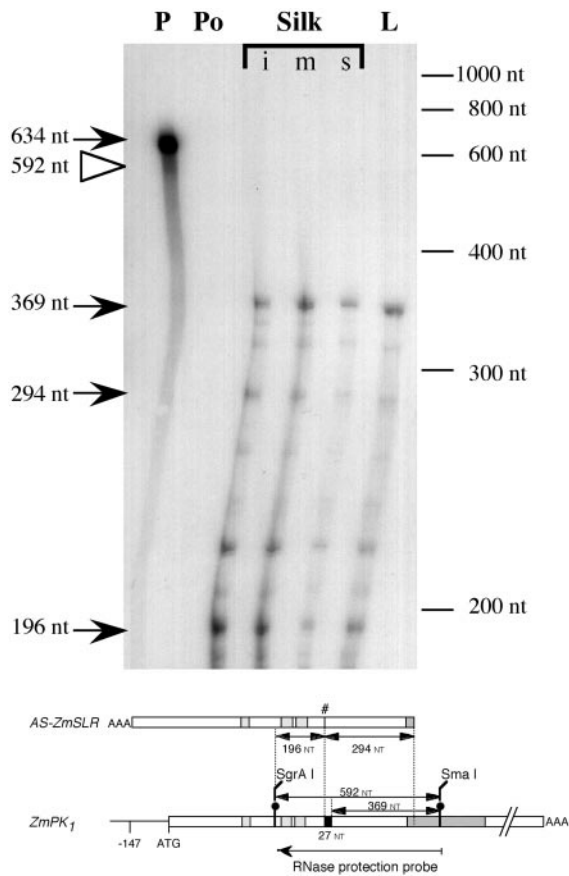


**FIG. 5. Ribonuclease protection analyses to test for the existence of antisense *S* transcripts in sexual and vegetative tissues.** A radiolabeled *AS-ZmSLR* RNA probe was hybridized to 100  $\mu$ g of total RNA isolated from mature pollen (*Po*) silks (*i*, immature non-receptive stage; *m*, mature fully receptive stage; *s*, senescent non-receptive stage) and leaf (*L*), digested with an RNase mix, and separated on a 5% sequencing gel. *P*, undigested probe. The autoradiograph was exposed for 3 days. The length in nucleotides of *in vitro* transcribed RNA size standards are indicated on the right, and the calculated sizes of probe and protected fragments on the left. In the lower part of the figure, the *AS-ZmSLR* and *ZmPK<sub>1</sub>* mRNAs are diagrammed, and the positions of the probe and the potential corresponding protected fragments are shown.

*S* transcripts using additional ribonuclease protection analyses. A single-stranded riboprobe identical to the antisense strand of *ZmPK<sub>1</sub>* was constructed by *in vitro* transcription of the *ZmPK<sub>1</sub>* cDNA. This probe contained a 27-nt insertion that was not present in the *AS-ZmSLR* sequence, which permitted this probe to discriminate between sense transcripts originating from *ZmPK<sub>1</sub>* or *AS-ZmSLR* gene loci. The results of RNase protection assays using this *ZmPK<sub>1</sub>* riboprobe with RNA samples from maize pollen, silks, and leaves are shown in Fig. 6. No band equivalent to the length of the region complementary between the riboprobe and *ZmPK<sub>1</sub>* sense transcripts, which would be 592 nt in length, was present at a detectable level in any of the tissues assayed. This indicates *ZmPK<sub>1</sub>* sense transcripts are absent from silks and leaves, where *AS-ZmSLR* have been shown (Fig. 5) to be present.

Although no *ZmPK<sub>1</sub>* transcripts could be detected in this RNase protection assay, bands corresponding closely to positions at 196 nt and 294 nt were protected from RNase digestion (Fig. 6). These protected bands occurred in tracks corresponding to all stages of silk development assayed and also to leaves, although not to mature pollen. These two band sizes are those that are expected from sense transcripts originating from *AS-ZmSLR* gene locus, which will entirely protect the *ZmPK<sub>1</sub>* riboprobe except over the 27-nt region that is not present in the *AS-ZmSLR* cDNA and over its 3' extremity, where *AS-ZmSLR* is truncated by comparison to *ZmPK<sub>1</sub>*. These results suggest, therefore, that sense transcription of *AS-ZmSLR* gene locus also occurs in maize silks and leaves in addition to antisense *AS-ZmSLR* transcripts whose presence was demonstrated in





**FIG. 6. Ribonuclease protection analyses showing that sense and antisense *S* transcripts could be *cis*-encoded at the same locus.** A radiolabeled *ZmPK<sub>1</sub>-S* domain RNA probe was hybridized to 100  $\mu$ g of total RNA isolated from a range of sexual and vegetative tissues (the same as in Fig. 5), digested with an RNase mix, and separated on a 5% sequencing gel. The autoradiograph was exposed for 7 days. The length in nucleotides of *in vitro* transcribed RNA size standards are indicated on the right, and the calculated sizes of probe and protected fragments are indicated on the left. The open triangle indicates the expected position of the protected fragment of *ZmPK<sub>1</sub>* transcripts. In the lower part of the figure, the *AS-ZmSLR* and *ZmPK<sub>1</sub>* mRNAs are diagrammed, and the positions of the probe and the potential corresponding protected fragments are shown. *Po*, mature pollen; Silks (*i*, immature non-receptive stage; *m*, mature fully receptive stage; *s*, senescent non-receptive stage); *L*, leaf; *P*, undigested probe.

the RNase protection assay (Fig. 5) and RACE-PCR experiments described above.

In addition to the 196- and 294-nt bands shown in Fig. 6, a prominent band at approximately 369 nt also appears in tracks corresponding to silk and leaf RNA. This cannot be protected by either sense *ZmPK<sub>1</sub>* transcripts, which are complementary to the riboprobe over 592 nt, or by the sense transcripts of *AS-ZmSLR* gene locus, which give bands of 196 and 294 nt. This band of 369 nt must, therefore, be ascribed to the sense transcript of an as yet uncharacterized member of the A188 maize *S*-gene family. It is striking that this fragment of approximately 369 nt would be produced by *AS-ZmSLR-like* transcripts that lack the 27-nt insertion characteristic of *ZmPK<sub>1</sub>* but which contain a region similar to the *ZmPK<sub>1</sub>* cysteine-rich region. This hypothetical protected fragment is indicated on the diagram presented in Fig. 6, aligned with the *ZmPK<sub>1</sub>* cDNA. Similarly, other bands ranging from 369 to 200 nt could be interpreted as sense transcripts of further *S*-family genes that partially protect the *ZmPK<sub>1</sub>* probe but which are truncated or divergent from *ZmPK<sub>1</sub>* at different points within the conserved cysteines region (5 conserved cysteines, 369 nt; 3 conserved cysteines, 320 nts; 1–2 conserved cysteines, 280 nt;

no conserved cysteines, 220 nt).

The RNase protection assays presented in Figs. 5 and 6 indicate that both antisense and sense transcripts sharing 100% identity with the *AS-ZmSLR* cDNA are present in silks and leaves of maize plants but not in mature pollen. This may be interpreted either as the bi-directional transcription of a single *AS-ZmSLR* gene or that more than one *AS-ZmSLR* gene is present and that different copies of this gene are transcribed in different directions to produce trans-encoded sense and antisense messages. An additional finding of these studies is that an uncharacterized gene closely similar, but not identical, to *AS-ZmSLR* and *ZmPK<sub>1</sub>* is transcribed to produce a sense transcript with a similar expression pattern to that of *AS-ZmSLR*. By contrast, no sense *ZmPK<sub>1</sub>* transcripts could be detected in pollen, silks, or leaves of maize plants by the use of RNase protection assays.

**Spatial Expression Pattern of Sense and Antisense *S* Transcripts in Maize**—Because ribonuclease protection analyses demonstrated a differential transcription pattern of *S* gene family in maize vegetative and sexual tissues, Northern blot analysis of leaves, seedling shoots and roots, mature silks, and pollen RNAs was conducted with strand-specific riboprobes to better define the spatial expression of *S* genes. This analysis was first conducted with the A188 inbred and then extended to three other maize lines. Except for slight quantitative differences, similar expression patterns were found in all lines. The general pattern observed is summarized in Table I. Antisense *S* transcripts were abundant in leaves and mature silks but absent from roots and mature pollen, whereas sense *S* transcripts were detected at a lower level in all the tissues tested. Northern analysis indicates that sense *S*-like transcripts do accumulate in mature pollen to a higher level than in other tissues. These are evidently not transcripts of the *ZmPK<sub>1</sub>* gene, as transcripts of that gene were shown to be absent in pollen by RNase protection analysis.

**Temporal Expression of Sense and Antisense *S* Transcripts during the Development of Sexual Tissues**—The developmental time course of *S* gene family expression and the size of sense and antisense transcripts produced in sexual tissues were examined in detail by Northern blot analysis with the same strand-specific riboprobes used for ribonuclease protection analyses (Fig. 7). Duplicate filters were hybridized, one with the probe specific for the antisense *S* mRNA (Fig. 7A) and the other with the probe specific for the sense *S* transcripts (Fig. 7B).

Antisense *S* transcripts are abundant in developing sexual tissues, because 25  $\mu$ g of total RNA and 1 day of autoradiographic exposure were sufficient to detect hybridizing bands (Fig. 7A). In contrast, sense *S* transcripts were much less abundant because they were barely detectable after 1 week of autoradiographic exposure (Fig. 7B). Two different transcripts hybridizing with the antisense-specific riboprobe derived from the *AS-ZmSLR* gene were expressed differentially during silk and anther development (Fig. 7A). High levels of a 1-kb transcript, corresponding to the predicted size of the cloned *AS-ZmSLR* cDNA, were detected in all developing sexual tissues except mature pollen. Slightly less abundant expression of a larger (2.8 kb) antisense transcript was detected in growing silks and in developing anthers but not in senescing silks and in tricellular or mature pollen. Because this larger antisense *S* transcript did not hybridize with the probe specific for antisense kinase transcripts (data not shown), it is not likely to represent transcription of the antisense strand of an *S* receptor kinase gene. The two antisense transcripts were also detected in leaves and seedling shoots but not in seedling roots; the 1-kb transcript was more abundant (data not shown).

TABLE I  
Expression pattern of antisense and sense S transcripts in maize

+, transcripts detected (the number of + symbols is qualitatively proportional to the level of transcript accumulation); -, no transcripts detected; ND, not determined.

Tissues	Maize inbred lines							
	Antisense S transcripts <sup>a</sup>				Sense S transcripts <sup>a</sup>			
	A188	B73	F546	F564	A188	B73	F546	F564
Leaves <sup>b</sup>	++++	++++	++++	++++	+	+	+	++
Shoots <sup>c</sup>	++	++	ND	ND	+	++	ND	ND
Roots <sup>c</sup>	-	-	ND	ND	+	++	ND	ND
Silks <sup>d</sup>	+++	+++	+++	+++	+	+	+	++
Pollen <sup>e</sup>	-	-	-	-	+++	++	++	+++

<sup>a</sup> Relative levels of antisense and sense S transcripts in a range of vegetative and sexual tissues as revealed by Northern blot hybridization with the same strand-specific RNA probes used for ribonuclease protection studies (see Figs. 3, 5, and 6).

<sup>b</sup> Youngest fully expanded leaves from a flowering maize plant.

<sup>c</sup> From 6-day-old germinated seedlings.

<sup>d</sup> Fully receptive mature silks.

<sup>e</sup> High quality mature pollen.

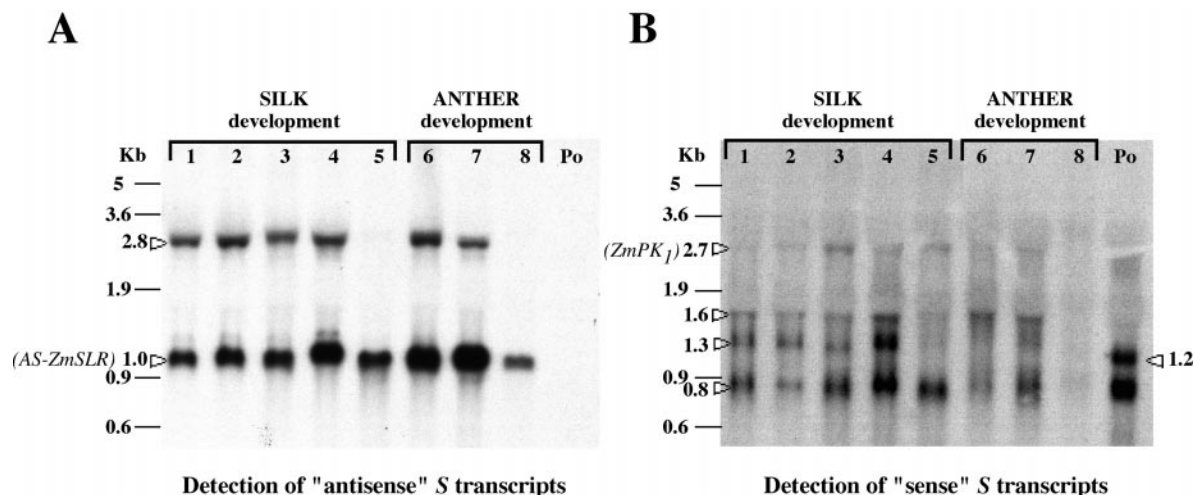


FIG. 7. Northern blot analysis of the temporal expression of antisense (A) and sense (B) S family transcripts during development of maize sexual tissues. Each lane was loaded with 25  $\mu$ g of total RNA isolated from silks at four developmental stages, from anthers containing immature pollen at three developmental stages, and from mature pollen: 1, silks from immature non-receptive ear (5-cm long ear with total silk length less than 4 cm); 2, silks from immature partially receptive ear (silk just emerging from husks); 3-4, silks from mature fully receptive ear (3, ears with external silk length of 13 cm; and 4, ears with external silk length of 15 cm); 5, senescent silks from non-receptive ear (ear with external silk length of 21 cm); 6, anthers containing uninucleate microspores; 7, anthers containing bicellular pollen grains; 8, anthers containing tricellular pollen grains; Po, mature pollen at anthesis. Strand-specific riboprobes used were the same as for RNase protection analyses, *i.e.* the same probe as in Figs. 5 (A) and 6 (B). Autoradiographs were exposed for 1 day (A) or 7 days (B). Numbers represent molecular length RNA markers and transcript sizes in kilobases.

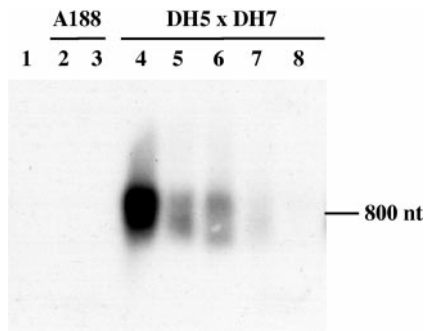
Quantification of the hybridizing signals indicated that the expression level of the larger antisense transcript was constant, whereas the intensity of the 1-kb transcripts was markedly tissue- and age-dependent. A basal level of *AS-ZmSLR* transcripts was detected in growing or senescing silks (Fig. 7A, lanes 1-3 and 5) and in anthers containing tricellular pollen (Fig. 7A, lane 8); a 2-fold increase of this basal level was detected in mature fully receptive silks and in anthers containing microspores (Fig. 7A, lanes 4 and 6). A 4-fold increase over the basal level was detected in anthers containing bicellular pollen (Fig. 7A, lane 7).

To determine if *AS-ZmSLR* transcripts originated from sporophytic tissues (anther) and/or from gametophytic tissues (developing pollen), 3' RACE-PCR was used on RNA from isolated gametophytes of the DH5xDH7 line and mature pollen from the A188 and DH5xDH7 lines (Fig. 8). Our results indicated that the accumulation of antisense S transcripts occurred in isolated gametophytes, the highest level being detected in microspores (lane 4), then declining in further stages of pollen development (lanes 5-7). Because this pattern of expression is different from that for developing anther (Fig. 7A), we expect that both sporophytic (anther) and gametophytic (pollen) tissues contribute differently to *AS-ZmSLR* transcript accumula-

tion during anther development. In addition, we confirmed the absence of antisense S transcripts in mature pollen, because no signal could be detected in cDNA synthesized either from total (Fig. 8, lanes 2 and 8) or poly(A)<sup>+</sup> (Fig. 8, lane 3) pollen RNA.

Fig. 7B shows that the sense-specific riboprobe derived from the *ZmPK<sub>1</sub>* gene detected a range of distinct transcripts differentially accumulated during silk and anther development. The 2.7-kb transcript corresponding to *ZmPK<sub>1</sub>* was barely detectable, with its highest level in mature, fully receptive silks. Three additional transcripts of 0.8, 1.3, and 1.6 kb in length were present in growing silks (Fig. 7B, lanes 1-4), whereas only the 0.8 band was revealed in senescing silks (Fig. 7B, lane 5). The 1.3-kb transcript appears to be silk-specific, as only the 0.8- and 1.6-kb hybridizing bands are detected in anthers containing microspores and bicellular pollen (Fig. 7B, lanes 6-7). The latest stage of anther development, where anthers contain tricellular pollen, did not accumulate any S transcripts (Fig. 7B, lane 8), contrary to mature pollen that showed a significantly higher abundance of sense transcripts (Fig. 7B, lane Po). Besides the common 0.8-kb transcript, a novel highly expressed sense transcript of 1.2 kb appeared to be pollen-specific. Furthermore, steady state levels of this sense transcript varied inversely to the antisense RNAs in that they were completely





**FIG. 8. RACE-PCR detection of antisense *S* transcripts in isolated male gametophytes at different developmental stages.** RNA was extracted from isolated male gametophytes or from mature pollen, reverse-transcribed in oligo(dT)-primed cDNA, then PCR-amplified with oligo(dT) and AS primers. After two rounds of amplifications, PCR products were detected on Southern blot by hybridization with *ZmPK<sub>1</sub>-S* domain double-stranded DNA probe. The inbred used is noted above the lane. 1, control amplification of non-reversed transcribed RNA extracted from isolated microspores; 2, mature pollen at anthesis, cDNA synthesized from total RNA; 3, mature pollen at anthesis, cDNA synthesized from poly(A<sup>+</sup>) RNA; 4, isolated microspores; 5, isolated bicellular pollen; 6, isolated early tricellular pollen; 7, isolated mid tricellular pollen; 8, mature pollen at anthesis.

absent from mature pollen. Taken together, these findings indicated that both receptive silk and mature pollen each had one tissue-specific sense *S* transcript. Northern analyses with total RNA and riboprobes gave clearer information concerning *S* gene expression in developing sexual tissues than Northern analysis with poly(A<sup>+</sup>) RNA and double-stranded DNA probes (Compare Figs. 2B and 7).

#### DISCUSSION

We have discovered novel members of the *S* gene family in maize and have shown that there are naturally occurring antisense *S* transcripts including *AS-ZmSLR*, whose molecular cloning and initial characterization we have performed. The expression of antisense *S* transcripts in maize is abundant and is not peculiar to the A188 maize line. Antisense *S* transcripts in maize co-exist with several less abundant sense *S* transcripts, and both are differentially regulated during pollen and silk development, some of them being tissue-specific. This work therefore greatly extends our knowledge about the *S* gene family in maize (7).

**Naturally Occurring *S* Antisense RNAs**—Naturally occurring antisense RNAs were first discovered in bacteria (38), and there is now ample evidence that posttranscriptional gene regulation via endogenous antisense transcripts controls diverse prokaryotic biological activities (39). Increasing numbers of naturally occurring antisense RNAs are also being found in a large variety of eukaryotic organisms, from slime molds to mammals (40–43). As these antisense RNAs are complementary to sense transcripts encoding proteins involved in extremely diverse functions and some of them are conserved between species, it strongly suggests that these endogenous antisense transcripts are not fortuitous and may play a general role in gene expression (40–43). To carry out this control, antisense transcripts may have diverse mechanisms of action and can control gene expression at several different levels: transcription (44, 45), processing or nucleocytoplasmic transport (46), mRNA modification (47), mRNA stability (48), and translation (49–52). Moreover, antisense transcripts can considerably vary in size; they may or may not be capable of encoding a protein, and they may be cis- or trans-encoded (40, 42, 43).

In plants, the first report of natural antisense transcripts concerned antisense  $\alpha$ -amylase RNAs in barley (53). Other

examples of endogenous antisense transcripts include the *Bz2* locus (54),  $\alpha$ -tubulin genes (55), and the *MuDR* regulatory transposon (56) in maize. Finally, at the *Brassica S* locus, a gene expressed only in anthers, *SLA* (57), and the *SRK* gene (58) have been shown to be transcribed in both directions. Very little attention has been paid to the roles of these endogenous antisense transcripts in plant gene expression (59) despite the deliberate use of exogenous sense or antisense RNA in transgenic plants for the down-regulation of specific genes, which sometimes also leads to transgene-induced gene silencing. Molecular mechanisms involved in such silencing still remain to be precisely defined (60–62). In some cases, endogenous genes appear to be silenced directly through the activity of antisense transcripts of a transgene (63, 64), whereas in other cases, the mechanism of silencing is more complex (65).

What are the roles of the antisense *S* transcripts? First, endogenous *S* antisense RNAs transcripts may have no natural function. However, this seems unlikely in the present one, because two different antisense *S* transcripts are abundantly expressed in maize vegetative and sexual tissues. Moreover, similar endogenous *S* antisense RNAs derived from the *SRK* and related genes were found to a lesser extent in *Brassica oleracea* (58). It therefore appears that antisense transcription associated with *S* gene family members represents an evolutionarily conserved feature. Similarly, antisense transcription has been conserved for the *bFGF* gene in *Xenopus*, chicken, rat, and humans (66–69), the *RAD10/ERCC-1* genes in yeast and humans (70), and the *c-myb* gene in chickens and humans (71), suggesting that antisense transcripts may be important for gene function.

Second, the antisense *S* transcripts might function by encoding protein(s). *AS-ZmSLR*, the major naturally occurring *S* antisense RNA in maize, has no long open reading frame and has some features characteristic of untranslated eukaryotic mRNAs. Nevertheless, our data do not address whether the two short *AS-ZmSLR* ORFs are translated *in vivo*. Recent papers have emphasized the role of oligopeptides in plant signaling (72, 73). For example, the gene *ENOD40*, expressed during the early stages of legume nodule development, was thought to act as a “riboregulator,” a novel class of untranslated RNA (74), until it was demonstrated that it encodes an oligopeptide of about 10 amino acids that probably has a primary role in the nodule organogenesis (75, 76).

Finally, antisense *S* transcripts might serve as natural antisense regulator RNAs of *ZmPK* and *ZmSLR* gene expression, either transcriptionally or post-transcriptionally. Because *AS-ZmSLR* is complementary to the 5' region upstream of the ATG of *ZmPK<sub>1</sub>* mRNA, it is possible that promoter occlusion could occur to prevent transcription. It is also interesting to note that *ZmPK<sub>1</sub>* was shown to be expressed predominantly in roots (6, 7) where we did not detect any antisense transcripts, suggesting a complementary pattern of sense/antisense regulation already reported for several genes in animals (44, 48, 77, 78). At least one set of antisense and sense transcripts was shown to be *cis*-encoded at the *AS-ZmSLR* locus, which would potentially allow regulation of accumulation of their respective messages. On another hand, it is likely that antisense *S* transcripts would not regulate splicing because *ZmPK<sub>1</sub>* has no intron (7). Further studies are required to determine how endogenous antisense *S* RNA transcripts could modulate the expression of *ZmPK* and other *ZmSLR* genes.

**Function of Multiple *S* Transcripts in Maize Flowers**—Despite their wide distribution in plants, precise functions of *S* gene family members have only been assigned for *SRK*, *SLG*, and *SLR* in *Brassica* (14, 18, 19, 79) and remain essentially speculative in other plant systems. The only other members of

the *S* gene family for which we have information concerning their possible function are two small families of *S* receptor-like kinases, *Arabidopsis* *ARK* (for *Arabidopsis* receptor kinase) involved in plant developmental processes (11, 80), and *Brassica* *SFR* (for *S* family receptor), involved in response to mechanical and biological attack (81). To propose putative functions of multiple *S* transcripts in maize flowers, we have compared our data concerning the structure of the cloned *S* members and their specific expression patterns with accumulated data from *Brassica* and other *S* gene family systems.

The structure of the cloned *AS-ZmSLR* silk cDNA appears to be similar to a small group of closely related *S* genes of the sub-family *SLR<sub>3</sub>* (*S locus* related 3), described in self-incompatible *B. oleracea* (82). These genes share with *AS-ZmSLR* a high similarity with the *S* domain of the corresponding receptor kinase and the fact that the putative encoded *S* protein differs from most other *S* family members by deletion of several of the highly conserved cysteine residues. In addition, *SLR<sub>3</sub>* and *AS-ZmSLR* genes show similar patterns of expression, being expressed in vegetative tissues as well as in stigmas and in young developing anthers. This *SLR<sub>3</sub>* sub-family is thought to arise from modification of receptor kinase genes by deletion for generation of genes encoding secreted *S* glycoproteins involved in several different cellular functions, perhaps as ancestral cell-cell communication systems adapted for multiple roles in the plant life cycle (82). Nothing is known on *SLR<sub>3</sub>* functions, but other secreted *S* glycoproteins as *SLR<sub>1</sub>* and *SLG* are now clearly involved in the pollen-stigma adhesion, as recently shown by a biomechanical assay in *Brassica napus* (18) and by isolation and characterization of pollen coat proteins that specifically bind to *SLR<sub>1</sub>* of *Brassica campestris* (19). Similarly, multiple *ZmSLR* silk transcripts can encode proteins that take part to pollen-stigma adhesion during maize pollination. The fact that higher levels of *S* transcripts are accumulated in developing silks at the maximum of female receptivity is consistent with this hypothesis.

Our study of *S* gene expression has revealed that the most abundant *S* transcripts in all maize tissues (except roots and mature pollen) were antisense transcripts. In silks, these transcripts co-exist with several different *S* sense transcripts. Thus, abundant natural antisense RNAs could modulate in maize tissues the expression and turnover of *ZmPK* and other *ZmSLR* genes by one of the possible mechanisms described above. Of particular interest in the developing male sexual tissues, the sense and antisense transcripts showed complementary expression patterns, with antisense RNAs decreasing in abundance as the anther matures, whereas sense *S*-like transcripts were accumulating at the highest level in mature pollen. The apparent reciprocal relationship between the abundance of sense and antisense *S* transcripts in mature pollen supports the possibility of a regulatory role for the antisense transcript in control of male reproductive development, as described previously in animals for several developmental processes (51, 52, 68, 78, 83).

Our study is the first to report *S*-like transcripts in mature pollen, one of which is pollen-specific. The maize pollen *S*-like transcripts are rather divergent from other known maize *S* sequences (detected by Northern blot hybridization, not by RNase protection assay), so that pollen-expressed *S* genes might have been missed in previous searches. Further experiments must be done to study in more detail this pollen *S* transcript, which could have a specific function either in pollen development and/or compatible pollination in maize.

To conclude, our study has evidenced in maize a new set of *S* gene family members showing, especially in flowers, a developmental and tissue-specific expression pattern and including

abundant antisense transcripts. It will be important to determine the direction of transcription for previously described *S* gene family members in other species. In *Brassica*, the presence of three different antisense *S* transcripts has been confirmed (58). Despite their low abundance in this species, it would be worthwhile to consider that these endogenous antisense transcripts could be involved in *S* gene family regulation. In particular, this endogenous regulation system could be disrupted/modified when sense or antisense *S* gene constructs are transgenically introduced in plants to modify pollen-pistil interactions. This phenomenon could contribute to the transgene-induced silencing or cosuppression of *S locus* genes and *S*-related genes in *Brassica* (84, 85). All these results allowed us to suppose that the regulation of *S* gene family expression is a very complex system in maize as in *Brassica*, which leads to the production of transcripts and proteins that can interact inside the *S* family or with other types of molecules to promote various mechanisms of cell-cell interactions.

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