The Plant Cell, Vol. 1, 173–179, February 1989, © 1989 American Society of Plant Physiologists

Characterization of a Pollen-Specific cDNA Clone from *Zea mays* and Its Expression

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A pollen-specific cDNA clone, Zmc13, has been isolated from a cDNA library constructed to poly(A) RNA from mature maize pollen. The cDNA as shown by primer extension analysis is a full-length copy of the mRNA. The cDNA has been sequenced and is 929 nucleotides in length plus a 47-nucleotide poly(A) tail. Putative polyadenylation signals are identifiable in the 3′-nontranslated region. The mRNA codes for a predicted polypeptide containing 170 amino acid residues and with a molecular mass of 18.3 kilodaltons. The hydropathy profile suggests a possible signal sequence on the amino terminus. A comparison of the nucleotide and deduced amino acid sequence with sequences in data banks has not shown homology to known molecules. In situ hybridizations using RNA probes show that the mRNA is located in the cytoplasm of the vegetative cell of the pollen grain and after germination is distributed throughout the pollen tube cytoplasm.

INTRODUCTION

The life cycle of angiosperms alternates between a diploid sporophyte and a haploid gametophyte generation. Pollen, as the male gametophyte, plays a vital role in the reproduction of flowering plants. The differentiation and development of the male gametophyte of angiosperms depends on the expression of the haploid genome following meiosis (Mascarenhas, 1988). Mature pollen grains of maize (Mascarenhas et al., 1984) and other plants (Frankis and Mascarenhas, 1980; Tupy, 1982) contain messenger RNAs (mRNAs) which appear to have functions during the late stages of maturation of pollen and during germination and early pollen tube growth (Mascarenhas, 1988). These mRNAs are the products of about 20,000 different genes (Willing and Mascarenhas, 1984; Willing et al., 1988). To understand the developmentally specific regulation and functions of pollen-expressed genes, a recombinant cDNA library was constructed to poly(A) RNA isolated from mature pollen of maize (Stinson et al., 1987). Differential screening of this library has resulted in the isolation of several clones that are pollen-specific, i.e. expressed in pollen but not in sporophyte tissues (Stinson et al., 1987).

In this study we present a detailed molecular characterization of one of these clones, Zmc13. This clone represents a gene that is present in a very few copies in the maize genome (Stinson et al., 1987). The Zmc13 mRNA was localized within the male gametophyte by in situ hybridization.

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RESULTS

Pollen Specificity of Zmc13 as Determined by RNA Gel Blot Analysis

Figure 1 is an RNA gel blot analysis of RNA isolated from mature pollen, shoots, roots, kernels, ovules, and silks and probed with Zmc13. Zmc13 is a pollen-specific clone. It hybridizes to an mRNA that is approximately 985 nucleotides in length and is present only in pollen RNA and not in the RNA from sporophyte tissues.

DNA Sequence Determination of Zmc13

Figure 2 describes the sequencing strategy used and Figure 3 shows the entire 929-nucleotide sequence of the cDNA and a putative amino acid sequence. In addition, the sequence includes a cloned poly(A) tail of 47 nucleotides and a poly(G:C) region of 27 base pairs (bp) located at the 5′ end of the clone (data not shown). The G:C tail was introduced during the construction of the cDNA library. A polyadenylation consensus sequence was identified centered around nucleotide 750. Two additional sequences that are quite similar to the polyadenylation consensus are present at nucleotides 875 and 886.

The longest open reading frame identified displays a pattern of codon usage that is highly conserved (80.1%) when compared with a codon usage pattern determined

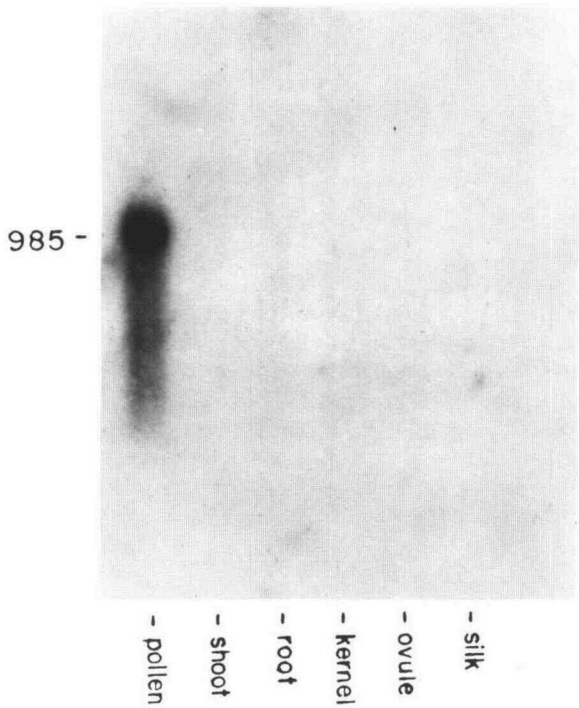

Figure 1. RNA Hybridization to Demonstrate Pollen Specificity of the Clone Zmc13.

Total RNA (5 μg per lane) isolated from the indicated tissues of corn was hybridized with ^{32}P-labeled Zmc13. The autoradiogram was overexposed to show clearly the lack of hybridization in the non-pollen lanes.

by analysis of 15 maize genes whose sequences have been published (data not shown). The next longest open reading frames found in any of the three reading frames were only 57.5% and 56.1% consistent with the calculated codon usage. The putative translatable sequence utilizes the first ATG codon of the message sequence at nucleotide 127, extends for 510 nucleotides, and ends with a TAA termination codon at nucleotide 637. Joshi (1987a) found that the initiation of translation occurs at the first AUG in 92% of 75 published genomic sequences from several higher plants. In Zmc13 the context of the first ATG is in close agreement with the consensus sequences proposed for plant genes by Lutcke et al. (1987) and Joshi (1987a). The predicted 170-residue amino acid sequence codes for an 18.3-kD polypeptide. Using the values and algorithm of Kyte and Doolittle (1982), a plot of its average hydrophobicity (Figure 4) reveals an 18-amino acid hydrophobic region at the amino terminus.

Primer Extension Analysis Indicates That Zmc13 Is a Full-Length cDNA

To locate the start of transcription, a synthetically prepared 17-residue oligonucleotide was prepared complementary to nucleotides 167 to 183 (Figure 3). This primer was ^{32}P-labeled at the 5′ end, annealed to total RNA extracted from mature pollen, and extended with reverse transcriptase. The same primer, unlabeled, was annealed to Zmc13, and also to a genomic subclone, Zmg13pBS0.8, which carries the start of transcription. These were then sequenced on an 8%, 8 M urea sequencing gel. Figure 5 is the resulting autoradiogram. The two relatively faint bands seen in the primer extension lane correspond to the first and second nucleotides, guanine and adenine, respectively, of the cDNA sequence ladder. The start of transcription is most likely represented by the second band of the doublet seen in the primer extension reaction that corresponds to an adenine residue in both the genomic and cDNA sequencing ladders. Joshi (1987a) has deduced a consensus for the transcription initiation site of higher plants that notes a conservation of adenine at the first position in 85% of those genes studied. The first band of the doublet seen in the primer extension reaction, which corresponds to a guanine residue, probably represents the fraction of the message population for which reverse transcriptase was successful in copying the methylated cap. This is supported by the fact that this guanine residue is not found in the genomic sequence. Alternatively, this result could indicate that there are two genes that are transcribing related messages but that differ from each other over this region by one nucleotide. Either explanation indicates that Zmc13 contains a full-length copy of the message.

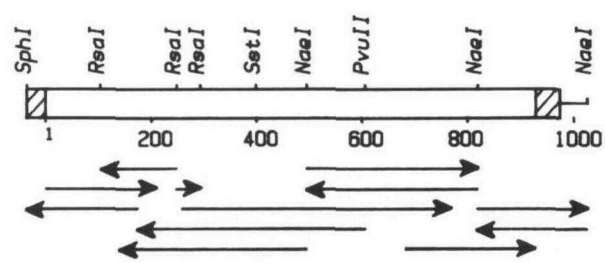

Figure 2. Sequencing Strategy and Partial Restriction Map of Zmc13.

The hatched areas represent poly G:C (left) and poly A:T (right) regions adjacent to the coding sequence of the clone. The arrows represent the extent and direction of sequencing.

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..A GGC CAG ATT TGC CAC CCT CGT CTC ACC CTC CCT CCC TCA CAC AAA TAA TAA GGA AAG   58

GTC CCG CCC TTT TCC TCC GAC ATC CAC AAG GGG GGA GGG GAA AAC ACG TAC ATT CAC CCG  118

GCG GCA ATA ATG GCC TCG GTT CCG GCT CCG GCG ACG ACG ACC GCC GCC GTC ATC CTA TGC  178
            Met Ala Ser Val Pro Ala Pro Ala Thr Thr Thr Ala Ala Val Ile Leu Cys   17

CTA TGC GTC GTC CTC TCC TGT GCC GCG GCT GAC GAC CCG AAC CTC CCC GAC TAC GTC ATC  238
Leu Cys Val Val Leu Ser Cys Ala Ala Ala Asp Asp Pro Asn Leu Pro Asp Tyr Val Ile   37

CAG GGC CGC GTG TAC TGC GAC ACC TGC CGC GCC GGG TTC GTG ACC AAC GTC ACC GAG TAC  298
Gln Gly Arg Val Tyr Cys Asp Thr Cys Arg Ala Gly Phe Val Thr Asn Val Thr Glu Tyr   57

ATC GCG GGC GCC AAG GTG AGG CTG GAG TGC AAG CAC TTC GGC ACC GGC AAG CTC GAG CGC  358
Ile Ala Gly Ala Lys Val Arg Leu Glu Cys Lys His Phe Gly Thr Gly Lys Leu Glu Arg   77

GCC ATC GAC GGG GTC ACC GAC GCG ACC GGC ACC TAC ACG ATC GAG CTC AAG GAC AGC CAC  418
Ala Ile Asp Gly Val Thr Asp Ala Thr Gly Thr Tyr Thr Ile Glu Leu Lys Asp Ser His   97

GAG GAG GAC ATC TGC CAG GTG GTG CTG GTG GCC AGC CCG CGC AAG GAC TGC GAC GAG GTC  478
Glu Glu Asp Ile Cys Gln Val Val Leu Val Ala Ser Pro Arg Lys Asp Cys Asp Glu Val  117

CAG GCG CTC AGG GAC CGC GCC GGC GTC CTG CTC ACC AGG AAC GTT GGC ATC TCC GAC AGC  538
Gln Ala Leu Arg Asp Arg Ala Gly Val Leu Leu Thr Arg Asn Val Gly Ile Ser Asp Ser  137

CTG CGC CCC GCC AAC CCG CTA GGC TAC TTC AAG GAC GTG CCG CTC CCC GTC TGC GCC GCG  598
Leu Arg Pro Ala Asn Pro Leu Gly Tyr Phe Lys Asp Val Pro Leu Pro Val Cys Ala Ala  157

CTG CTC AAG CAG CTG GAC TCG GAC GAC GAC GAC GAC CAG TAA ACT ATA CCA CGG CGG CGT  658
Leu Leu Lys Gln Leu Asp Ser Asp Asp Asp Asp Asp Gln                              170

CGC GGA CAT GCT GCA CAA AAC TAC AAC GAT ACA GAG CGA ACG CAT GGC ATG GAT AGC AGT  718

ATC TAC GGA AAG GAA AGG AAG AAA AGG AAA ATA AAA AAT GTA TCA GAG TGC TTG ATT CAC  778

TTG CTG CTG TCA CCC ATT CCC CGT TCT TAA CAT AAC ATG TGG GCC GGC TTG GCC CAG GCA  838

CAA GCC CAT CTA CGC ATG GCC TAC GGT CCG CTA AAA TAT AGC CCT AAT TAT GAG CCG TGT  898

TGT GCC GTC ACA TGG ATC GAT CCA GCG GCA TAA AAA AAA AAA AAA ...                  929
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Figure 3. cDNA Sequence and Predicted Amino Acid Sequence for Zmc13.

The underlined sequences indicate possible polyadenylation signals.

Messenger RNA Localization as Identified by in Situ Hybridization

In situ hybridizations of pollen and anther tissue sections utilized single-stranded ^{35}S-labeled riboprobes. The in situ hybridizations show that the Zmc13 mRNA is present in the vegetative cell cytoplasm in both the pollen grain and the pollen tube (Figure 6). Figure 6, A, B, and C, are sections of anthers about 3 to 4 days prior to anthesis. The pollen grains have completed the generative cell division and are trinucleate. Figure 6A and the inset, Figure 6C, show sections that have been hybridized with the antisense strand. A high silver grain density is seen uniformly over the pollen grain cytoplasm. Figure 6C, which is the identical section as Figure 6A but focused on the surface of the cells of the anther wall, shows the same

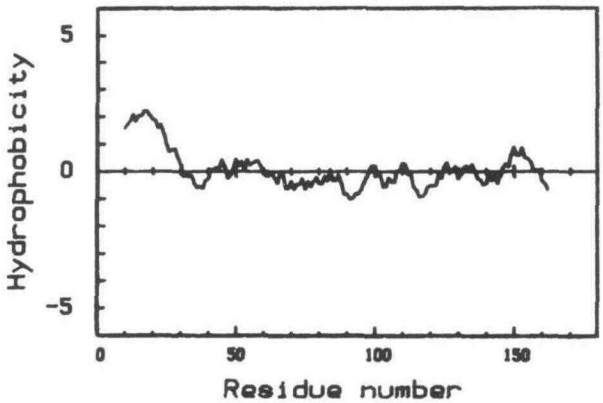

Figure 4. Hydropathy Plot of the Deduced Amino Acid Sequence of Zmc13.

Hydrophobicity was averaged over a window of 19 amino acids. Note the hydrophobic region at the N terminus.

density of silver grains as in the background of Figure 6A, indicating that Zmc13 mRNA is not present in the cells of the anther wall. Figure 6B is an anther section that has been hybridized to the sense strand probe. The focus is on the cut surface of the pollen grains and shows background level silver grains. The Zm13 mRNA is distributed uniformly throughout the pollen tube cytoplasm (compare Figure 6D [antisense probe] with Figure 6E [sense probe]), and when the focus is on the pollen grain (data not shown), the mRNA is found also to be in the cytoplasm within the pollen grain. Dark-field illumination was not useful because the pollen grains contain a large number of light-scattering cytoplasmic granules, probably starch grains. In fact it is necessary to use high magnification even with bright-field illumination to distinguish between the granules and silver grains.

DISCUSSION

Zmc13 is the first pollen-specific sequence to be characterized. The cDNA clone is a full-length copy of the mRNA. The context of the start site of translation in Zmc13 is in close agreement with the consensus sequences described for plant genes (Joshi, 1987a; Lutcke et al., 1987).

An unusual feature of Zmc13 is the relative distance of the putative polyadenylation signal from the actual site of poly(A) addition. The polyadenylation signals of plant messages are known to be more complex than those of animals since there are often two or more in each 3′-untranslated region of the message (Lycett et al., 1983). The sequence of Zmc13 contains the consensus motif AATAAA located at the unusually large distance of 180 nucleotides upstream from the site of polyadenylation, and 110 bp downstream from the presumptive stop codon. In most plant mRNAs this motif is located 27 ± 9 bases 5′ to the poly(A) site (Joshi, 1987b), although longer distances of 56 to 104 bases have been reported for some plant genes (Geraghty et al., 1981; Hyldig-Nielsen et al., 1982; Lycett et al., 1984). The alcohol dehydrogenase gene (Adh1) of maize, which is expressed in pollen although it is not pollen-specific, also exhibits a long region between the consensus AATAAA signal and the actual polyadenylation sites (Sachs et al., 1986). Two variant hexanucleotide sequences occur nearer to the polyadenylation site: AATATA centered 55 bp and AATTAT centered 44 bp upstream, respectively. It is interesting that Adh1 also has a sequence AATTAT centered 44 bp upstream of one of the polyadenylation sites (Sachs et al., 1986). It would be of interest to determine whether these sequence motifs occur in other pollen-expressed genes.

The cloned cDNA has a poly(A) tail of 47 nucleotides, making the cDNA total sequence 976 nucleotides. Because the size of the mRNA determined by RNA gel blot analysis (Figure 1) was 985 nucleotides, this discrepancy

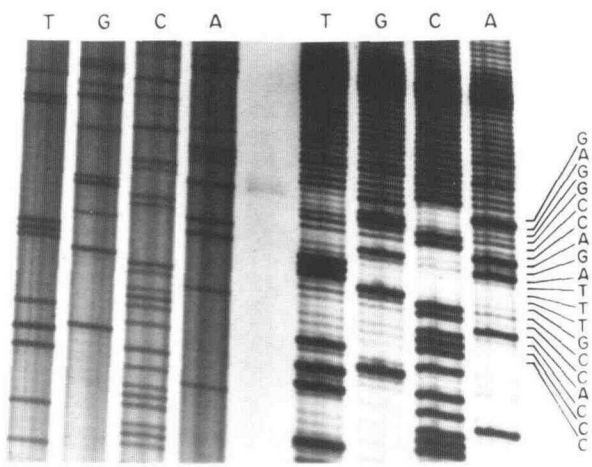

Figure 5. Primer Extension Mapping of the Start of Transcription of Zmc13.

Autoradiogram of a sequencing gel used to analyze a ^{32}P-labeled DNA primer annealed to total RNA extracted from mature corn pollen and extended by reverse transcriptase (center lane). The sequencing ladders of the coding strand were generated using the same DNA primer by dideoxy sequencing of the cDNA clone (righthand ladder) and of a genomic clone containing the start of transcription (Zmg13pBS0.8, lefthand ladder). The poly G:C stretch is apparent at the end of the cDNA sequence.

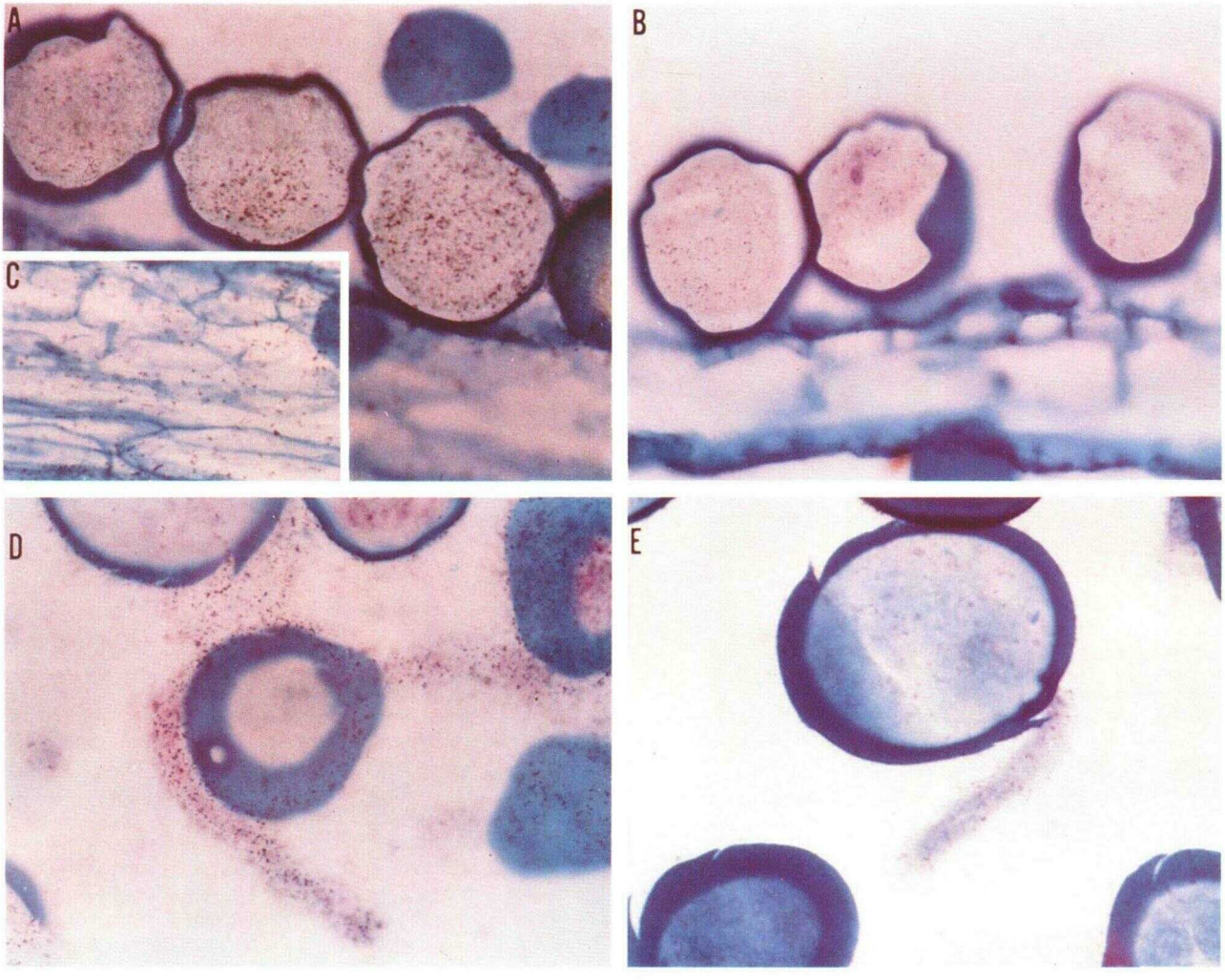

Figure 6. Localization of Zmc13 mRNA in Pollen and Germinating Pollen Tubes.

(A) Bright-field photograph of pollen (3 to 4 days prior to anthesis) hybridized with antisense Zmc13 RNA. Dark spots (developed silver grains) represent regions of RNA/RNA hybridization.
(B) Pollen hybridized using sense RNA (control).
(C) Background hybridization of antisense RNA to anther wall tissue [same slide as (A)].
(D) Hybridization of antisense Zmc13 RNA to germinating pollen tubes.
(E) Hybridization of sense RNA (control) to germinating pollen tubes.

in size can be accounted for by a longer average poly(A) segment in the mRNA population.

All of the sequence represented in the cDNA clone has been sequenced in a genomic clone that has been isolated from a genomic library, and it was found to be completely colinear with the mRNA; no introns were observed (D.A. Hamilton, D.M. Bashe, and J.P. Mascarenhas, unpublished data). Comparison of the cDNA sequence with the sequence of the genomic clone shows only a single difference over the 929-nucleotide region of the message: a G to A transition at nucleotide 731, which lies outside of the coding region. This may stem from the fact that the two libraries (genomic and cDNA) were made from the tissues of two different strains of corn, the pure line W-22 and the hybrid Goldcup, respectively. Because of the unusually high degree of polymorphism observed in corn at the level

of DNA sequence (Evola et al., 1986), one might have expected to find a larger sequence variation between the genomic and cDNA clones.

The molecular mass of the putative protein encoded by the open reading frame of the mRNA is about 18,000 D. The most conspicuous feature of the hydropathy profile (Figure 4) is a strongly hydrophobic region near the amino terminus, which may indicate the presence of a signal peptide. The amino acid sequence of this region does meet the major criteria for a signal sequence (von Heijne, 1987). We have no information yet about the identity or function of the protein product of Zmc13. A computer search of the National Biomedical Research Foundation's Protein Sequence Database and the nucleotide and deduced amino acid GenBank sequences using both the nucleotide and deduced amino acid sequences of Zmc13 has not revealed any meaningful homologies with known proteins.

In situ hybridizations (Figure 6) show convincingly that the Zmc13 mRNA is present in the vegetative cell cytoplasm and is most likely a product of transcription of the vegetative cell nucleus. Sections stained with mithramycin to locate the sperm cells do not show any increase in silver grain density over the sperm cells (data not presented). It is thus unlikely that the mRNA is present in the sperm cells. This localization of the mRNA to the vegetative cell might be expected because all the clones from the cDNA library that have been studied represent genes that are activated after microspore mitosis and before sperm cell formation (Stinson et al., 1987). The Zmc13 mRNA is distributed throughout the cytoplasm of the pollen tube and is not confined to any one region of the tube, such as the region at the tip.

METHODS

Isolation of RNA and RNA Gel Blot Hybridization

Total RNA was isolated from mature pollen, roots, shoots, and other portions of the plant of the inbred line of corn W-22 (Illinois Foundation Seeds), as described earlier (Willing and Mascarenhas, 1984). Total RNA was electrophoretically separated in a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to Gene Screen hybridization transfer membrane (New England Nuclear Research Products), and hybridized as described previously (Stinson et al., 1987).

Subcloning and Sequencing of DNA

cDNA fragments were generated by NaeI, PvuII, or RsaI digestion of Zmc13, recovered after gel electrophoresis in low-melting-point agarose, and ligated into either pUC13 or M13mp11. Single-stranded DNA template from M13mp11 was prepared as described in the M13 Cloning and Sequencing Manual (New England Biolabs Inc.). Double-stranded template DNAs were prepared and sequenced according to the Sequenase® sequencing kit manual (U.S. Biochemical). Both strands were sequenced using universal primers. In addition, oligonucleotides (17-mers) were synthesized (Operon Technologies) according to sequence information obtained and used directly as primers for further sequencing.

Determination of the Start Site of Transcription by Primer Extension Analysis

A 17-residue oligonucleotide complementary to nucleotides 167 to 183 (Figure 3) was radiolabeled by 5′-phosphorylation using T4 polynucleotide kinase. The primer extension technique was similar to that used by Belfort et al. (1985). Briefly, the labeled primer was annealed to 25 μg of total RNA extracted from pollen in annealing buffer (50 mM Tris-HCl, pH 8, 60 mM NaCl, 10 mM DTT) for 3 min at 60°C. A cDNA was made by extending the primer with avian myeloblastosis virus reverse transcriptase. The primer-extended molecules were sized in an 8% polyacrylamide sequencing gel.

Preparation of Anther Tissue and Pollen Tubes for in Situ Hybridization

Anthers collected from tassels of field-grown W-22 plants were fixed in 1% glutaraldehyde in 0.05 M sodium cacodylate (pH 7.0) buffer for 3 hr. Freshly collected pollen grains were germinated for 1 hr in 5-cm sterile plastic Petri plates containing 1.2 ml of Pfahler's (1973) medium containing 0.6% agar. The material was fixed directly in the plates with 1% glutaraldehyde in 0.05 M cacodylate buffer for 3 hr. After removing the fixative and washing with 0.05 M cacodylate buffer, the surface of the plate was then covered with a very thin layer of growth medium containing 0.6% low-melting-point agarose. Small blocks of agar with pollen tubes were cut out. Anthers and agar blocks with pollen tubes were dehydrated through an alcohol series into xylene and embedded in Paraplast Plus (Fisher Scientific). Sections of 10μm were cut and mounted on slides.

The in situ hybridizations were performed essentially as described by Cox et al. (1984), following a detailed protocol for plant material kindly provided by Dr. Robert B. Goldberg. In brief, the mounted slides were hybridized with 15 to 25 ng of RNA/100 μl/slide for 14 hr at 42°C in 50% formamide, 0.3 M NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 × Denhardt's, 10% dextran sulfate, and 70 mM DTT. The probe for the in situ hybridizations consisted of ^{35}S-labeled antisense RNA made to a 718-bp fragment from the corresponding genomic clone, designated Zmg13pBS0.8, with the sense strand serving as the control. The 718-bp fragment was subcloned in the pBS+ vector (Stratagene) such that the T7 RNA polymerase reaction produced the antisense strand and the T3 RNA polymerase reaction produced the sense strand. The riboprobes were made utilizing the Stratagene RNA Transcription Kit. Before hybridization, both probes were sheared to approximately 0.15 kb. Following hybridization, the sections were incubated with RNase A at 50 μg/ml, washed with 0.1 × SSC at 55°C, and coated with Kodak NTB-2 emulsion. The slides were developed after 4 to 8 days and stained with 0.5% toluidine blue prior to mounting in Kleermount (Carolina Biologicals Supply Co.).

ACKNOWLEDGMENT

This research was supported by National Science Foundation Grant DCB88-10165.

Received December 2, 1988.

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