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Cloning and characterization of a *COBRA*-like gene expressed *de novo* during maize germination

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Abstract

The search for germination-specific genes has been a laborious and unrewarding task, since many of the genes expressed during germination are also expressed in embryogenesis or in other developmental stages. By using mRNA differential display of transcript populations from maize (*Zea mays* L.) embryo axes, germinated for different times with or without a previous osmopriming treatment, a 682 bp cDNA was isolated that was present only after 24 h germination, and absent during osmopriming or during early germination. Screening of a cDNA library using the 682 bp probe yielded a 1554 bp cDNA that contained an open reading frame coding for 436 amino acids. This gene, referred to as *ZmAA9-24*, was expressed in root tissues, but was not detected in shoot or leaf tissues. Expression of *ZmAA9-24* occurred earlier during germination (by 15 h) if embryo axes were imbibed in the presence of cytokinins or if seeds were previously osmoprimed. The predicted protein sequence of *ZmAA9-24* is 39.6% identical to the product of the recently identified *Arabidopsis* gene *COBRA* (54.5% in the central region), which appears to participate in the regulation of cell expansion, particularly in roots, and belongs to the glycosylphosphatidylinositol (GPI)-anchored protein family. *ZmAA9-24* expression might be regulated by both cell expansion and the cell cycle, processes that have a central role during seed germination.

Keywords: *ZmAA9-24* gene, *COBRA* gene, cell elongation, *Zea mays* L., priming, benzyladenine

Introduction

When orthodox seeds are imbibed in water, there is a gradual reactivation of cells and tissues resulting in eventual cell enlargement, followed by radicle protrusion, cell proliferation and seedling establishment. In contrast, dormant seeds, which cannot germinate under standard hydration conditions, will only exhibit cell enlargement but no proliferation (Bewley and Black, 1994). Seeds will also fail to germinate when imbibed in polyethylene glycol (PEG) solutions. However, during this treatment, called osmopriming, cells remain in an active, metabolic state (Heydecker and Coolbear, 1977; Bradford, 1986; Cruz-García *et al*., 1995), but the germination process is blocked until the osmopriming agent is removed.

Osmopriming can elicit faster and more uniform germination than the conventional imbibition of seeds (Bradford, 1986; Cruz-García *et al*., 1995). The synchronizing effect of osmopriming can also be achieved by the addition of exogenous cytokinins to imbibing seeds (Reyes *et al*., 1991). Inhibitors of transcription and translation prevent the promotive effects of cytokinins if applied during early germination (Vázquez-Ramos and Reyes-Jiménez, 1990). Germination events and seedling establishment are also blocked by inhibitors of transcription (Jendrisak, 1980; Sánchez de Jiménez and Aguilar, 1984; Sánchez-de-Jiménez *et al*., 1997) Thus, apparently the accumulation (or appearance) of newly synthesized mRNA would be a prerequisite for germination to occur.

It has been of great interest to those working on seed biology to understand what triggers the germination process, as very little is known about the early biochemical events. The presence and abundance of storage and late embryogenesis proteins (and their transcripts) in seeds (Galau *et al*., 1986; Baker *et al*., 1988; Bewley and Black, 1994) has probably hampered the finding of regulatory, germination-specific gene expression that would be essential for triggering and controlling germination.

Since exogenous cytokinins or osmopriming accelerate germination of maize, these treatments could potentially be used to identify *de novo* expressed genes during early germination. We have performed subtractive hybridization of a cDNA

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library from maize embryo axes imbibed for 5 h and treated with benzyladenine (BA), and RNA differential display from maize embryo axes imbibed for different periods of time in the presence/absence of benzyladenine. However, neither of these strategies provided evidence of *de novo* promotion of gene expression; mostly, increased and decreased amounts of transcripts already present were observed (Zúñiga-Aguilar *et al*., 1995).

However, RNA differential display from maize embryo axes imbibed for 0, 10 and 24 h after seed osmopriming revealed the existence of a cDNA corresponding to an authentic *de novo* expressed mRNA during germination. This cDNA fragment, named AA9, was used as a probe to isolate a cDNA from a root and shoot library. In this paper we describe the isolation of a 1554 bp AA9 cDNA and its conceptual translation product, as well as the expression pattern of the transcript, and discuss the possible identity of the corresponding protein.

Materials and methods

Germination and osmopriming of maize

Maize caryopses (*Zea mays* cv. Chalqueño) or embryo axes were used. For germination experiments, maize axes (10–50 axes/plate) were surface sterilized with 0.5% NaClO (v/v, 2 ml) for 1 min and then washed thoroughly with sterile distilled water. After drying with Whatman paper No. 1, axes were imbibed in the dark at 27°C with sterile imbibition buffer, consisting of 50 mM KCl, 10 mM $MgCl_2$, 50 mM Tris/HCl pH 7.6, 2% sucrose and 10 µg ml^{-1} chloramphenicol. When needed, benzyladenine was added at a 1×10^{-6} M concentration. For osmopriming, maize caryopses (three replicates of 40 kernels each) were put into Petri dishes containing two discs of sterile Whatman paper No. 1 and 40 ml of a –1.7 MPa PEG-8000 solution; caryopses were incubated at 25°C in an incubator for 10 d (Cruz-García *et al*., 1995). Caryopses had been disinfected previously by rinsing with sterile water and then soaking in a 0.25% Captan solution for 10 min. After removing excess water with Whatman paper No. 1, caryopses were dried in a cabinet at 30°C for 3 h. For germination after osmopriming experiments, maize axes were removed from recently osmoprimed caryopses and incubated as above.

Chemicals

RNase-free DNase I, *Taq* DNA polymerase and RNase inhibitor (recombinant non-competitive inhibitor to neutral pancreatic ribonucleases) were from Boehringer-Mannheim (Mannheim, Germany); Trizol® reagent and Super Script II Reverse Transcriptase were from Gibco BRL, Life Technologies (Gaithesburg, MD, USA); primers were from Operon Technologies (Alameda, CA, USA); [α-^{35}S]dATP (15 TBq $mmol^{-1}$) was from New England Nuclear (Boston, MA, USA); the thymine/adenine (T/A) cloning system was from Invitrogen (San Diego, CA, USA), and the Thermosequenase kit was from Amersham International PLC (Little Chalfont, Bucks, UK).

Total RNA isolation and mRNA differential display

Total RNA was obtained using the Trizol® reagent (according to the manufacturer's instructions), and the tissues used were embryo axes from unimbibed and 10 d osmoprimed caryopses, or from axes isolated from caryopses after 24 h in imbibition buffer following osmopriming. Additionally, we isolated total RNA from root tips, leaves and shoots of maize seedlings after 4 d of germination. To eliminate contaminant DNA, total RNA (50 µg) was treated with 10 U of DNase I and 10 U of RNase inhibitor for 30 min at 37°C. RNA samples were extracted with phenol/$CHCl_3$, the supernatant was ethanol-precipitated in the presence of 0.3 M sodium acetate and RNA was dissolved in diethyl pyrocarbonate-treated water. mRNA differential display was performed according to Liang and Pardee (1992). Single-stranded cDNA was synthesized from total RNA (1 µg). Additionally, we included a cDNA synthesis reaction without adding reverse transcriptase, to discriminate between PCR products originating from contaminant DNA and those from the cDNA. Likewise, duplicate reactions were performed with the same amount of RNA to minimize both the possibility of losing rare RNAs and the introduction of errors in the PCR procedure, which could produce spurious bands.

For this particular case, we used four different anchor primers: oligo$(dT)_{11}$AA, oligo$(dT)_{11}$GA, oligo$(dT)_{11}$GC and oligo$(dT)_{11}$CA (1 mM each). PCR amplification to produce double-stranded cDNA was performed in the presence of [α-^{35}S]dATP, using 200 nM of either of two different 10-mer primers with sequences 5′ GTCTTGCGGA 3′ (OPM-9) and 5′ AGGTCTTGGG 3′ (OPM-20) as follows: 94°C, 30 s; 40°C, 2 min; 72°C, 30 s for 40 cycles and a final extension at 72°C, 10 min; a Perkin Elmer 480 Thermal Cycler was used. The amplified cDNAs were then electrophoresed for 2 h at 55°C and 60 W on a 6% DNA sequencing gel. Gels were dried under vacuum at 80°C and exposed to BioMax film at –70°C for 48 h.

Recovery and re-amplification of cDNA probes

After developing the film, cDNA bands of interest were located and cut from the gel. Gel slices were placed on 3MM Whatman paper strips, incubated

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with 100 µl water for 10 min, boiled for 15 min and then centrifuged. The supernatant was ethanol-precipitated in the presence of 0.3 M sodium acetate, 5 µl glycogen (20 mg ml^{-1}) as a carrier, and dissolved in 10 µl water. The eluted cDNAs (2 µl) were reamplified in a 40 µl reaction volume using the same primer set and PCR conditions as used for mRNA differential display (Ausubel *et al*., 1994).

Cloning and sequencing of cDNA probes

Reamplified cDNAs were cloned into the pCR II vector, using the T/A cloning system. Plasmid DNA sequencing was performed with either SP6 or T7 primers, using the Thermosequenase kit, according to the manufacturer's instructions.

Northern blot analysis

Total RNA (10 µg) was heat denatured, subjected to electrophoresis on agarose gels with 2.2 M formaldehyde at 50 V for 4–5 h, and transferred to nylon membranes. Blots were UV-crosslinked, pre-hybridized at 65°C in 250 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8), 1 mM EDTA, 7% SDS, 1% albumin, and hybridized to random primer-labelled cDNA probes. After hybridization, the blots were washed twice at 65°C for 15 min with a washing solution containing 100 mM Na_2HPO_4/NaH_2PO_4 (pH 6.8), 1 mM EDTA, 0.1% SDS, and exposed to X-O-Mat film at –70°C (Ausubel *et al*., 1994) .

Isolation of ZmAA9-24 cDNA clones

A cDNA library from root and shoot meristems from 9-day-old maize seedlings, constructed in the λ gt11 vector, was screened. As a probe, a 682 bp cDNA (identified as AA9) was used; this was isolated after differential display of mRNA populations derived from embryo axes excised from maize kernels after 24 h incubation in imbibition buffer. Nearly 400,000 clones were screened. The clone with the largest cDNA insert (ZmAA9-24), showing the strongest hybridization signal, was selected and then subjected to three more screenings, yielding virtually 100% hybridization-positive colonies after the fourth screening. The ZmAA9-24 cDNA clone contained a DNA fragment of *c*. 1.6 kbp. The complete sequence was obtained with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems) at the DNA core facility of the Instituto de Fisiología Celular, UNAM, México.

Results

A cDNA was preferentially amplified from an RNA population from embryonic axes after 24 h of incubation on imbibition buffer, following caryopsis osmopriming; this transcript was not detectable in RNA from unimbibed axes or from axes osmoprimed for 10 d (Fig. 1). The sequence of this cDNA, named AA9, corresponded to a 682 bp DNA fragment, which in the first analyses showed no identity or similarity to any known sequence in databases. When the AA9 cDNA was used as a probe to screen a cDNA library from maize root and shoot meristems, a 1554 bp cDNA was isolated and cloned. The DNA sequence (Fig. 2) contained an open reading frame flanked by a 63 bp 5′-UTR and a 183 bp 3′-UTR. The predicted protein encoded by this cDNA, named ZmAA9-24, was 436 amino acids in length, with a molecular mass of 49 kDa. An in-frame stop codon was located 39 bp upstream of the putative ATG, suggesting that this

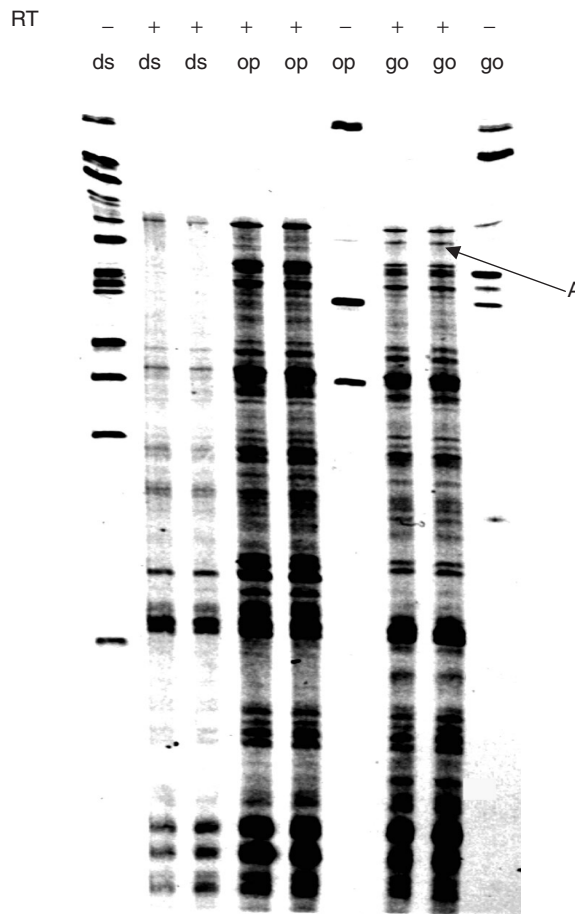

Figure 1. Differential display of mRNA populations extracted from maize axes. RNA samples (in duplicate) were obtained from dry caryopses (ds), after 10 d osmopriming (op) in a –1.7 MPa PEG solution at 25°C or after 24 h in imbibition buffer at 27°C following osmopriming (go). Reverse transcriptase was omitted in some samples (RT–) as a negative control. The arrow indicates the position of AA9 in the gel.

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agcatgctatgcatacatggttcc**tga**tttcccataaaatacctcctgcaggctgttgtc    60
aca**ATG**tttaattatcaacaatttcggcatatcggcgcacctggttggcagcttgggtgg   120
    M  F  N  Y  Q  Q  F  R  H  I  G  A  P  G  W  Q  L  G  W     19
acatgggcaaagaaggaggttatatggtcaatggttggggctcagaccactgaacagggc   180
 T  W  A  K  K  E  V  I  W  S  M  V  G  A  Q  T  T  E  Q  G     39
gactgctcaaagttcaagagcagcccaccccattgctgcaagaaagatccaacaattgtc   240
 D  C  S  K  F  K  S  S  P  P  H  C  C  K  K  D  P  T  I  V     59
gatttacttccaggcactccatacaacatgcaaattgccaattgctgcaaggcaggagtt   300
 D  L  L  P  G  T  P  Y  N  M  Q  I  A  N  C  C  K  A  G  V     79
gtaaatacctttaaccaggacccagcaaatgctgcttcctccttccagatcagtgttggt   360
 V  N  T  F  N  Q  D  P  A  N  A  A  S  S  F  Q  I  S  V  G     99
cttgctggaactaccaataaaactgttaaggtgcccaggaacttcactcttaagactcca   420
 L  A  G  T  T  N  K  T  V  K  V  P  R  N  F  T  L  K  T  P    119
ggccctgggtacacatgtgggcgtgccattgttggcaggcctacgaagtttttcaccgcg   480
 G  P  G  Y  T  C  G  R  A  I  V  G  R  P  T  K  F  F  T  A    139
gacgggcgcagggcaacccaagctctaatgacatggaatgtgacctgcacatattcccaa   540
 D  G  R  R  A  T  Q  A  L  M  T  W  N  V  T  C  T  Y  S  Q    159
tttcttgctcagaagactccatcctgctgtgtatctctatcatcgttttataatgacaca   600
 F  L  A  Q  K  T  P  S  C  C  V  S  L  S  S  F  Y  N  D  T    179
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agaaaagatgagcgatgccaagaaaaagtcgatatgtctatgtgtttaagt**taa**agggcc  1380
 R  K  D  E  R  C  Q  E  K  V  D  M  S  M  C  L  S  -          436
aaaatgtatttcttgtttggtata**taa**cagccctacaacactttggtgaacttagttact  1440
gcagat**tag**gt***aatta***cagttgcacctttttgtattttatagcaaacccagaatttttcat  1500
tggattctacgactgcccctcttgtagtaaaaaaaaaaaagcggccgcgaattc          1554
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Figure 2. Nucleotide sequence of *ZmAA9-24* (AFF24189) and its encoded protein. The *ZmAA9-24* gene consisted of 1554 bp with an open reading frame corresponding to 436 amino acids, having a molecular mass of 48.967 kDa. The initial codon is indicated in bold capital letters and stop codons in bold lower-case letters. A putative poly-A signal is indicated in bold lower-case italic letters.

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ATG may be the initiation codon. At the 3′ end, several in-frame stop codons were identified immediately after the last amino acid, and a putative polyadenylation (AATTA) signal was located 76 bases from the 3′ end of the sequence (Fig. 2).

Translation of the predicted amino acid sequence and analysis using the BLAST-P program showed that ZmAA9-24 has a 39.6% identity to a recently reported protein called COBRA (Schindelman *et al*., 2001) from *Arabidopsis thaliana* (Fig. 3). However, identity increases to 54.5% if the amino end in COBRA (around 65 amino acids) and the carboxy end in ZmAA9-24 (around 70 amino acids) are not considered. Other related *Arabidopsis* COBRA-like sequences can be retrieved from the databases. Sequences BAB02996, T51392, AJ006787 and AAF02128 (Fig. 3) share similar identity values to both COBRA and maize ZmAA9-24. It is interesting that all six sequences have two high-identity regions separated by a central region.

A very interesting feature in all maize and *Arabidopsis* sequences is the perfect conservation of 17 cysteine residues, which should be important for protein folding (Bernfield *et al*., 1999) and could be responsible for the metal-chelating phenotype originally found in BAB02996.

The main differences found between COBRA and ZmAA9-24 protein sequences were an N-terminal signal sequence for secretion and a stretch of residues found in proteins with processing in the C-terminal in which a glycosylphosphatidylinositol (GPI) group is attached (Schindelman *et al*., 2001), both sequence motifs being present in COBRA but not in ZmAA9-24 (Fig. 3). BAB02996 and AAF02128 sequences may also possess a N-terminal signal sequence for secretion, and sequences AJ006787 and AAF02128 also have a GPI-anchoring sequence (Fig. 3). COBRA, ZmAA9-24 and BAB02996 also possess a similar C-terminal hydrophobic region (Fig. 4).

ZmAA9-24 expression pattern

Expression of *ZmAA9-24* was followed during maize germination, either under control conditions, after stimulation of germination by cytokinins or during and after an osmopriming treatment. The *ZmAA9-24* transcript was not detected in unimbibed caryopses or during osmopriming (Fig. 5A), but it was present by 24 h of germination under control conditions (Fig. 5B). Treatments such as osmopriming or addition of cytokinins to germinating embryo axes (isolated axes exposed to BA) shifted the gene expression pattern during germination, so that *ZmAA9-24* transcript was now detectable by 15 h (Fig. 5A, C). Under these conditions, the amount of transcript declined by 24 h. It should be noted that no visible germination (radicle protrusion) occurred under these experimental conditions (Cruz-García *et al*, 1995). Radicle protrusion takes place after 32 h and 20 h in control and osmoprimed maize caryopses, respectively.

Tissue expression of *ZmAA9-24* was also followed in roots, shoots and leaves of 4-day-old seedlings. Figure 5D shows that *ZmAA9-24* was expressed in root tips, and a faint band could be seen in shoot tips, but could not be detected in leaves.

Discussion

Previous results from our laboratory and others demonstrated that mRNA synthesis is required for seed germination and seedling establishment to occur, indicating that *de novo* appearance of the resulting gene products is also a prerequisite (Jendrisak, 1980; Sánchez de Jiménez and Aguilar, 1984; Vázquez-Ramos and Reyes-Jiménez, 1990; Sánchez-de-Jiménez *et al*., 1997). We began a search for such transcripts by means of differential display experiments using embryo axis mRNAs from post-osmoprimed germinating maize caryopses, comparing them with transcripts present in germinating controls. However, most of the transcripts we found were present in dry seeds, and their expression either increased or decreased during the first 15 h of germination (Zúñiga-Aguilar *et al*., 1995; our unpublished observations). In this screen, only one mRNA (ZmAA9-24) was identified that was not present in dry or in caryopses osmoprimed for 10 d, but was expressed late in the germination time course. It is relevant that the transcript was not present during osmopriming, a condition that allows germination to start but, at least for maize, blocks germination before the S phase is triggered (Cruz-García *et al*., 1995).

Until recently assigning a role in germination for the *ZmAA9-24* gene sequence had been puzzling. The high identity with the cDNA sequence BAB02996 from *A. thaliana*, which rescued *Schizosaccharomyces pombe* mutant cells with a defect in phytochelatin synthesis and high Cd^{2+} sensitivity (Leuchter *et al*., 1998), suggested that this sequence would code for a putative phytochelatin synthetase. However, a *bona-fide* phytochelatin synthetase from *Arabidopsis* has been cloned and sequenced (Vatamaniuk *et al*., 1999), and the sequence shows no identity or homology with cDNA sequence BAB02996 or with *ZmAA9-24*.

The protein product of the *Arabidopsis COBRA* (*Cob*) gene, which affects the orientation of cell expansion in the root, is a GPI-anchored protein (Schindelman *et al*., 2001), and its sequence is 39.6% identical to ZmAA9-24. *COBRA* was isolated as a mutant with abnormally expanded roots (Benfey *et al*., 1993). Roots in the mutant appear to be expanded more in a radial than in a longitudinal orientation, suggesting that COBRA participates in regulating the

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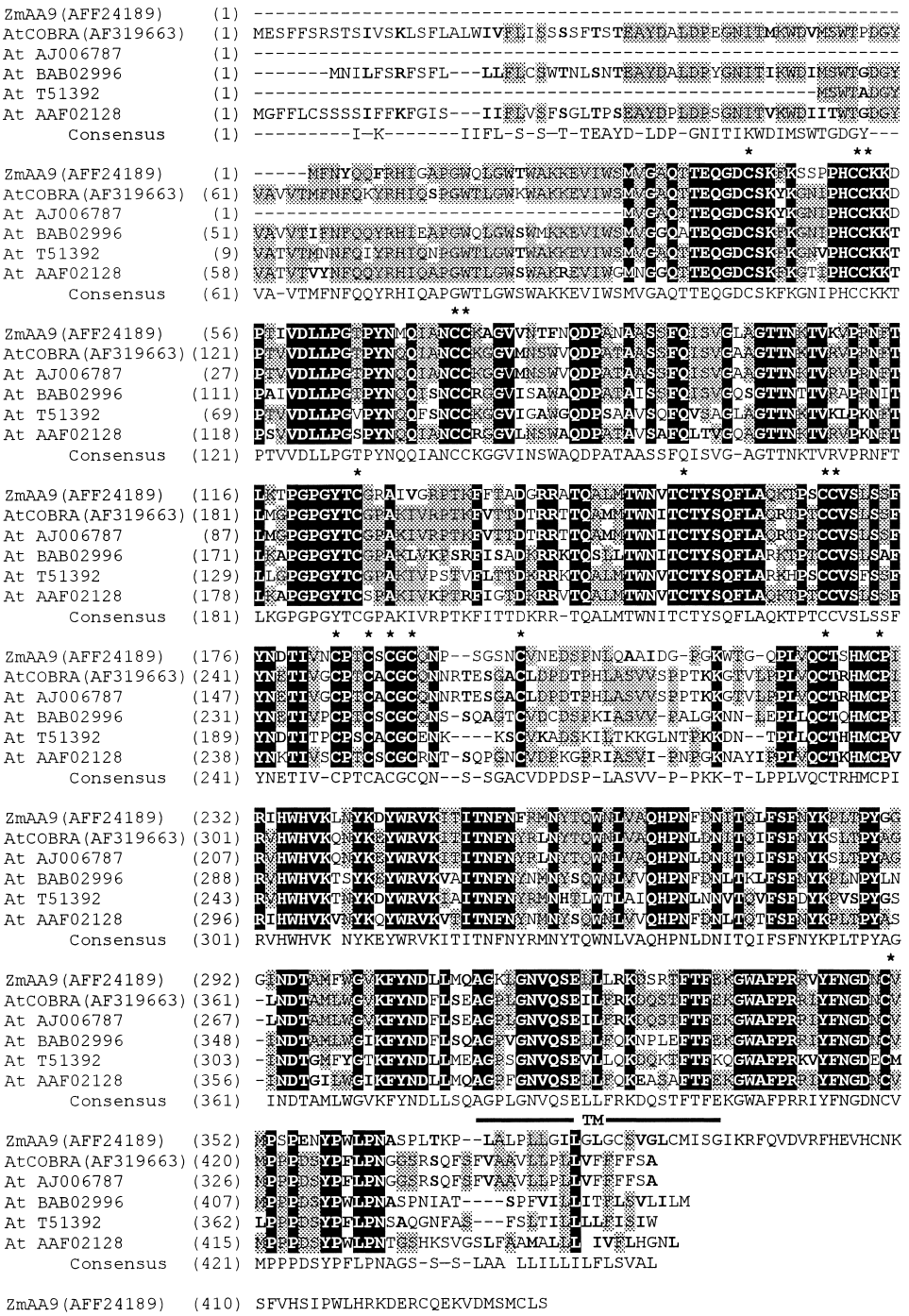

Figure 3. Alignment of the predicted amino acid sequences of ZmAA9-24 from maize axes, COBRA and four COBRA-related proteins from *A. thaliana*; accession numbers are given in parentheses. The alignment was adjusted to maximize amino acid similarity and minimize gaps by using the molecular biology software Vector NTI. Identical amino acid blocks are in black shadow; conservative replacements are in black bold, and similar amino acids are in grey shadow blocks. The identity percentage was 36.3. Conserved cysteines are marked with an asterisk. Transmembranal domains (TM), predicted by SMART (website: http://smart.embl-heidelberg.de/smart/show_motifs.pl), were localized at the C-terminus; in ZmAA9-24, 371–393 (ALPLLGILGLGCSVGLCMISGI); in COBRA, 438–455 (SFVAAVLLPLLVFFFFS); At AJ006787, 344–361 (SFVAAVLLPLLVFFFFS); and At BAB02996, 423–441 (IATSPFVILLITFLSVLIL). GPI-anchors were predicted by the GPI Prediction Server (website: http://mendel.imp.univie.ac.at/gpi/cgi-bin/gpi_pred.cgi) and these include COBRA amino acids 433–438, At AJ006787 amino acids 338–343 and At AAF02128 amino acids 428–433.

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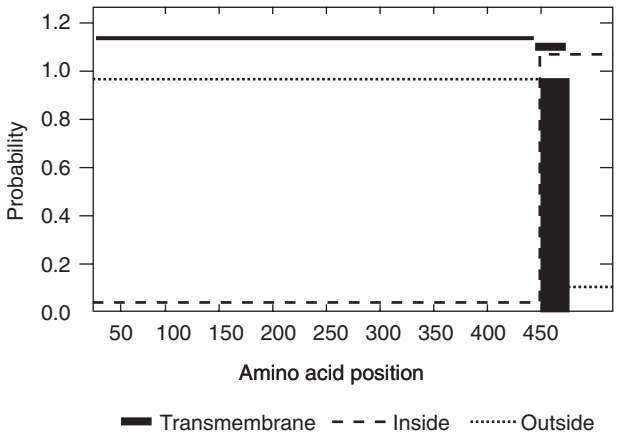

Figure 4. Prediction of **trans**membranal **h**elices with a hidden **M**arkov **m**odel, TMHMM (Krogh *et al.*, 2001; website: http://www.cbs.dtu.dk/services/TMHMM/). Analysis predicted that ZmAA9-24 is primarily a cytoplasmic protein with a transmembranal helix at the carboxyl terminal (black vertical band, positions 444–465).

orientation of cell expansion. The finding that COBRA localizes primarily in the plasma membrane, in cells undergoing highly oriented longitudinal expansion, is consistent with a role in regulating oriented cell expansion (Schindelman *et al.*, 2001).

COBRA appears to be part of a family of related proteins, as shown by the retrieval of at least four other highly related cDNA sequences from *Arabidopsis* databases. Although these COBRA-related sequences show some differences from COBRA, they do possess some of the hallmarks that define COBRA, i.e. an N-terminal signal peptide for secretion, 17 conserved cysteines and/or a C-terminal GPI addition sequence. In contrast, the predicted maize ZmAA9-24 protein (Fig. 2) seems to lack both the N-terminal signal peptide and the C-terminal GPI addition sequence; otherwise, it is very similar to COBRA, particularly in the central region, in which identity increases to 54.5%. Noteworthy are the hydrophobic sequence in the C-terminal domain, also shared by the other *Arabidopsis* COBRA-like sequences, and the 17 perfectly conserved cysteines, which could be related to a disulphide-linked compact domain, with a likely globular structure, perhaps involved in protein–protein interactions (Fig. 2).

The lack of a signal peptide in the ZmAA9-24 predicted protein is controversial, since immediately upstream of the ATG starting codon, the sequence would predict four more amino acids (AVVT) that are also present in the signal peptide sequence in COBRA. We performed a screening of a maize DNA genomic library, using the AA9 cDNA as a probe, and isolated several clones. The largest one (ZmAA-9g) was 1.4 kbp and encoded the first 148 amino acids of *ZmAA-9* cDNA, including the AVVT sequence found upstream the initial ATG codon; this genomic clone also contained the in-frame stop codon at –39 (data not shown). Additionally, this genomic clone contained two other in-frame stop codons upstream of the –39 stop codon. If the ATG codon in *ZmAA-9* cDNA is the initial methionine, then we have a full cDNA. We could not find any other in-frame ATG in 950 bp of UTR sequence in this genomic clone, which could restore an amino acid sequence that resembled COBRA or its related proteins. It is still possible that we have an incomplete cDNA, interrupted by a very large intron. It should be noted, however, that a similar gene seems to be present in the *A. thaliana* genome (AAF02128). This cDNA has an ORF that does not appear to encode a signal peptide at the 5′end.

COBRA-like members may belong to a superfamily of proteins called heparan sulphate proteoglycans (HSP) (Bernfield *et al.*, 1999), which have been implicated in the modulation of action of extracellular ligands, since HSPs are localized on the surface of all adherent cells in animals. Members of this family have been divided in two main groups: syndecans and glypicans. Both are extracellular proteins attached to the plasma membrane. Syndecans are bound to membranes by a transmembranal domain in the C-terminal end, while glypicans are bound by a GPI anchor (Turnbull *et al.*, 2001).

Glypican-type proteins share at least three characteristics: (1) they have a signal peptide at the amino end; (2) they have 14 conserved cysteines spaced in the whole sequence; and (3) they have a signal sequence for a GPI anchor at the carboxyl terminus. All the COBRA-like proteins discussed here have some or all of these characteristics.

The transmembrane domain in the ZmAA9-24 carboxyl end allows us to suggest that the ZmAA9-24 protein is also attached to the plasma membrane. It may be that COBRA and its related proteins form complexes that are membrane bound and, thus, participate in biochemical processes such as orientation of cellulose deposition on the cell surface (Schindelman *et al.*, 2001).

ZmAA9-24 was isolated as a gene that is expressed during germination and in root tissues, but with no evident expression in leaves and residual expression in shoots. Moreover, *ZmAA9-24* could not be detected in unimbibed caryopses, during osmopriming or during the early stages of germination. Cell elongation and division in embryo meristems are events that take place late during maize germination, before or together with radicle protrusion (Baíza *et al.*, 1989). Mitotic figures can be seen by 28–30 h under standard germination conditions, and root protrusion

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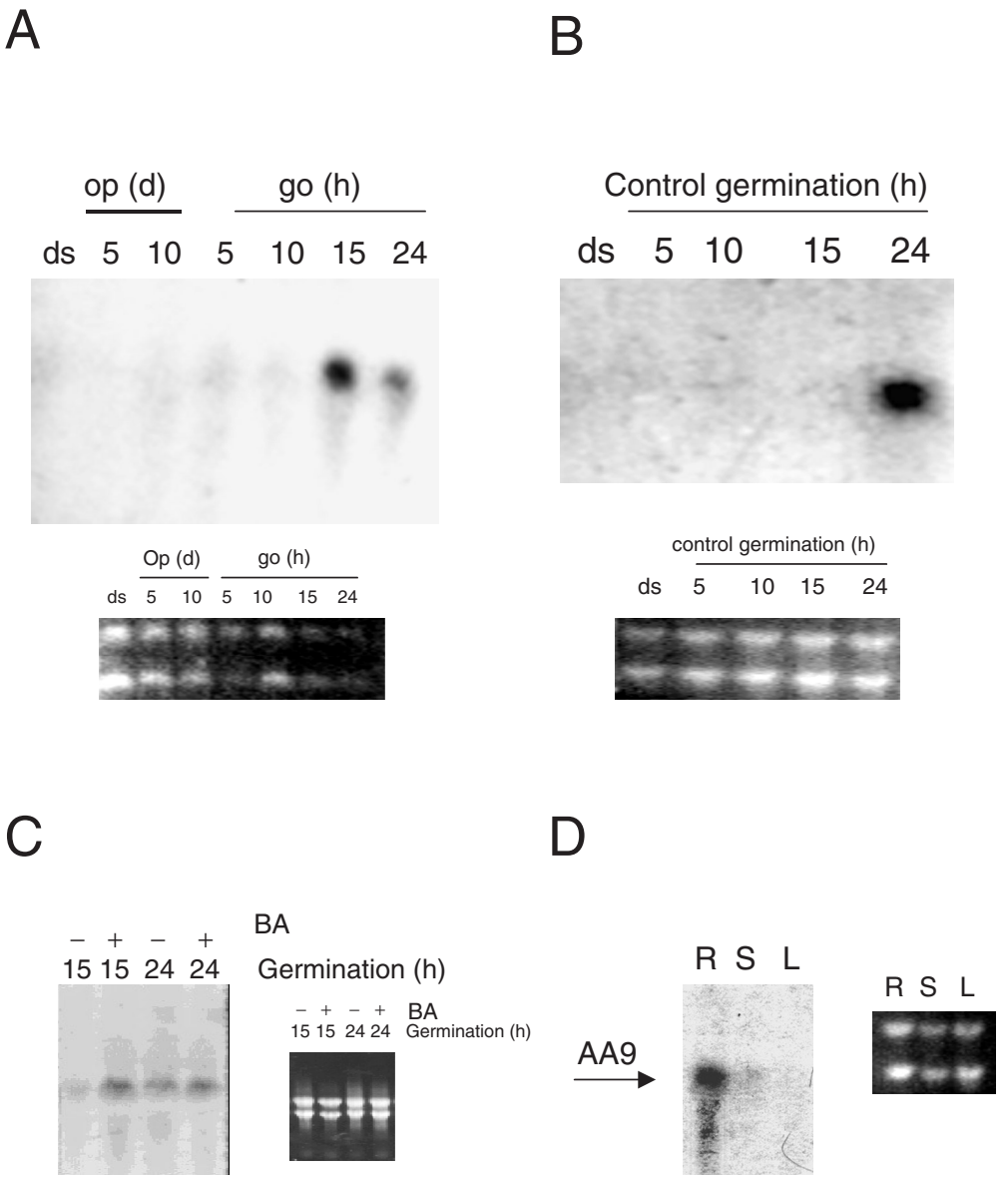

Figure 5. *ZmAA9–24* expression during maize germination. (A) mRNA expression in embryonic axes from dry caryopses (ds), during 5 or 10 d (d) osmopriming with a –1.7 MPa PEG solution at 25°C (op) and after 5, 10, 15 and 24 h germination in imbibition buffer at 27°C following osmopriming (go); (B) mRNA expression during control germination at 0 (ds), 5, 10, 15 and 24 h of germination; (C) effect of cytokinins (benzyladenine, 10^{-6} M) on *ZmAA9-24* expression after 15 and 24 h of germination; (D) *ZmAA9-24* expression in maize roots (R), stems (S) and leaves (L) from caryopses germinated for 4 d.

occurs a few hours later (Baíza *et al*., 1989). The first mitotic figures are observed by 15–18 h if germination is accelerated by adding cytokinins or by germinating osmoprimed kernels (Reyes *et al*., 1991; Cruz-García *et al*., 1995). *ZmAA9-24* gene expression would precede the appearance of mitotic figures, probably coinciding with the beginning of cell elongation and some hours before radicle protrusion is visible. Thus, it is noteworthy that during maize osmopriming, when embryo axis cells are blocked in the G_1 phase of the cell cycle (Cruz-García *et al*., 1995), the *ZmAA9-24* gene is not expressed.

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