

Full Length Research

Cloning and characterization of maize *ZmSPK1*, a homologue to nonfermenting1-related protein kinase2

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SnRK2s play important roles in plant stresses responses. One full-length cDNA encoding a *SnRK2b* homologue was isolated from maize by RT-PCR and named as *ZmSPK1* (for stress-induced protein kinase). The *ZmSPK1* protein has 364 amino acids with an estimated molecular mass of 41.8 KD and an isoelectric point of 5.8. The deduced protein sequence has the closest identities to the members of *SnRK2b* group. RT-PCR analysis showed that the *ZmSPK1* expression was induced by mannitol, salt and abscisic acid (ABA). Furthermore, in different tissues the *ZmSPK1* showed different expression patterns and was most abundant in reproductive organs. These results suggested that *ZmSPK1* might play multiple roles in abiotic stress resistance pathways, as well as in plant reproductive development.

Key words: *Zea mays* L., SnRK2b, expression pattern, abiotic stress

INTRODUCTION

Plants often encounter various environmental stresses including high or low temperature, drought and salinity. These stresses impose osmotic stress on plants, causing a series of morphological, physiological, biochemical and molecular changes in plants. To survive these stresses, plants have evolved complex mechanisms to perceive external signals and to manifest adaptive responses with proper physiological, morphological and molecular changes (Bohnert et al., 1995; Bray, 1997; Xiong et al., 2002; Zhu, 2002). Protein phosphorylation / dephosphorylation plays important roles in the responses to stresses including the perception of extracellular stimuli and the subsequent activation of defense responses (Yang et al., 1997).

The mitogen-activated protein kinase (MAPK) cascade is one of the well-known signal transduction factors conserved among eukaryotes (Hirt, 1997; Kultz, 1998). MAPK cascade is composed of MAPK, MAPKK and

MAPKKK in which each component is activated by phosphorylation by the upstream kinase. The putative *Arabidopsis* MAPK cascade is consisted of AtMEKK1, AtMEK1/AtMKK2, and AtMPK4 identified by using two-hybrid analysis and yeast complementation (Ichimura et al., 1998). AtMEKK1 is induced by salt stress and can activate AtMPK4 *in vitro* (Ichimura et al., 2000; Huang et al., 2000). AtMPK4 can also be activated by cold, low humidity, osmotic stress, touch, and wounding (Ichimura et al., 2000). Additionally, pairs of MAPK and MAPK kinase involved in osmotic stress signaling have been identified in other plant species; for example, SIMKK-SIMK in alfalfa (Kiegerl et al., 2000) and NtMEK2-SIPK/WIPK in tobacco (Yang et al., 2001; Zhang et al., 2001). All these suggest that those MAPK cascades may function in stresses signal pathways and participate in the plant protection responses.

Osmotic and other abiotic stresses can cause increase in cytosolic Ca²⁺ concentration (Knight, 2000). Ca²⁺-dependent protein kinase (CDPK) currently known only in plants and protozoa can perceive and transduce the stress-induced Ca²⁺ signals and play important roles in

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signaling pathways in response to stresses such as drought, salt and cold (Urao et al., 1994; Saijo et al., 2000). Expression of a constitutively active form of AtCDPK1 can activate an osmotic stress-related promoter (Sheen, 1996). Furthermore, overexpression of OsCDPK7 confers cold, salt and drought tolerance on rice (Saijo et al., 2000). It also indicates that the kinase does not participate in the early response to stress but rather in the adaptive process (Martín et al., 2001).

SNF1-related protein kinases (SnRKs) in plants have a catalytic domain similar to that of sucrose non-fermenting-1 (SNF1) of yeast *Saccharomyces cerevisiae* and AMP-activated protein kinase (AMPK) of animals. SNF1 is activated in response to low cellular glucose levels and is required for the derepression of genes that are repressed by glucose (Gancedo, 1998; Ronne, 1995). SNF1 can also directly modulate the phosphorylation state of a number of metabolic enzymes (Woods et al., 1994; Hardy et al., 1994). AMPK can be activated by AMP in the higher AMP/ATP ratio and by its upstream protein kinase AMP-activated protein kinase kinase (AMPKK). It is indicated that activation of AMPK is in response to a variety of conditions of stress in mammalian cells (Sato et al., 1993; Corton et al., 1994; Kudo et al., 1995). Interestingly, some of SnRKs in plants appear to be highly conserved with those in yeast and animals, and suggest their very similar roles (Halford et al., 1998). In plants, three SnRKs subfamilies have been identified: SnRK1, SnRK2 and SnRK3. The SnRK1 subfamily shares direct structural and functional homologues of the SNF1/AMPK family (Halford et al., 1998). To date, SnRK1 genes have been identified and characterized in many plant species after the first plant SnRK1 sequence being reported from a rye endosperm cDNA library (Halford et al., 1998; Alderson et al., 1991). Based on amino acid sequence similarity and expression patterns, the SnRK1 gene subfamily of cereals can be subdivided further into two groups, SnRK1a and SnRK1b (Halford et al., 1998). SnRK2 and SnRK3 subfamilies, with less sequence similar to SNF1 and AMPK than SnRK1, are unique to plants and may be involved in the response to environmental stresses. SOS2, as a well known member of SnRK3 subfamily, is involved in conferring salt tolerance (Halfter et al., 2000; Liu et al., 2000).

SnRK2, a relatively small plant-specific gene family, has been recently identified as osmotic-stress-activated protein kinases in plants. PKABA1 from wheat is the first cloned SnRK2 subfamily member (Anderberg et al., 1992). After that other members of the SnRK2 subfamily are demonstrated to be involved in osmotic signaling, for example, AAPK (induced by ABA in guard cells in response to drought) from fava bean and SPK-1, SPK-2, SPK-3 and SPK-4 (response to hyperosmolarity or salinity) from soybean (Monks et al., 2001; Yoon et al., 1997). Very recently, Kobayashi et al. (2004) analyzed all members of the SnRK2 subfamily encoded by the rice genome and find that all 10 members are activated by hyperosmotic

stress and that three of them are also activated by ABA. Additionally, Boudsocq et al. (2004) analyzed 10 *Arabidopsis* SnRK2s and demonstrate that hyperosmolarity activate at least four members of the family *in vivo*. Furthermore, using protoplast transient expression assays they find that hyperosmotic and saline stresses activate all SnRK2s, except SnRK2.9.

Maize is an important crop cultivated in many areas in the world. In the many maize-growing areas, drought, salinity and extreme temperature frequently influence maize growth and production. It has been one of the main objects for the researchers to elucidate the plant stress resistance signal pathway and improve the ability of crops resistance to the stresses. In this study, we reported the cloning of a *SnRK2b* member *ZmSPK1* (GenBank accession no, AY722708) from maize and its expression patterns in various maize tissues and in roots treated with drought, high salt and ABA. The possible function of the *ZmSPK1* was discussed based on the experimental results.

MATERIALS AND METHODS

Plant material, growth conditions, and experimental treatments

Seeds of maize (*Zea mays* L.) Jingyu7 were surface-sterilized for 5 min in 1% (w/v) sodium hypochlorite, and were finally washed in distilled water. The sterilized seeds were soaked in distilled water for 12 h. Fully imbibed seeds were germinated at 28°C in the dark. The germinating seeds were then sown on a layer of hydrophilic cotton in trays and covered with two layers of wet hydrophilic pledget. When the primary roots were about 1.5 cm in length, the roots and coleoptiles were harvested for RT-PCR. The left seedlings were then transferred to a chamber with 70% relative air humidity, 30°C/28°C day/night temperature, a day/night cycle of 16 h/8 h, and a 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux density. When the ligules of the second leaves were visible the seedlings were subjected to stress as following treatments.

ABA, mannitol, or NaCl treatments were performed by transferring the seedlings onto the trays watered with 100 μM ABA, 600 mM mannitol or 150 mM NaCl solution. For ABA treatment the seedlings was sprayed with the same concentration of ABA at the same time of transferring to ABA solution. Roots were harvested from the treated seedlings at 2, 6, 12 and 24 h just after transferring. Roots from untreated seedlings were also sampled as a control (0 h).

Mature leaves, immature tassels, silks and ovaries were sampled from field-grown maize. In detail, immature tassel were sampled approximately 4 to 5 cm in size; several days later, immature silks were sampled before emergence out of the bract, ovaries were excised from the central part of the ears, and mature leaves were also sampled at the same time.

All the above samples were put into liquid N₂ just after harvested, and stored in -80°C.

Cloning of cDNA encoding *ZmSPK1*

Using the *Arabidopsis SnRK2.4 (ASK1)* gene as a query probe, a highly homologous maize EST contig (*ZMtuc03-08-11.10376*) was obtained from maize EST TUGs database. According to the EST contig sequence, two primers, 5'-AAAACCCAGCGAATTCC-3' (forward primer) and 5'-TCTCAGCACCACAACTACAACA-3' (reverse primer; synthesized at Sunbiotech, China) were used to

MEKYELLKD IGSNFGVARLMRNKDTKELVAMKYIPRGLKIDENVAREI INHRSLRHHNI IRFKE 65
VVLTPTHLAI VMEYAAGGELFDRIC SAGRFSSEDEARYFFQQL IGVSYCHLMQ***ICHRDLKLENTL*** 130
ZDGSAPRLKICDFGYSKSSLLHSPKSTVGTPAYIAPEVLSRREYDGKMDVWSCGVTLVVMLV 195
GAYPFEDPDDPKNFRKTIGRIVSIQYQIPEYVHISQDCRQLLARIFVANPAKRITIREIRNHPWF 260
LRNLPRELTEAAQAKYYKDNSAPTFSQTVVEIMKIVEEARTPPQSSSTPVACFGWAEEDDEQEDG 325
KRSDDDEQYGEDEDYDGEDEYDKQVKVHVASGDFQHLK 364

Figure 1. ZmSPK1 protein sequence deduced from cloned gene *ZmSPK1*. The underlined amino acid is the protein kinase catalytic domain. Protein kinase ATP-binding region signature is shown in gray box. Serine/Threonine protein kinases active-site signature is shown in bold and italic type. The sequence in frame is potential N-myristoylation site. The potential transmembrane spanning region is indicated by dots.

amplify DNA encoding ZmSPK1 by PCR. The first strand cDNA was obtained by RT using M-MLV Reverse Transcriptase (Promega, USA) in a 20 µl reaction volume on RNA prepared from maize roots treated with 150 mM NaCl for 24 h. RNA was isolated using TRIzol Reagent (Invitrogen, USA). The RT-PCR product was cloned into pGEM-T vector (Promega, USA) and sequenced (Sangon, China).

RT-PCR analysis

Reverse transcription-polymerase chain reaction (RT-PCR) was used to semi-quantitatively determine the expression profile of the *ZmSPK1* gene. 5 µg of RNA from different samples was reverse-transcribed into cDNA as described above. The specific primers were designed according to the cDNA sequence. The sense primer is 5'-CCAGCGAATTCCCCACG-3' and the antisense primer is 5'-TCTTCTCCTCAGCCCAACAA-3'. The expected length of the amplified fragment is 1011 bp. The total volume of PCR reaction is 25 µl, containing 1 µL of the first-strand cDNA, 0.4 µM of each primer, 1×PCR Buffer, 0.2 mM of dNTP and 1 U of Taq DNA polymerase. The reaction was denatured at 94°C for 3 min, and then subjected to 28 cycles of 94°C for 45 s, 59.6°C for 1 min and 72°C for 2 min, plus a final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gel and quantified using the Higher Performance Ultraviolet Transilluminator (GDS-8000, Gel Documentation System, UVP, USA). Maize *18S rRNA*, amplified with primers 5'-CCATAAACGATGCCGA-3' (forward primer) and 5'-CACCACCCATAGAATCAAGA-3' (reverse primer) were used as the internal standard in the experiment. The experiments were repeated three times with the similar results and one of them is presented. The ratio of the target band intensity to the *18S rRNA* band intensity represented the relative expression level of the target gene.

RESULTS

Isolation and sequence analysis of ZmSPK1

Using the *SnRK2.4* as a query probe, a highly homologous maize EST contig (*ZM1uc03-08-11.10376*) was obtained from maize EST TUGs database. By analyzing the contig

sequence in <http://au.expasy.org/tools/dna.html>, a putative ORF with the length of 1095 bp was predicted. The analysis result also showed that there was an in-frame stop codon TAA upstream from the first initiation codon ATG at the 5'-end. And there was also an in-frame stop codon TGA at the 3'-end (data not shown). These indicated that it was a full-length gene. RT-PCR product from salt treated maize roots using the primers as described in material and methods was cloned into pGEM-T vector and sequenced. Based on the sequencing result, further analysis via PlantP program (Gribskov et al. 2001) indicated that the deduced protein, ZmSPK1, was a Ser/Thr protein kinase contained 364 amino acids with an estimated molecular mass of 41.8 KD and an isoelectric point of 5.8. A protein kinase catalytic domain was also found. Additionally, in this catalytic domain one potential N-myristoylation site GVSYCH, one Serine/Threonine protein kinase active-site ICHRDLKLENTLL, one protein kinase ATP-binding region IGSNFGVARLMRNKDTKELVAMK and one potential transmembrane spanning region MADVWSCGVTLVVMLVGAY were also found (Figure 1).

Comparison of ZmSPK1 with other related SnRK2s

The predicted kinase contained 11 conserved kinase subdomains that were typical of protein kinases (Hanks et al., 1988) plus a stretch of acidic amino acids at the C-terminus presented in most of the known kinases of the SnRK2b group. Blasting protein homologies in NCBI by the deduced amino acid sequence of ZmSPK1 showed the greatest identities to protein kinases assigned to the SnRK2b group, i.e. SPK3 and SPK4 from soybean, SnRK2.1 and SnRK2.4 from *Arabidopsis* and NtOSAK from tobacco (Figure 2), suggesting that ZmSPK1 is a member of this group. Analysis by generation of a

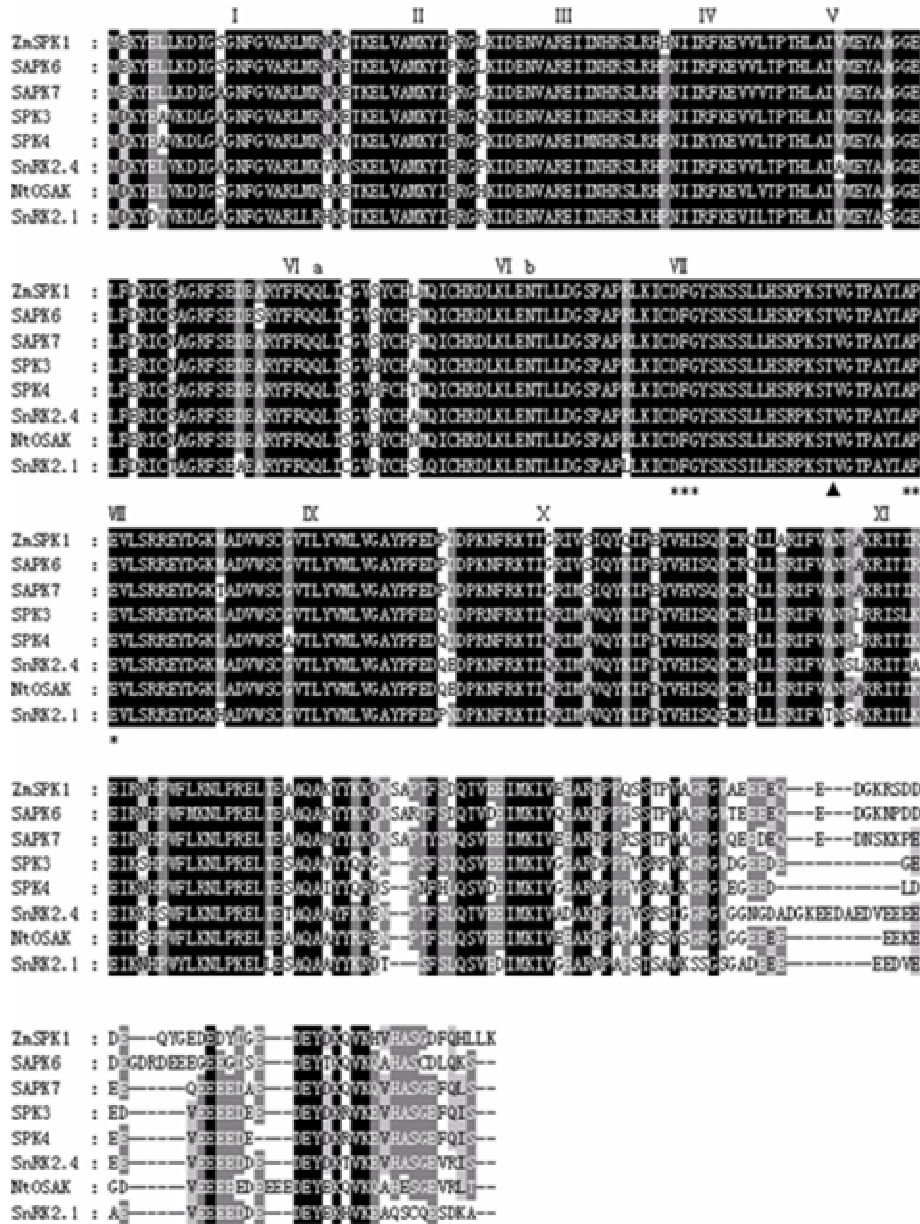


Figure 2. Alignment of amino acid sequence of ZmSPK1 with that of other closely related sequences from plant: rice SAPK6 and SAPK7 (GenBank accession numbers AB125307 and AB125308), soybean SPK3 and SPK4 (GenBank accession numbers L19361 and L38855), *Arabidopsis* SnRK2.1 and SnRK2.4 (GenBank accession numbers NM_120946 and NM_100969), tobacco NtOSAK (GenBank accession number AY081175). The positions of the catalytic subdomains according to Hanks and Quinn are indicated above the sequences with roman numerals. In the consensus sequence, black boxes indicate positions at which the residues are identical and gray boxes highlight residues that are similar. DFG and APE conserved motifs are indicated by asterisk (*), and the conserved T between these motifs was indicated by '▲'. Sequences were aligned using Clustal X 1.8 and GeneDoc.

phylogenetic tree with all 10 rice and 10 *Arabidopsis* SnRK2s and other closely related and functionally characterized SnRK2s confirmed that ZmSPK1 has the closest relationship with SAPK6 and SAPK7 from rice (Figure 3).

Additionally, by multiple sequence alignment we also

found the conserved Thr residue between the DFG and APE conserved motifs of subdomain VII and VIII where many other protein kinases were activated by its phosphorylation (Johnson et al. 1996). This indicates that the conserved Thr residue in the putative activation loop could be the critical target site for phosphorylation by an

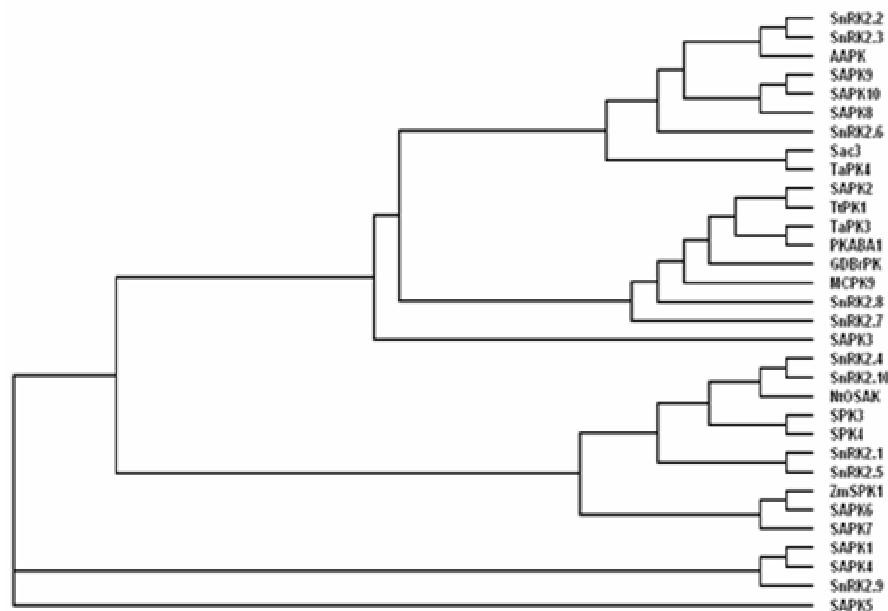


Figure 3. Phylogenetic tree based on an alignment of maize *ZmSPK1* with all 10 rice and 10 *Arabidopsis* SnRK2s and other closely related SnRK2s: SAPK1-10 from rice (GenBank accession number AB125302-11), SnRK2.1-10 from *Arabidopsis* (GenBank accession numbers NM_120946, NM_114910, NM_126087, NM_100969, NM_125760, NM_119556, NM_120165, NM_106478, NM_127867 and NM_104774), AAPK from fava bean (GenBank accession number AF186020), Sac3 from *Chlamydomonas* (GenBank accession number AF100162), TaPK3, TaPK4, PKABA1 and TtPK1 from wheat (GenBank accession numbers U29094, AF519805, BAB61735 and AY714526, respectively), GDBrPK from grapevines (GenBank accession number AF178575), MCPK9 from *Mesembryanthemum crystallinum* (GenBank accession number Z26846), NtOSAK from tobacco and SPK3 and SPK4 from soybean. The phylogenetic tree was created with Clustal W and TreeView programs.

upstream activating kinase(s).

Expression characterization of *ZmSPK1*

As shown in Figure 4 A, *ZmSPK1* showed different expression level in different maize tissues. The *ZmSPK1* transcript was most abundant in tassels and ovaries and at a lower level in mature leaves, whereas it was lowest in roots and silks. Additionally, we did not amplify the corresponding band from coleoptiles (data not shown).

RT-PCR analysis was also performed to investigate the *ZmSPK1* expression in response to various abiotic stresses treatments. The results presented in Figure 4B-D indicated that the *ZmSPK1* could be induced by NaCl, mannitol and ABA. However, the induction patterns were different among those of the three treatments. For NaCl and mannitol treatments, the transcript level was increased within 2 h, reached the maximum at 6 h, and then decreased. But for ABA treatment, the transcript level gradually increased and reached the maximum level at 24 h.

DISCUSSION

In this study, a maize cDNA designated as *ZmSPK1* was

cloned and characterized. The deduced amino acid sequence showed 90 and 89% identity with SAPK6 and SAPK7 from rice, respectively, 79 and 78% identity with SPK3 and SPK4 from soybean, respectively, 79 and 78% identity with SnRK2.1 and SnRK2.4 from *Arabidopsis*, respectively, and 80% identity with NtOSAK from tobacco, which all belong to SnRK2b group. However, a phylogenetic tree analysis further revealed that the *ZmSPK1* were closely related to the protein kinases which are known to be the typical SnRK2b group. All these above indicate that *ZmSPK1* is a member of SnRK2b.

Using the PlantsP program, a protein kinase catalytic domain and a Ser/Thr protein kinase active-site was found indicating it is a Ser/Thr protein kinase. N-terminal myristoylation plays a vital role in membrane targeting and signal transduction in plant responses to environmental stress, and this modification is essential for protein function to mediate membrane association or protein-protein interaction (Podel et al., 2004; Ishitani et al., 2000). In the catalytic domain of *ZmSPK1*, one potential N-myristoylation site and one potential transmembrane spanning region were found by PlantsP program. This strongly suggests that *ZmSPK1* may interact with cell-membrane system and response to stress. However, precise roles and subcellular localization of *ZmSPK1* remain to be identified.

The expression of *ZmSPK1* varied in the various maize

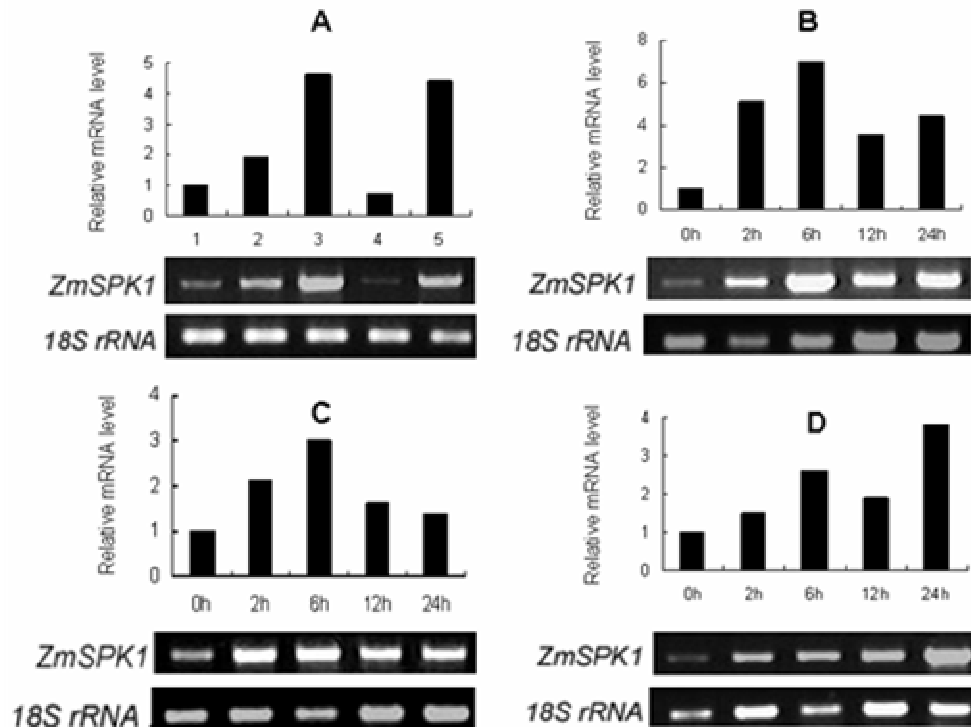


Figure 4. Expression patterns of the *ZmSPK1* gene in various tissues and in response to various treatments. A, Expression patterns of *ZmSPK1* in various tissues: (1) roots, (2) mature leaves, (3) tassels, (4) silks, and (5) ovaries. The ratio value of the *ZmSPK1* intensity to the *18S rRNA* intensity in roots was arbitrarily set to 1 and all the other values were compared with it. B-D, Time-course of *ZmSPK1* expression in maize roots upon NaCl, mannitol and ABA treatment, respectively. For NaCl, mannitol and ABA treatment the ratio value of the *ZmSPK1* intensity to the *18S rRNA* intensity at 0 h was arbitrarily set to 1 and all the other values were compared with it.

tissues indicating its function in plant development. Interestingly, *ZmSPK1* transcript was most abundant in tassels and ovaries which belong to reproductive organs. This may suggest that *ZmSPK1* may play a role in reproductive development and physiology. Stress adaptation in plants can be mediated either by ABA-dependent or ABA-independent pathways (Shinozaki et al., 1996). In this study we found that the *ZmSPK1* was induced not only by NaCl and mannitol but also by ABA. At first sight it would appear that the induction of *ZmSPK1* was dependent on ABA. However, induction of *ZmSPK1* by NaCl and mannitol was more rapid than induction by ABA. This may suggest that other factor(s), other than ABA, may also be involved in *ZmSPK1* induction and/or that ABA does not induce *ZmSPK1* directly. In addition, compared to control the degree of induction of *ZmSPK1* by NaCl was higher than that achieved by mannitol and ABA. This may indicate that *ZmSPK1* has the different sensitivity to different stresses treatments and more sensitive to salt. However, further research, including the biochemical properties, the precise roles in stress signal transduction and in plant development, will be needed to reveal its function in plants.

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