The MsK family of alfalfa protein kinase genes encodes homologues of shaggy/glycogen synthase kinase-3 and shows differential expression patterns in plant organs and development

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Summary

This paper reports on the isolation of a novel class of plant serine/threonine protein kinase genes, MsK-1, MsK-2 and MsK-3. They belong to the superfamily of cdc2-like genes, but show highest identity to the Drosophila shaggy and rat GSK-3 proteins (65-70%). All of these kinases share a highly conserved catalytic protein kinase domain. Different amino-terminal extensions distinguish the different proteins. The different plant kinases do not originate from differential processing of the same gene as is found for shaggy, but are encoded by different members of a gene family. Similarly to the shaggy kinases, the plant kinases show different organ-specific and stage-specific developmental expression patterns. Since the shaggy kinases play an important role in intercellular communication in Drosophila development, the MsK kinases are expected to perform a similar function in plants.

Introduction

Segment polarity genes are required for the establishment of the metaneric pattern of the embryonic cells within the segments (Nusslein-Volhard and Wieschaus, 1980). The number, position and orientation of metaneric units is dictated by the products of maternal and zygotic segmentation genes. The positional information that has been derived for the cells that give rise to the segment-polarity genes has been termed the segment polarity pattern (SPP). The position of some of the segment-polarity genes is conserved, which suggests that these genes are required for the establishment of the segment polarity pattern. In Drosophila, the genetic information for the establishment of the segment polarity pattern is transmitted by the related genes shaggy (shg) and fushi tarazu (ftz). The segment polarity pattern is established by the action of the shaggy gene, which encodes a protein kinase involved in the regulation of the cell cycle (Bouros et al., 1990; Siegfried et al., 1990). The shaggy gene is expressed in the posterior region of the embryo, where it is regulated by the expression of the zygotic segmentation gene fushi tarazu (ftz). Although the different kinases have identical catalytic domains, different amino-terminal extensions are added by differential processing.

Shaggy shows 75% identity to glycogen synthase kinase-3/Factor A (Woodgett, 1990). Glycogen synthase kinase-3 (GSK-3) was first identified as a protein kinase that phosphorylates glycogen synthase (Embi et al., 1980; Hemmings et al., 1982). GSK-3 is also known as a factor that regulates the activity of the glycogen synthase-3/Factor A, the activator protein of protein kinase phosphatase-1 (Hemmings et al., 1982; Vandenheede et al., 1992; revised 6 November 1992; accepted 20 November 1992.)
three clones. Alignment of the predicted protein sequences of the three clones showed a co-linear arrangement of the amino acids over the entire length, as shown in Figure 2. The open reading frames of all MsK clones were very similar. Overall, identities of 83% for MsK-1/2, 88% for MsK-2/3 and 83% for MsK-1/3 were obtained. Although the majority of amino acid differences were found at the N- and C-terminal ends, the presence of conserved domains within these regions was still observed. Analysis of the precursor amino acid sequences revealed that all three genes contain the conserved sequence domains of serine/threonine protein kinases.

Results

Isolation of MsK-1, MsK-2 and MsK-3

Three redundant oligonucleotides with sequences deduced from the conserved domains of the cdc2 protein kinases were synthesized (for nucleotide sequences, see Experimental procedures). These oligonucleotides were 32P-labelled and used as probes to screen an alfalfa suspension culture cDNA library. With one of the oligonucleotides (directed at the sequence H-R-D-L-K-P-Q-N-L), one clone showed strong hybridization at washing conditions for oligonucleotides, with a fit of more than 16 nucleotides. Sequencing of this clone indicated a 5'-truncated cDNA encoding a putative protein kinase gene. When another cDNA library prepared from somatic embryos was screened with the truncated clone, 32 positive clones were isolated. Restriction analysis indicated that three different classes of clones had been isolated. The longest inserts of each class had a length of 1.6 kb (MsK-1), 1.5 kb (MsK-2) and 1.8 kb (MsK-3).

MsK-1, MsK-2 and MsK-3 belong to a family of highly related protein kinases

When the sequenced cDNA clones were analysed for open reading frames, putative proteins of 411, 411 and 412 amino acids were identified for MsK-1, MsK-2 and MsK-3 as shown in Figure 1, respectively. Within the open reading frames, the H-R-D-L-K-P-Q-N-L sequence, highly conserved in the cdc2 homologues, is interrupted by an arginine in all three clones. Alignment of the predicted protein sequences of the three clones showed a co-linear arrangement of the amino acids over the entire length, as shown in Figure 2. The open reading frames of all MsK clones were very similar. Overall, identities of 83% for MsK-1/2, 88% for MsK-2/3 and 83% for MsK-1/3 were obtained. Although the majority of amino acid differences were found at the N- and C-terminal ends, the presence of conserved domains within these regions was still observed. Analysis of the precursor amino acid sequences revealed that all three genes contain the conserved sequence domains of serine/threonine protein kinases.
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**Figure 1.** Nucleotide and predicted amino acid sequences of the alfalfa MsK cDNA clones. (a) MsK-1; (b) MsK-2; (c) MsK-3.
(b) Msk-2

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\begin{align*}
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Figure 2. Alignment of the predicted alfalfa MsK-1,-2 and -3 proteins.
Amino acids are represented in the single letter code. Identical amino acids are indicated by dots. Where necessary, gaps were introduced and are denoted by hyphens.

Figure 3. Alignment of the predicted alfalfa MsK-2, rat GS3 and Drosophila shaggy proteins.
Amino acids are represented in the single letter code. Identical amino acids are indicated by dots. Where necessary, gaps were introduced and are denoted by hyphens.

RNA was pretreated and cDNA was synthesized using the 3X oligo-dT primer.

poly(A)+ RNA

Analysis of the transcription levels of MsK-1,-2 and -3 genes, Northern analysis was performed with poly(A)+ RNA from alfalfa suspension cultures. To avoid cross-hybridization, 3′ end specific fragments were synthesized by PCR. MsK-1,-2 and -3 genes were found to encode mRNAs of approximately 1.6 kb, 1.5 kb and 1.8 kb, respectively. Whereby MsK-1 was found to be strongly expressed, MsK-2 and MsK-3 transcription levels were very low (data not shown).

To analyse the expression of the three MsK genes in various vegetative organs of alfalfa plants, Northern analysis with both total RNA or poly(A)+ RNA was performed and identity bands were detected by hybridization with probes coding for MsK homologous DNA. Resulting bands could be detected only in leaves, petioles, stems, and roots. No hybridization was observed in roots, stems, or flowers. Since we knew from Northern analysis that MsK-2 and MsK-3 transcription levels were very low, transcript levels of all three genes were quantitatively determined by reverse transcription PCR of RNA samples and PCR amplification with specific primer pairs.

The following controls were performed. First, to confirm equal amounts of total RNA were used in the reverse transcription reactions, the RNA samples were quantified by spectrophotometry and ethidium bromide staining of denaturing agarose gels, as shown in Figure 4A. Second, all reactions were performed at the same time in parallel. Third, to test whether the RNA preparations contained contaminating genomic DNA traces, PCR was performed with and without the use of reverse transcriptase.

The identity bands were detected by hybridization with probes coding for MsK homologous DNA.
Figure 4. Reverse transcribed PCR amplification.
(a) To prove that equal amounts of RNA were used per organ, total RNA was isolated from alfalfa leaves, stems, roots, nodes, flower buds, developing flowers, unopened mature flowers, green seed pods and globular, heart-shaped and torpedo-shaped somatic embryos (lanes 1 to 11, respectively). After spectrophotometric quantification, 10 μg of each sample was loaded onto a 1% denaturing agarose gel and stained with ethidium bromide.
(b) To prove that reverse transcription is necessary for PCR amplification of transcripts, total RNA was isolated from suspension cultured alfalfa cells and treated with or without DNase (lanes 1, 2 and 3, 4, respectively) and with or without reverse transcriptase before amplification with PCR (lanes 1, 3 and 2, 4, respectively). 32P-end-labelled cdc2 primers were used. PCR products for the cdc2 gene were separated on a 5% polyacrylamide gel.
(c) To prove linearity of the amplification system, 0.1, 0.5, and 1 μg of total RNA was used for each reaction (lanes 1 to 3, respectively). PCR products for the cdc2 gene were separated on a 2% agarose gel, blotted to nylon filters and hybridized with a radiolabelled cdc2 fragment.

For this purpose, primers for the alfalfa cdc2 gene (Hirt et al., 1991) were mixed with the MsK amplifications. Finally, the amplified products were separated on agarose gels, blotted to nylon filters and hybridized with gene-specific probes. The result of such an analysis is shown in Figure 5. MsK-1 appeared to be constitutively expressed in all five organs (Figure 5a, lanes 1 to 5). In contrast, MsK-2 was found to be absent in leaves and petioles but expressed at similar levels in stems, roots and nodes (Figure 5b, lanes 1 to 5, respectively). MsK-3 transcripts were completely absent in leaves and petioles, hardly detectable in stems and roots and moderately expressed in nodes (Figure 5c, lanes 1 to 5, respectively). The amplification products of the internal control cdc2 gene (Figure 5d) show that the observed differences in the MsK transcript levels are representative of the steady-state levels of the mRNAs within the different organs.

Changing pattern of MsK-1, -2 and -3 transcript levels during flowering

To analyse whether MsK-1, -2 and -3 genes may play a specific role during flower and seed development, several stages of flowers were quantitatively analysed. For this purpose, total RNA was isolated from flower buds (corolla enclosed in the sepals), developing flowers (corolla protruding 2 mm from sepals), unopened mature flowers and green seed pods (lanes 1 to 5 in Figure 6, respectively). MsK-1 appeared to be constitutively expressed as in...
present in flower buds (Figure 6b, lane 1). MsK-3 transcript levels were high in early flower development, gradually decreased in later stages and were absent after fertilization (Figure 6c, lanes 1 to 5, respectively).

**MsK-1, -2 and -3 transcripts in somatic embryos**

In *Drosophila*, shaggy is developmentally transcribed during embryogenesis, giving rise to different mRNAs in each stage. To analyse the situation of the MsK genes in plants, we determined mRNA levels of the three MsK genes in various stages of alfalfa somatic embryos: globular embryos (lane 1), heart-shaped embryos (lane 2) and torpedo-shaped embryos (lane 3). MsK-1 transcript levels did not change appreciably (Figure 7a). MsK-2 transcripts could only be observed in globular embryos (Figure 7b, lane 1) while MsK-3 transcripts were moderate in globular embryos and decreased in the later stages (Figure 7c, lanes 1 to 3, respectively).

**Discussion**

*Shaggy* belongs to the class of segment-polarity genes, necessary for correct pattern formation within each segment. It also plays a role in epidermal cell differentiation in the adult fly (Bourouis et al., 1989; Simpson et al., 1988). Mutations in segment-polarity genes lead to deletions or deficiencies in each segment boundary, thought to act coordinately to control gene expression.

Figure 6. MsK-1, -2 and -3 transcript levels during alfalfa flower and seed development. Total RNA was isolated from alfalfa flower buds (lane 1), immature developing flowers (lane 2), unopened mature flowers (lane 3), fully opened flowers (lane 4) and green seed pods (lane 5). After first strand synthesis, MsK-1, -2, -3 or cdc2 transcripts were amplified with specific primers by PCR. PCR products were separated on 2% agarose gels, blotted to nylon membranes and hybridized to 3' probes specific for: (a) MsK-1; (b) MsK-2; (c) MsK-3; (d) cdc2.

Figure 7. MsK-1, -2 and -3 transcript levels in somatic embryos. Total RNA was isolated from three fractions of somatic embryos: globular-shaped embryos (lane 1), heart-shaped embryos (lane 2) and torpedo-shaped embryos (lane 3). After first strand synthesis, MsK-1, -2, -3 or cdc2 transcripts were amplified with specific primers by PCR. PCR products were separated on 2% agarose gels, blotted to nylon membranes and hybridized to 3' probes specific for: (a) MsK-1; (b) MsK-2; (c) MsK-3; (d) cdc2.
shown). Finally, all three MsK genes were found to give rise to single transcripts in Northern analyses.

Comparison of the predicted protein sequences of the two mammalian GSK-3 genes and the two characterized Drosophila shaggy transcripts revealed high conservation over most of the sequence. However, sequence similarity of the two GSK-3 proteins disappeared in the N-terminal direction at the same conserved amino acid motif where the differentially processed shaggy gene products diverge from each other. Alignment of the MsK proteins with the GSK-3 and shaggy sequences revealed a similar picture. The N-terminal protein sequences of all three MsK genes were very different and showed no similarity to either the shaggy or the GSK-3 proteins. Homology started from the same conserved amino acid domain as was found in the comparison of the Drosophila and mammalian kinases.

All serine/threonine protein kinases including the cdc2-like kinases contain eleven conserved domains (Hanks et al., 1988) which are found in the carboxyl part of all shaggy, GSK-3 and MsK protein kinases. These proteins thereby resemble amino-terminally extended versions of a catalytic kinase subunit. These extensions appear to be conserved during evolution since GSK-3β and the ovarian product of shaggy have highly similar amino termini and must be considered to be true functional homologues of each other.

Since the catalytic domain carries all the relevant sequence domains for a functional protein kinase, the N-terminal modules may have a regulatory function. Each amino-terminal module may interact with specific proteins that couple the catalytic core module to different regulators. Alternatively, different N-termini could induce different conformational states of the respective kinases resulting in an altered substrate specificity. Taken together, attachment of different amino terminal sequences to a common serine/threonine protein kinase domain might mediate external signals to a variety of different substrates, including activation of transcription factors that switch on particular target genes involved in a particular developmental or metabolic pathway.

In Drosophila, different shaggy transcripts were found to be expressed in a developmental stage-specific manner. Similarly, the alfalfa MsK genes were found to be transcribed in an organ- and developmental stage-specific pattern. Therefore, it is an intriguing possibility that the MsK genes could perform analogous functions in plants to those which the shaggy transcripts do in insects. In particular, the highly regulated pattern of the MsK-2 and MsK-3 genes during somatic embryogenesis is reminiscent of the short-lived embryonic forms of shaggy. In contrast, the constitutive expression of the MsK-1 gene is suggestive of a late developmental role similar to the shaggy form.

kinase will be indispensable. For this purpose, we are presently producing peptide antibodies against the amino-terminus of each kinase. Furthermore, although we have obtained clear evidence that the MsK genes are differentially transcribed in various vegetative organs and during flowering, it will be important to identify the particular tissues or cells that actively synthesize the kinases. Finally, by expression of mutant versions of the MsK genes, we hope to interfere with their normal function in plants. It will be seen whether, in analogy to Drosophila or mammals, embryogenesis, organogenesis or specific metabolic pathways will be affected by this approach.

**Experimental procedures**

**Plant tissue culture**

A suspension culture of Medicago sativa L. (genotype RA3) was used (Györgyey et al., 1991). The suspension culture was derived from callus tissue which originated from stem segments and was grown in Schenck and Hildebrandt (1972) medium supplemented with 15 μM naphthalene acetic acid and 10 μM kinetin. Induction of somatic embryos was performed as described (Györgyey et al., 1991).

Poly(A)⁺ RNA was isolated from somatic embryos (Györgyey et al., 1991). A lambdaZAP-cDNA library was prepared according to the manufacturer (Stratagene, La Jolla, CA). Two degenerate oligonucleotides were synthesized: a (5'-GAA TTC GGN GAA/G GGI AGT CAT/G CNG GTG) and b (6'-AAG CTG TTT IGG TCT/TT IAAG A/GTC NCG/TA/GTG-3'). They correspond to two highly conserved regions of the superfamily of cdc2 kinases and encode either the nucleotide binding domain (gly-glu-glu-tyr-tyr-gly-val) or domain VI of the catalytic domain (his-arg-aspart-leu-lys-pro-gln-asn-ala). After gel elution from 10% denaturing polyacrylamide gels, 50 ng of each batch of oligonucleotides was 32P-labelled with 50 μCi gamma-ATP and poly nucleotide kinase. Approximately 300 000 colonies were screened with both probes. Putatively positive clones were rescreened to a minimum fit of 16 nucleotides according to Wood et al. (1985). The only positive clone was sequenced from both directions with the T7 polymerase sequencing kit (Pharmacia, Uppsala). The insert from this clone was used as a probe to rescreen the library. Fifteen positive clones were identified. They could be classified into three groups, called MsK-1, -2 and -3. The longest members of each group were sequenced as above.

**RNA extraction and PCR quantification of transcript levels**

RNA from suspension culture cells or plant tissue was prepared as described (Hirt et al., 1991). The quantification of MsK-1, -2 and -3 mRNA levels was principally done according to Scheres et al. (1980) with the modification that a DNase treatment of the total RNAs was included. This was found to be necessary since PCR amplification was obtained also when no reverse transcriptase was used. However, after DNase treatment, no amplification was
heating the reaction mixture for 3 min at 65°C. The 3’ primers for the reverse transcription were: for 3’ MsK-1 (CAT GTA AAG GAC GAT TCC TAT GTA GGA), for 3’ MsK-2 (TGT TAC TAA CAT TGG TAC TAG T), for 3’ MsK-3 (AAC TCG GAA CAC ATG ACA T) and for 3’ cdc2 (GGC TGC GAG AAA CTG AAG TCG CT). Annealing of the primers to 0.5 µg of DNA-treated RNA was done by heating to 85°C for 3 min, incubation at 61°C for 30 min and gradually cooling to 37°C. PCR was performed with the above 3’ end primers and the following 5’ end primers: for 5’ MsK-1 (AGG AAC TAC AGT CCA GAC GCT GTT G), for 5’ MsK-2 (GTG TAC TAA CAT TGG TAC TAG T) and for 5’ MsK-3 (TGG TAC TAA CAT TGG TAC TAG T). PCR was done for 15 cycles of 1 min at 92°C, 2 min at 61°C and 3 min at 72°C. The amplified fragments were run on 2% agarose gels, blotted to nylon membranes and hybridized to 32P-labeled 3’-specific MsK-1, MsK-2, MsK-3 and cdc23 probes.


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**References**


