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
In Drosophila, the segment-polarity genes are required for the establishment of the metameric pattern of the embryo, specifying positions of cells within the segments (Nüsslein-Völlhard and Wieschaus, 1980). The number, position and polarity of the metameric units is dictated by the products of interacting maternal and zygotic segmentation genes. The activity of the segment-polarity proteins has been suggested to control the cellular interactions which convey the positional information to the involved cells (Martinez-Arias et al., 1988). The predicted protein sequences of some of the segment-polarity genes are consistent with a function in a signal transduction process involved in the determination of cell identity within the segmental unit (Hooper and Scott, 1989; Nakano et al., 1989; Van den Heuvel et al., 1989).

Intercellular interactions also appear to be involved in the determination of fate during early embryogenesis. For example, the central nervous system arises from a number of neuroblasts that segregate from initially ectodermal cells. If a neuroblast is removed experimentally, a neighbouring cell will change its fate and become a neuroblast (Doe and Goodman, 1985). A process called lateral inhibition normally prevents neighbour cells from becoming neuroblasts. Lateral inhibition is thought to be mediated by a signal transduction pathway that converts the extra-cellular signals to changes in gene expression. The protein sequences of several neurogenic genes involved in these processes are compatible with such a function (Campos-Ortega, 1988).

Drosophila shaggy mutants show two seemingly unrelated phenotypes with characteristics similar to both of the above described processes. One type of shaggy mutants results in the segregation of excess numbers of neuroblasts in the embryo and bristle mother cells in the imago (Bourois et al., 1989; Simpson et al., 1988). Functional shaggy has been shown to be necessary for the intercellular communication process which mediates the formation of only one bristle mother cell (Simpson and Carteret, 1989). The other type of shaggy mutants form abnormal embryos in that all cells develop neural characteristics (Bourois et al., 1989). The molecular identification of shaggy has revealed that the gene encodes several distinct serine/threonine protein kinases which are developmentally regulated (Bourois et al., 1990; Siegfried et al., 1990). 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 was also shown to
al., 1980). GSK-3 was reported to phosphorylate the transcription factors c-jun and c-myc. While phorbol esters cause specific dephosphorylation of c-jun, phosphorylation by GSK-3 inhibits its DNA binding activity (Woodgett, 1990).

We report here the isolation of a novel class of plant serine/threonine protein kinases (MsK-1, MsK-2 and MsK-3) which are 65–70% identical to the shaggy and GSK-3 proteins. All of these genes belong to the superfamily of cdc2-like genes. GSK-3, shaggy and the plant genes share a highly conserved catalytic protein kinase domain. Different amino-terminal extensions distinguish different family members. In contrast to shaggy, the plant transcripts do not originate from differential processing of the same gene but are encoded by different members of a gene family. Individual members of this family of plant kinases were found to undergo different transcriptional regulation in various organs and during flower development. Since different shaggy transcripts perform strikingly different functions in Drosophila, the transcription pattern of the different plant homologues suggests a similar diversity of roles in plant development.

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 1.6 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-I-K-P-Q-N-L sequence, highly similar to the motive used for screening, was found 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 predicted amino acid sequences revealed that all three genes contain the conserved sequence domains of serine/threonine protein kinases (Hanks et al., 1988).

MsK-1, MsK-2 and MsK-3 are homologues of GSK-3/shaggy and belong to the cdc2 superfamily of protein kinases

A homology search in current protein sequence databanks showed highest similarity to the rat GSK-3 and to the Drosophila shaggy proteins. All predicted MsK proteins were found to have identities of 65–70% to different members of the GSK-3 and shaggy kinases. Significant homology was also observed to the cdc2 kinases (35–40%) from yeast (Hindley and Phear, 1984; Lörincz and Reed, 1984), humans (Lee and Nurse, 1986), and plants (e.g. Hirt et al., 1991) and to the yeast protein kinases KSS1 (Courchesne et al., 1989), FUS3 (Ellon et al., 1990) and KIN28 (Simon et al., 1986) (33, 32 and 34%, respectively) and to extracellular signal-regulated kinase-1 (ERK-1; Boulton et al., 1990) with 31% identity. All of these kinases belong to the superfamily of cdc2 genes. Comparison of the predicted MsK, GSK-3 and shaggy proteins with the cdc2 kinases revealed that all kinases have a similar arrangement of the nucleotide binding region and the catalytic kinase core domain. However, the PSTAIRED motive, which all true cdc2 kinases contain interspersed between these two domains, is completely missing in GSK-3, shaggy and MsK-type kinases. Moreover, the GSK-3, shaggy and MsK kinases contain N-terminal extensions not found in the cdc2 kinases.

Shaggy and GSK-3 proteins are highly similar (identities between 73 and 78% for different pairs of the family members). The divergence between the two members of the GSK-3 family has been found to arise from the presence of completely different amino-terminal sequences (Woodgett, 1990). In Drosophila, seven different shaggy transcripts are produced, each at a specific stage in embryogenesis (Bourouis et al., 1990; Siegried et al., 1990). Comparison of maternal and zygotic shaggy cDNA clones suggests that the N-terminal divergence arose through differential processing of 5’ exons (Siegfried et al., 1990). Alignment of the predicted MsK-2 amino acid sequence with maternal shaggy and GSK-3b reveals that the sequences are very similar over nearly the entire length, excluding the amino-
Figure 1. Nucleotide and predicted amino acid sequences of the alfalfa MsK cDNA clones.
(a) MsK-1; (b) MsK-2; (c) MsK-3.
Figure 1. Continued.
Alfalfa protein kinases homologous to shaggy/GSK-3

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Figure 1. Continued.
terminal regions as shown in Figure 3. Also within the Msks, homology is much reduced in the amino-terminal regions (Figure 2). Including these divergent amino-terminal regions, amino acid identities between the Msk and shaggy/GSK-3 kinases amount to scores between 65 and 70% for different pairs.

Highly different transcript levels of MsK-1, -2 and -3 in vegetative alfalfa organs and suspension culture cells

To determine the size of the mRNAs of the 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 transcript levels were very low (data not shown).

To analyse the expression of the three MsK genes in various vegetative organs of alfalfa plants, RNA was prepared from leaves, petioles, stems, roots and nodes. Northern analysis with both total RNA or poly(A)' RNA resulted in detection of constitutive transcript levels of MsK-1. However, no transcripts could be observed for MsK-2 or MsK-3, even after 2 weeks of exposure (data not shown). Since we knew from Northern analysis of suspension cultured cells that MsK-2 and MsK-3 transcript levels were very low, transcript levels of all three genes were quantitatively determined by reverse transcribing the RNA samples and PCR amplification with specific primers. The following controls were performed. First, to certify that equal amounts of total RNA were used in the reverse transcription reactions, the RNA samples were quantified by spectrophotometry and by 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 without using the
total RNA. Since we found that amplification could be obtained without reverse transcriptase, the total RNAs were DNase treated before reverse transcription. Inclusion of this step led to no amplification without the use of the reverse transcriptase, as shown in Figure 4b. Fourth, to test whether our PCR protocol results in linear amplification of the transcripts, we performed the reactions with 0.1, 0.5 and 1 μg of total RNA as starting material. In all cases, we obtained linearity in terms of concentration-dependent amplification (Figure 4c). Fifth, to test whether the MsK-1, MsK-2 and MsK-3 amplified signals are representative of the situation in the investigated organs, the PCR reactions were standardized with a constitutively expressed gene.

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 vegetative tissues (Figure 6a). MsK-2 levels were only
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 (Bouwmeester et al., 1991). In plants, MsK genes are expressed in the developing seed, where they are thought to act co-ordinately by means of intercellular communication. The transcription factors c-jun and c-myc, glycogen synthase and phosphoprotein phosphatase-1 are substrates of GSK-3. Since both GSK-3 and shaggy are mediators of extracellular signals, they can be envisioned to integrate diverse extracellular signals into a conserved pathway of signal transduction.

In this paper, we report on the isolation of several cDNA clones which encode a novel family of serine/threonine protein kinases in plants. Comparison with current data banks revealed highest homology to the Drosophila shaggy and rat GSK-3 protein kinases (65–70% identities). Significant identity scores were obtained with several other protein kinase genes (30–35%), all of which belong to the cdc2/CDC28 superfamily of protein kinases.

The shaggy gene in fruit fly gives rise to seven different transcripts which are expressed in a developmentally regulated fashion (Bouwmeester et al., 1990; Siegfried and others, 1990). A 50 kb long region constitutes the shaggy gene from which all five transcripts appear to be synthesized by the use of alternative 5′ exons. The same catalytic domain is thus covalently linked to various amino-terminal domains. In mammals, the two GSK-3 transcripts do not arise by differential processing but are the products of different genes. For the following reasons, we think that the diversity of the MsK protein kinase genes is also achieved by the transcription of individual members of a gene family.
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 motive 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-3p 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 which is responsible for the epidermal differentiation in the adult fly.

To investigate the role of the shaggy-like kinases in plants, the study of the substrate specificity of each MsK kinase will be indispensable. For this purpose, we are presently producing peptide antibodies against the aminotermus 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 Schenk 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).

**Library screening and sequence analysis**

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 ACI TAT/C GGN GT-3') and b (5'/AAG CTT TCG IGG T/C T IAAG/A GTC NCG/A TG-3'). They correspond to two highly conserved regions of the superfamily of cdc2 kinases and encode either the nucleotide binding domain (gly-glu-gly-thr-tyr-gly-va)

**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. (1990) 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 obtained. For this purpose, 5 µg total RNA were digested for 30 min at 37°C with 3 units of RNase-free DNasel (Pharmacia) in 40 mM Tris-HCl, pH 7.5, 6 mM MgCl₂, containing 5 units of RNasin (Sigma). After incubation, the DNase was inactivated by...
heating the reaction mixture for 3 min at 65°C. The 3′-primers for
the reverse transcription were for 3′ MsK-1 (CAT CTA GAA CGT-
TCA TAT GGA A), for 3′ MsK-2 (TAG AAT TCA CAA TGG TTC
TAG T), for MsK-3 (AAC TCG AGT GAA CAC ATC AGA T) and
for 3′ cdcc2 (GGC TGC AGC AAA CTG AAG TCC CT). Annealing
of the primers to 0.5 µg of DNase-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 AAT TCC AGT CGA GAC CCT G), for 5′ MsK-2 (ATA AGC
TTA GAA TGC TGT CCT G), for 5′ MsK-3 (TGC TGC AGC CCT
GTG TAG TTA TTA) and for 5′ cdcc2 (GGA AGC TTA ATG GTA
AAG GTG T). PCR was done for 20 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-labelled 3′-specific MsK-1, MsK-2, MsK-3 and cdcc2 probes.

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