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Regulation of the kinase activity of the MIK GCK-like MAP4K by alternative splicing

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Abstract Alternative splicing of introns is essential to ensure the complexity of mammalian genome functions. In particular, the generation of a high number of different isoforms by alternative splicing is an important characteristic of genes coding for signalling proteins such as mitogen activated protein kinases (MAPKs). This is thought to allow these proteins to transduce multiple stimuli in a highly regulated manner. Plant genes are also subjected to alternative splicing. Nevertheless, clear examples of the functional consequences of this phenomenon are still scarce in plants. *MIK* is a maize gene coding for a GCK-like MAP4K that can be activated by interaction with maize atypical receptor kinase (MARK), an atypical receptor kinase. Here we show that *MIK* is subjected to alternative splicing. Expression of *MIK* leads to, at least, 4 different mature mRNAs that accumulate with particular expression profiles during maize development. Our results show that the polypeptides encoded by the different *MIK* mRNAs display different kinase activity and are differentially activated by interaction with the MARK receptor. Two MIK isoforms display constitutive kinase activity, one isoform is inactive but can be activated by MARK, and the fourth MIK isoform is inactive and cannot be activated by MARK. Our results constitute a clear example of the biochemical consequences of alternative splicing in plants. The selective conservation during evolution of the intron–exon structure of the region coding for the regulator domain of MIK, as well as the maintenance in maize, rice and Arabidopsis of the alternative splicing of some of these introns, are strong indications of its functional importance.

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Introduction

Alternative splicing of introns is a process that allows genomes to generate multiple transcripts from a single gene. More than 40% of human (Modrek and Lee 2002) and mouse (Okazaki et al. 2002) genes are subjected to alternative splicing, and is supposed to be important to ensure the complexity of mammalian genome functions (Brett et al. 2002). Mitogen Activated Protein Kinases (MAPKs) are Ser/Thr kinases that play a key role in signal transduction in eukaryote systems. MAPKs modulate many different processes, including cell proliferation, differentiation, development or responses to different stress situations. The existence of multiple genes for each type of MAPKs explains, in part, their capacity to transduce different types of signals, but the generation of a high number of different isoforms by alternative splicing is thought to be an important characteristic of MAPK genes. Indeed, alternative splicing is a common feature to all MAPKs, leading to isoforms with altered capacities to interact with substrates or effectors (Kramer et al. 1996), different subcellular localisation (Yung et al. 2001) or dominant negative effects (Cameron et al. 2004). GCK-like kinases are a subfamily of MAPK kinase kinase kinase (MAP4K), which often connect membrane-located receptors to MAP kinase modules (Kyriakis 1999; Dan et al. 2001). These kinases are also regulated by alternative splicing (see for example Hu et al. 2004; Machida et al. 2004).

Plant genes are also subjected to alternative splicing, although to a lesser extent. Analyses of full-length cDNAs have shown that in both Arabidopsis (Iida et al. 2004) and rice (Kikuchi et al. 2003) alternative splicing affects 10% of the pre-mRNAs. Nevertheless, clear examples of the functional consequences of this phenomenon are still scarce in plants (Kazan 2003; Lorkovic et al. 2000).

MAP kinases are also important players in plant signal transduction, and mediate stress, hormone and developmental related processes (Jonak et al. 2002; Tena et al. 2001). Nevertheless, although the expression of a large number of MAPK has been analysed in plants, only two examples of possible alternative splicing of MAPKs transcripts have been described to date, an Arabidopsis MAPK kinase kinase (Nishihama et al. 1997), and a rice MAPK (Xiong and Yang 2003), and very little is known on their regulation and biological relevance.

Mark Interacting Kinase (*MIK*) is a maize gene coding for a kinase related to the GCK subgroup of MAP4Ks (Llompart et al. 2003). We have previously shown that MIK interacts with Maize Atypical Receptor Kinase (MARK), an atypical receptor expressed during embryogenesis and in the meristems of maize. Interestingly, the interaction with the intracellular domain of MARK (MARK-ID) results in MIK activation, and we have already proposed that this interaction could release MIK from a possible auto inhibited state (Llompart et al. 2003). Here we show that MIK pre-mRNA is subjected to alternative splicing leading to, at least, 4 different mature mRNAs that accumulate with particular expression profiles during embryogenesis and in the meristem of adult maize plants. Interestingly, the polypeptides encoded by the different *MIK* mRNAs display different kinase activity and are differentially activated by interaction with MARK. Two MIK isoforms are truncated proteins that only contain the MIK Kinase Domain (MIK-KD) and display constitutive kinase activity; one isoform, containing MIK-KD and the complete Regulatory Domain (MIK-RD) is inactive but can be activated by MARK; and the fourth MIK isoform, which contains a truncated MIK-RD, is inactive and cannot be activated by MARK. The analysis of the biochemical properties of the different MIK polypeptides has allowed us to get new insight on the mechanisms of MIK activation by MARK, and suggests that differential splicing could be an important control of MIK activity.

Experimental procedures

Plant material

Zea mays cv. W64A plants were grown at 26°C on a 16-h light/8-h dark cycle.

RNA extraction and PCR amplification

Total RNA was isolated from the indicated tissues using TRIzol® reagent (Invitrogen), and contaminating DNA was removed using DNase I. Two microgram of total RNA was used for first-strand cDNA synthesis with M-MLV reverse transcriptase (GibcoBRL), using a modified oligo(dT) as described (Casacuberta et al. 1995). The first-strand cDNA was used as a template for PCR amplification. Reaction conditions were as follows: amplification with F3 (5′-CAC GAT CTT CAA GGT CAA GG C-3′) and R3 (5′-CAG AAG CTT AAC AGA GTT AAG ATC TTG AG-3′), 30 cycles (45 s 94°C, 45 s 58°C, 1 min 72°C); amplification with F2 (5′-GGA GCT TCT CAA GCA TCG TTT C-3′) and R4 (5′-CTC TGA CTG CAG ATT TCC TAA C-3′), 30 cycles (45 s 94°C, 45 s 56°C, 40 s 72°C). PCR fragments were subcloned into pCRII-vector (Invitrogen), and sequenced. PCR controls were performed in the absence of added reverse transcriptase to ensure RNA samples were free of DNA contamination.

For quantitative PCR, Real-time PCR experiments were done in the ABI Prism 7000 sequence detection system (Applied Biosystems) using SYBR-Green® (Applied Biosystems) as a fluorescent reporter. Relative quantification was performed using the ΔCt method (Relative quantification of gene expression. Applied Biosystems, user bulletin #2 ABI PRISM 7700, sequence detection system 2001) using *Ubiquitin* (*UBQ*) as a house-keeping gene. Primers for quantitative PCR (see below) were designed using Primer Express 2.0 program (Applied Biosystems), and in order to check the specificity of annealing of the nucleotides, a dissociation kinetics was performed at the end of the experiment. Primers used were: MARK-RT-F (5′-CGC CTC AAG GAC GTG ACT CT-3′), MARK-RT-R (5′-GGA AAT GCG TTC ACG GAA CT-3′), UBQ-RT-F (5′-GGT CGT TTA AGC TGC CGA TG-3′), UBQ-RT-R (5′-CAT ATG GAG AGG GCA CCA GAC-3′), E11-F (5′-CTC CCT TCC CTG AAA GAG GCT A-3′), E11-R (5′-AAC AGC AGG CCG ATC AAA CT-3′), I11-F (5′-TGC AGT TAT CTC ACC TCA TGA TGG-3′), I11-R (5′-GAA TCC TTT GAG CTG CGG G-3′), I4-F (5′-TGC ACT CGA ATG TTT ATA TCA TTC TG-3′), I4-R (5′-ACC AAT AAC AGT AAT AGA AGG CAC ACA-3′), I5-F (5′-TTT TGG TGT GTC TTC ACT TAC CTG A-3′) and I50-R (5′-TTC CTT GAC TAC AGT AGA AAA AAG AAT CA-3′).

Transient expression, immunoprecipitation, immune complex kinase assay and Western blotting

The full-length MIK sequence was obtained by RACE PCR, as described (Llompart et al. 2003). The partial cDNA corresponding to MIK-KD (1090 bp, 363 aa) was obtained deleting the *Bgl*II fragment of the full-length MIK

sequence. MIK2 sequence was obtained substituting a *Hin*dIII fragment of the full-length MIK sequence, by the F2-MIKR3H3 amplified fragment of the *MIK2* corresponding clone. MIK1, MIK2 and MIK-KD sequences were subcloned into pCMV-HA (Clontech) and pFLAG-CMV (Sigma) expression vectors.

Mammalian 293T cells were transfected at approximately 70% confluence using lipofectamine transfection reagent (Invitrogen) according to the manufacturer's recommendation. Transfected cells were lysed 24 h after transfection in 0.5 ml immunoprecipitation buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 1 μg/ml E-64 and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 10,000 rpm for 10 min and the supernatant was recovered. Five hundred micrograms of the extract were pre-cleared with 12 μl of a 25% slurry of protein G-Sepharose 4B fast flow (Sigma) and incubated with anti-HA mouse monoclonal antibody (clone number 16B12) (Covance MMs-101R) or anti-FLAG mouse monoclonal antibody (Sigma F3165) for 2 h at 4°C. Twenty-five microliters of a 25% slurry of protein G-Sepharose 4B fast flow (Sigma) was added to the mixture and incubated for 1 h at 4°C. Immunocomplexes were washed three times with immunoprecipitation buffer. Cell lysates or immunoprecipitates were separated on a 10% SDS-PAGE gel before electrophoretic transfer onto a PVDF membrane. Proteins were detected with the respective antibodies.

Kinase assays of the different MIK polypeptides were performed as follows. After immunoprecipitation, immunocomplexes were treated with alkaline phosphatase at 30°C during 60 min and washed twice with immunoprecipitation buffer and twice with buffer P (20 mM Tris 7.5, 50 mM NaCl, 0.01% triton, 1 mM DTT). Three different dilutions (1/2) were tested for each MIK polypeptide. Kinase reactions were performed in 20 μl buffer P containing 10 mM $MgCl_2$, 2 μg of MBP, 50 μM ATP and 10 μCi of [γ-^{33}P] ATP 3000Ci/mmol (Amersham). The protein kinase reactions were performed at 30°C for 10 min and the reactions were stopped by adding 2× loading buffer. After phosphorylation, proteins were separated in a 11% SDS-PAGE. The gel was stained with Coomassie Brilliant Blue and dried. Radioactive signals were quantified with a PhosphoImager using the Quantity One program (Bio-Rad). To verify equal loading of the different MIK constructs, equivalent amounts of the immunoprecipitated were separated on a 10% SDS-PAGE gel before electrophoretic transfer onto a PVDF membrane. Proteins were detected with antibodies raised against the MIK-KD polypeptide.

Kinase assays for the analysis of MIK1 and MIK2 activation by MARK were performed as follows. The FLAG-MARK-ID protein obtained from 6 different immunoprecipitations was eluted at 4°C with 50 μl of buffer P containing 0.1 mg/ml of FLAG peptide. Kinase assays were performed with 1/5 of the HA-MIK1, HA-MIK2 immunocomplexes in 20 μl of buffer P containing 10 mM $MgCl_2$, 2 μg of MBP, 50 μM ATP and 10 μCi of [γ-^{33}P] ATP 3000Ci/mmol (Amersham), containing 0, 1 or 4 μl of eluted FLAG-MARK-ID. A control kinase reaction was performed using immunocomplexes from non-transfected cells. The reactions were performed and analysed as described for the different MIK polypeptides.

Recombinant proteins, production of antibodies and gel filtration assays

A partial cDNA corresponding to the last 325 amino acids of MARK protein, which coincide with its putative intracellular domain (ID-MARK), and two partial MIK cDNAs corresponding to the last 143 amino acids of MIK (Ab 2899) and to the entire MIK-RD polypeptide (Ab 165) were cloned into a pET28 vector and the recombinant His_6-tagged-ID-MARK and His_6-tagged-MIK were obtained by standard methods. Antibodies against ID-MARK and C-MIK were obtained in rabbits inoculated with the His_6-tagged-ID-MARK and His_6-tagged-C-MIK recombinant proteins. Gel filtration experiments were performed as previously described (Llompart et al. 2003).

Results

Different transcripts of *MIK* are generated by differential splicing

Characterisation of *MIK* expression in maize immature embryos by RT-PCR gave amplified bands of different molecular sizes (Fig. 1A and not shown). The cloning and sequencing of these bands, and their comparison with the *MIK* genomic sequence, revealed the presence of intron-like sequences in these cDNA fragments. This suggests the existence of partially spliced *MIK* transcripts. The possible contamination with genomic DNA was ruled out with control amplification experiments where no reverse transcriptase was added to the samples (not shown). Moreover, first-strand cDNA synthesis was performed with oligo(dT) primer and, more importantly, correct splicing events for other predicted introns were depicted in the cloned partial cDNAs, confirming that they indeed correspond to alternatively spliced *MIK* mRNAs. We describe here 4 predicted *MIK* mRNA species (Fig. 1B): *MIK1* results from the complete splicing of the 12 *MIK* introns (partial cDNA: DQ390216); *MIK-KD1* is the result of intron 4 retention

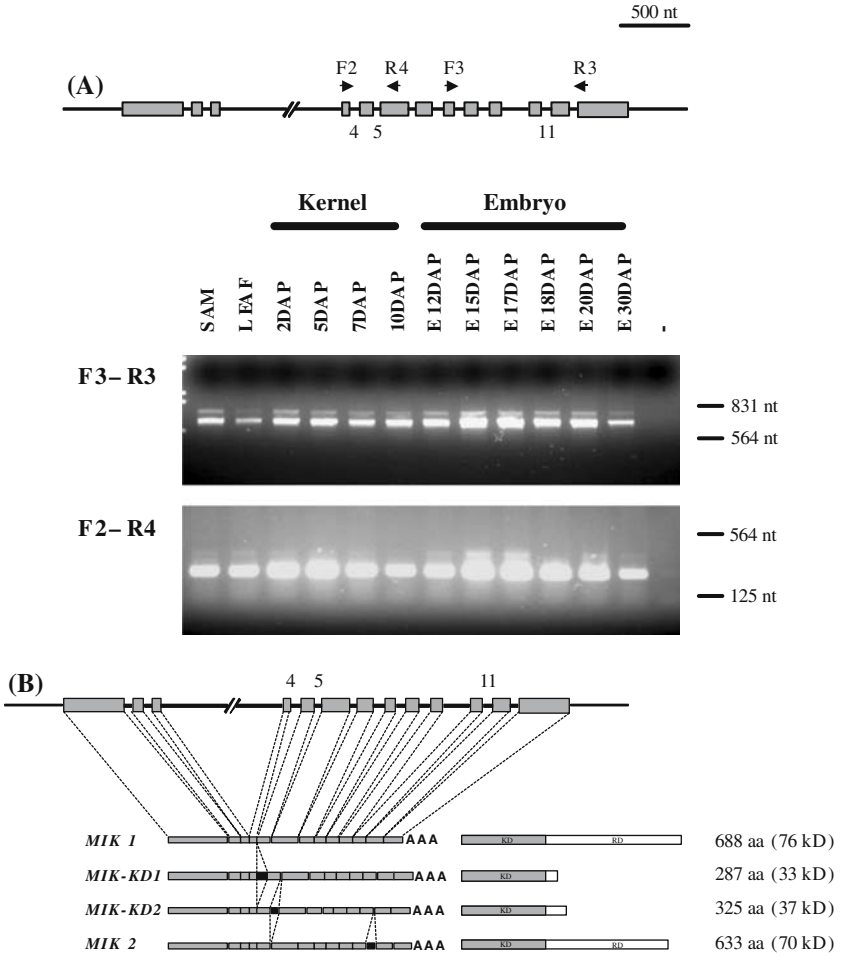

Fig. 1 *MIK* structure and splice variants. (**A**) RT-PCR using different MIK primers with RNAs obtained from different maize tissues (shoot apical meristem, SAM; leaf; 2, 5, 7 and 10 days after pollination (DAP) kernels; 12, 15, 17, 18, 20 and 30 DAP embryos). A negative control without RNA (-) is also shown. The names of the primers used are shown on the left of each amplification experiment and the migration position of two relevant molecular weight markers are shown on the right. The structure of the *MIK* gene, the position of the primers used and the number of the introns alternatively retained are shown on the top. Exons are indicated by grey boxes and introns by solid lines. (**B**) Structure of the MIK gene (top) and *MIK* splice variants and predicted polypeptides (bottom). Intron-like sequences retained in *MIK* transcripts are shown as black boxes. The kinase domain (KD) and the regulatory domain (RD) of MIK are shown by filled and open boxes, respectively. The number of residues of each predicted polypeptide is shown on the right, and the predicted molecular weight is shown between brackets

(partial cDNA: DQ390218); *MIK-KD2* contains intron 5 (partial cDNA: DQ390219), and *MIK2* results from the retention of intron 11 (partial cDNA: DQ390217). The three new *MIK* splice variants here described were generated by the retention of an intron, and in all the cases this generated a premature STOP codon. Intron retention is not infrequent in plants. It has been recently reported that, in Arabidopsis, 30% of alternatively spliced transcripts are generated by intron retention (Ner-Gaon et al. 2004).

The predicted *MIK* mRNAs here described potentially code for four different polypeptides: MIK1, the previously described MIK protein, which contains an N-terminal kinase domain (MIK-KD), and a C-terminal regulator domain (MIK-RD) (Llompart et al. 2003); MIK-KD1 and MIK-KD2 that are truncated proteins that only contain MIK-KD; and MIK2 that lacks the last 67 amino acids of the C-terminal region of the RD (Fig. 1B). Using antibodies raised against different regions of MIK-RD we were able to detect two different MIK-related bands with electrophoretic mobilities coinciding with those of recombinant MIK1 and MIK2 (see Fig. 3B), confirming that different alternative spliced *MIK* mRNAs were translated into proteins.

Analysis of the kinase activities of the peptides encoded by the different *MIK* splice variants

MAP4Ks very often contain a kinase-unrelated domain that usually inhibits their kinase activity (Creasy et al. 1996; Eichinger et al. 1998; Guertin and McCollum 2001). Activation of these MAP4Ks involves the release of auto

inhibition by phosphorylation or protein–protein interaction-induced conformational changes or by cleavage of the regulatory domain (Glantschnig et al. 2002; Guertin and McCollum 2001). We have previously proposed that the regulatory domain of MIK, MIK-RD, could negatively regulate MIK activity, and that the interaction of MIK, through the RD, with the MARK receptor could activate MIK by releasing from its auto-inhibited state (Llompart et al. 2003). Two of the *MIK* splice variants here described (*MIK-KD1* and *MIK-KD2*), were predicted to code for MIK isoforms where most of the RD is absent. We tested the auto- and trans-phosphorylation activity of a MIK polypeptide in which we deleted most of the RD (named MIK-KD), which makes it similar to the polypeptides encoded by *MIK-KD1* and *MIK-KD2* splice variants. This kinase activity was compared to that of the complete MIK (MIK1) and the polypeptide encoded by *MIK2*, which contains a truncated RD (Fig. 1). The different constructs were transfected into mammalian 293T cells, immunoprecipitated with antibodies against the HA epitope present in the N-terminus of all polypeptides, and different dilutions of the proteins were subjected to auto- and trans-phosphorylation assays. Coomassie staining of the SDS-PAGE gels, as well as immunoblots blots with an antibody raised against the MIK-KD polypeptide, were used to compare the amount of proteins loaded (Fig. 2 top and bottom). The results presented in Fig. 2 showed that the auto and trans-phosphorylation activity of MIK-KD is higher than the one displayed by equivalent amounts of MIK1 and MIK2 proteins. These results showed that MIK is auto-inhibited by its RD, and that the shorter RD present in MIK2 is sufficient for this auto-inhibition. In vitro phosphorylation experiments in which increasing concentrations of the MIK-RD were added to the MIK-KD phosphorylation reactions showed that the inhibitory effect of MIK-RD could also be accomplished *in trans* (not shown).

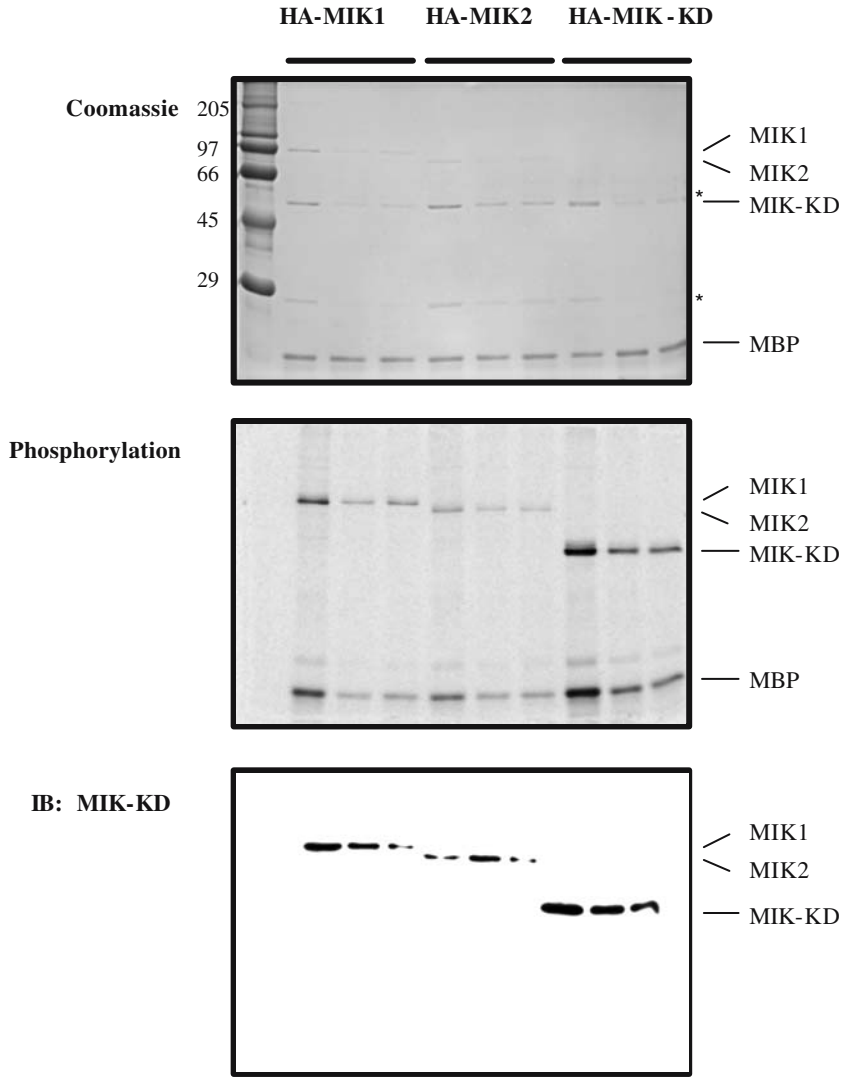

Fig. 2 Analysis of the kinase activity of MIK1, MIK2 and MIK-KD. Mammalian 293T cells were transfected with the indicated clones. Serial dilutions (1, 1/2 or 1/4) of the anti-HA immunoprecipitates were assayed for auto-phosphorylation and phosphorylation activity on myelin basic protein (MBP). Reaction products were separated by SDS-PAGE, stained with Coomassie blue (upper panel) and visualised by autoradiography (middle panel). The amount of MIK1, MIK2 and MIK-KD proteins in the immunocomplexes was determined by immunoblotting (IB) with antibody against MIK-KD (lower panel). Immunoglobulin light chain and heavy chain, which partially overlaps with MIK-KD, are indicated by asterisks. Molecular weight markers (kD) are shown on the left of the upper panel

Interaction with and activation by MARK of the different MIK isoforms

We have already shown that the C-terminal 143 amino acids of MIK are sufficient to specifically interact with MARK-ID, and that MARK-MIK interaction results in MIK activation (Llompart et al. 2003). Nevertheless, the minimal MIK region necessary for the interaction was not determined, and nothing was known on the possible interaction of MARK-ID with other MIK domains. We thus compared the interaction of MIK1 and MIK2, which lacks the 67 amino acids of the C-terminus, with MARK-ID by co-transfecting HA-MIK1 or HA-MIK2 together with Flag-MARK-ID tagged proteins into mammalian 293T cells. Figure 3A shows that MIK2 failed to co-immunoprecipitate MARK-ID in the conditions in which MIK1 protein efficiently did. This result demonstrates that the C-terminal part of MIK-RD is the only domain of MIK that can interact with MARK-ID, and that the last 67 amino acids of MIK are necessary for the interaction.

We have already shown that MARK and MIK form stable complexes in vivo, as they co-fractionate when a protein extract obtained from 15 DAP embryos is separated on a Superose 6 gel filtration column (Llompart et al. 2003). As MIK2 does not interact with MARK it should be absent from high molecular weight MARK-MIK complexes. We thus reanalysed the Superose 6 gel filtration protein fractions with two different antibodies raised against MIK-RD. Figure 3B shows that the two MIK-RD antibodies immunoreact with two different polypeptides with electrophoretic mobilities coinciding with those of recombinant MIK1 and MIK2. The polypeptide corresponding to MIK1 co-fractionated with MARK while the polypeptide corresponding to MIK2 did not, suggesting that it is absent from MARK–MIK complexes in vivo.

As the interaction of MARK with MIK results in the activation of the phosphorylation activity of the latter, we analysed the ability of MARK-ID to activate the kinase activity of MIK1 and MIK2 proteins. Figure 4 shows that MIK1 auto- and trans-phosphorylation activity was highly induced by MARK-ID, in accordance with our previous observations (Llompart et al. 2003). On the contrary, the auto- and trans-phosphorylation activity of MIK2 was not affected by the presence of increasing concentrations of the MARK-ID protein.

Differential expression of MIK splice variants

In order to analyse the possible regulation of the splicing of the *MIK* pre-mRNA, we measured the expression of the different splice variants. We designed specific oligonucleotides for the amplification of introns 4, 5 and 11 by quantitative Real-Time RT-PCR, and we compared the accumulation of these mRNA species with that of the total *MIK* mRNA (analysed by amplification of an invariant exon with specific oligonucleotides). The expression of the different *MIK* splice variants, as well as the total *MIK* expression, were referred to the expression of the *Ubiquitin* gene and were compared with the expression of the *MARK* gene. Figure 5 shows that the total *MIK* expression

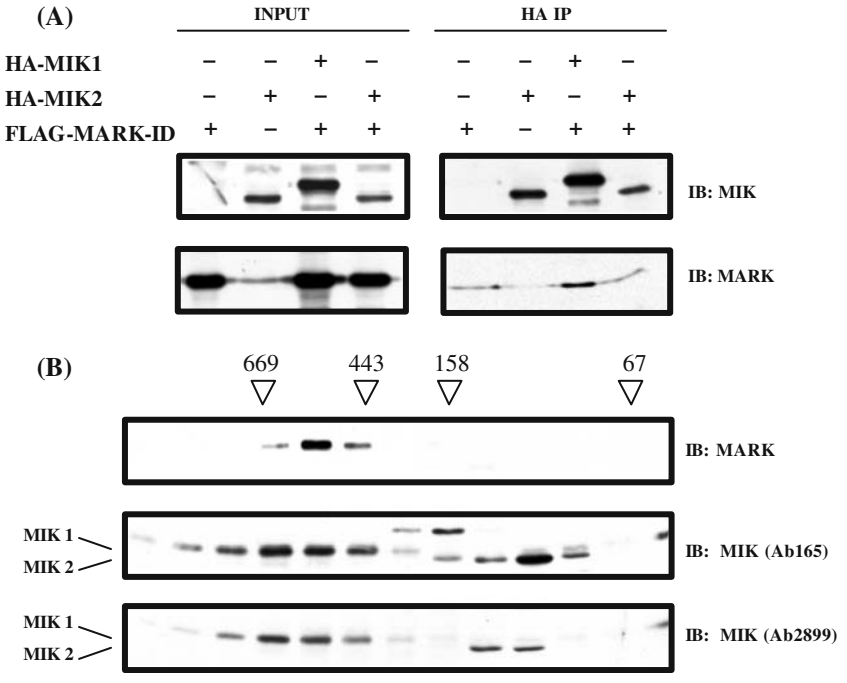

Fig. 3 Analysis of MARK-MIK interaction. (**A**) Mammalian 293T cells were transfected with clones corresponding to the indicated proteins. About 1/10 of the extracts (INPUT, left panels) and 1/3 of the anti-HA immunoprecipitates (HA IP, right panels) were subjected to immunoblot (IB) against MIK (upper panels) and MARK (lower panels). (**B**) Total protein extract from 15 DAP maize embryos were fractionated on a Superose 6 column. Fractions were analysed by Western blot using the antibodies indicated to the right of each panel. The bands showing the same mobility as the recombinant MIK1 and MIK2 are indicated to the left of each panel. Arrows indicate where size standards eluted from the column

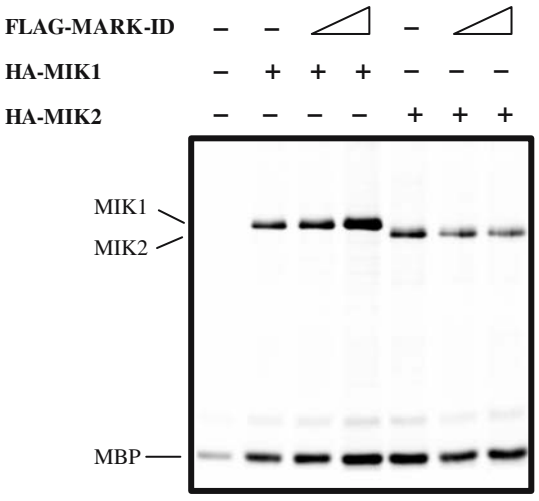

Fig. 4 Analysis of MIK1 and MIK2 activation by MARK. Mammalian 293T cells were transfected with clones coding for HA-MIK1, HA-MIK2 and FLAG-MARK-ID, and those recombinant proteins were immunoprecipitated using anti-HA or anti-FLAG antibodies. MIK1 and MIK2 immunoprecipitates were assayed for autophosphorylation and phosphorylation activity on MBP in the presence of increasing concentrations of MARK-ID. Reaction products were separated by SDS-PAGE, visualised by autoradiography

increased during early embryogenesis, with a maximum at 15 Days After Pollination (DAP), and was reduced afterwards, it being high in meristems while remaining low in adult leaves (Fig. 5 lower panel). This pattern of expression is similar to that of *MARK* (Fig. 5 upper panel), in accordance with its role as MIK activator. The analysis of the expression of the different *MIK* splice variants revealed that the frequency of intron retention varied along embryogenesis. At the beginning of the process, intron 11 was frequently found in *MIK* mRNA (65% of *MIK* mRNAs contained intron 11) while at mid embryogenesis, when the expression of MARK, a protein that can activate MIK through its C-terminal domain, reaches the maximum, intron 11 was efficiently spliced (in embryos of 15 DAP, e15, only 26% of *MIK* mRNAs contained intron 11). In order to confirm these data by a different approach, we amplified by conventional RT-PCR the entire MIK-RD coding region of *MIK* mRNAs from e15 RNA and we analysed 30 independent clones. Nine out of 30 (30%) partial cDNAs contained intron 11, the rest containing only the predicted exonic sequences, which perfectly coincides with the results obtained by quantitative Real-Time RT-PCR.

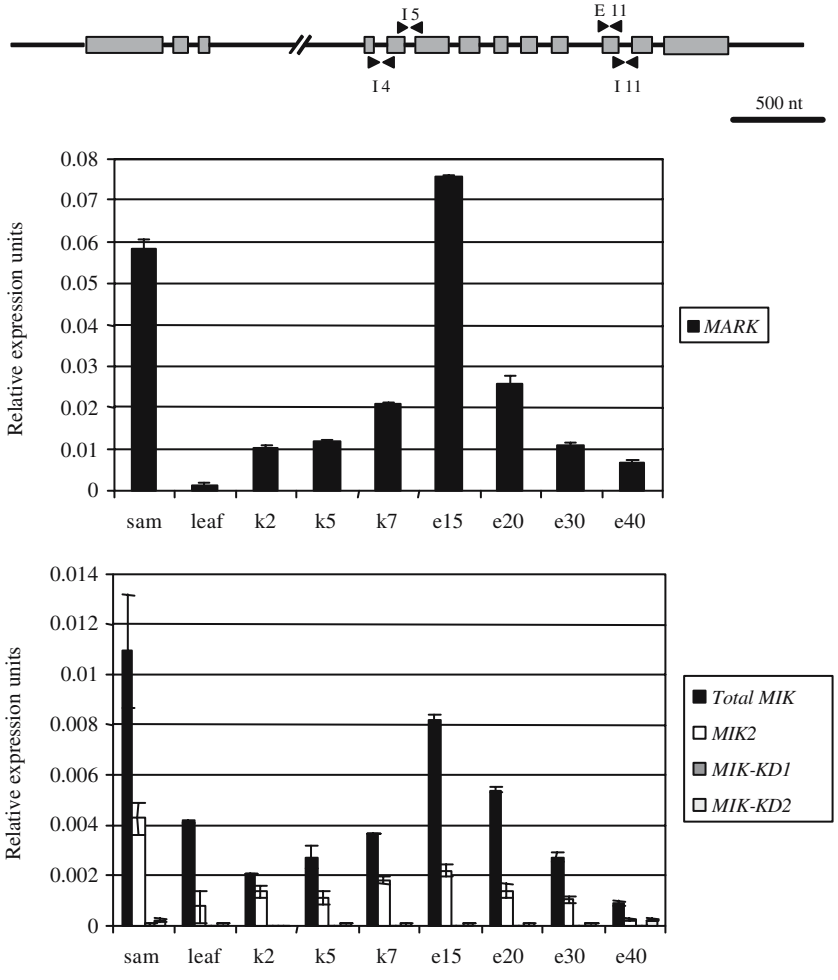

Fig. 5 Analysis of the expression of *MIK* splice variants. Real time RT-PCR analysis of the expression of *MARK* (upper pannel) and *MIK* splice variants (lower pannel) was performed on shoot apical meristems (sam), leaves and developing seeds (k: kernel and e:embryo). The expressions are shown relative to that of the *Ubiquitin* house-keeping gene and standard deviations are indicated. The structure of the *MIK* gene, the position of the primers used and the number of the introns alternatively retained are shown on the top. Exons are indicated by grey boxes and introns by solid lines

On the other hand, although we have been able to detect *MIK-KD1* and *MIK-KD2* mRNAs, the splicing of introns 4 and 5 seems highly efficient in the conditions tested, and both splice variants are maintained during embryogenesis at very low levels.

Conservation of *MIK* differential splicing in different species

MIK has a possible homologue in rice (AP005127), and two possible homologues in Arabidopsis *MAP4K1* and *MAP4K2*, also known as *AtMAP4Kα*1 and *AtMAP4Kα*2 (Champion et al. 2004). We thus asked whether the intron–exon structure of *MIK*, and its alternative splicing, is conserved in different species. Figure 6 shows that the intron–exon structure of the rice *MIK* gene and the two Arabidopsis *MIK* homologues is perfectly conserved. On the contrary, the maize *MIK* gene does not contain the first 8 introns, the rest being perfectly conserved. Thus, the maize *MIK* gene has a composite structure with a compact 5′region (coding for most of the kinase domain) and an intron-rich 3′region (coding for the regulatory domain). Although the intron–exon structure of genes is frequently conserved among related species, introns can also be lost or gained during evolution, intron loss being much more frequent than intron gain (Roy et al. 2003). Intron loss is probably the result of a gene conversion of the genomic copy of a gene by the reverse transcriptase product of a spliced transcript (Roy and Gilbert 2005 and references therein), and this process usually leads to the simultaneous loss of multiple introns, as it seems to be the case for the maize *MIK* gene. A recent study in *Caenorhabditis* has shown that intron losses are not randomly distributed along genes and that the preferential retention of particular introns could be an indication of its functional importance (Cho et al. 2004). The selective conservation of the intron–exon structure of the region coding for MIK-RD thus suggests that the alternative splicing of these introns could have been maintained during evolution. Interestingly, in addition to the completely spliced transcript (AK102538), the data bases contain a full-length cDNA corresponding to a splice variant of the rice *MIK* homologue that retains the last intron and potentially codes for a protein similar to MIK2 (AK106514) (Fig. 6). We did not find in the Arabidopsis data base ESTs that could represent alternatively spliced transcripts of the Arabidopsis *MIK* homologues. We thus searched them by an RT-PCR approach. We cloned a *MAP4K1* splicing variant that retains intron 18 (corresponding to intron 11 in the maize gene) and that would code for a protein homologous to MIK2 (DQ390220), and a *MAP4K2* variant that retains part of intron 11 (corresponding to intron 4 in the maize gene) and that would code for a protein homologous to MIK-KD1 (DQ390221) (Fig. 6), confirming the existence of equivalent alternatively spliced *MIK* mRNAs in maize, rice and Arabidopsis.

Discussion

The results presented here show that when the C-terminal kinase-unrelated domain of MIK, MIK-RD, is eliminated the kinase activity of MIK is increased, suggesting that MIK-RD inhibits MIK kinase activity. The auto-inhibition by regulatory domains is common among MAP4Ks, and their activation usually requires induced conformational changes, or the cleavage of this regulatory domain, to liberate the kinase active site (Creasy et al. 1996; Eichinger et al. 1998; Glantschnig et al. 2002; Guertin and McCollum 2001). The existence of *MIK* splice variants coding for MIK isoforms devoid of MIK-RD suggests that constitutive active forms of MIK can be generated in maize. The regulation of the splicing of introns 4 and 5, to give the splicing variants *MIK-KD1* and *MIK-KD2* here described, could thus be a singular way to regulate the activity of the MIK protein, and could constitute a new mechanism to selectively overcome the auto-inhibition of MAP4Ks.

We have previously reported that MIK can interact with the intracellular domain of the MARK receptor, this

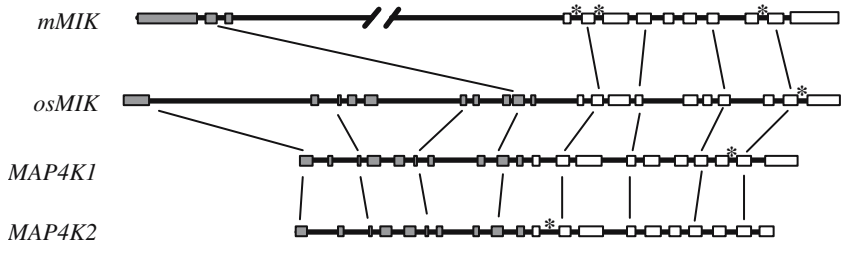

Fig. 6 Intron–exon structure of MIK genes in maize (*mMIK*), rice (*osMIK*) and Arabidopsis (*MAP4K1* and *MAPK4K2*). Exons coding for MIK-KD are shown as filled boxes; exons coding for MIK-RD are shown by open boxes; introns are shown by solid lines; maize MIK intron 3 has not been completely sequenced due to the presence of multiple transposable elements (intron length >1000 nt). Correspondence of selected exons among the 4 different genes is indicated for clarity. Introns retained in *MIK* mRNAs are marked by an asterisk

interaction resulting in the activation of MIK kinase activity (Llompart et al. 2003). Here we show that MARK–MIK interaction is restricted to the most C-terminal part of MIK (67 amino acids). When the C-terminal part of MIK is eliminated, to generate the MIK2 isoform, the interaction with MARK-ID, and the consequent activation, is abolished. On the other hand, the MIK2 isoform displays low kinase activity, similar to that of the non-activated entire MIK protein, suggesting that the truncated MIK-RD of MIK2 is sufficient to inhibit MIK activity. All these results support a model in which the N-terminal part of MIK-RD inhibits MIK kinase activity, and the interaction of the C-terminal region of MIK-RD with MARK-ID liberates MIK protein from its auto-inhibition. The results presented here show that the auto-inhibition of plant MAP4Ks, like their animal counterparts, can be released by protein–protein interactions. MIK2 protein might be activated by other receptors or regulatory proteins. This would not be surprising as it has been shown that most MAPK cassette components can perform very different functions in different pathways (Jonak et al. 2002). Our results thus show that differential splicing of the MIK primary transcript could be an important mechanism to modulate the activity of the MIK protein and to regulate the signal transduction pathway(s) in which this protein is potentially involved.

On the other hand, the results presented here show that the different *MIK* splice variants accumulate with different patterns of expression in maize, showing that *MIK* splicing is highly regulated. *MIK*, and its activator *MARK*, are expressed during embryogenesis and in the meristems of the plant, with similar patterns of expression, and we have already proposed that MIK and MARK could participate in proliferation/differentiation signalling transduction pathways (Llompart et al. 2003). Interestingly, the efficiency of intron 11 splicing, that gives rise to a MARK-activatable MIK isoform, increases during embryogenesis reaching a maximum at mid embryogenesis (e15) when *MARK* expression is maximal. This suggests that MIK activation could be particularly high at this stage. The regulation of *MIK* splicing could thus contribute to the precise control of MIK activity during development. If this is the case, interfering in splicing regulation should result in developmental abnormalities. Interestingly, it has been recently shown that the overexpression of the LAMMER kinase PK12, which is potentially involved in splicing regulation, results in developmental alterations in Arabidopsis, suggesting that, indeed, splicing regulation plays an important role in developmental control in plants (Savaldi-Goldstein et al. 2003). The *MIK* splice variants here described constitute a good model to analyse splicing regulation at the molecular level, as well as the role that alternative splicing could play in the control of signal transduction and plant development. The selective conservation during evolution of the intron–exon structure of the *MIK* region coding for MIK-RD, and the maintenance of the alternative splicing of some of these introns in maize, rice and Arabidopsis, highlight the functional importance of this phenomenon.

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References

Brett D, Pospisil H, Valcarcel J, Reich J, Bork P (2002) Alternative splicing and genome complexity. Nat Genet 30:29–30

Cameron SJ, Abe J, Malik S, Che W, Yang J (2004) Differential role of MEK5alpha and MEK5beta in BMK1/ERK5 activation. J Biol Chem 279:1506–1512

Casacuberta JM, Vernhettes S, Grandbastien MA (1995) Sequence variability within the tobacco retrotransposon Tnt1 population. EMBO J 14:2670–2678

Champion A, Jouannic S, Guillon S, Mockaitis K, Krapp A, Picaud A, Simanis V, Kreis M, Henry Y (2004) AtSGP1, AtSGP2 and MAP4K alpha are nucleolar plant proteins that can complement fission yeast mutants lacking a functional SIN pathway. J Cell Sci 117:4265–4275

Cho S, Jin S-W, Coen A, Ellis RE (2004) A phylogeny of *Caenorhabditis* reveals frequent loss of introns during nematode evolution. Genome Res 14:1207–1220

Creasy CL, Ambrose DM, Chernoff J (1996) The Ste20-like protein kinase, Mst1, dimerizes and contains an inhibitory domain. J Biol Chem 271:21049–21053

Dan I, Watanabe NM, Kusumi A (2001) The Ste20 group kinases as regulators of MAP kinase cascades. Trends Cell Biol 11:220–230

Eichinger L, Bahler M, Dietz M, Eckerskorn C, Schleicher M (1998) Characterization and cloning of a Dictyostelium Ste20-like protein kinase that phosphorylates the actin-binding protein severin. J Biol Chem 273:12952–12959

Glantschnig H, Rodan GA, Reszka AA (2002) Mapping of MST1 kinase sites of phosphorylation. Activation and autophosphorylation. J Biol Chem 277:42987–42996

Guertin DA, McCollum D (2001) Interaction between the noncatalytic region of Sid1p kinase and Cdc14p is required for full catalytic activity and localization of Sid1p. J Biol Chem 276:28185–28189

Hu Y, Leo C, Yu S, Huang BC, Wang H, Shen M, Luo Y, Daniel-Issakani S, Payan DG, Xu X (2004) Identification and functional characterization of a novel human misshapen/Nck interacting kinase-related kinase, hMINK beta. J Biol Chem 279:54387–54397

Iida K, Seki M, Sakurai T, Satou M, Akiyama K, Toyoda T, Konagaya A, Shinozaki K (2004) Genome-wide analysis of alternative pre-mRNA splicing in *Arabidopsis thaliana* based on full-length cDNA sequences. Nucleic Acids Res 32:5096–5103

Jonak C, Okresz L, Bogre L, Hirt H (2002) Complexity, cross talk and integration of plant MAP kinase signalling. Curr Opin Plant Biol 5:415–424

Kazan K (2003) Alternative splicing and proteome diversity in plants: the tip of the iceberg has just emerged. Trends Plant Sci 8:468–471

Kikuchi S, Satoh K, Nagata T, Kawagashira N, Doi K, Kishimoto N, Yazaki J, Ishikawa M, Yamada H, Ooka H, Hotta I, Kojima K, Namiki T, Ohneda E, Yahagi W, Suzuki K, Li CJ,

Ohtsuki K, Shishiki T, Otomo Y, Murakami K, Iida Y, Sugano S, Fujimura T, Suzuki Y, Tsunoda Y, Kurosaki T, Kodama T, Masuda H, Kobayashi M, Xie Q, Lu M, Narikawa R, Sugiyama A, Mizuno K, Yokomizo S, Niikura J, Ikeda R, Ishibiki J, Kawamata M, Yoshimura A, Miura J, Kusumegi T, Oka M, Ryu R, Ueda M, Matsubara K, Kawai J, Carninci P, Adachi J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Hayatsu N, Imotani K, Ishii Y, Itoh M, Kagawa I, Kondo S, Konno H, Miyazaki A, Osato N, Ota Y, Saito R, Sasaki D, Sato K, Shibata K, Shinagawa A, Shiraki T, Yoshino M, Hayashizaki Y, Yasunishi A, Rice Full-Length cDNA Consortium, National Institute of Agrobiological Sciences Rice Full-Length cDNA Project Team, Foundation of Advancement of International Science Genome Sequencing & Analysis Group, RIKEN (2003) Collection, mapping, and annotation of over 28,000 cDNA clones from japonica rice. Science 301:376–379

Kyriakis JM (1999) Signaling by the germinal center kinase family of protein kinases. J Biol Chem 274:5259–5262

Kramer RM, Roberts EF, Um SL, Borsch-Haubold AG, Watson SP, Fisher MJ, Jakubowski JA (1996) p38 Mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets. Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. J Biol Chem 271:27723–27729

Lorkovic ZJ, Wieczorek Kirk DA, Lambermon MH, Filipowicz W (2000) Pre-mRNA splicing in higher plants. Trends Plant Sci 5:160–167

Llompart B, Castells E, Rio A, Roca R, Ferrando A, Stiefel V, Puigdomenech P, Casacuberta JM (2003) The direct activation of MIK, a germinal center kinase (GCK)-like kinase, by MARK, a maize atypical receptor kinase, suggests a new mechanism for signalling through kinase-dead receptors. J Biol Chem 278:48105–48111

Machida N, Umikawa M, Takei K, Sakima N, Myagmar BE, Taira K, Uezato H, Ogawa Y, Kariya K (2004) Mitogen-activated protein kinase kinase kinase kinase 4 as a putative effector of Rap2 to activate the c-Jun N-terminal kinase. J Biol Chem 279:15711–15714

Modrek B, Lee C (2002) A genomic view of alternative splicing. Nat Genet 30:13–19

Ner-Gaon H, Halachmi R, Savaldi-Goldstein S, Rubin E, Ophir R, Fluhr R (2004) Intron retention is a major phenomenon in alternative splicing in Arabidopsis. Plant J 39:877–885

Nishihama R, Banno H, Kawahara E, Irie K, Machida Y (1997) Possible involvement of differential splicing in regulation of the activity of Arabidopsis ANP1 that is related to mitogen-activated protein kinase kinase kinases (MAPKKKs). Plant J 12:39–48

Okazaki Y, Furuno M, Kasukawa T, Adachi J, Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H, Yamanaka I, Kiyosawa H, Yagi K, Tomaru Y, Hasegawa Y, Nogami A, Schonbach C, Gojobori T, Baldarelli R, Hill DP, Bult C, Hume DA, Quackenbush J, Schriml LM, Kanapin A, Matsuda H, Batalov S, Beisel KW, Blake JA, Bradt D, Brusic V, Chothia C, Corbani LE, Cousins S, Dalla E, Dragani TA, Fletcher CF, Forrest A, Frazer KS, Gaasterland T, Gariboldi M, Gissi C, Godzik A, Gough J, Grimmond S, Gustincich S, Hirokawa N, Jackson IJ, Jarvis ED, Kanai A, Kawaji H, Kawasawa Y, Kedzierski RM, King BL, Konagaya A, Kurochkin IV, Lee Y, Lenhard B, Lyons PA, Maglott DR, Maltais L, Marchionni L, McKenzie L, Miki H, Nagashima T, Numata K, Okido T, Pavan WJ, Pertea G, Pesole G, Petrovsky N, Pillai R, Pontius JU, Qi D, Ramachandran S, Ravasi T, Reed JC, Reed DJ, Reid J, Ring BZ, Ringwald M, Sandelin A, Schneider C, Semple CA, Setou M, Shimada K, Sultana R, Takenaka Y, Taylor MS, Teasdale RD, Tomita M, Verardo R, Wagner L, Wahlestedt C, Wang Y, Watanabe Y, Wells C, Wilming LG, Wynshaw-Boris A, Yanagisawa M, Yang I, Yang L, Yuan Z, Zavolan M, Zhu Y, Zimmer A, Carninci P, Hayatsu N, Hirozane-Kishikawa T, Konno H, Nakamura M, Sakazume N, Sato K, Shiraki T, Waki K, Kawai J, Aizawa K, Arakawa T, Fukuda S, Hara A, Hashizume W, Imotani K, Ishii Y, Itoh M, Kagawa I, Miyazaki A, Sakai K, Sasaki D, Shibata K, Shinagawa A, Yasunishi A, Yoshino M, Waterston R, Lander ES, Rogers J, Birney E, Hayashizaki Y, FANTOM Consortium; RIKEN Genome Exploration Research Group Phase I & II Team (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420:563–573

Roy SW, Fedorov A, Gilbert W (2003) Large-scale comparison of intron positions in mammalian genes show intron loss but no gain. Proc Natl Acad Sci USA 100:7158–7162

Roy SW, Gilbert W (2005) The pattern of intron loss. Proc Natl Acad Sci USA 102:713–718

Savaldi-Goldstein S, Aviv D, Davydov O, Fluhr R (2003) Alternative splicing modulation by a LAMMER kinase impinges on developmental and transcriptome expression. Plant Cell 15:926–938

Tena G, Asai T, Chiu WL, Sheen J (2001) Plant mitogen-activated protein kinase signalling cascades. Curr Opin Plant Biol 4:392–400

Xiong L, Yang Y (2003) Disease and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. Plant Cell 15:745–759

Yung Y, Yao Z, Aebersold DM, Hanoch T, Seger R (2001) Altered regulation of ERK1b by MEK1 and PTP-SL and modified Elk1 phosphorylation by ERK1b are caused by abrogation of the regulatory C-terminal sequence of ERKs. J Biol Chem 276:35280–35289