A mutant ankyrin protein kinase from *Medicago sativa* affects *Arabidopsis* adventitious roots

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Abstract. A family of plant kinases containing ankyrin-repeats, the Ankyrin-Protein Kinases (APKs), shows structural resemblance to mammalian Integrin-Linked Kinases (ILKs), key regulators of mammalian cell adhesion. *MsAPK1* expression is induced by osmotic stress in roots of *Medicago sativa* (L.) plants. The *Escherichia coli*-purified MsAPK1 could only phosphorylate tubulin among a variety of substrates and the enzymatic activity was strictly dependent on Mn^{2+}. MsAPK1 is highly related to two *APK* genes in *Arabidopsis thaliana* (L.), *AtAPK1* and *AtAPK2*. Promoter-GUS fusions assays revealed that the *Arabidopsis APK* genes show distinct expression patterns in roots and hypocotyls. Although *Medicago truncatula* (L.) plants affected in *MsAPK1* expression could not be obtained using *in vitro* regeneration, *A. thaliana* plants expressing MsAPK1 or a mutant MsAPK1 protein, in which the conserved aspartate 315 of the kinase catalytic domain was replaced by asparagines (DN-lines), developed normally. The DN mutant lines showed increased capacity to develop adventitious roots when compared with control or MsAPK1-expressing plants. APK-mediated signalling may therefore link perception of external abiotic signals and the microtubule cytoskeleton, and influence adventitious root development.

Additional keywords: *Arabidopsis thaliana*, tubulin phosphorylation.

Introduction

Plant root architecture is determined by environmental abiotic conditions, such as soil nutrient availability, local stress constraints, as well as by biotic interactions (symbiotic or pathogenic). Notably, various morphological responses for the adaptation of roots to modified environmental conditions are retained across different phylogenetic groups such as Brassicaceae (including the model plant *Arabidopsis thaliana* L.) and Leguminosae (including the model legume *Medicago truncatula* L., related to the legume crop alfalfa, *Medicago sativa* L.). An example of such diversity is the ability of leguminous plants to interact with specific *Rhizobium* strains in the soil to develop, in the absence of combined nitrogen, a new root-derived symbiotic organ, the nitrogen-fixing nodule (Crespi and Galvez 2000). In contrast, Brassicaceae and Leguminosae as dicots share the capacity to develop secondary roots derived from main roots (lateral roots) or, in particular conditions, from hypocotyls or stems (adventitious roots) that allow them to adapt their root architecture to diverse environmental conditions.

Lateral root organogenesis has been studied in various plants and notably in *A. thaliana*, either based on a careful histological description of the process (Malamy and Benfey 1997), physiological experiments (Reed *et al.* 1998; Zhang and Forde 2000; Casimiro *et al.* 2001; Malamy and Ryan 2001) or genetic analysis (Celenza *et al.* 1995). These data revealed involvement of exogenous environmental factors such as nitrate, phosphate and sulfate availability (Lopez-Bucio *et al.* 2003), and the sucrose/nitrogen ratio (Malamy and Ryan 2001) as well as endogenous cues, such as abscisic acid, auxin and cytokinin (Casimiro *et al.* 2003; Aloni *et al.* 2006). In contrast, for adventitious root development, little information is available. Differences in the ability to form adventitious roots have been attributed to differences in auxin metabolism (Alvarez *et al.* 1989; Epstein and Ludwig-Müller 1993; Blazkova *et al.* 1997). The *alf1* (*aberrant lateral root formation1*)/*superroot1*/*rooty* and *superroot2* mutants affected in auxin homeostasis (Boerjan *et al.* 1995; Celenza *et al.* 1995; King *et al.* 1995; Delarue *et al.* 1998) and a mutant altered in a G protein β-subunit exhibiting modified auxin sensitivity (Ullah *et al.* 2003), are also affected in adventitious root development. A systematic screening for adventitious rooting mutants has led to the identification of nine mutants (Konishi and Sugiyama 2003). One of those, *rid5*

(for *root initiation defective*), was affected in the MOR1/GEM1 (MICROTUBULE ORGANISATION 1/GEMINI POLLEN 1) protein previously characterised as a Microtubule-Associated Protein (Whittington *et al*. 2001). These data suggest the involvement of the microtubule cytoskeleton in adventitious root organogenesis that may be itself controlled by auxin (Konishi and Sugiyama 2003).

In plants, type 2A serine/threonine protein phosphatases (PP2As) are critical in controlling the phosphorylation state of proteins involved in auxin transport (Smith and Walker 1996). Tissue culture experiments show that regulatory subunit B of PP2A was differentially expressed during adventitious root formation in *Arabidopsis* and suggest that polar auxin transport also plays a role in this process (Ludwig-Muller *et al*. 2005). The *Arabidopsis* mutant *rcn1* affected in another regulatory subunit of PP2A was isolated using an assay for alterations in differential root elongation in the presence of the auxin transport inhibitor NPA (Deruère *et al*. 1999). Probably, PP2A subunits are coordinately expressed and PP2A might play a role in the regulation of auxin transport during adventitious rooting by altering the phosphorylation status of proteins involved in this organogenesis thus most likely acting upstream of auxin transport (Ludwig-Muller *et al*. 2005).

In *M. sativa*, we have identified a gene encoding a novel putative serine/threonine kinase with an ankyrin-repeat domain in its N-terminal region, therefore named *MsAPK1* (for *Ankyrin Protein Kinase 1*; Chinchilla *et al*. 2003). This gene, expressed at low level in all plant organs, was rapidly induced in alfalfa roots grown in hyperosmotic conditions. Three similar genes were identified in *A. thaliana*, two of them were found expressed in various tissues (*AtAPK1* and *AtAPK2*), whereas the third one may correspond to a pseudogene (Chinchilla *et al*. 2003).

Proteins structurally related to these plant APKs that contain both ankyrin-repeats and kinase domains exist in animals, the Integrin-Linked Kinases (ILK; Wu and Dedhar 2001). Integrins are heterodimeric cell-surface molecules that link the actin cytoskeleton to the cell membrane and mediate cell-matrix interactions. In response to signals perceived from the extracellular matrix (ECM), the ILKs are recruited and activated from the cytoplasm to focal adhesion plates to interact with the cytoplasmic domain of β-integrin subunits and the actin cytoskeleton (Wu and Dedhar 2001). ILKs belong to the tyrosine kinase-like (TKL) group and have been classified as pseudokinases because they lack the HRD and DFG motifs in its kinase domain (Boudeau *et al*. 2006). However, there is considerable debate whether they have catalytic activity and different reports show that ILKs are active kinases (Delcommenne *et al*. 1998; Naska *et al*. 2006; Tabe *et al*. 2007). Overexpression of a dominant-negative ILK form was sufficient to abolish rearrangements of actin filaments induced by fibronectin peptides (PHSRN) as well as cell migration and invasion (Qian *et al*. 2005). The occurrence of integrin-like proteins associated to plasma membrane in plants has been suggested by immunological studies using antibodies raised against animal integrins (Sakurai *et al*. 2004). However, these putative plant integrin-like proteins have low similarity with animal integrins.

In this work, we first assayed the kinase activity of MsAPK1 in protein extracts from *Escherichia coli* expressing this gene and showed that it was able to phosphorylate tubulin but not actin *in vitro* among a variety of substrates indicating that it is an active kinase. *In situ* expression pattern of the two closely related *Arabidopsis AtAPK* genes was analysed using transcriptional GUS fusions, revealing that *AtAPK2* was induced in roots and hypocotyls, notably in root apexes. Whereas mutations affecting a single APK gene in *Arabidopsis* had no obvious phenotype, a dominant negative mutant affecting the kinase catalytic site developed more adventitious roots. However, the overall root architecture remained unaffected in these mutants even under hyperosmotic conditions. Our results suggest that APKs have a specific function in controlling the formation of adventitious roots.

Material and methods

Plant material and treatments

Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) was used as wild-type control for phenotypic comparison and to generate transgenic plants using floral dip transformation (Bechtold and Pelletier 1998). For adventitious rooting assays, *A. thaliana* seedlings were germinated and grown on MS medium (Sigma, Saint Louis, MO, USA) supplemented with 1% sucrose (w/v), 0.7% Bacto-agar (w/v) and 10^{-8} M 2,4-dichlorophenoxyacetic acid (2,4-D) for 3 days in the dark followed by 10 days under light conditions (photoperiod 16 h, 22°C). Alternatively, adventitious roots were obtained growing *A. thaliana* seedlings on MS medium (Sigma) supplemented with 1% sucrose (w/v) and 0.7% Bacto-agar (w/v) for 12 days, then the main root was completely removed and de-rooted plantlets were transferred to fresh medium. In both cases, adventitious roots were counted or tested for GUS staining. For the analysis of lateral root formation, *A. thaliana* plants were germinated and grown on MS medium for 10 days to 2 weeks before scoring for lateral roots. We consider a lateral root when a lateral root primordium emerges from the parent root. In all physiological assays, at least 30 *A. thaliana* plants were used per line per condition, and three biological replicates were performed.

Sequence analysis

Homology searches were done using the NCBI (http://www.ncbi.nml.nih.gov, accessed 20 October 2007) and TIGR BLAST (http://www.tigr.org, accessed 20 October 2007) servers. Search for protein kinase motifs was performed using either the SMART (http://smart.embl-heidelberg.de, accessed 2 May 2007), the eMotif (http://motif.stanford.edu, accessed 2 May 2007) or www.kinase.com databases (accessed 2 May 2007). Alignments were done using GCG software package (Genetic Computer Group, WI, USA).

Expression, purification of 6His::MsAPK1 and kinase assays

Full length MsAPK1 cDNA was cloned into the pQE31 vector (QIAexpressionist, Qiagen, Courtaboeuf Cedex, France) in frame with an N-terminal 6His tag. Its expression was induced in transformed M15 *Escherichia coli* cell cultures with 1 mM IPTG during 4 h at 30°C. Proteins were extracted from pellet with denaturing conditions (50 mM TRIS-HCl pH 8.0, 5 mM DTT, 2 mM PMSF, 6 M urea) and eluted using a pH gradient, according to the manufacturer's instructions. Fractions were tested in 12% SDS–PAGE, transferred to nitrocellulose membrane and

analysed by western blot using the RGS His antibody (dilution 1 : 2000; Qiagen). After three sequential dialysis steps performed with extraction buffer containing 4 M, 2 M and 0 M urea, a functional protein was recovered.

Protein kinase activity was assayed at 30°C during 5 or 10 min according to Raices *et al.* (2003). Aliquots (2 μg) of renatured 6His::MsAPK1 protein were incubated in a reaction mixture containing 20 mM TRIS-HCl pH 7.5, 10 mM β-mercaptoethanol, 10 mM $MnCl_2$ or 10 mM $MgCl_2$ and 5 μM ^{32}P-γATP (specific activity 1000 cpm pmol^{-1}) with different protein substrates (0.1 mg/mL): myelin basic protein and histone H1 (Calbiochem, Darmstadt, Germany); protamine, casein, tubulin and actin (Sigma). To test calcium dependence the assays were carried in the presence of 1 mM $CaCl_2$ or 10 mM EGTA. The reactions were stopped with the addition of cracking buffer (50 mM TRIS-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and boiled 3 min. The incubation mixture was electrophoresed on 12% SDS polyacrylamide gels stained with 0.25% (w/v) Coomassie Brilliant Blue, dried and exposed to X-OMAT Kodak films.

In addition, different peptides (Syntide-2, Sigma; Glycogen Synthase 1–8 and Kemptide, Calbiochem) were used as phosphate acceptors at a final concentration of 25 μM. Reactions were initiated by the addition of [γ-^{32}P]-ATP and stopped as described previously (Ulloa *et al.* 1991).

D^{315}N-MsAPK1 *overexpression*

D^{315}N-MsAPK1 substitution was performed on the full-length cDNA of *MsAPK1* using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and the primers DN5 (5′-CCACTGTAATTTAAAGCCAAAAAATA TTTTGCTGG-3′) and DN3 (5′-CCAGCAAAATATTTTTTG GCTTTAAATTACAGTGG-3′). Both wild type and modified cDNAs were cloned into the pCP60 vector (Crespi *et al.* 1994) under the control of the 35SCaMV promoter. *Agrobacterium tumefaciens* (EHA105) mediated transformation of Col-0 *A. thaliana* plants was performed using the floral dip method and transformant T0 seeds were selected on kanamycin 50 μg/mL. Transgene expression levels were tested by semiquantitative RT–PCR in 15 lines of T1 plants. Total RNAs were extracted from rosette leaves using RNeasy Plant Mini kit (Qiagen). After DNase treatment (RNase-free DNase; Promega, Madison, WI, USA), cDNAs were prepared using reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, USA). Expression of the D^{315}N-MsAPK1 transgene and ubiquitin (used as a constitutive gene) was analysed as described by Chinchilla *et al.* (2003).

GUS *transcriptional fusions and assays*

We defined promoter regions of *AtAPK1* (At2g43850) and *AtAPK2* (At2g31800) as the sequence in between the polyA signal from the previous ORF and the ATG of the corresponding *AtAPK* gene. These sequences were PCR amplified from Col-0 genomic DNA using primers: 5PH1 (5′-ATCGCTGAACGACG TCGTCCCGATTGC-3′) and 3PX1 (5′-CGTCTAGAAAAAGT TTCTTCCTTTACGCAAATTCTC-3′) for *AtAPK1* promoter; and 5PH2 (5′-CGCAAGCTTGCTACTGAAGACTGACGACG ACGAAACGGC-3′) and 3PX2 (5′-GCTCTAGATCTCT CTTTCGTCTTCTTCTGCG-3′) for *AtAPK2* promoter. Each fragment (818 bp and 575 bp long, respectively) was cloned

in the pPR97 vector using XbaI and *Hin*dIII restriction sites (Szabados *et al.* 1995). Col-0 *A. thaliana* plants were *in vivo* transformed with *A. tumefaciens* (EHA105) and were selected on kanamycin 50 μg/mL. T1 plants were genotyped and homozygous plants were confirmed by PCR with primers directed against the T-DNA vector and primers 3PX1 and 3PX2, respectively. In both cases, six independent lines were tested for GUS activity using a histochemical assay (Jefferson *et al.* 1987). Seedlings or roots were incubated at 37°C from 2 to 24 h in GUS assay buffer (50 mM NaH_2PO_4 pH 7, 1 mM EDTA, 0.1% triton X-100 (v/v), 0.1% sarcosyl (w/v), 0.05% SDS (w/v) and 1 mM X-Gluc). Tissues were cleared (chlorohydrate/water/glycerol 8 : 3 : 1 w/w/w) and observed with a binocular Nikon SMZ800 (Nikon, Champigny sur Marne, France) equipped with a CCD Pixelink camera or a Reichert Polyvar (Leica, Rueil, France) microscope equipped with a Nikon DXM1200 CCD camera (Nikon). At least 20 plants per transgenic line were tested. The pictures shown were taken from a single transgenic line for each *AtAPK* gene, but similar results were obtained in three independent lines (data not shown).

Results

APKs contain three ankyrin-repeat domains

In a first structural analysis, we identified only one ankyrin-repeat in the N-terminal portion of the APK proteins (Chinchilla *et al.* 2003). The increasing number of ankyrin-repeats found in various proteins has led us to refine our analysis, resulting in the identification of two additional motifs in MsAPK1 (Fig. 1*A*). Even though the latter repeats are more variable in sequence, they still match the consensus (Sedgwick and Smerdon 1999). Alignment of MsAPK1 with the three closest *A. thaliana* relatives (*AtAPK1*, *AtAPK2*, *AtAPK3*; Chinchilla *et al.* 2003) revealed that the three repeats were conserved in all these proteins (Fig. 1*B*). Ankyrin repeats, found in multiple copies in proteins (from 2 to 20 repeats) are considered critical for proper functional three-dimensional conformation of the domain (Sedgwick and Smerdon 1999) and enhance the stability and folding rate of an ankyrin repeat containing protein (Tripp and Barrick 2007).

MsAPK1 *phosphorylates tubulin* in vitro

To analyse the functionality of this kinase, a 6His::MsAPK1 recombinant protein was expressed in *E. coli*. Although it accumulated in inclusion bodies (data not shown), the protein was successfully purified in denaturing conditions (Fig. 2*A*), and subsequently renatured by progressive dialysis. The ability of the kinase to phosphorylate various substrates was then analysed. Among nine substrates tested (see Materials and methods), MsAPK1 was only able to phosphorylate tubulin but not actin, and kinase activity was strictly dependent on manganese (Fig. 2*B*). An APK variant with a mutation in the kinase catalytic subdomain VIb (the quasi-invariant residue aspartate D315 present in the catalytic loop of the kinase was replaced with an asparagine amino-acid D315N) did not show this activity after renaturation. A recombinant CDPK (Gargantini *et al.* 2006) purified in the same denaturing conditions was nevertheless able to phosphorylate MAP (positive control, data not shown). Unfortunately, different antibodies prepared against MsAPK1 (using either APK-specific peptides or the purified protein) failed
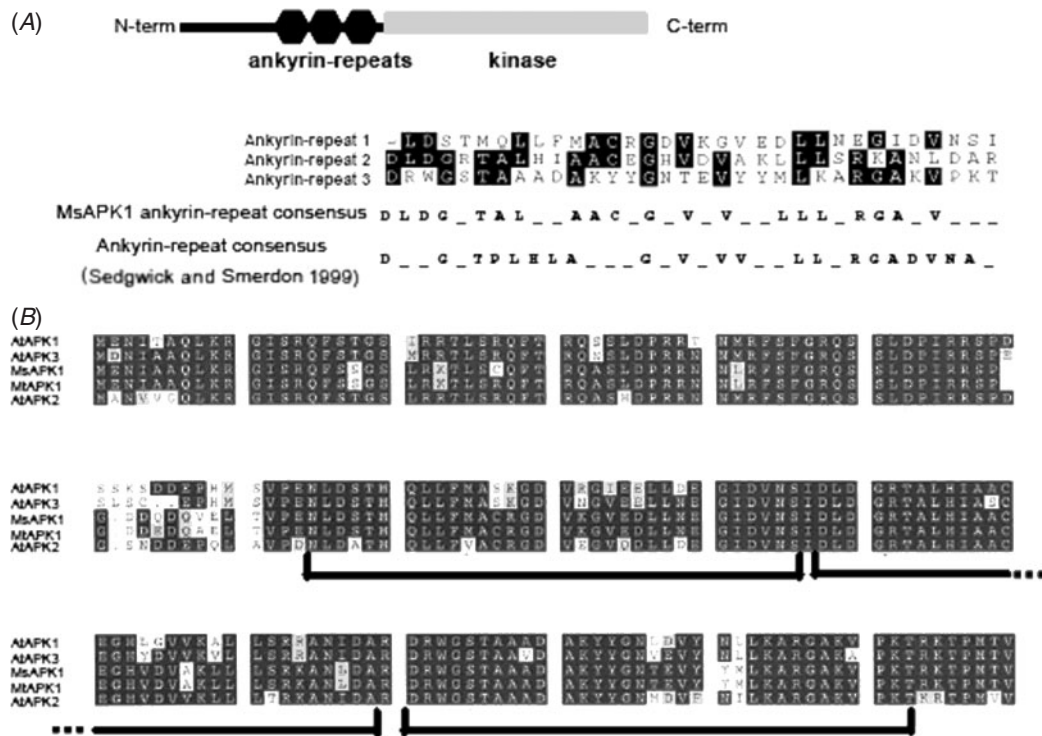
Fig. 1. Ankyrin kinase structure and genomic organisation. (*A*) Representation of the MsAPK1 protein. The three ankyrin-repeats were aligned using Prettybox (GCG, University of Wisconsin, Madison, WI, USA) to define a consensus for MsAPK1. This consensus is compared with the canonical one, proposed by Sedgwick and Smerdon (1999). (*B*) Homology between *Medicago truncatula*, *Medicago sativa* and *Arabidopsis thaliana* APKs. N-terminal region containing ankyrin-repeats of APKs was aligned using prettybox (GCG). Amino acid sequences used are from *A. thaliana* (AtAPK1, AtAPK2, and AtAPK3), *M. truncatula* (MtAPK1) and *M. sativa* (MsAPK1). Black boxes show identity, and grey boxes indicate similarity between residues of the different sequences. The sequences of the three ankyrin repeats are underlined.

to reveal specific bands in western blot analysis that could be linked to this protein activity or to immunoprecipitate APK activity (data not shown). This may be related to the very low abundance of these transcripts (Chinchilla *et al.* 2003). For this reason, we could not analyse up to what extent MtAPK1 activity contributes to tubulin phosphorylation *in vivo*.

AtAPK1 and AtAPK2 show differential expression patterns in roots

The tissue-specificity of the two *A. thaliana* genes, *AtAPK1* and *AtAPK2* whose expression was confirmed by RT–PCR (Chinchilla *et al.* 2003) was analysed. The GUS transcriptional fusions prepared (Fig. 3*A*) revealed that both promoters were active in various plant organs in correlation with our previous RT–PCR experiments. In the aerial portion of 2-week-old plants, both genes were expressed in the shoot apical meristem, in leaf vasculature, in flower stamens and in the abscission zone of siliques (data not shown). Beside this redundant expression, the two genes were differentially regulated in hypocotyls and roots of 1-week-old seedlings: *AtAPK1* was weakly expressed in the root stele, and also faintly at the base of the hypocotyl (Fig. 3*B*), but not in the root apex (Fig. 3*C*). In contrast, *AtAPK2* was detected at high levels in the hypocotyl (Fig. 3*F*) and in the root apex region (Fig. 3*G*). In addition, lateral roots from 2-week-old plants showed a pattern of *AtAPK2* gene expression in the apex

similar to the one observed in main roots (Fig. 3*H*) whereas no expression of the *AtAPK1* gene was detected (Fig. 3*D*).

A more detailed analysis of *AtAPK2* expression in lateral roots showed that *AtAPK2* was always restricted to the apexes (Fig. 3*J*). No expression was found in other parts of lateral roots and only a faint staining was observed at the surface of the root epidermis during early stages of lateral root development. In adventitious roots, *AtAPK1* expression remains undetectable (Fig. 3*E*), although a faint signal was occasionally observed in the hypocotyl at the initiation site of the root (data not shown). In contrast, *AtAPK2* is strongly expressed in adventitious root tips as well as in the hypocotyl region from which the adventitious root originates (Fig. 3*I*).

These data suggest that both genes have distinct patterns of expression, *AtAPK2* being induced mainly in primary, adventitious and lateral root apexes.

Expression of a $D^{315}N$-MsAPK1 mutant affected in the kinase catalytic site positively affects adventitious rooting

To gain further insight in the function of the *APK* genes, we developed functional approaches based on transgenic plants and reverse genetics. Blast searches (http://www.tigr.org/tdb/e2k1/mta1/, accessed 20 October 2007; http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi, accessed 20 October 2007)
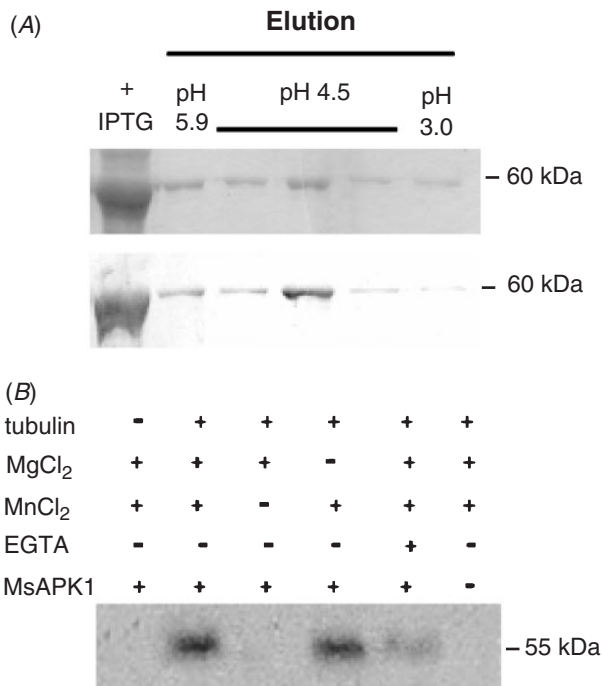
Fig. 2. Purification of the recombinant 6His::MsAPK1 and kinase assays. (*A*) MsAPK1 protein tagged with an N-terminal 6His-peptide was expressed and purified in denaturing conditions from *Escherichia coli*. Purity of various pH-eluted protein fractions was assayed using SDS–PAGE, followed by Coomassie brilliant blue staining (upper panel) and western blotting using anti-His antibodies (lower panel). In both cases a single band for 6His::MsAPK1 was observed at the expected size (60 kDa). (*B*) MsAPK1 kinase activity *in vitro*. 6His::MsAPK1 kinase activity has been tested using ^{32}P-γATP and various protein and peptide substrates and ionic conditions as indicated. MsAPK1 was able to phosphorylate only tubulin in a Mn^{2+}-dependent manner (lanes 2 and 4) and EGTA abolished this activity (lane 5).

revealed that only one APK gene (96% identical to MsAPK1) is present in the *M. truncatula* genome. *M. truncatula* plants were transformed with the *MsAPK1* gene under the control of the strong $2 \times 35S$ CaMV promoter or a portion of *MsAPK1* in antisense orientation according to Frugier *et al.* (2000). However, we were unable to regenerate viable transgenic plants, suggesting that the abnormal expression of the transgene could affect plant viability.

Therefore, two *Atapk2* T-DNA lines were analysed (SALK_083275 and SALK_571204), but no major phenotype was observed in the homozygote mutants in a wide variety of conditions. This may be due to functional redundancy, however double mutants could not be obtained because the genes were genetically linked. Alternatively, these insertions may not be loss of function mutants since they are 100 bp before the ATG or in a 3′ region of the gene (11th exon). The very low level of expression of *Atapk2* makes it difficult to detect reduction of gene expression in these plants.

Since *MsAPK1* is very similar to the *AtAPK* genes from *A. thaliana* (at least 80% identity at whole protein level), we expressed the full-length *MsAPK1* cDNA in *A. thaliana* where no *in vitro* regeneration steps are required to generate transgenic plants. On the other hand, we expressed the MsAPK1

variant ($D^{315}N$ substitution; Fig. 4*A*) that presented no protein kinase activity in order to produce a dominant negative mutant effect. A related D to N substitution in the *A. thaliana* CDC2 protein kinase resulted in a dominant negative mutation (Taylor *et al.* 1993; Hemerly *et al.* 1995). Different transgenic lines expressing *MsAPK1* or its mutated form were obtained (Fig. 4*B*, left and right panels), however, these plants did not show any obvious developmental phenotype, even under various osmotic stress conditions (data not shown). Several independent lines expressing a $D^{315}N$ version of *MsAPK1* were selected based on their high transgene expression levels (DN-6, DN-12 and DN-15; Fig. 4*B*, right panel). In comparison to Col-0 plants expressing an empty vector or wild type MsAPK1, 12-day-old plants from the selected DN lines from which the primary root was removed (de-rooted plants) showed a significant (ANOVA test, $P < 0.05$ and post-hoc comparison: Col-0/DN-6: $P = 0.038$; Col-0/DN-12: $P = 0.00027$; Col-0/DN-15: $P = 0.04$) increase in the development of adventitious roots (Fig. 4*C*, data not shown). Similarly, seedlings from Col-0 control plants or DN6 and DN12 transgenic lines subjected to a treatment involving dark and 2,4-D to induce adventitious root development, also produced a significantly higher number of adventitious roots per hypocotyl (ANOVA test, $P < 0.05$, and post-hoc comparison: Col-0/DN-6: $P = 0.027$; Col-0/DN-12: $P = 0.000018$; Fig. 4*D*). This difference correlated with a decrease in lateral root formation (Fig. 4*E*), which may reveal the integration of adventitious root formation into a global regulation of root meristem number.

These results therefore suggest that perturbation of APK-mediated signalling affects the regulation of root architecture, notably through the control of adventitious root development. The latter observation was obtained independently of the experimental condition used to induce lateral root formation (de-rooting or dark/auxin treatment).

Discussion

In this work, we have analysed the function of a plant ankyrin kinase family from *A. thaliana* and *Medicago* spp. at biochemical and physiological levels. The presence of three ankyrin-repeats in the APK proteins tends to support the functionality of this domain and reinforces the structural resemblance with mammalian ILKs that generally carry three N-terminal ankyrin-repeats fused to its kinase domain.

A computational analysis of the kinase domain present in MsAPK1 was performed using different databases. SMART database indicated two possible representations due to overlapping domains, that of a Pfam kinase or of a Tyr-kinase. MsAPK1 presents a tyrosine kinase catalytic domain (residues 359-**SS**L**Y**VA**PE**IYRGDVFDRSV**D**AYS**FG**LIVVY**EM**-389; conserved residues are indicated in bold and the tyrosine phosphorylation consensus site is underlined) and according to KinBase, it could be classified within the TKL group. The ATP-Mn^{2+} requirement for MsAPK1 catalytic activity suggests a possible relationship to a tyrosine kinase activity as serine/threonine kinases prefer ATP-Mg^{2+}.

Tyrosine-phosphorylation was reported for the first time in plants when *Catharanthus roseus* L. roots were transformed by *Agrobacterium rhizogenes* (Rodríguez-Zapata and Hernández-Sotomayor 1997). It has been proposed to play a role
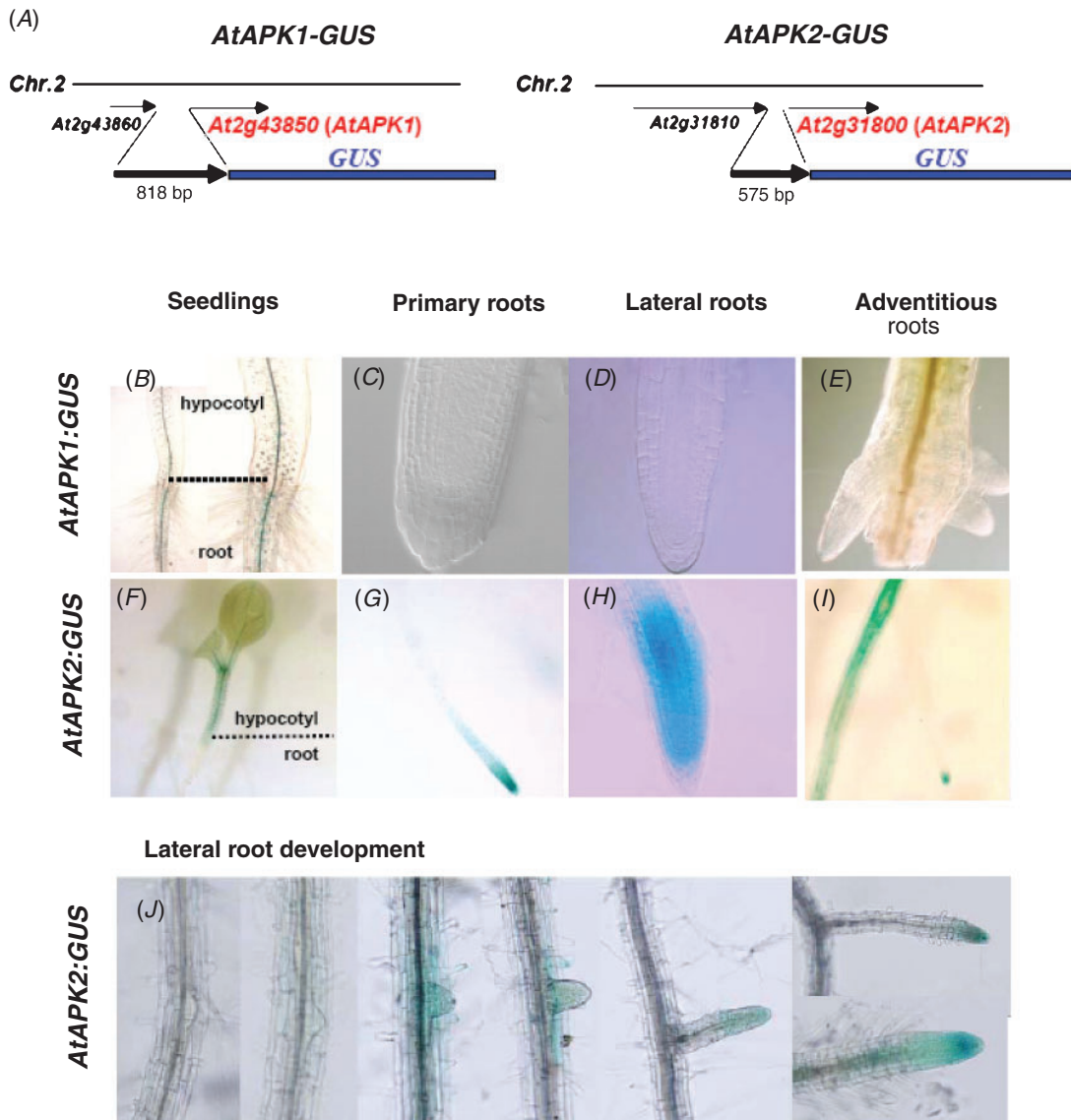
Fig. 3. Expression patterns of *AtAPK1* and *AtAPK2* genes in different roots types. (*A*) Transgenic plants expressing promoter-GUS transcriptional fusions for the *AtAPK1* and *AtAPK2* genes were prepared and analysed. The putative promoter regions used are presented, including the sequence in between the polyA signal from the upstream gene and the ATG of the *APK* gene. (*B*–*E*) *AtAPK1::GUS*; (*F*–*I*) *AtAPK2::GUS*; expression patterns in (*B*, *F*) seedlings, 7 days after germination, DAG; (*C*, *G*) primary roots, 7 DAG; (*D*, *H*) lateral root tips, 12 DAG; and (*E*, *I*) adventitious roots. (*J*) Detailed analysis of *AtAPK2::GUS* expression in lateral roots at different developmental stages. *AtAPK1* was expressed mainly in (*B*) root stele of seedlings. However, expression was not detectable in root tip of (*C*) primary roots, (*D*) lateral roots or (*E*) adventitious roots. *AtAPK2* was expressed in hypocotyls of (*F*) seedlings and in (*G*) the root apex. Similar expression pattern was found in (*H*, *J*) lateral root tips, as well as in (*I*) adventitious roots (tips and at the base of the initial hypocotyl-derived root). Similar results have been obtained with at least three independent lines for each construct.

in plant development and embryogenesis (Islas-Flores *et al*. 1998; Barizza *et al*. 1999), in petiole bending in *Mimosa pudica* L. (through actin phosphorylation (Kameyama *et al*. 2000)) and in callus cell proliferation in *Arabidopsis* hypocotyls (Huang *et al*. 2003). However, genome-wide analysis of *Arabidopsis* using the delineated tyrosine kinase motifs from animals revealed the presence of only dual-specificity kinases, raising an intriguing possibility that plants lack classical tyrosine kinases (Rudrabhatla *et al*. 2006).

The *A. thaliana* dual-specificity protein kinase (AtSTYPK) exhibits strong preference for manganese over magnesium for its kinase activity (Reddy and Rajasekharan 2006), while MsAPK1 activity is strictly dependent on the presence of manganese.

Even though we cannot rule out that other substrates may be phosphorylated *in vivo* by APKs, MsAPK1 was only able to phosphorylate tubulin *in vitro*. In animals, phosphorylation is an essential mechanism controlling microtubule dynamics (Aletta
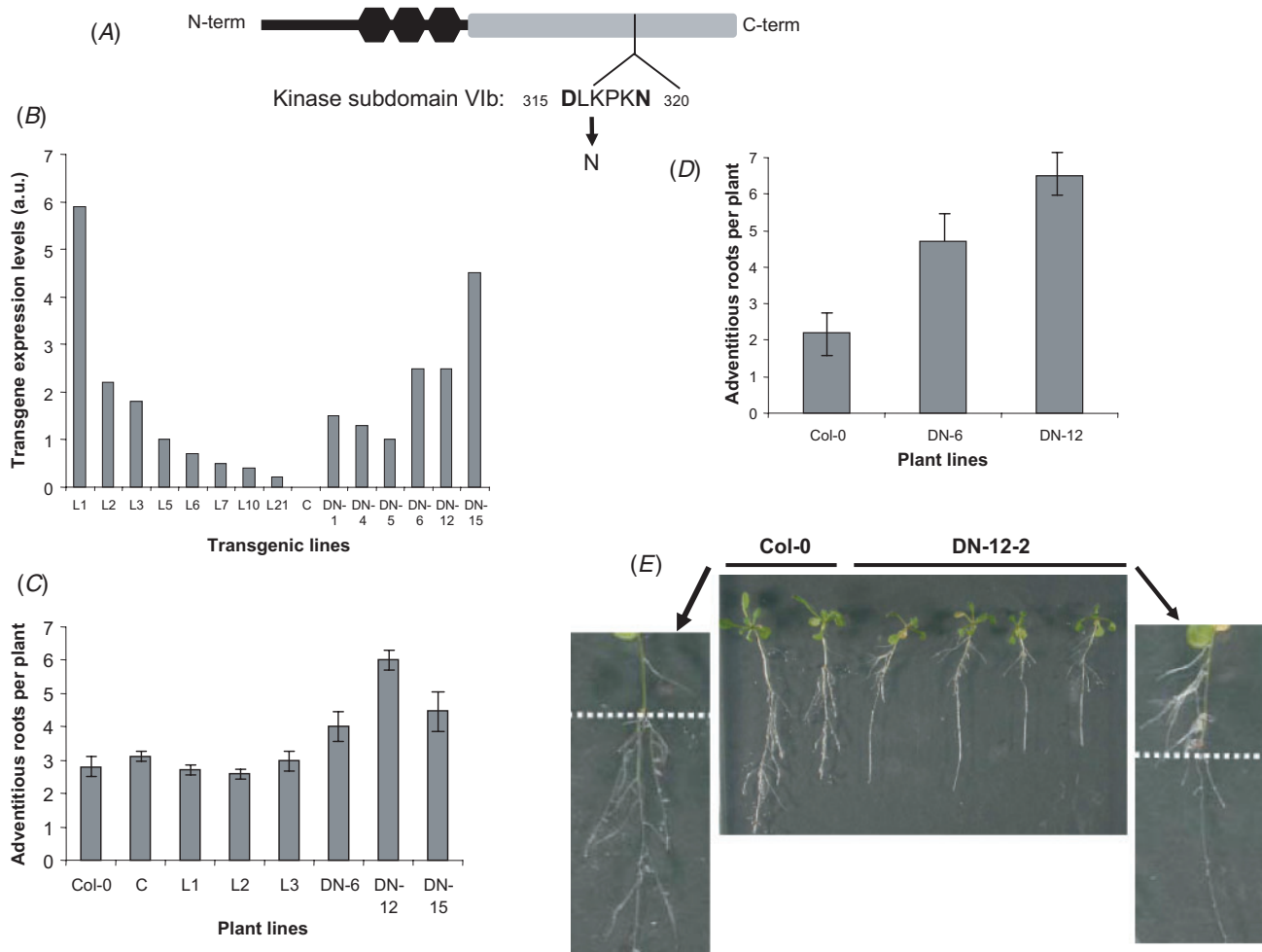
Fig. 4. D^{315}N-APK1 mutants are affected in their adventitious rooting ability. (*A*) A substitution (D to N) was introduced by point mutation in the MsAPK1 kinase subdomain VIb (catalytic loop) at the conserved position 315 essential for kinase activity, to generate an altered kinase mutant. (*B*) Expression of 35S::*MsAPK1* (lines L) or 35S::*D^{315}N-MsAPK1* (lines DN) in transgenic *Arabidopsis thaliana* lines (a.u., arbitrary units). Plants transformed with the empty vector were used as control (C). * Indicates the lines selected to perform the experiments depicted in (*C*). (*C*) Number of adventitious roots per plant in Col-0 control line, plants containing an empty vector (C), three independent *MsAPK1* lines (L1, L2, L3) or three independent *D^{315}N-MsAPK1* lines (DN-6, DN-12, and DN-15). Adventitious roots were induced using de-rooting of main root and counted 12 days after de-rooting. Error bars represent standard deviations from three independent experiments (*n* > 30). (*D*) Number of adventitious roots per hypocotyl was determined in seedlings from Col-0 control plants or the transgenic DN6 and DN12 lines expressing the D^{315}N variant at 13 days after germination. Adventitious roots were induced using a 2,4-D treatment (10^{-8} M) on seedlings grown for 3 days in the dark and then 10 days in long-day conditions. (*E*) Root architecture of representative Col-0 and DN12–2 plants grown on a 2,4-D 10^{-8} M medium as mentioned in (*D*). Bars on details indicate the limit between hypocotyl and root organs. Error bars represent Confidence of Interval (α = 0.01) from one representative experiment (*n* > 30). Values for transgenic lines expressing *MsAPK1* or an empty vector were not statistically different from the ones obtained with Col-0 (data not shown).

1996; MacRae 1997). Several kinases from different families are able to phosphorylate tubulin *in vitro* or *in vivo*. Most of these kinases, such as Polo Like Kinases (PLK), are involved in cell cycle control, where the regulation of microtubules dynamics is crucial (Tavares *et al.* 1996; Feng *et al.* 1999). However, biological significance of *in vivo* tubulin phosphorylation remains poorly understood: in *Saccharomyces cerevisiae*, β-tubulin, one of the main constituents of centrosomes, is regulated by phosphorylation and mimicking a phosphorylated tyrosine residue in this tubulin using an aspartate substitution results in changes in microtubule organisation (Vogel *et al.* 2001).

In plants, pharmacological studies indicate that phosphorylation regulates cortical microtubules dynamics in *A. thaliana*, and alters roots morphology (Baskin and Wilson 1997). Moreover, *ton2* mutant affected in a PP2A phosphatase regulatory subunit shows abnormal cortical microtubules in root cells (Camilleri *et al.* 2002). However, direct tubulin phosphorylation in plants has been rarely established (Koontz and Choi 1993) and its biological function remains mostly unknown. Several kinases have been reported to be associated with microtubules such as the MAP-Kinase (MAPK) MMK3 (Bogre *et al.* 1999), CDC2 (Hemsley *et al.* 2001) or NPK1 for Nicotiana Protein Kinase 1 (Nishihama *et al.* 2002) but it

is not known whether such interaction leads to microtubule rearrangements and control of their polymerisation status. A 46 kDa protein resembling immunochemically the mammalian dually phosphorylated p38-MAPK was detected in wheat root cells under hyperosmotic conditions (Komis *et al*. 2004). The authors suggest that this kinase is probably involved in tubulin cytoskeleton reorganisation induced by hyperosmotic stress as well as in protoplast volume regulation and osmotic tolerance of wheat root cells. MsAPK1 is able to phosphorylate tubulin (Fig. 2) and its expression is induced by osmotic stress (Chinchilla *et al*. 2003). Preliminary experiments suggest that an MsAPK1-GFP fusion relocalises into a microtubule-like network under osmotic stress (data not shown). Microtubule reorganisation during plasmolysis, e.g. after an osmotic stress, may be also associated to modifications of adhesion sites connecting cell wall, plasmalemma and cytoskeleton (Fowler and Quatrano 1997). APK and ILK proteins present structural similarity (3 amino-terminal ankyrin-repeats fused to a kinase domain), but have differential substrate specificity (tubulin *vs*. actin). According to published data, *Arabidopsis* genome does not contain genes coding for integrins; however, immunocytochemical data suggest that there might be some integrin-like proteins in plants. We may speculate that APKs could be involved in connecting the cytoskeleton (via tubulin phosphorylation) to outer cues, through integrin-like proteins.

Adventitious rooting is a developmental program poorly documented in plants, notably compared with other types of root organogenesis. This process is induced in peculiar environmental conditions, such as abiotic stresses or injury. Only few mutants affected in adventitious rooting have been reported, and a systematic search lead to the identification of nine mutants altered in different stages of the process (Konishi and Sugiyama 2003). An interesting mutant, *rid5* was perturbed in auxin signalling in relation with its rooting capacity, and turned to be allelic to *mor1/gem1* mutants (Whittington *et al*. 2001). Knowing that the function of MOR1 is associated to the microtubule cytoskeleton, it is tempting to speculate whether a similar pathway is affected in *mor1/rid5* and in $D^{315}N$ APK mutants. It would be of interest to determine whether both proteins are indeed involved in a common pathway, or in independent and parallel ones. Moreover, our results suggest that adventitious rooting is dependent on a process that may require the involvement of MOR1 and AtAPK2. Furthermore, in the DN-plants a compensatory effect on lateral root number may counteract the presence of an excess of adventitious roots. The total number of root-related meristems may be regulated in plants and linked to carbon allocation into these growing sinks (Malamy and Ryan 2001). Interestingly, AtAPK2 is expressed in the apex of all root-related meristems in *Arabidopsis*.

The use of mutants altered in their kinase catalytic domain can serve to affect signalling pathways (Taylor *et al*. 1993; Hemerly *et al*. 1995). Recent work has shown that a point mutation in the autophosphorylation site of the calcium calmodulin dependent protein kinase (CCaMK) lead to spontaneous nodule initiation (Tirichine *et al*. 2006). In addition, *Arabidopsis* T-DNA mutant lines carrying null alleles of wall-associated kinase 2 (WAK2), a newly identified effector of invertases, had reduced vacuolar invertase activity in roots and alter root growth when osmolyte

supplies are limiting (Kohorn *et al*. 2006). The identification and manipulation of critical sites in proteins has provided new ways to rebuild the proteins and engineer plants to obtain desired traits (Yang *et al*. 2007). This approach can be particularly useful when functional redundancy in gene families may complicate detailed genetic analysis as it may be the case of the various *AtAPK* genes present in *A. thaliana*. Indeed, single mutants in the *AtAPK1* gene did not display any detectable phenotype (data not shown), in contrast to the expression of the $D^{315}N$-APK in DN lines which developed more adventitious roots.

The increase in adventitious roots observed in the DN lines was independent of the method used to obtain them (e.g. removal of main root or induction with 2,4-D). *AtAPK2* expression in hypocotyls could be correlated with the phenotype observed in the $D^{315}N$ APK mutants on adventitious root development since this organogenesis is initiated in hypocotyls. Interestingly, the closest relative to MsAPK1 in *A. thaliana* is AtAPK2 (Chinchilla *et al*. 2003). Even though, double APK1 and APK2 loss of function mutants would be required to definitely confirm this conclusion, our results suggest that perturbation of AtAPK2 function by the $D^{315}N$ APK isoform may be responsible for the observed phenotype. Alternatively, we cannot exclude that the dominant effect observed in adventitious rooting could be linked to neomorphic consequences on potential substrates of MsAPK1 such as microtubules (Konishi and Sugiyama 2003).

Our data suggest that APKs are a new regulatory element related to cytoskeleton changes and involved in adventitious rooting.

Acknowledgements

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