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A CDPK isoform participates in the regulation of nodule number in *Medicago truncatula*

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Summary

***Medicago* spp. are able to develop root nodules via symbiotic interaction with *Sinorhizobium meliloti*. Calcium-dependent protein kinases (CDPKs) are involved in various signalling pathways in plants, and we found that expression of *MtCPK3*, a CDPK isoform present in roots of the model legume *Medicago truncatula*, is regulated during the nodulation process. Early inductions were detected 15 min and 3–4 days post-inoculation (dpi). The very early induction of *CPK3* messengers was also present in inoculated *M. truncatula dmi* mutants and in wild-type roots subjected to salt stress, indicating that this rapid response is probably stress-related. In contrast, the later response was concomitant with cortical cell division and the formation of nodule primordia, and was not observed in wild-type roots inoculated with *nod*$^-$ strains. This late induction correlated with a change in the subcellular distribution of CDPK activities. Accordingly, an anti-*Mt*CPK3 antibody detected two bands in soluble root extracts and one in the particulate fraction. CPK3::GFP fusions are targeted to the plasma membrane in epidermal onion cells, a localization that depends on myristoylation and palmitoylation sites of the protein, suggesting a dual subcellular localization. *MtCPK3* mRNA and protein were also up-regulated by cytokinin treatment, a hormone linked to the regulation of cortical cell division and other nodulation-related responses. An RNAi-CDPK construction was used to silence CPK3 in *Agrobacterium rhizogenes*-transformed roots. Although no major phenotype was detected in these roots, when infected with rhizobia, the total number of nodules was, on average, twofold higher than in controls. This correlates with the lack of *MtCPK3* induction in the inoculated super-nodulator *sunn* mutant. Our results suggest that CPK3 participates in the regulation of the symbiotic interaction.**

Keywords: CDPK, *Medicago truncatula*, nodulation, cytokinins, stress.

Introduction

Nitrogen is a critical element required for plant growth. Most legume plants are capable of entering into symbiosis with nitrogen-fixing bacteria (collectively called rhizobia) to form the root nodule. In this symbiotic organ, the bacteria, differentiated into bacteroids, fix nitrogen for the plant host (Gálvez *et al.*, 2000). This developmental process starts with a molecular recognition system that enables root hairs to promote the entry of rhizobia. Nodule development is controlled by an exchange of signals between the two partners. Rhizobia induce nodule morphogenesis on the plant by producing a lipochitoligosaccharide signal molecule, the Nod factor (Crespi and Galvez, 2000; Gresshoff, 2003). Nod factors act as primary morphogenetic signals for nodulation, and trigger events related to infection (e.g. root hair deformation) and organogenesis (e.g. cortical cell division). These Nod factors are probably recognized by specific LysM domain-containing receptor kinases (Limpens *et al.*, 2003; Ben Amor *et al.*, 2003) in *Medicago truncatula*, proteins that are analogous to NFR1 and NFR5 in *Lotus japonicus* (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003).

These LysM receptor kinases trigger a signal-transduction cascade that is essential to induce all early symbiotic events. In addition to the LysM receptor kinases, several other components of this Nod factor-induced signalling cascade have been identified, and, in *M. truncatula*, these components include the putative cation channel DMI1 (Ané *et al.*, 2004) that is analogous to CASTOR and POLLUX in *L. japonicus* (Imaizumi-Anraku *et al.*, 2005), the leucine-rich-repeat-containing receptor kinase DMI2 (Endre *et al.*, 2002) that is orthologous to SYMRK in *L. japonicus* (Stracke *et al.*, 2002), and the calcium/calmodulin-dependent kinase DMI3; (Ca/CaMK; Lévy *et al.*, 2004; Mitra *et al.*, 2004). In addition, characterization of a super-nodulator mutant revealed that function of a *clavata* 1-related kinase (Clark *et al.*, 1997) was essential for control of the number of nodules (auto-regulation; Schnabel *et al.*, 2005).

When added to legume roots, Nod factors induce two phases of calcium changes that can be observed in root hair cells. One is a rapid influx of Ca^{2+} (Ca^{2+} flux) that is immediately followed by membrane depolarization. A few minutes later, oscillations in the cytosolic Ca^{2+} concentration (a phenomenon known as Ca^{2+} spiking) are induced. The putative Ca/CaMK could integrate this oscillatory signal into the nodulation pathway, through the phosphorylation of regulatory proteins (Lévy *et al.*, 2004). Nod factor-induced Ca^{2+} flux has been observed in a diversity of legumes (*Pisum sativum*, *M. sativa*, *M. truncatula*, *Phaseolus vulgaris*), indicating that this response is commonly found, although the induction is variable and may depend on the developmental status of the root-hair cell (Oldroyd and Downie, 2004).

While cytosolic Ca^{2+} fluctuations have been observed in response to a number of stimuli (Sanders *et al.*, 2002), the downstream signal transduction pathways that connect Ca^{2+} fluctuations to changes in metabolism and gene expression are largely unknown. Transient increases in cytosolic calcium, transduced via calcium-binding proteins, probably affect protein kinases to transduce various external signals. Calcium-dependent calmodulin-independent protein kinases (CDPKs) have been implicated as important sensors of calcium flux in plants in response to stresses (Ludwig *et al.*, 2004). CDPKs are members of the serine/threonine protein kinase family that are composed of a single polypeptide chain with a catalytic kinase domain and a calmodulin-like domain (CLD) with four EF-hand Ca^{2+} binding sites. These enzymes require micromolar concentrations of free Ca^{2+} for their activity (Harmon *et al.*, 1986; Harper *et al.*, 1991). A junction domain lies between the kinase domain and the CLD. This junction domain acts as an auto-inhibitor in a pseudo-substrate fashion (Harper *et al.*, 1991,1994; Weljie *et al.*, 2000). Many CDPKs also have additional N-terminal domains comprised of highly variable amino acid sequences.

Although CDPKs have been implicated as key regulators in many signalling pathways, very little is known about which particular CDPK acts in each case (Ludwig *et al.*, 2004). In *M. sativa*, *Ms*CPK3 expression was found in cultured cells linked to auxin treatment (Davletova *et al.*, 2001), and in *M. truncatula*, another distantly related CDPK gene *Mt*CDPK1 has been shown to be involved in root development. *Mt*CDPK1 was proposed to modulate cell expansion or cell wall synthesis (Ivashuta *et al.*, 2005). Consequently, mutants affected in this CDPK cannot interact with symbionts.

CDPKs are regulated at the transcriptional level by phytohormones and stress conditions (Cheng *et al.*, 2002), for example *Nt*CDPK1 mRNA accumulates in cytokinin-treated tobacco leaves (Yoon *et al.*, 1999). Interestingly, phytohormonal treatments of roots can mimic responses induced by rhizobia at the initiation of nodule organogenesis (Fang and Hirsch, 1998), suggesting that Nod factors may act by creating phytohormonal imbalances inside root tissues. Exogenous cytokinin is able to induce the expression of various early nodulin genes in various legumes, and promotes cortical cell division in alfalfa, in a similar way to Nod factors (Dehio and de Bruijn, 1992; Hirsch and Fang, 1994; Bauer *et al.*, 1996; Gronlund *et al.*, 2005).

In this paper, we analyse the expression of *Mt*CPK3 and its homologue in *M. sativa*, *Ms*CPK3, during the nodulation process and in response to cytokinin treatment. Even though there is no global change in total CDPK activity, the subcellular distribution of CDPK activity varies concomitantly with a transient increase in the expression of these isoforms during the very early stages of the *Medicago–Rhizobia* interaction and later during nodule formation. The very early induction of *CPK3* mRNAs was also present in *M. truncatula dmi* mutants inoculated with *Rhizobia* spp. and in wild-type roots subjected to salt stress, suggesting a rapid stress-responsive role for this kinase. In contrast, the late response observed was concomitant with the division of cortical cells from the nodule primordia. Furthermore, *Mt*CPK3 mRNA and protein were rapidly up-regulated by cytokinin, a hormone linked to the regulation of cortical cell division and nodulation, whereas CPK3-RNAi *M. truncatula* transgenic roots, showing strongly reduced *MtCPK3* expression, were affected in the control of nodule number. Our results suggest that this specific CDPK isoform participates in the regulation of the symbiotic interaction.

Results

Expression and immunoblot analysis of MtCPK3 *in roots*

A gene encoding a CDPK isoform has been identified in alfalfa (*Ms*CPK3; Davletova *et al.*, 2001), which is closely related to the model legume *M. truncatula*. In the *M. truncatula* EST database (TIGR, http://www.tigr.org/tdb/e2k1/mta1/), at least 21 different CDPK genes were found to be expressed in various tissues. Among them, the partial tentative consensus

sequences (TCs) TC101857 and TC103686 were detected in roots. TC101857 shared 97% identity in protein sequence with the catalytic domain of *Ms*CPK3 (Davletova *et al.*, 2001) and high similarity with its N-terminal variable domain, while TC103686 was 100% identical to the protein sequence of the CLD domain of *Ms*CPK3, suggesting that both clones correspond to the same CDPK isoform in *M. truncatula*.

The complete cDNA sequence of this CDPK isoform was amplified by RT-PCR using RNA from *M. truncatula* roots as template and specific primers designed against the 5′ region of TC101857 and the 3′ region of TC103686. The full-length clone, designated *Mt*CPK3 (GenBank accession number DQ454073), has an open reading frame that is 1617 nt long from the ATG to the stop codon, encoding a predicted protein of 538 amino acids with an estimated molecular mass of 60.6 kDa. The *Mt*CPK3 amino acid predicted sequence shares the highest homology (95–98%) with *Ms*CPK3 from *M. sativa* (GenBank accession number X96723.1; EMBL accession number DQ454073) and CDPK isoforms from *Glycine max* (GenBank accession numbers AAB80693 and AY247756.1).

*Mt*CPK3 includes subdomains I–XI from serine/threonine kinases (Hanks and Quinn, 1991), the junction domain and the four EF-hand calcium-binding motifs of the CLD. The *Mt*CPK3 N-terminal variable domain only shares high identity with that of *Ms*CPK3 (Davletova *et al.*, 2001), and presents a myristoylation consensus and a putative palmitoylation site. The predicted protein also contains numerous phosphorylation sites, suggesting that its activity may be regulated by reversible phosphorylation.

The expression of this isoform was analysed in roots, stems, leaves and flowers of *M. truncatula* plants by real-time RT-PCR (qRT-PCR). The *M. truncatula* CDPK isoform was predominantly expressed in roots (Figure 1a). Developing roots from *M. truncatula* A17 plants were harvested at various times (4, 5 and 7 days after germination, dag). Specific antibodies developed against the N-terminal variable domain of *Mt*CPK3 detected two specific bands of 60 and 63 kDa in crude protein extracts. Further separation into soluble and particulate fractions revealed that the slower migrating band was predominant in soluble root extracts, while the faster migrating form was present in the particulate fraction (Figure 1b). Although it is difficult to assess the amount of CPK3 protein present in each fraction, our data suggest that it is enriched in the particulate fraction (Figure 1b, upper panel). In certain Western analyses using larger amounts of total proteins (50 μg), a second weak band could be detected in the soluble fraction (Figure 1b, lower left panel).

The different bands could correspond to different CDPK isoforms (even though the immunogenic peptide revealed no hit in the available databases, i.e. TIGR and NCBI, when a BLASTX search for short sequences, allowing mismatches, was performed), or to post-translationally modified versions

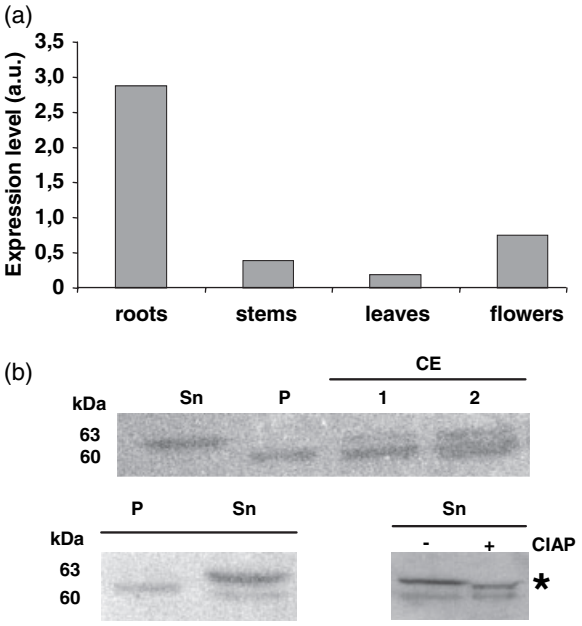

Figure 1. Expression and biochemical analysis of *MtCPK3* in roots.
(a) Quantitative RT-PCR was performed on RNAs obtained from roots, stems, leaves and flowers of *M. truncatula* plants using specific primers for the *MtCPK3* isoform. The same amounts of total RNA were used in each experiment. Values are expression levels in arbitrary units (a.u.) of two technical replicates.
(b) Western blot assays of *M. truncatula* (A17) roots were performed using an anti-*Mt*CPK3-specific antibody (1:1000). Upper panel: soluble (Sn, 45 μg) and particulate (P, 15 μg) fractions or crude extracts (CE; lane 1, 10% of total protein; lane 2, 20% of total protein). Lower left panel: particulate (15 μg) and soluble (50 μg) fractions. Lower right panel: soluble fraction (90 μg) incubated or not with 100 U of calf intestinal alkaline phosphatase (CIAP) for 15 min at 37°C. The asterisk indicates a mobility shift. Molecular weights (kDa) are indicated on the left.

of the same kinase. Among these post-translational modifications, calcium-dependent auto-phosphorylation of *Ms*CPK3 recombinant protein has been reported (Davletova *et al.*, 2001). Indeed, *M. truncatula* soluble root extracts incubated with 10 μM γ^{32}P-ATP revealed several phosphorylated polypeptides including the 63 kDa band recognized by the anti-CPK3 antibody (as confirmed by Western analysis of the same membrane, data not shown). The soluble fraction was incubated or not with alkaline phosphatase, and was then analysed by Western blot. Although two bands were still revealed in the phosphatase-treated extract, the slower migrating form showed a mobility shift (Figure 1b, lower right panel). Resistance to phosphatase treatment has already been reported for other kinases (Humphries *et al.*, 2005), so it may be that complete dephosphorylation of the protein could not be achieved. Western analysis of the auto-phosphorylated soluble extract showed that the slower migrating form becomes enriched (data not shown). Our data suggest a phosphorylation-dependent transition between the two forms as reported for other CDPKs (Romeis *et al.*, 2001); however, we cannot discard the possibility that

other post-translational modifications may also affect *Mt*CPK3 mobility.

Calcium-dependent protein kinase activity in M. truncatula *roots*

CDPK activity was determined in soluble and particulate protein extracts from *M. truncatula* (A17) roots using Syntide-2 or histone H1 as substrates (Figure 2a, upper and middle panels). Both extracts phosphorylated the substrates in a calcium-dependent manner, and this activation was reversed by the addition of EGTA or calmodulin antagonists such as clorpromazine (CPZ) and compound 48/80 (Figure 2b). Calmodulin antagonists inhibit CDPKs because of the similarity of their CLD to calmodulin. The kinase inhibitor staurosporine (ST) reduced the activity in both extracts, while the addition of okadaic acid, a potent inhibitor of protein phosphatases from the PP1 and PP2A family, increased CDPK activity in the particulate fraction (Figure 2b).

In terms of total CDPK activity, the greater part was associated with the soluble fraction (Figure 2a, lower panel); however, the specific activity of the particulate isoform was higher than that of the soluble one (5 and 7 dag; Figure 2a, upper panel). We cannot conclude that *Mt*CPK3 is exclusively responsible for the detected CDPK activity; however, our immunoblot data (Figure 1b) suggest that this isoform is present in particulate and soluble extracts of developing roots.

Expression analysis of MsCPK3 in S. meliloti*-inoculated alfalfa roots*

Considering the tissue specificity of this isoform, it was interesting to determine whether it could play a role in the symbiotic process of nodulation. Both TCs were detected in nodulated roots and in roots collected 24 h after inoculation with *Sinorhizobium meliloti* (http://www.tigr.org/tdb/e2k1/mta1/). *M. sativa* nodulates faster than *M. truncatula*; hence, after confirming the presence of *MsCPK*3 mRNA in roots of *M. sativa* plants (data not shown), an initial kinetic analysis of *CPK3* expression during nodule morphogenesis was performed in alfalfa roots infected with the wild-type *S. meliloti* Sm41 strain. The semi-quantitative RT-PCR assays were performed using the mRNAs obtained from the early stages of the symbiotic interaction using either a pair of primers directed against conserved CDPK regions (subdomain VIb and junction domain) or a specific primer against the N-terminal domain of *Ms*CPK3 together with the junction domain primer. While using conserved primers, no induction of CDPK expression was observed along the process, the other primer pair revealed that *MsCPK3* expression was enhanced 4 days after infection (Figure 3a) concomitantly with the formation of nodule primordia.

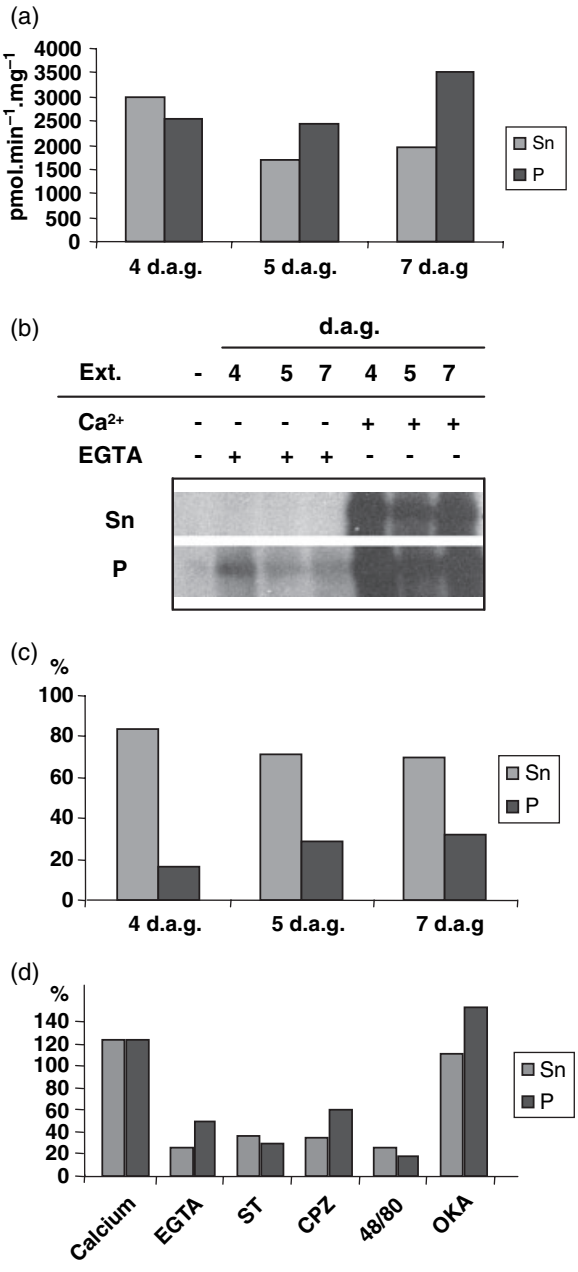

Figure 2. CDPK activity in M. truncatula roots.
CDPK activity was determined in soluble (Sn) and particulate (P) protein extracts (2 μg) from M. truncatula (A17) roots harvested at different times after germination (4, 5 and 7 days after germination). As phosphate acceptors, 25 μg Syntide-2 (a) or 1 mg/ml Histone H1 (b) were used. CDPK activity is the difference between the kinase activity detected with 1 mM calcium and the one detected with 5 mM EGTA. Specific activity is expressed as pmol. $min^{-1}.mg^{-1}$.
(c) Percentage of CDPK activity in soluble or particulate fractions is plotted.
(d) Soluble and particulate protein extracts (2 μg) from roots (7 days after germination) were pre-incubated for 30 min in an ice bath with 25 μM calcium and the following inhibitors: 1 μM staurosporine (ST), 0.5 mM clorpromazine (CPZ), 0.1 mg ml^{-1} compound 48/80 or 100 nM okadaic acid (OKA), or with 5 mM EGTA. Full (100%) CDPK activity corresponds to 1430 pmol min^{-1} mg^{-1} for the soluble fraction (Sn) or 1540 pmol min^{-1} mg^{-1} for the particulate fraction (P).
These results are representative of three independent experiments.

Total CDPK activity was then analysed during the early stages of nodule development using the previously described phosphate acceptors. Soluble and particulate extracts of control and inoculated roots were used as enzyme sources. In the particulate fraction of inoculated roots, an increase in calcium-dependent histone H1 phosphorylation was observed 3–4 days post-inoculation (dpi; Figure 3b, upper panel), correlating with *MsCPK3* induction. Histone phosphorylation was significantly reduced in the presence of EGTA, suggesting that one or more CDPKs were responsible for the increase observed (Figure 3b, lower panel).

*Ms*CPK3 has myristoylation and palmitoylation sites in its N-terminus, therefore acylation of the protein may promote membrane association (McCabe and Berthiaume, 1999; Thompson and Okuyama, 2000). The role of these sites in *Ms*CPK3 localization was studied in epidermal onion cells bombarded with a vector over-expressing wild-type *Ms*CPK3, or its mutant versions with altered myristoylation (Gly-2 to Ala-2) and/or palmitoylation (Cys-3 to Gly-3) sites, fused to the green fluorescent protein, GFP (Figure 4). Confocal analysis showed that the *Ms*CPK3 fusion was efficiently targeted to the cell periphery

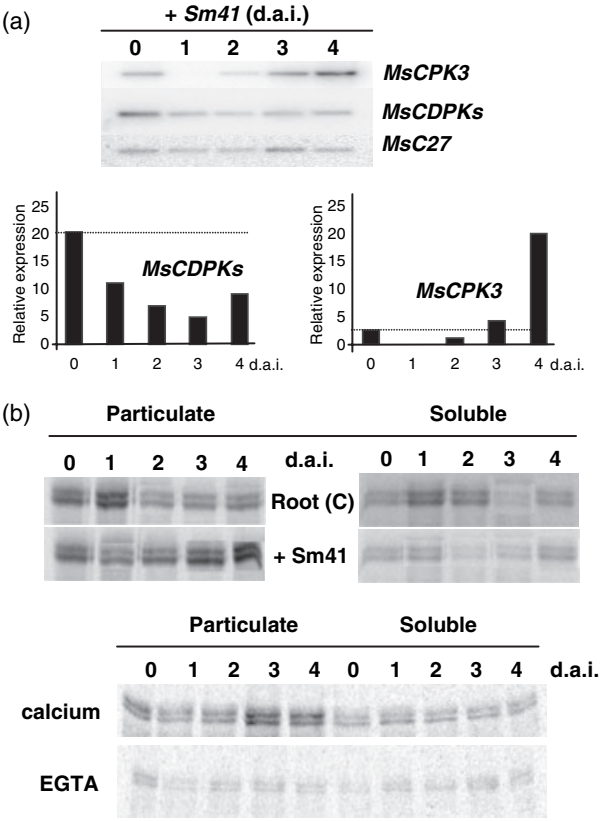

Figure 3. CDPK expression and activity during symbiosis.
(a) Expression analysis of *MsCDPK*s and *MsCPK3* in *M. sativa* roots infected with the *S. meliloti* Sm41 strain using spot inoculation and harvested at the indicated days post-inoculation (dpi). Semi-quantitative RT-PCRs were performed using conserved primers for CDPKs and specific primers for the *MsCPK3* isoform. The constitutive gene *MsC27* was used as control to normalize between samples.
(b) Upper panel: histone phosphorylation. CDPK activity was determined in *M. sativa* control (C) and inoculated (+Sm41) roots in the presence of 1 mM calcium. Lower panel: CDPK activity in *M. sativa*-inoculated (Calcium) roots: the assay was performed in the presence of 1 mM calcium or 1 mM EGTA. Soluble and particulate protein extracts (2 μg) were used as the enzyme source. Roots were harvested 1–4 days after inoculation.

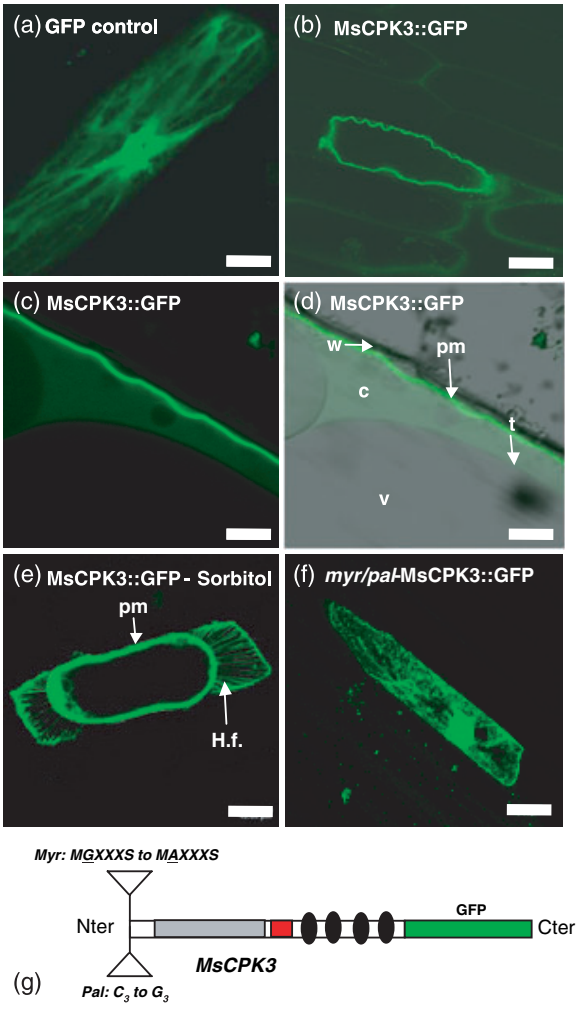

Figure 4. Subcellular localization of MsCPK3::GFP fusion proteins in onion epidermal cells.
Wild-type *MsCPK3* and myristoylation and palmitoylation *MsCPK3* mutants (*myr*, *pal*) were transiently expressed under the control of 2 x 35S promoters as C-terminal GFP fusion proteins. Confocal images were taken 24 h after bombardment.
(a) GFP control.
(b, d) *Ms*CPK3::GFP.
(e) *Ms*CPK3::GFP after cell treatment with 0.5 M sorbitol; plasma membrane (pm) and Hechtian filaments (H.f.) are indicated in the plasmolysed cell.
(f) *myr/pal Ms*CPK3::GFP fusion.
(g) Schematic representation of the mutant CPK3::GFP fusion protein used in this study. The grey area represents the kinase domain, red the junction domain, black the four EF hands, and green the GFP.
(c) and (d) are magnifications of (b) (bars for (c) and (d) = 8 μm). The superposition shown in (d) (with a bright-field image) allows confirmation of the membrane localization (pm) of the fusion protein (w, cell wall; c, cytoplasm; v, vacuole; t, tonoplast). Three bombardment experiments were performed for each construction. Bars = 40 μm for (a), (b), (e) and (f).

(Figure 4b–d). Plasma membrane localization was further confirmed by treating the epidermal cells with sorbitol to promote cell plasmolysis (Figure 4e). In contrast, *Mg*CPK3–GFP fusions mutated at either only the myristoylation site (*myr-*), the palmitoylation site (*pal-*) (data not shown) or both sites (*myr-/pal-*) (Figure 4f) showed a incorrect localization of the *Ms*CPK3 protein. Hence, the integrity of the N-terminal myristoylation and palmitoylation consensus is essential for plasma membrane targeting of CPK3 fusions in onion cells. This suggests that these post-translational modifications could regulate CPK3 subcellular localization.

Expression analysis of Mt*CPK3 in mutants unable to interact with symbionts*

Calcium-mediated transduction signalling pathways may be activated at various points during nodule organogenesis, from minutes (e.g. calcium spiking 10 min after Nod factor treatment) to days after infection when complex morphogenetic processes occur (e.g. cortical cell division, reorganization of the cytoskeleton). In contrast to alfalfa, *M. truncatula* mutants affected in the early signalling events have been identified. We first confirmed that the pattern of expression of *MtCPK3* at various stages of the symbiotic interaction (from 10 min to 14 dpi) correlated with that determined for the *M. sativa* isoform. *M. truncatula* (A17) roots were spot-inoculated with *S. meliloti* and the infected region was harvested at various time points. Using a qRT-PCR approach, we observed a large increase in *MtCPK3* mRNA accumulation around 1 h post-inoculation (hpi). This increase was transient, and messenger levels were reduced again at 3 hpi (Figure 5a). Concomitant with this expression pattern, the subcellular distribution of CDPK activity changed in inoculated roots 1 h after infection, but showed no difference in its distribution at 1 dpi (Table 1).

The temporal correlation with early calcium signalling suggested that *Mt*CPK3 could be directly involved at the very early steps of nodulation. However, qRT-PCR analysis on *M. truncatula dmi1*, *dmi2* and *dmi3* mutants (Oldroyd and Downie, 2004) revealed that a three- to fivefold enhancement was observed at an early stage in all *dmi* mutants (Figure 5b). Thus this very early induction may be associated with a stress response induced by the infection treatment and not with nodulation signalling. A second induction of *MtCPK3* expression was observed at 4 dpi (Figure 5a,c), confirming our previous results in *M. sativa* plants. When roots were inoculated with rhizobial *nod*$^-$ strains, no induction of *MtCPK3* was observed (Figure 5c). Western blot analysis of inoculated roots also revealed an increase in *Mt*CPK3 protein content at this time point, concomitant with the formation of the nodule primordia (Figure 5c, inset). This induction occurs after the activation of *Mtenod40* expression, a known early marker of cortical cell division leading to nodule formation (Figure 5d).

Biochemical analysis of CDPK activity in *M. truncatula* plants again showed a subcellular redistribution of CDPK

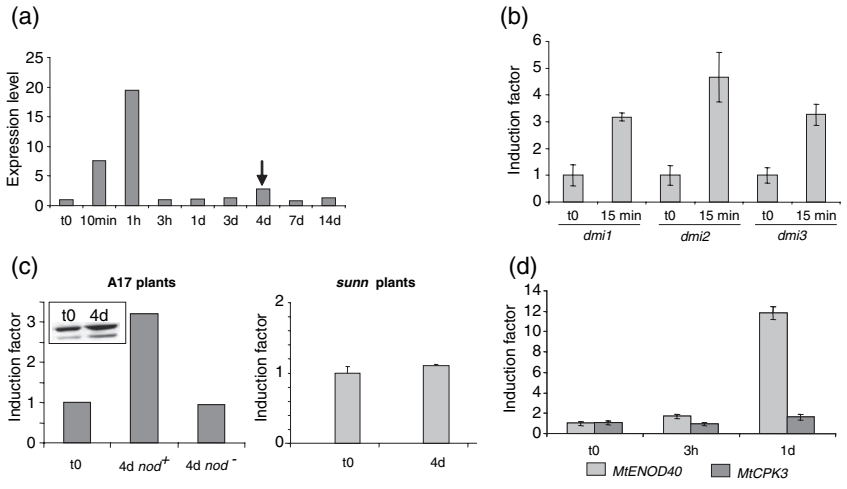

Figure 5. Real-time quantitative RT-PCR analysis of MtCPK3 during nodulation.
Roots of *M. truncatula* (A17) plants were inoculated with the *S. meliloti* strain Sm1021 using spot inoculation. Infected regions were harvested at the times indicated and total RNA was obtained from the treated roots.
(a) *MtCPK3* expression at different times after inoculation. The arrow indicates induction at 4 days post-inoculation (dpi).
(b) *MtCPK3* expression in *M. truncatula dmi1*, *dmi2* and *dmi3* mutant plants (15 min after infection).
(c) *MtCPK3* expression in wild-type roots inoculated with Nod$^+$ or Nod$^-$ *S. meliloti* and analysed at 4 days post-inoculation. Inset: Western analysis of *M. truncatula* root extracts (15 μg) with anti-*Mt*CPK3-specific antibody (1:1000). *M. truncatula* mutant *sunn* roots were inoculated with the wild-type *S. meliloti* strain Sm1021 using spot inoculation. Infected regions were harvested at 4 days post-inoculation.
(d) *MtENOD40* and *MtCPK3* expression during early stages of the nodulation process.
To compare the results of controls (t0) with those for treated roots, the relative mRNA expression ratios of controls were set as 1.0. The ratios *MtCPK3*/*MtACTIN11* or *MtENOD40*/*MtACTIN11* are plotted. These results are representative of two independent experiments. Data plotted are the average values of technical duplicates.

Table 1 Distribution of total CDPK activity in soluble and particulate extracts

Time post-inoculation	CDPK activity* (%)			
	Control roots		Inoculated roots	
	Soluble	Particulate	Soluble	Particulate
1 hpi	90	10	63	37
1 dpi	75	25	82	18
3 dpi	78	22	57	43

activity. At 3 dpi, 43% of CDPK activity was detected in the particulate fraction in inoculated roots versus 22% in controls (Table 1). This suggests that localization of CDPK activity during nodulation may also contribute to the translation of calcium fluxes into physiological responses.

To further reinforce a possible association of *Mt*CPK3 with stress responses, two different abiotic stresses, 100 mM NaCl or cold temperature (4°C), were applied to 5-day-old *M. truncatula* plantlets for 1 h. A significant increase in mRNA levels (ninefold) was observed after salt exposure, while no difference was observed with the cold treatment (Figure 6a). Kinetic analysis of *MtCPK3* expression in response to salt stress (Figure 6b) showed that transcription reached its maximum 1 h after salt exposure and diminished slowly (it was still fourfold higher than controls at 6 h), while Western analysis showed an increase in *Mt*CPK3 protein content at 6 h (Figure 6c). These data suggest that *Mt*CPK3 can rapidly respond to an abiotic stress, and that the very early response triggered by *S. meliloti* infection is probably not specific to the nodulation process. Altogether, these results suggest a late and specific role of *Mt*CPK3 in developed nodule primordia, distinct from an early stress-related signalling event in roots.

Analysis of MtCPK3 *mRNA and protein levels after cytokinin treatment*

As cytokinins mimic certain morphogenetic responses induced by rhizobia at the initiation of nodule organogenesis, we assessed whether *Mt*CPK3 could be linked to cytokinin action in *M. truncatula*. Total RNAs were obtained from *M. truncatula* roots treated with 10^{-7} and 10^{-8} M benzylaminopurine (BAP) for 15 min to 6 h to analyse *MtCPK3* expression by qRT-PCR. A transient induction was observed at 30 min (Figure 7a), which correlated with a significant increase in *Mt*CPK3 protein content after 1 and 6 h of treatment (Figure 7b). This activation was weaker than that induced by salt stress or bacterial infection. However, we feel that this is not related to a stress response induced by the hormone treatment because: (a) other early nodulins (e.g. *Mtenod40*) were activated in these samples; (b) controls were similarly treated except for hormone addition, and

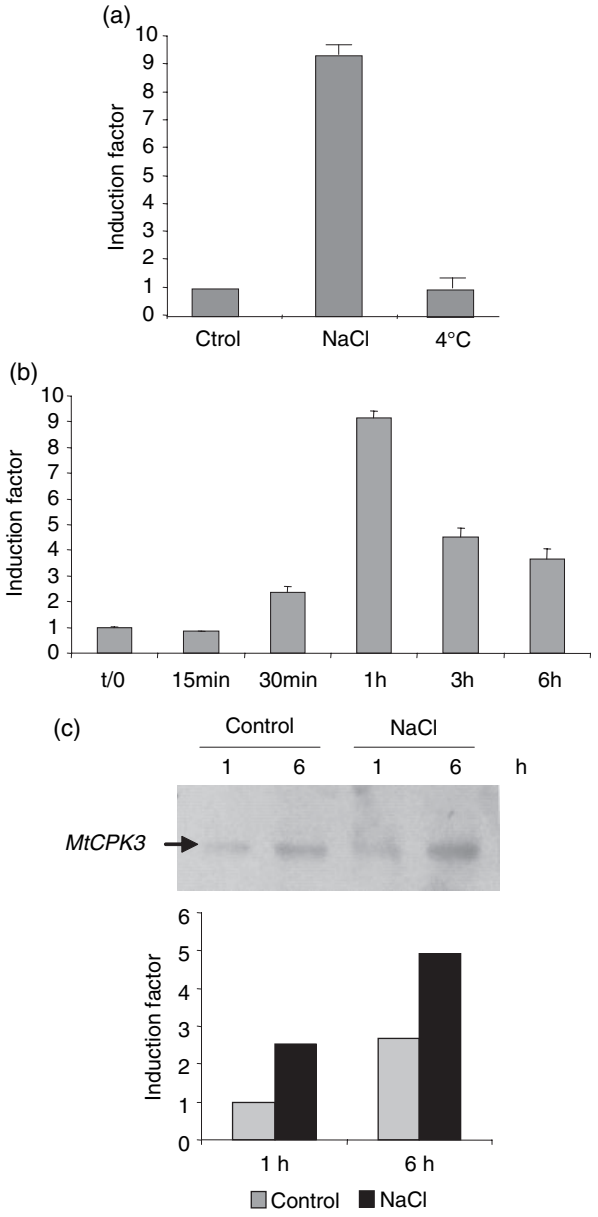

Figure 6. Expression analysis of MtCPK3 in roots exposed to high salt (100 mM NaCl) or cold temperature (4°C).
(a) Five-day-old *M. truncatula* plantlets were placed in flasks containing Fahreus medium plus 100 mM NaCl and incubated with agitation for 1 h at 24°C. For cold stress, *M. truncatula* plantlets were transferred to 4°C for 1 h. Error bars represent the standard deviation between two technical replicates.
(b) Kinetic analysis of salt response: Five-day-old *M. truncatula* plantlets were transferred to flasks containing Fahreus medium plus 100 mM NaCl and incubated with agitation (15 min to 6 h). Real-time quantitative RT-PCR was performed using specific primers for *MtCPK3* and specific primers for *MtACTIN11* as a constitutive control. Error bars represent the standard deviation between two technical replicates.
(c) Three-day-old *M. truncatula* plantlets were transferred to plates containing Fahreus medium plus 80 mM NaCl and incubated for 1 or 6 h at 24°C. CDPK was detected by Western blot using 50 μg of total plant protein extract and a specific antibody directed against *Mt*CPK3 (1:1000). Relative amounts of *Mt*CPK3 protein levels are plotted underneath.

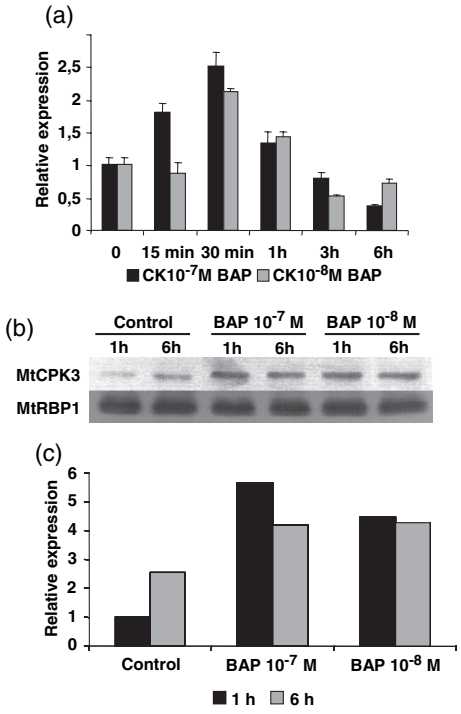

Figure 7. Analysis of MtCPK3 mRNA and protein levels after cytokinin treatment.
(a) Real-time RT-PCR analysis. Total RNAs were obtained from *M. truncatula* roots treated with 10^{-7} and 10^{-8} M BAP for 15 min to 6 h. The ratios *MtCPK3*/*MtACTIN11* are plotted. These results are representative of two independent experiments, and data plotted are the average values of technical duplicates.
(b) Western blot analysis. Protein extracts from *M. truncatula* (A17) roots treated with 10^{-7} and 10^{-8} M BAP for 1 or 6 h were obtained and immunoblotted (50 μg) with anti-*Mt*CPK3 antibody (1:1000). Immunoblot data were normalized relative to RNA-binding protein (*MtRBP1*) expression (Campalans *et al.*, 2004).

(c) at these hormone concentrations, root growth is much less perturbed when compared to abiotically stressed roots. These data indicate that cytokinins can modulate *MtCPK3* expression at the mRNA and protein levels, and suggest that this CDPK isoform could be linked to the induction of cortical cell division and formation of nodule primordia.

Mt*CPK3-RNAi* A. rhizogenes-*transformed* M. truncatula *roots show an increased number of nodules*

In order to elucidate *Mt*CPK3 function during the nodulation process, *Mt*CPK3-RNAi *M. truncatula* transgenic roots were obtained. A specific 3′ sequence of *Mt*CPK3 was subcloned into the pFRN vector using Gateway technology. This construction and a control one (pFRN-GUS) were used to transform *A. rhizogenes* Arqua1 (Quandt and Hynes, 1993), and transgenic roots were generated using *M. truncatula* A17 seeds (Boisson-Dernier *et al.*, 2001). Western analysis of the transgenic roots confirmed the efficient silencing of *MtCPK3* (Figure 8a).

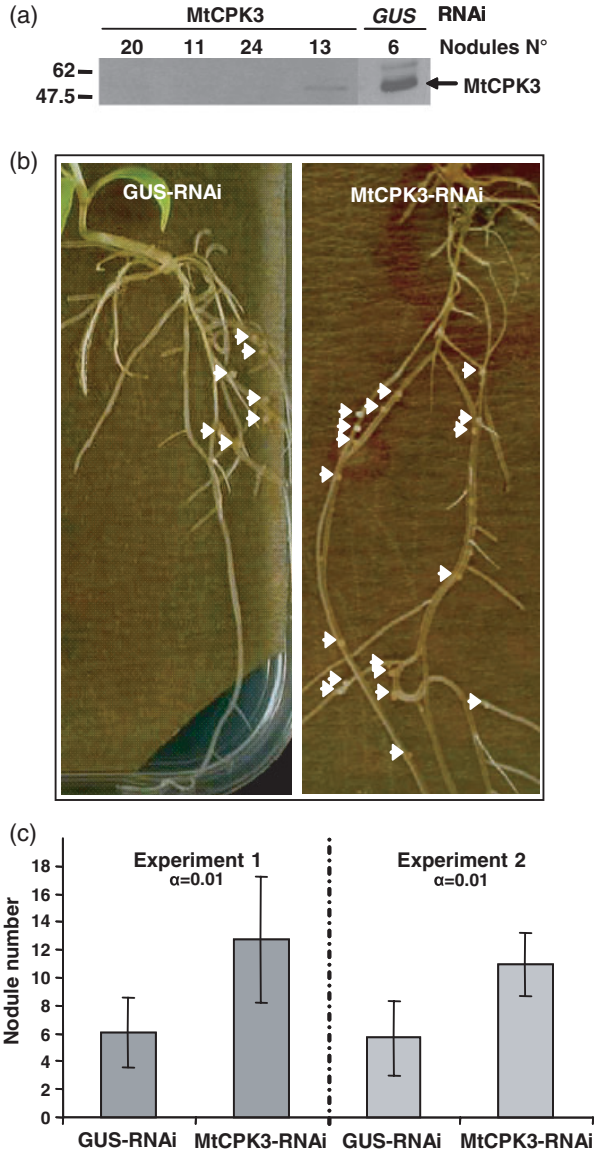

Figure 8. Analysis of *MtCPK3*-RNAi M. truncatula transgenic roots.
(a) Western blot analysis of *Mt*CPK3. Protein extracts (50 μg) from *M. truncatula* individual representative *MtCPK3*-RNAi transgenic lines were obtained and immunoblotted with anti-*Mt*CPK3 antibody (1:1000). GUS-RNAi transgenic roots were used as controls. The total nodule number per transgenic root is indicated.
(b) *GUS*-RNAi and *MtCPK3*-RNAi transgenic roots. Arrows indicate nodule positions on the roots.
(c) Total nodule number of *MtCPK3*-RNAi and GUS-RNAi transgenic roots determined 15 days after inoculation with the Sm1021 strain. Data plotted represent two independent experiments. Error bars represent confidence intervals, $\alpha = 0.01$.

Recently, Ivashuta *et al.* (2005) reported that silencing of another *M. truncatula* CDPK, *MtCDPK1*, resulted in significantly reduced root hair growth and root cell lengths, and also affected both symbiotic interactions with mycorrhizal fungus and *Rhizobium* bacteria. In contrast, out of 41 *Mt*CPK3-RNAi transgenic roots screened, no significant

differences in root phenotypes were seen when compared with controls (transgenic roots carrying vector sequences or uninfected control roots), and root hair growth or cell size were not affected by our construct (data not shown). In addition, no differences in root growth were observed after cytokinin or salt treatments (data not shown). These transgenic roots were then inoculated with the wild-type Sm1021 strain. While they were able to produce normal nitrogen-fixing nodules, the number of nodules was significantly higher than those in GUS-RNAi control plants (Figure 8b,c), suggesting that this CDPK isoform participates in the negative control of nodule number in *M. truncatula*.

Analysis of CPK3 expression was then performed in the *M. truncatula* hyper-nodulation mutant, designated *sunn* (super-numeric nodules). *S. meliloti*-inoculated roots of *sunn* plants were harvested 4 days after inoculation. In contrast to wild-type plants, no increase in MtCPK3 expression was observed at this time point (Figure 5c), further linking a reduction of CPK3 expression with increased nodulation.

Discussion

CDPKs are key elements in calcium signalling, and many CDPK genes are expressed in most of the plant tissues examined. This is the case for the recently described *M. truncatula* CDPK1 isoform that was detected in diverse plant organs (Ivashuta *et al.*, 2005). However, certain CDPKs display an isoform-specific expression pattern that is not only organ- or tissue-specific but is also dependent on growth conditions (reviewed in Hrabak, 2000). The *Ms*CPK3 and *Mt*CPK3 isoforms are more strongly expressed in developing roots of *Medicago* plants. Western blot analysis of soluble and particulate fractions from root tissues using a specific anti-CPK3 antibody detected a 60 kDa protein, corresponding to the expected molecular weight of this isoform, suggesting that CPK3 could contribute to the CDPK activity detected in both fractions. The additional 63 kDa band observed in soluble root extracts may correspond to a differentially phosphorylated version of the same 60 kDa protein, even though we cannot exclude the possibility that the antibody cross-reacts with another CDPK isoform in this fraction. The ratio between the 60/63 kDa bands changes in phosphorylated versus non-phosphorylated protein root extracts: the slower migrating form is predominant in phosphorylated extracts. As *Ms*CPK3 encodes an active CDPK that undergoes *in vitro* auto-phosphorylation (Davletova *et al.*, 2001), auto-phosphorylation or phosphorylation by an upstream protein kinase present in this extract could post-translationally modify this kinase *in planta*. In addition to Ca^{2+}, intramolecular auto-phosphorylation on Ser/Thr residues plays a role in influencing the activity of CDPKs, as shown by Romeis *et al.* (2001) for *Nt*CDPK2.

CDPKs have been implicated in many signalling pathways; however, very little is known about which particular CDPK acts in each case (Ludwig *et al.*, 2004). Semi-quantitative RT-PCR assays showed that *MsCPK3* expression peaked 3–4 days after infection (early nodule primordia), whereas no change could be detected by monitoring several CDPK isoforms with conserved primers. Co-ordinately with this increase, a calcium-dependent kinase activity was enhanced in the particulate fraction of inoculated alfalfa roots. In addition, the overall distribution of CDPK activity in *M. truncatula* roots changed after infection with *Rhizobium*, in temporal correlation with the increase in *MtCPK3* expression. This suggests that *Medicago* CPK3 isoforms may participate in calcium-dependent phosphorylation events triggered during nodule formation. The required specificity of a CDPK signalling pathway may be achieved by differential substrate affinity, by temporally and spatially restricted patterns of expression, or by targeting of CDPK isoforms to specific subcellular compartments. Protein acylation is important for the subcellular localization of a variety of proteins, and it has been implicated in CDPK targeting. Both N-terminal myristoylation and palmitoylation are required for plasma membrane targeting of CPK3–GFP fusion proteins, similarly to other CDPKs (Martin and Busconi, 2000; Lu and Hrabak, 2002; Rutschmann *et al.*, 2002; Dammann *et al.*, 2003; Raices *et al.*, 2003). Co-translational *N*-myristoylation enables subsequent palmitoylation, a dynamic and reversible process. The subcellular redistribution of CDPK activity upon nodule formation suggests that *in vivo*, post-translational acylation mechanisms could be involved in targeting *Mt*CPK3 isoforms to membrane compartments, thus affecting downstream pathways.

Real-time RT-PCR assays confirmed the increase in CPK3 mRNA accumulation observed at 4 dpi, but also revealed an earlier induction 10–15 min after infection that temporally correlated with the early Nod factor-dependent calcium responses (Wais *et al.*, 2002). However, experiments with *dmi* mutant plants revealed that this induction was probably not related to nodule signalling but to a stress response. Non-pathogenic rhizobacteria induce an enhanced defensive capacity against a broad spectrum of plant pathogens after root colonization (Verhagen *et al.*, 2004). It is thought that to achieve efficient colonization of plant roots, symbionts may later suppress this defence response (Garcia-Garrido and Ocampo, 2002; Mithofer, 2002). It may be hypothesized that this very rapid activation of *Mt*CPK3 is stress-dependent. Induction of TC101857 and TC103686 has been reported in cell cultures from root tissues treated with yeast cell wall extracts or in roots treated with oligogalacturonides, in roots inoculated with *Phytophtora medicaginis*, in roots colonized with *Glomus versiforme* and in response to aphid infection (http://www.tigr.org/tdb/e2k1/mta1/). In addition, we observed that *MtCPK3* is induced by salt stress, as for *AtCDPK1* and *AtCDPK2* (Urao *et al.*, 1994),

OsCDPK7 (Saijo *et al.*, 2000) and *McCDPK1* (Patharkar and Cushman, 2000), but not by low temperature. Calcium is a major point of signalling cross-talk because it can be elicited by numerous abiotic as well as developmental, hormonal and biotic stress cues (Sanders *et al.*, 2002). Therefore, this calcium-dependent kinase is possibly involved in various responses.

In contrast, the second activation observed 3–4 days after infection was nodulation-specific and was not observed in control roots at any time point or in roots infected with nodulation-deficient bacteria (Figure 5c). In addition to exogenous signals, endogenous signals, such as plant hormones, are important for proper nodule development. In particular, the induction of nodule organogenesis may be mediated by local changes in the auxin/cytokinin ratio (for review, see Hirsch and Fang, 1994). Transformation of Nod factor-deficient *S. meliloti* strains with a gene for cytokinin synthesis (i.e. the trans-zeatin secretion gene) confers the ability to induce nodule-like structures on host alfalfa plants (Cooper and Long, 1994). Furthermore, cytokinin treatments rapidly induce *ENOD2* expression in *Sesbania rostrata* (Dehio and de Bruijn, 1992), cortical cell division, and *ENOD12* and *ENOD40* expression in alfalfa (Bauer *et al.*, 1996; Fang and Hirsch, 1998). Exogenous cytokinin application induced *ENOD40* expression in white clover root in a spatial and temporal pattern mirroring that induced by Nod factors (Mathesius *et al.*, 2000) and increased nodulation in pea roots (Lorteau *et al.*, 2001). Finally, functional analysis of cytokinin oxidases in *Lotus japonicus* confirmed the key role of cytokinins in nodule regulation (Lohar *et al.*, 2004).

Several years ago, it was reported that the addition of cytokinins induced an increase in cytosolic calcium in the moss *Funaria hygrometrica* as a result of extracellular calcium uptake (Hahm and Saunders, 1991), and that calmodulin inhibitors and calcium antagonists block cytokinin-induced bud formation in *Funaria* (Saunders and Hepler, 1983). Calmodulin inhibitors and calcium antagonists inhibit CDPK activity (Harper *et al.*, 2004, Figure 2b), so it may be hypothesized that a CDPK is involved in the cytokinin response in *Funaria*. More recently, cytokinin treatment has been reported to induce expression of *NtCDPK1* (*Nicotiana tabacum*; Yoon *et al.*, 1999). Our results demonstrating that CPK3 expression and protein are rapidly up-regulated by cytokinins in *M. truncatula* roots, suggest that this isoform could be a common player in the signal transduction cascades triggered by *Rhizobium* signals, cytokinins and abiotic stresses.

The function of CPK3 in the *M. truncatula–S. meliloti* interaction was explored using an RNAi approach. While *MtCPK3*-RNAi transgenic roots displayed normal root growth and produced nitrogen-fixing nodules, nodule number was significantly higher than in control plants. This result suggests that CPK3 could be involved in the control of nodule number. In legume nodulation, the plant can control and optimize nodule initiation (Gresshoff, 2003) to maintain the appropriate carbon/nitrogen ratio. Inhibition of root nodule formation on leguminous plants by already induced or existing root nodules is called auto-regulation. It seems to be linked to the ability of these plants to support the initiation of carbon 'sinks' in their roots, and the establishment of cell-to-cell communication between the phloem and the cortical cells (Complainville *et al.*, 2003). Indeed, nodule primordia are carbon 'sinks', and auto-regulation seems to depend on organ number but not on their capacity to fix nitrogen.

In several legumes, putative receptor kinases involved in auto-regulation have been identified (Krusell *et al.*, 2002; Nishimura *et al.*, 2002; Searle *et al.*, 2003; Penmetsa *et al.*, 2003). Very recently, van Noorden *et al.* (2006) showed that *sunn* mutants had an increased amount of auxin transported from the shoot to the root compared to the wild-type. After inoculation, wild-type seedlings showed decreased auxin loading from the shoot to the root; however, the *sunn* mutant failed to reduce the amount of auxin loaded. Those authors propose a new model for the role of auxin during auto-regulation of indeterminate legumes: (1) high levels of endogenous auxin are correlated with increased numbers of nodules, (2) inoculation of roots reduces auxin loading from the shoot to the root, and (3) subsequent reduction of auxin levels in the root inhibits further nodule initiation. In this paper, we show that *Mt*CPK3 expression is enhanced by cytokinins, while Davletova *et al.* (2001) observed that *Ms*CPK3 (its alfalfa homologue) is rapidly enhanced by auxins in cell suspensions. It is tempting to speculate that CPK3 will respond to a particular ratio of auxin to cytokinin in legume roots; therefore, if the auxin/cytokinin ratio were biased in the *sunn* mutant, expression of this kinase would be turned off and would not regulate nodule number.

The signalling pathways triggered by these receptor kinases probably involve phosphorylation cascades, and *Mt*CPK3 could be a relevant player in this scenario. According to Wopereis *et al.* (2000), the auto-regulatory response relies, at least in part, on the mechanism regulating cell division in relation with 'sink' formation. This process has been linked to cytokinin action and cell-to-cell communication processes (Bauer *et al.*, 1996; Complainville *et al.*, 2003). The fact that *MtCPK3* expression is regulated both by nodulating rhizobia and by cytokinins, together with the evidence revealed by *Mt*CPK3-RNAi transgenic roots suggests that this active kinase may negatively regulate the action of plant signals controlling source–sink interaction in root tissues.

Our results show that nodulation signals promote CPK3 transcription; in addition, the data presented in this paper suggest that the CPK3 protein could undergo different post-translational modifications such as reversible phosphorylation or palmitoylation. Palmitoylation, like phosphorylation, is likely to be a regulatory mechanism in the cell (for a

review, see Mumby, 1997). These modifications have evolved to facilitate the communication of external stimuli across cell surfaces, subsequently leading to alterations in the activities and functions of other intracellular proteins. Future experiments are necessary to understand the fine regulation of this kinase; however, our data reinforce the importance of calcium-binding proteins in the nodulation process of the model legume *Medicago truncatula*. Together with the calcium/calmodulin-dependent protein kinase DMI3 (Lévy *et al.*, 2004; Mitra *et al.*, 2004) and the novel family of six calmodulin-like proteins (CaMLs) expressed specifically in root nodules (Liu *et al.*, 2006), *Mt*CPK3 adds to the complexity of calcium signalling cascades that are recruited to regulate the development of this unique plant organ, the root nodule.

Experimental procedures

Plant material

Medicago sativa cv. Sitel, *Medicago truncatula* cv. Jemalong A17 and *Medicago truncatula* mutant seeds (*dmi1*, *dmi2*, *dmi3* and *sunn*) were surface-sterilized (20 min in 2% w/v Bayrochlore solution; Bayrol, Mundolsheim, France) and rinsed with ten volumes of sterile water. Seeds were sown in Petri plates (0.75% agar in water) and stored in the dark at 4°C for 2 days and then transferred overnight at 24°C for germination. After germination, the seeds were transferred to square plates with Fahreus medium (Boisson-Dernier *et al.*, 2001) without nitrogen and grown vertically in a chamber at 24°C under a 16 h light period for 4 days before treatments.

Cytokinins, salt and cold stress treatments

After germination, *M. truncatula* plantlets were grown for 5 days in flasks containing Fahreus liquid medium. Then 10^{-7} or 10^{-8} M 6-benzyl-aminopurine (BAP) or 100 mM NaCl were added to the medium and incubated with agitation for 15 min to 6 h at 24°C. Alternatively, plantlets were incubated for 1 h at 4°C. Controls were kept in the same medium at 24°C.

Inoculation of symbiotic bacteria

M. sativa and *M. truncatula* seeds were grown for 4 days in Fahreus medium without nitrogen before inoculation with *Sinorhizobium meliloti* 41, 2011 or 1021 strains. *S. meliloti* strains were grown in TA medium (Orosz *et al.*, 1973) overnight at 30°C, centrifuged at 2000 ***g***, and resuspended in 10 mM $MgSO_4$ until an OD_{600} of approximately 0.8 was obtained. The culture was mixed with agar (0.8% in sterile water) to a final OD_{600} of approximately 0.4, and was used to inoculate approximately 2 cm of the roots excluding the root tip region.

MtCPK3 *cloning procedure*

Total RNA was extracted from frozen roots following the instructions supplied with the RNeasy Plant Mini Kit from Qiagen Inc. (Valencia, CA, USA). Total RNA (2 μg) was reverse-transcribed with reverse transcriptase (Superscript II RNase H reverse transcriptase; Gibco BRL, Eragny, France) using an oligo(dT) primer. *M. truncatula* cDNA was amplified with specific primers directed against the 5′ end of TC101857 (5′-AGAATCTGAAAAAATGGGG-3′) and the 3′ end of TC103686 (5′-TAAAGTACTTCTGAGAAGTGC-3′). The amplified PCR fragment was cloned in pGEM-TEasy vector system I (Promega, Madison, WI, USA) according to the manufacturer's instructions. Double-stranded phagemid DNA for automated sequencing was prepared using a Qiagen Plasmid Midi Kit. Automated sequencing was performed by the Macrogen Sequencing Facility (Seoul, Korea). Assembly and analysis of DNA sequence data were performed using the NCBI's Advanced BLAST sequence similarity search, designed to support the analysis of nucleotide and protein databases.

Agrobacterium rhizogenes *root transformation and inoculation*

A specific region of *Mt*CPK3 3′ sequence (300 nt) was subcloned into the vector pFRN (derived from pFGC5941; NCBI AY310901) using Gateway technology (Invitrogen, Cergy-Pontoise, France). The primers used were *Mt*CPK3-FOR (5′-TGCTGAATCTTCTGGTATG-CAC-3′) and *Mt*CPK3-REV (5′-TCCTCTTGGAAAT TGACTTGC-3′). A control GUS-RNAi fragment was amplified using the following primers: GUS-FOR (5′-GGCCAGCGTATCGTGCTGCG-3′) and GUS-REV (5′-GGTCGTGCACCATCAGCACG-3′), and cloned into the same RNAi destination vector. Both constructions were used to transform the *A. rhizogenes* ARqua1 strain. Transformed *M. truncatula* A17 roots were prepared as described by Boisson-Dernier *et al.* (2001). Two weeks later, transgenic roots were inoculated with 10 ml of *S. meliloti* 2011 suspension (OD_{600} approximately 0.05) for 45 min. Nodule formation was followed for 15 days.

Semi-quantitative RT-PCR analysis

Total RNA was extracted from frozen roots following the instructions supplied with the RNeasy Plant Mini Kit. Total RNA (2 μg) pretreated with DNase (RQ1 RNase-free DNase; Promega, Charbonnieres, France) was reverse-transcribed with reverse transcriptase (Superscript II RNase H- reverse transcriptase) using an oligo(dT) primer. To amplify the *MsCPK3* isoform, the following primers were used: P1 directed against the N-terminal domain (5′-AATCATGAA-CAAGTCAACACATCAAG-3′) and P3 (5′-CTTTCTAGAATGAAGCAA-TTCAGAGC-3′) directed against the junction domain. Another PCR was performed using primers P2, directed against subdomain VIb (5′-GATCTCAAGCCAGAGAATTTCTTGTTG-3′), and P3, to amplify several CDPKs as checked by subcloning and sequencing of PCR products. *MsC27* was used as an RNA-loading control in *Medicago* spp. roots (Pay *et al.*, 1992). Amplified cDNAs were subjected to electrophoresis, blotted to nylon membrane (Hybond N+; Amersham, Saclay, France) and hybridized to specific radiolabelled DNA probes. Probes were generated by random priming using the Megaprime labelling system kit (Amersham Pharmacia Biotech, Uppsala, Sweden) for PCR products amplified from cDNA clones.

Real-time RT-PCR analysis

RNA extraction and cDNA synthesis were performed as described above. The primers were designed using the LightCycler® probe design software (Roche, Meylam, France) with approximately 95% efficiency: CDPK-Q-52 (5′-ACCAACCAAGCCTTCA-3′), CDPK-Q-32 (5′-AGCATAAAGCAATCCAGT-3′), E40-FORWARD (5′CCTACACAC-TCTCCCTCCA-3′), E40-REVERSE (5′-CACAAAAAGGCAATGGATT-CCT-3′), Actin11-F (5′-ACGAGCGTTTCAGATG-3′) and Actin11-R (5′-ACCTCCGATCCAGACA-3′). The LightCycler FastStart DNA

Master SYBR Green I reaction mix was used according to manufacturer's instructions for the LightCycler instrument. At least two cDNAs were tested for each point. *MtACTIN11* was used as a standard gene.

Protein extraction and CDPK activity

Treated and control *Medicago* roots were harvested, ground in a mortar cooled with liquid nitrogen, and extracted with 50 mM Tris-HCl, pH 7.5, 2 mM β-mercaptoethanol, 1 mM EDTA, 20% glycerol and protease inhibitors as described previously (Chico *et al.*, 2002). The suspensions (1 ml buffer/g of wet tissue) were centrifuged for 10 min at 2500 ***g*** and the pellet was discarded. The supernatant (crude extract) was centrifuged for 1 h at 20000 ***g***, and soluble and particulate fractions were obtained. Pellets were washed with 0.5 ml EB, and were then resuspended in 30 μl EB. Total protein from RNAi transgenic roots was extracted as described by Chico *et al.* (2002) but with a buffer containing 125 mM Tris-HCl, pH 8.8, 1% SDS, 10% glycerol and 50 mM $Na_2S_2O_5$.

CDPK activity was assayed in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 μM [γ-^{32}P]-ATP (specific activity 100 cpm pmol^{-1}), 10 mM β-mercaptoethanol, 25 μM Syntide-2, with the addition of 1 mM EGTA or 1 mM $CaCl_2$, as described by MacIntosh *et al.* (1996). In addition, activity was assayed in the presence of the following inhibitors: 1 μM staurosporine (Ser/Thr protein kinase inhibitor), 1 nM okadaic acid (inhibitor of phosphatases from the PP2A family), 0.5 mM chlorpromazine and 0.1 mg/ml compound 48/80 (calmodulin antagonists). Alternatively, fractions were incubated for 5 min at 30°C with 0.1 mg/ml histone H1 and 10 μM [γ-^{32}P]-ATP (specific activity 500 cpm/pmol) in the presence of 1 or 5 mM EGTA or 0, 1 or 1 mM $CaCl_2$. Reactions were stopped as described previously (Chico *et al.*, 2002), and subjected to 12% SDS-PAGE. Signal was scanned with a Storm 820 phosphorimager (Amersham Pharmacia Biotech, Saclay, France), and quantified using ImageQuant software (University of Virginia, Virginia, USA). Protein contents were determined using bovine albumin as standard with Bio-Rad protein assay reagent (Bio-Rad, Marnes-La-Coquette, France). SDS-PAGE was carried out as described by MacIntosh *et al.* (1996). Pre-stained SDS-PAGE standards from Invitrogen (Carlsbad, CA, USA) were used as molecular weight markers.

Phosphatase treatment

Soluble protein fractions from root extracts (90 μg) were incubated or not with 100 units of calf intestinal alkaline phosphatase (CIAP; Invitrogen) for 15 min at 37°C in a reaction buffer containing 100 mM Tris-HCl, pH 9.5, 50 mM $MgCl_2$ and 100 mM NaCl. The reaction was stopped using cracking buffer (10% v/v glycerol, 1.5% p/v weight/volume DTT, 2% p/v SDS, 0.08 M Tris-HCl, pH 6.8, and 0.001% p/v bromophenol blue), boiled for 5 min and subjected to 10% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes and were subjected to Western analysis.

Western blotting

Western blot analysis of protein samples from root extracts (15 μg) or transgenic roots (25 μg) was performed using affinity-purified polyclonal antibodies (1:1000, Eurogentec, Seraing, Belgium) directed against an N-terminal peptide (IPTKPSSISAPSPKP) present both in *Ms*CPK3 and in *Mt*CDPK3 but not in other CDPKs or proteins present in the TIGR or NCBI databases (using BLASTX for small sequences allowing mismatches). Blots were developed with ECL reagent (Amersham) according to the manufacturer's procedure. For normalization, the same Western blots were probed with antibodies against *Mt*RBP1, a constitutive RNA-binding protein (Campalans *et al.*, 2004).

Construction of Ms*CPK3-GFP fusions and transient expression in onion cells*

Translational C-terminal fusions with GFP to full-length *Ms*CPK3 (Davletova *et al.*, 2001) were performed by cloning PCR products corresponding to *Ms*CPK3 downstream of a duplicated 35S promoter at the *Xho*I and *Nco*I restriction sites of the pPK100 vector (Despres *et al.*, 2001). The 5′ primer used to amplify the wild-type *Ms*CPK3 contained the *Xho*I restriction site and the codons corresponding to the first six residues of the kinase. Mutated forms of *Ms*CPK3 with an altered myristoylation site, an altered palmitoylation site, or both, were generated by PCR amplifications with 5′ oligonucleotides carrying point mutations. A common 3′ primer containing the *Nco*I restriction site (5′-CATGCCATGGGGTTT CCACTTCTCATCATTGCAC-3′) was used for all the PCRs. Mutated versions of *Ms*CPK3 were cloned in the same vector at the same position (*myr-Ms*CPK3::GFP, *pal-Ms*CPK3::GFP and *myr/pal-Ms*CPK3::GFP). The four constructs were delivered into onion (*Allium cepa*) epidermal cells (Scott *et al.*, 1999) using a biolistic PDS-1000/HeTM particle gun (Bio-Rad). Three bombardments were performed for each construction (approximately 15 transformed cells per experiment). Cells were observed 24 h later using a laser scanning confocal microscope (Leica DM RXA2; Rueil-Malmaison, France). To confirm plasma membrane localization, cells were plasmolysed with 0.5 M sorbitol. GFP fluorescence was monitored with a band pass filter (488 nm excitation line of a krypton-argon laser). Typically 20–30 focal sections (1 μm each) were obtained for each cell and projected along the *z*-axis.

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