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Short Communication

An active form of calcium and calmodulin dependant protein kinase (ccamk) of *Medicago truncatula*

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The *DMI3* gene of the model legume *Medicago truncatula* encodes a calcium and calmodulin dependent protein kinase (CCaMK) involved in the signalling pathways leading to the establishment of both mycorrhizal and rhizobial root symbiosis. The removal of the auto-inhibitory domain that negatively regulates the kinase activity in *M. truncatula* results in a constitutively-active form, inducing symbiotic responses in the absence of bacterial signals. In this study, we verified the functionality of a *DMI3* variant and its ability to induce spontaneous nodules in *M. truncatula dmi3* mutant. Our results based on enzymatic radio activity assay using [γ-^{33}P] ATP, suggests that the DMI3-311 variant is active and its corresponding gene (*DMI3-311*) when introduced in the *dmi3* mutant, by *Agrobacterium rhizogenes* transformation, induced in the formation of a few spontaneous nodules.

Key words: *Medicago truncatula*, CCaMK; *DMI3* variant; spontaneous nodules.

INTRODUCTION

Legumes can form a nitrogen fixing symbiosis with soil bacteria called rhizobia (the RL symbiosis). They can also like most plants, form symbiotic associations with arbuscular mycorrhizal (AM) fungi, which facilitate plants' phosphate nutrition. In both interactions, the symbionts are hosted inside the plant root. Nitrogen-fixing rhizobia are housed in intracellular symbiotic structures within nodules, while AM fungi form intracellular symbiotic structures called arbuscules, within cortical root cells and can also develop external mycelium which extends from around the root. Molecular genetics studies performed on the model legumes *Medicago truncatula* and *Lotus japonicas* have shown that the establishment of the nodulation and the mycorrhization processes share a common signaling pathway, required for the initiation of endosymbiotic programs in host plants. In *M. truncatula*, three genes called the *DMI* genes (which does not make infection) are involved in this pathway (Catoira et al., 2000). The common signaling pathway diverges after *DMI3* which represents the last known gene common to both symbiosis. *DMI3* encodes calcium and calmodulin dependent protein kinase (CCaMK), located in the nucleus, and is supposed to perceive and transduce calcium signals generated upon perception of the symbiotic signals. Therefore, it has been hypothesized that *DMI3*, depending on its activation determined by the calcium signature, could phosphorylate substrates involved either in nodulation or mycorrhization (Levy et al., 2004).

CCaMKs has been studied in other plants and its activity depends on an auto-inhibitory domain that negatively

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regulates the kinase activity. Removal of this domain or point mutations at the auto-phosphorylation site respectively, in *M. truncatula* and *L. japonicus*, leads to a constitutively-active form of CCaMK that can induce spontaneous symbiotic responses and nodulation in the absence of Nod factors (Gleason et al., 2006; Tirichine et al., 2006). Otherwise, a protein interacting with *DMI3*, named IPD3 (interacting protein of *DMI3*) in *M. truncatula* and CYCLOPS in *L. japonicus*, has been identified (Messinese et al., 2007; Yano et al., 2008). CYCLOPS, which is phosphorylated *in vitro* by the CCaMK seems to be important for the infection process in both symbioses, but is dispensable for nodule organogenesis, suggesting that CCaMK has different molecular targets during the nodulation process. Recently, Rival et al. (2012, 2013) showed that both rhizobial and mycorrhizal symbiosis are controlled by *DMI3* in a cell autonomous way. Nodule organogenesis was not observed when *DMI3* expression was restricted either to the epidermis or to the cortex, but was restored when *DMI3* was expressed in both tissues. Moreover, it has been shown that transcription factor NIN downstream DMI3, negatively regulates infection but positively regulates nodule organogenesis during the course of the symbiosis (Yoro et al., 2014). Through all these observations, the infection process needs the full activation of CCaMK *via* the calcium spiking generated by the common signalling pathway and possibly an additional calcium signal. In this context, the use of a constitutively-active form of *DMI3* could be a valuable tool to search for downstream targets which could potentially include further components of the common symbiotic signaling pathway or components specific to Nod or Myc signalling. From previous work, *DMI3* variant (DMI3-311) in terms of production was described as a tool for identifying substrates potentially involved in nodulation or mycorrhization (Kassouar and Baba Hamed, 2011). In this study, we verified the functionality of this variant and its ability to induce spontaneous nodules in the *M. truncatula dmi3* mutant.

MATERIALS AND METHODS

Enzymatic radio activity assay

The catalytic activity of the recombinant DMI3-311 was examined by a test of auto-phosphorylation of the protein. Indeed, a volume of 40 µL purified fraction was added to a reaction buffer (10 mM Mg Cl_2, 50 µM [γ-^{33}P] ATP, 1mM DTT and 10 mM $MnCl_2$), the mixture was then incubated at 25°C for 1 h. After centrifugation, the labeled proteins were separated by SDS-PAGE gel electrophoresis (10% acrylamide). The labeling of phosphorylated proteins was revealed on X-Ray film (Amersham) after 4 to 5 days of exposure at -80°C.

M. truncatula transformation by *Agrobacterium rhizogenes*

Transformation of *dmi3* mutant by *Agrobacterium rhizogenes* using pCambia2202 binary vector with *DMI3 1-311* construction (the tagged form: StrepTagII and no tagged form were used separately) under control of its own promoter (*pDMI3-DMI3 1-311*) (Godfroy et al., 2008), began with (TRV25 allele) *dmi3* mutant germination. After the step of scarifying by H_2SO_4 (95%), surface-sterilised seeds were sown on agar plates and placed for 3 days in the dark at 4°C then left overnight at 25°C to germinate. After approximately 30 h germination, when seedlings had a radicle length of approximately 10 mm, the radicle was sectioned approximately 3 mm from the root tip with a sterile scalpel. Sectioned radicles were inoculated by coating the freshly cut surface with *A. rhizogenes* grown on TY solid medium (Boisson-Dernier et al., 2001). Thereafter, the inoculated sectioned seedlings were placed on slanted agar (Laboratoire Industriel de Biologie, Avignon, France) containing a modified Fahraeus medium supplemented with kanamycin (1mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.7 mM KH_2PO_4, 0.8 mM Na_2HPO_4, 50 µM FeEDTA, 0.5 mM NH_4NO_3, supplemented with 0.1 mg of $MnSO_4$, $CuSO_4$, $ZnSO_4$, H_3BO_3, and Na_2MoO_4 per liter) in square Petri dishes (12 × 12 cm). After several incisions in the Parafilm seal allowing gas exchange, the Petri dishes were placed vertically in a growth chamber at 20°C for 1 week (16-h photoperiod and a light intensity of 70 µE/s/m^2) to optimize the transformation frequency, and then transferred to a 25°C growth chamber (identical light conditions) for 2 weeks.

For nodulation assays, transformed plants were transferred separately in two different environments: nto growth pouches, with 7 ml of Fahraeus liquid medium (with nitrates) (3 to 4 plants/pouch), and incubated at 25°C for 3 to 4 weeks; and to sepiolite (Agrauxine, Quimper)/sand (2:1 volume mix) pots and grown at 25°C with 18 h light/6 hour dark cycles (Catoira et al., 2000).

Microscopy methods

The presence of spontaneous nodules was first observed by optical microscope (immersion x100) (Zeiss Axiophot, Carl Zeiss, Germany). Transformed roots were cleared with 3% sodium hypochlorite for 15 min and observed with a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany).

RESULTS AND DISCUSSION

In order to identify targets DMI3, a biochemical study based on obtaining a truncated form of DMI3 displaying constitutive activity was chosen to overcome stimulation by Nod factors and providing DMI3 homogeneous samples that would be permanently in its interaction with potential targets. For this we followed Gleason et al. (2006) who showed that expression of a truncated form of DMI3 in the roots of *M. truncatula* induces symbiotic responses in the absence of symbiont. These researchers present the construction of a set of partially-deleted mutants to DMI3, 1-326 and 1-311, corresponding to the kinase domain without two regulatory domains (visinin domain and central domain). *In vitro* phosphorylation tests on artificial substrate showed constitutive activity, independent calcium, 1-326 and 1-311 forms of DMI3, corresponding to about 30 and 40%, respectively of the activity of the whole protein in the presence of calcium and calmodulin. From previous work done by authors, a truncated form of DMI3 was produced, the variant DMI3-311. Here, the catalytic activity of this variant was first verified, *in vitro*, by autophosphorylation assay of the protein.

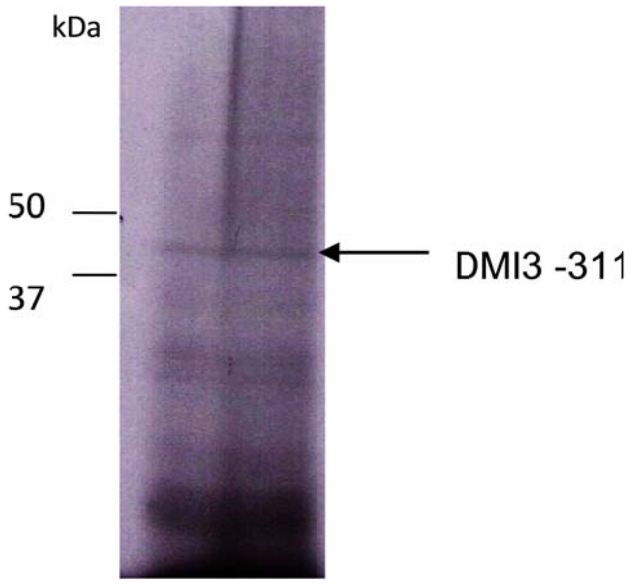

Figure 1. Enzymatic radio activity assay. Radioenzymatic labeling of the protein with [γ-^{33}P] ATP, reveals the presence of phosphorylation at the expected molecular weight about 39 to 42 kDa confirming the functionality of the DMI3-311 protein.

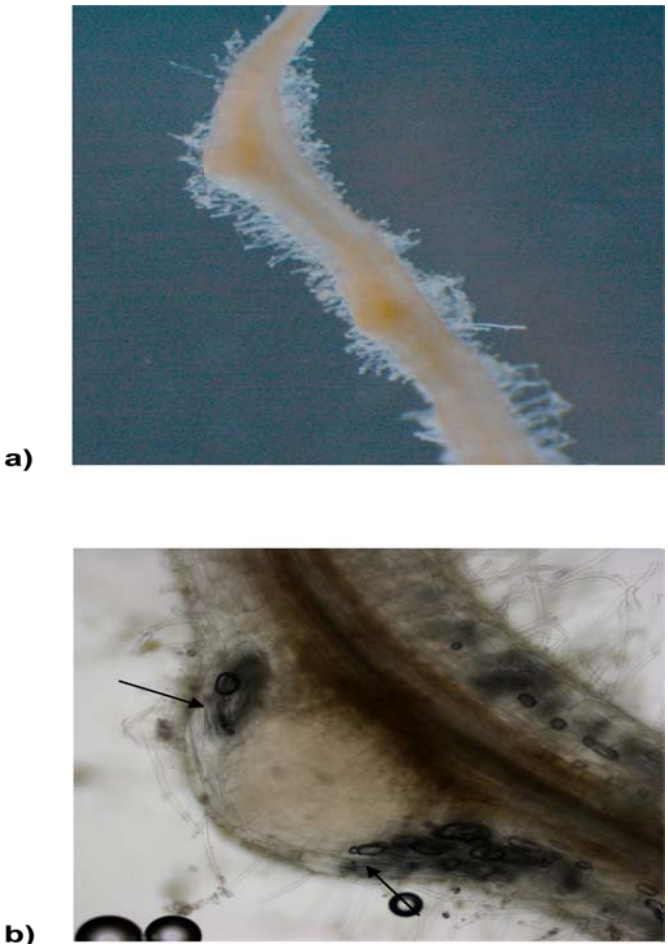

Figure 2. Spontaneous nodules in the *M. truncatula dmi3* mutant grown on sepiolite. After transformation of *dmi3* mutant by *Agrobacterium rhizogenes* using the binary vector *pCambia*2202 with *DMI3 1-311* construction under control of its own promoter (*pDMI3-DMI3 1-311*), complement plants were then transferred to sepiolite (Agrauxine, Quimper)/sand (2:1 volume mix) pots and grown at 25°C with 18 h light/6 h dark cycles. Spontaneous nodules observation in the *dmi3* mutant transformed with *DMI3-311* no tagged construction: **a)** by optical microscopy (immersion x100) (Zeiss Axiophot, Carl Zeiss, Germany), allowing us to visualize transparent rounded nodules. **b)** after roots clarification (3% sodium chloride for 15 min) and microscopic observation by a Leica MZFLIII stereomicroscope (Leica Microsystems, Wetzlar, Germany). The presence of peripheral vascular bundles (arrow) is characteristic of nodule anatomy in *M. truncatula*.

Catalytic activity of the DMI3-311 protein

The presence of auto-phosphorylation was detected using a radioenzymatic labeling of the protein with [γ-^{33}P] ATP. It is important to know that at the end of the N-terminal kinase domain is found the binding domain to ATP and at its C-terminal the Thr267, auto-phosphorylation site of the protein (Sathyanarayanan et al., 2000; Sathyanarayanan and Poovaiah, 2002). Figure 1 reveals the presence of phosphorylation at the expected molecular weight about 39 to 42 kDa confirming that the DMI3-311 recombinant protein is active.

Spontaneous nodulation

We tested, *in planta*, the ability of the variant DMI3-311 to induce spontaneous nodules in absence of bacterial symbiont and Nod factor. For this, we performed tests of complementation of *dmi3* mutant using the binary vector *pcambia*2202 with *DMI3 1-311* construction under control of its own promoter (*pDMI3-DMI3 1-311*) (Godfroy et al., 2008) (the tagged form: Strep TagII, and no tagged form were used separately) by *Agrobacterium rhizogenes* transformation. Composite plants were then transferred separately in two different environments, into growth pouches with Fahraeus liquid medium and to sepiolite pots. The transformation efficiency, in absence of any stimulation symbiotic (Nod factors or bacterial symbiont) results first, by the early nodulin *ENOD11*gene expression sion conferring a blue coloration to root epidermic tissue (data not shown) and the formation of structures with the anatomy of a nodule. Few spontaneous nodules were observed in plants complemented with the no tagged *DMI3 1-311* construction and grown in sepiolite (Figure 2). Microscopic observations revealed transparent rounded nodules (Figure 2a) characterized by the presence of peripheral vascular bundles characteristic of nodule anatomy in *M. truncatula* (Figure 2b). These results were confirmed after histological assay (data not

shown). However, no nodules were observed in plants complemented with the StrepTagII *DMI3 1-311* construction.

In light of these results, we can claim to be in possession of a constitutively active truncated form of the kinase DMI3, this molecular tool will allow us to achieve phosphorylation *in vitro*, first in extracts of cell nuclei suspensions of *M. truncatula* A17 (wild strain) and the mutant *dmi3* (as suspension cells are easy to grow) and in extracts of root, in order to identify substrates or DMI3 molecular partners involved in the process of nodulation or mycorrhiza. Preliminary *in vitro* phosphorylation studies, on cell suspensions nuclei extracts of *M. truncatula A17* (wild type) and *dmi3* mutant are encouraging but need to be confirmed.

Conclusion and recommendation

By decoding calcium signals, *DMI3*/CCaMK could play a central role in orientating the signalling pathway leading to nodulation or mycorrhization. The possibility to produce active DMI3-311 gives the opportunity to search for its substrates by performing *in vitro* phosphorylation assays using plant extracts.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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