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Full Length Research Paper

Heterologous expression and partial purification of calcium and calmodulin dependant protein kinase (CCaMK) of *Medicago truncatula*

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In legumes, the establishment of symbioses with arbuscular mycorrhizal fungi and nitrogen-fixing bacteria share a common signalling pathway. One of the shared components is a calcium and calmodulin dependant protein kinase predicted to perceive and transduce the calcium signals generated upon perception of the symbiotic signals. The removal of the autoinhibitory domain that negatively regulates the kinase activity in *Medicago truncatula*, results in a constitutively-active form, inducing symbiotic responses in the absence of bacterial signals. Here, the heterologous production and partial purification of *DMI3* variant as a tool for identifying substrates potentially involved in nodulation or mycorrhization was described.

Key words: *Medicago truncatula*, CCaMK, heterologous protein expression.

INTRODUCTION

Plant legumes have the ability to establish two agronomically and ecologically important root endosymbioses, namely: the rhizobia-legume symbiosis and the arbuscular mycorrhizal symbiosis. In past decade, molecular genetics studies peformed on the model legumes, *Medicago truncatula* and *Lotus japonicus* have shown that the establishment of the nodulation and the mycorrhization processes share a common signalling pathway, required for the initiation of endosymbiotic programs in host plants. In *M. truncatula*, three genes called the *DMI* genes (which does not cause infection) are involved in this pathway (Catoira et al., 2000). The common signalling pathway diverges after *DMI3* which represents the last known gene common to both symbioses. *DMI3* encodes calcium and calmodulin dependant 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 autoinhibitory domain that negatively regulates the kinase activity. Removal of this domain, or point mutations at the autophosphorylation 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). Recently, a new 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. Recent work performed on *L.*

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japonicus demonstrates that a gain of function mutation of CCaMK, which results in the induction of nodule organogenesis, does not restore infection in mutants altered in the putative Nod factor receptors NFR1 and NFR5 (Hayashi et al., 2010). As proposed by this study, 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 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 signalling pathway or components specific to Nod or Myc signalling. In this study, the production and purification of a *DMI3* variant was described.

MATERIAL AND METHODS

Bacterial expression and preparation of the protein extracts

Escherichia coli BL21 (DE3) cells were cultivated in selective LB medium at different temperatures, in the presence or absence of isopropyl β-D-1-thiogalactopyranoside (IPTG) as inducer. As indicated, *E. coli* BL21 (DE3) cells have been cultivated in Overnight Express^{TM} Instant TB Medium (Merck Chemicals) at 37°C. In all cases, the bacteria were collected by centrifugation at 10 000 xg and the resulting pellet was resuspended in 500 µl of lysis buffer (100 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 1 mM DTT, 0.6 µg/ml DNase) and further extracted using the FRENCH Press (working pressure: 20,000 psi). After centrifugation at 100 000 xg, the soluble (S) and the insoluble (P) fractions were obtained and analysed by 1-D SDS PAGE.

Protein analysis by SDS-PAGE and Western blotting

Samples were separated on polyacrylamide resolving gels by using the buffer system of Laemmli, and the resolved polypeptides were electro-transferred on nitrocellulose membrane and stained with Ponceau -S. The membrane was then blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS (40 mM Na_2HPO_4, 8 mM NaH_2PO_4, 150 mM NaCl (pH 7,4)) and probed with a Strep-Tactin Horse Radish Peroxidase conjugated antibody, used at 1:10 000 dilution in PBS/milk/Tweeen-20, and revealed by ECL (GE Healthcare).

Purification of DMI3-311 by affinity chromatography method

Four hundred and fifty millilitres of BL21 DE3 cells were grown at 28°C for 90 min in the presence of 0.1 mM IPTG. Bacteria were collected by centrifugation at 10 000 xg and the resulting pellet was extracted as described earlier. After centrifugation at 100 000 xg, the soluble (S) and the insoluble (P) fractions were obtained. The soluble fraction (15 ml) was diluted to a final volume of 60 ml with the equilibration buffer column (150 mM NaCl, 100 mM Tris buffer, pH 8.0) and loaded on a prepacked 5 ml StrepTactin™ Sepharose™ column, previously equilibrated in this buffer. After a fixation step and a washing step in the same buffer, the proteins were eluted in one step using a 2.5 mM desthiobiotin solution. The fraction containing the affinity eluted proteins was collected and further analysed by 1-D gel electrophoresis (15% acrylamide).

RESULTS AND DISCUSSION

Production of the recombinant and truncated protein DMI3-311

Based on the truncated versions of *DMI3* that have been reported to have constitutive activity, we have exploited the full-length genomic and cDNA clones that were generated during the cloning of *DMI3* to construct a truncated and tagged (His tag and Strep TagII) form in pET52b(+) under the control of the T7 promoter (Godfroy et al., in preparation). The corresponding encoded protein, predicted to correspond just to the kinase domain, contains 311 amino acids exhibiting a theoritical molecular weight of 39 KDa. The construct has been introduced in *E. coli* BL21 (DE3) and the bacteria have been grown in different culture medium, temperature and concentrations of IPTG used as inducer.

In a first set of experiments, it was determined that the best temperature for the production of DMI3-311 in a soluble form was 28°C, as compared to 37 or 16°C (data not shown). Then the effect of the concentration of IPTG used to induce the expression of the protein was examined. Figure 1 represents the SDS-PAGE analysis of the soluble (S) and insoluble (P) protein fractions prepared from *E. coli* BL21 DE3, grown at 28°C to reach an O.D of 0.6, and then treated by 1 or 2 mM IPTG for 30 or 120 min. It appears that the production of the total amount of DMI3-311, either in its soluble or insoluble form, increased with the duration of the cultivation but did not depend on the concentration of IPTG. Similar results were obtained for lower concentrations, namely: 0.1, 0.25 and 0.5 mM IPTG (data not shown). Since the protein was mainly recovered in the insoluble protein fraction, corresponding to inclusion bodies, the production of DMI3-311 in the classical LB medium after induction with 0.1 mM IPTG for 90 min were compared with that obtained in a special medium (Overnight Express^{TM} Instant TB Medium, Merck Chemicals) designed to facilitate a slower production of the protein favouring its solubility without induction by IPTG. Protein extracts corresponding to the soluble protein fraction (S) or the insoluble protein fraction (P) were analysed by SDS-PAGE electrophoresis and Western blot using Strep-Tactin antibodies as primary antibodies to detect the recombinant protein *via* the Strep-Tag previously introduced at the N-terminus of the sequence. As reported in Figure 2a, an immune-reactive signal was detected at a molecular weight corresponding to that of DMI3-311 in all the tested conditions. However, the use of the auto-inducing medium (lanes 1, 3, 5 and 2, 4, 6 corresponding to the insoluble and soluble fraction, respectively) did not result in a higher yield of soluble protein in comparison to the LB medium supplemented with 0.1 mM IPTG (lanes 7 and 8). This was confirmed by the staining of the nitrocellulose membrane by Ponceau-S (Figure 2b). Taken together, these results showed that the DMI3-311 recom-

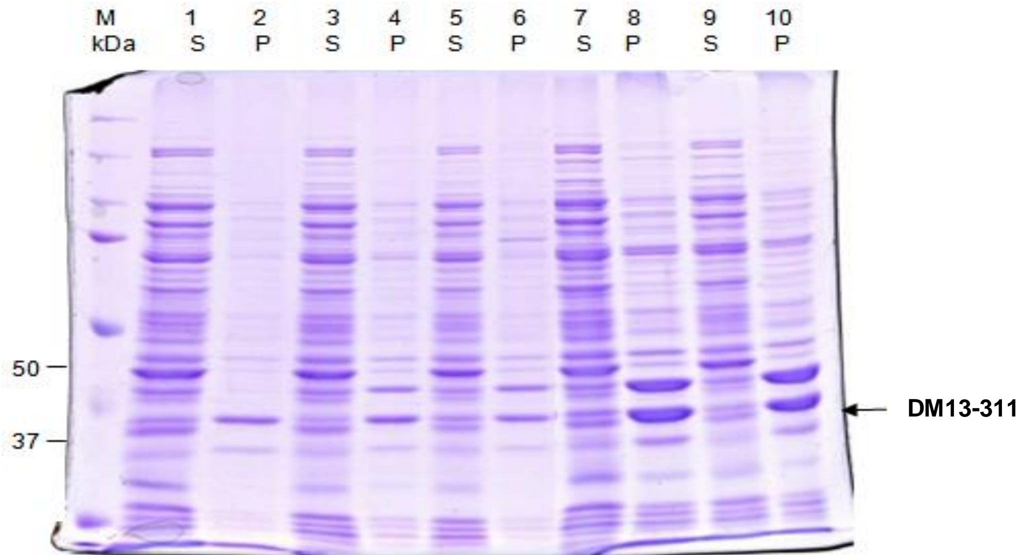

Figure 1. IPTG induction of the recombinant DMI3-311 expression in *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) cells were cultivated at 28°C for 30 and 120 min in the presence of 1 or 2 mM IPTG. Bacteria were collected by centrifugation at 10000 xg and the resulting pellet was resuspended in 500 µl of lysis buffer (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM AEBSF, 1 mM DTT, 0.6 µg/ml DNase) and further extracted using the FRENCH Press (working pressure: 20,000 psi). After centrifugation at 100 000 xg, the soluble (S) and the insoluble (P) fractions were obtained and analysed by 1-D SDS PAGE (12.5% acrylamide). Lanes 1 and 2: not induced cells; lanes 3, 4, 5 and 6: cells grown for 30 min with 1 mM IPTG (lanes 3 and 4) and 2 mM IPTG (lanes 5 and 6); lanes 7, 8, 9 and 10: cells grown for 120 min with 1 mM IPTG (lanes 7 and 8) and 2 mM IPTG (lanes 9 and 10).

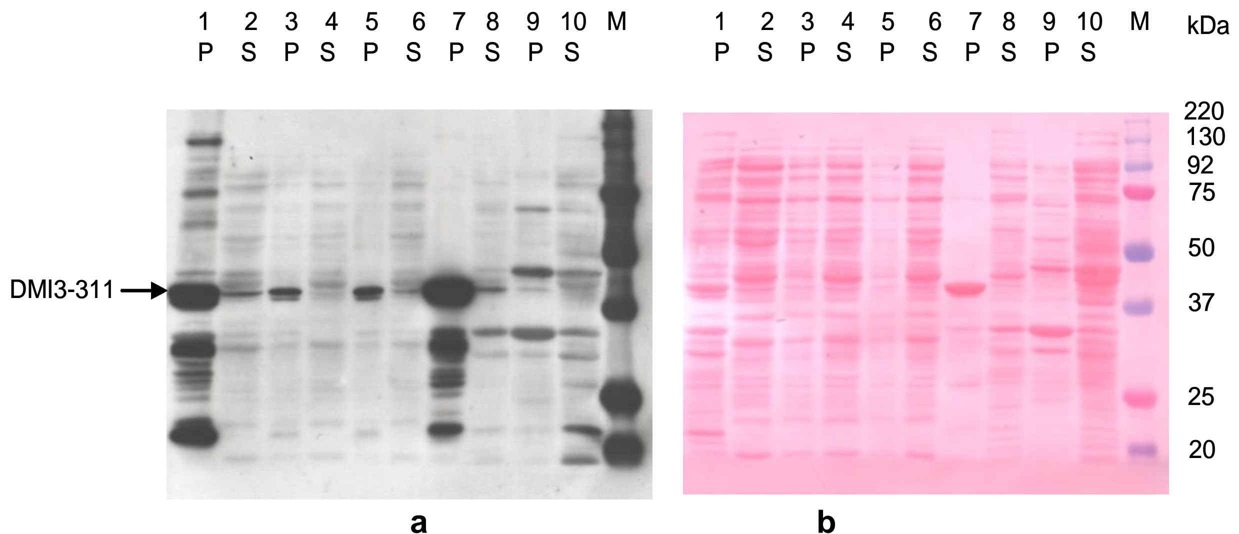

Figure 2. DMI3-311 expression in *E. coli* BL21 (DE3) in selective LB medium and auto-inducing medium. The soluble protein fraction (S) and the insoluble protein fraction (P) were prepared as previously described from bacterial cultures gown in LB medium or auto-inducing medium. The fractions were analysed by SDS–PAGE (12.5% acrylamide) and Western blot. After gel electrophoresis, the polypeptides were transferred on nitrocellulose membrane, stained with Ponceau red then probed with a Strep-Tactin Horse Radish Peroxidase conjugated antibody used at 1:10 000 dilution and revealed by ECL. (a): Lanes 1, 3 and 5: insoluble protein fractions obtained from bacteria cultured in respectively 5 and 2.5 ml (two independent experiments) of Overnight Express^{TM} Instant TB Medium; lanes 2, 4 and 6: corresponding soluble protein fractions. Lanes 7 and 8: cells grown in LB medium at 28°C and treated with 0.1 mM IPTG for 90 min. Lanes 9 and 10: not induced cells. (b): Ponceau-S staining of the same nitrocellulose membrane.

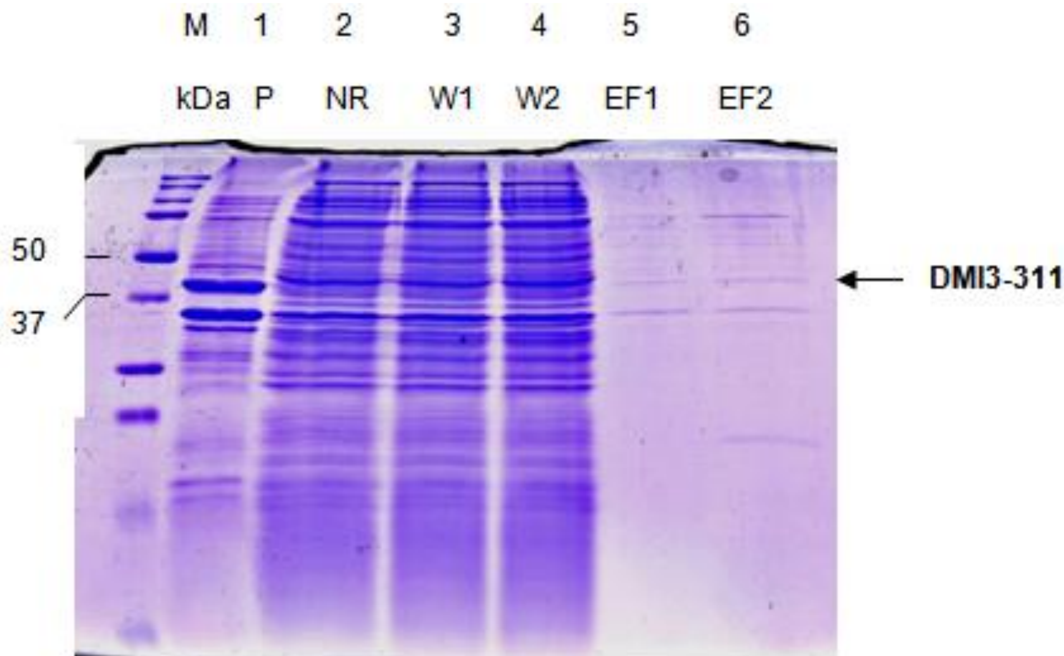

Figure 3. Obtaining enriched soluble DMI3-311 fraction after affinity matrix purification. 450 ml of BL21 DE3 cells were grown at 28°C for 90 min in the presence of 0.1 mM IPTG. Bacteria were collected by centrifugation at 10 000 xg and the resulting pellet was extracted using the FRENCH Press. After centrifugation at 100 000 xg, the soluble (S) and the insoluble (P) fractions were obtained. The soluble fraction (15 ml) was diluted to a final volume of 60 ml with the equilibration buffer column (150 mM NaCl, 100 mM Tris buffer, pH 8.0) and loaded on a prepacked 5 ml StrepTactin™ Sepharose™ column, previously equilibrated in this buffer. After a fixation step and a washing step in the same buffer, the proteins were eluted in one step using a 2.5 mM desthiobiotin solution. The fraction containing the affinity eluted proteins was collected and further analysed by 1-D gel electrophoresis (15% acrylamide). Lane 1: insoluble protein fraction (P) used as contrrol; lane 2: non retained fraction (NR); lane 3: first wash (W1); lane 4: second wash (W2); lanes 5 and 6: desthiobiotin eluted protein fractions (EF1 and EF2).

binant protein was produced in low amounts as a soluble protein. However, this amount was sufficient to carry out a further purification step.

Purification of DMI3-311

In order to purify the protein from the soluble fraction, an affinity chromatography method was used. When affinity purification, based on the presence of the His tag was carried out using IMAC columns, no interaction of the protein with the matrix was observed, suggesting that the tag was masked when located at the C-terminus end. Therefore, we took advantage of the presence of the StrepTagII at the N-terminus of the protein to interact with a matrix based on StrepTactin™ Sepharose™. The fractions containing the affinity eluted proteins (monitored by the absorbance at 280 nm) were collected and analysed by 1-D gel electrophoresis. The resolved polypetides were visualised using a PageBlue™ Protein Staining Solution (Fermentas, Inc.). As shown in Figure 3, a polypeptide with the expected molecular weight was enriched in the fraction eluted by the desthiobiotin. The band was excised from the gel and digested in-gel with trypsin according to Borderies et al. (2003). Peptide mass mapping was performed on a MALDI TOF mass spectrometer (Voyager-DE STR, PerSeptive Biosystems, Framingham, MA, USA) as previously described (Borderies et al., 2003). Protein identification was performed by mining a non-redundant protein sequence database (NCBI) using MS-FIT (Protein Prospector, http://prospector.ucsf.edu) program. With a monoisotopic mass accuracy fixed at 20 ppm, six peptides matched with the DMI3-311 sequence representing 30% coverage.

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 DMI3-311 gives the opportunity to search for its substrates by performing *in vitro* phosphorylation assays

using plant extracts.

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