Calcium regulates the production of nodulation outer proteins (Nops) and precludes pili formation by *Sinorhizobium fredii* USDA257, a soybean symbiont

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Abstract

Sinorhizobium fredii USDA257 forms nitrogen-fixing nodules on primitive soybean cultivars such as 'Peking' but is unable to establish efficient symbiosis with North American cultivars. USDA257 when grown in presence of genistein, a potent *nodD*-inducing isoflavonoid, secretes at least six *n*odulation *o*uter *p*roteins (NopX, NopB, NopL, NopP, NopA and NopC) to the extracellular milieu through a type III secretion system. These proteins regulate legume nodulation in a host-specific manner. Here, it is demonstrated that calcium prevents the accumulation of NopB and NopA, and drastically reduces that of NopX and NopL. The inhibitory effect on Nops accumulation appears to be mediated specifically by calcium since other divalent cations such as Mg^{2+} and Mn^{2+} had no detectable effect. Calcium does not appear to interfere with the secretion of these proteins since Western blot analysis revealed that these Nops do not accumulate inside the cell. The inhibitory effect of calcium on Nops production is mediated at the posttranscriptional level. Studies by the authors indicate that the production of Nops, which function as determinants of host-range, is regulated by calcium.

Introduction

Sinorhizobium fredii, mainly known as soybean symbiont, can nodulate a wide array of legumes resulting in the formation of nitrogen-fixing nodules under appropriate conditions (Pueppke & Broughton, 1999). One strain, USDA257, has been intensively studied because of its ability to form nodules on soybean roots in a cultivar-specific manner. USDA257 forms nodules on primitive soybean cultivars such as 'Peking' but fails to induce nodules on agronomically improved 'McCall' cultivar (Annapurna & Krishnan, 2003). A cluster of genes located on an 8.0 kb DNA fragment located on the symbiosis (*sym*) plasmid was shown to be involved in regulating the cultivar-specific nodulation on soybean (Meinhardt *et al.*, 1993; Kovacs *et al.*, 1995). This region was designated as the soybean cultivar-specificity locus.

Some of the genes located within the soybean cultivar-specificity locus code for the structural components of the type III protein secretion system (T3SS). In these symbiotic bacteria, genes encoding the type III secretion apparatus are clustered within a 35–47 kb region (Marie *et al.*, 2001). The genes encoding T3SS components of symbiotic rhizobia are designated '*rhc*' (for *r*hizobia *c*onserved) and are presumably involved in the formation of Rhc secretion apparatus. The existence of a functional T3SS in USDA257 and *Rhizobium* sp. strain NGR234 and its role in regulating nodulation has been documented (Krishnan *et al.*, 2003; Marie *et al.*, 2003). Both USDA257 and NGR234 utilize T3SS to secrete several *n*odulation *o*uter *p*roteins (Nops) into the extracellular medium. Some of the Nops that are secreted by T3SS include NopA, NopB, NopC, NopL, NopP and NopX (Viprey *et al.*, 1998; Krishnan *et al.*, 2003; Marie *et al.*, 2003; Lorio *et al.*, 2004; Deakin *et al.*, 2005). In *Bradyrhizobium japonicum*, USDA257 and NGR234, T3SS is under the control of the transcriptional regulator TtsI (Krause *et al.*, 2002). Putative regulatory motifs termed *tts*-boxes are found upstream of *ttsI* (Krause *et al.*, 2002). Similar sequences are also located upstream of *nopB*, *nopC*, *nopL*, *nopP* and *nopX*, proteins secreted by T3SS (Marie *et al.*, 2003). Abolition of Nops secretion results in altered nodulation phenotype in a host-dependent manner. For example, USDA257 *rhcN* mutant forms nitrogen-fixing nodules on McCall soybean, while the wild-type strain is unable to nodulate this soybean cultivar (Krishnan *et al.*, 2003). Similarly, NGR234 *rhcN*

mutant, in contrast to the wild-type strain, formed an increased number of nodules on *Pachyrhizus tuberosus* and fewer nodules on *Tephrosia vogelii* (Viprey *et al.*, 1998). These observations suggested an important role for T3SS in nodulation and the inclusion of Nops as host-specificity determinants.

USDA257, when grown in yeast extract mannitol (YEM) media in presence of flavonoids, secretes several Nops into the extracellular media. However, on several occasions it was observed that USDA257 failed to secrete Nops even under inductive conditions. Investigation into the conditions responsible for this inconsistency revealed that yeast extract, a component in the YEM, was a major contributor. Because T3SS in *Yersinia* is regulated by calcium (Straley *et al.*, 1993), and yeast extract is the only source of calcium in YEM media, it was suspected that this divalent cation could play a role in regulating Nops production. In this study, it was demonstrated that Nops production and formation of pilus-like surface appendages in USDA257 are regulated by calcium. Results of this study indicate that the T3SS in USDA257 is subject to regulation at multiple levels.

Materials and methods

Microbiological techniques

USDA257 was grown in a defined gluconate–mannitol (GM) medium supplemented with $CaCl_2$ as necessary (Bhuvaneswari *et al.*, 1977) or in YEM medium (Vincent, 1970) on a reciprocal shaker at 30 °C. Yeast extract is the only source of Ca^{2+} in YEM. The concentration of Ca^{2+} in the YEM was determined by calculating the stability constant of Ca^{2+} EGTA ($5.4 \times 10^{-3}\,mM^{-1}$) and Mg^{2+} EGTA ($8.97 \times 10^{-3}\,mM^{-1}$) (Bartfai, 1979). Chemical analysis performed revealed the concentration of calcium in YEM to be 12.5 μM (Balatti *et al.*, 1991). The effect of Ca^{2+} on Nops production was studied by growing USDA257 in YEM liquid medium to which 1 mM Ca^{2+} was added.

Isolation of extracellular proteins and bacterial surface appendages

Bacterial cells were induced with 1 μM genistein using starter cultures as inoculum then grown for 48 h at 30 °C (Krishnan & Pueppke, 1993). After removal of the cells by centrifugation (12 000 ***g***, 30 min), proteins in the supernatant were precipitated by the addition of two volumes of ice-cold acetone. Precipitated proteins were recovered by centrifugation (8000 ***g***, 4 °C, 30 min), then resuspended in sodium dodecyl sulfate (SDS)-sample buffer. Surface appendages of genistein-induced USDA257 were isolated by ultracentrifugation as described earlier (Krishnan *et al.*, 2003). Proteins associated with the surface appendages were identified by resuspending the pellet from the ultracentrifugation step directly in SDS-sample buffer (2% SDS, 10% glycerol, 0.125 M Tris-HCl pH 6.8, 0.1% bromophenol blue and 5% β-mercaptoethanol). Samples were boiled for 3 min and aliquots resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970). Proteins were visualized by staining with Coomassie Brilliant Blue (Sigma, St Louis, MO). Western blot analysis was performed utilizing Nops-specific antibodies at a final dilution of 1 : 10 000. Immunoreactive polypeptides were identified following the horseradish peroxidase color-development procedure provided by the manufacturer (Bio-Rad, Hercules, CA).

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA from rhizobia was isolated as described earlier (Jiang *et al.*, 2001). RNA was first treated with DNase I (Invitrogen, Carlsbad, CA) to remove contaminating DNA. RT-PCR was performed in a volume of 50 μL with a OneStep RT-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. The thermal cycler program was 50 °C for 30 min, 95 °C for 15 min, 30 cycles at 94 °C (1 min), 58 °C (1 min) and 72 °C (1 min), followed by a final 10 min at 72 °C. Oligonucleotide primers specific for *nopA* (forward primer 5′-ATGTCTAAAATAGGTACTGTCACGAG-3′ and reverse primer 5′-TCACTGTACGCGTTCATCCGCCGC CTTC-3′), *nopB* (forward primer 5′ATGATGCTGCCTGTC ACGTCAATCTC-3′ and reverse primer 5′-TCACCCTTC CTTTAATAGTTTGTTTAC-3′), *nopL* (forward primer 5′-ATGGATATCAATTCAACCCGCCCAC-3′ and reverse primer 5′-TCAAATGTCAAAATCCAGCGATGGCC-3′), *nopX* (forward primer 5-ATGTCGGCCAGCAACCTTTTACCA ATG-3 and reverse primer 5′-GCGCGTAGCTTTG CGC TTGGCTTTCC-3′) and *gltA* (forward primer 5′-ATGTCAG-GAACAAACGCGACGATCTC-3′ and reverse primer 5′-TCAGCGCTTGGAGAGCGGCACGTAATC-3′) were synthesized by Integrated DNA Technologies (Coralville, IA). The PCR products were separated on 0.8% agarose gel.

Results

Calcium abolishes the secretion of Nops

USDA257 secretes several proteins into the extracellular milieu when grown in presence of flavonoids (Krishnan & Pueppke, 1993; Krishnan *et al.*, 2003). Some of these proteins including NopA, NopB, NopL, NopP and NopX are secreted by T3SS (Viprey *et al.*, 1998; Krishnan *et al.*, 2003; Marie *et al.*, 2003; Lorio *et al.*, 2004; Deakin *et al.*, 2005). Previously it has been demonstrated by the authors that calcium is required for optimal growth of *S. fredii* (Balatti *et al.*, 1991). To test the effect of calcium on Nops, USDA257 was grown in a defined GM medium supplemented with various amounts of calcium (Fig. 1). Genistein-induced

USDA257 grown in the presence of 0.01 mM Ca^{2+} revealed the presence of several extracellular proteins including the Nops (Fig. 1). However, the addition of increasing amounts of Ca^{2+} to growth medium resulted in a significant decrease in the accumulation of extracellular proteins including the Nops. Addition of calcium to the growth medium promoted the accumulation of two abundant proteins of 34 and 36 kDa (Fig. 1).

Because the production of Nops in YEM medium was higher than in calcium-free GM medium and all previous studies by the authors were conducted with YEM medium, the effect of calcium on Nops production in YEM medium was examined. Addition of 1 mM Ca^{2+} to YEM media containing 1 μM genistein clearly inhibited the secretion of most of the Nops by USDA257 but promoted the accumulation of 34 and 36 kDa proteins (Fig. 2a). To determine if the inhibition of Nops accumulation is specific to calcium, the affect of divalent cations, Mg^{2+} and Mn^{2+}, on Nops production was tested. Neither of these cations at 1 mM concentration had any apparent affect on Nops accumulation (Fig. 2a). Western blot analysis utilizing *Sinorhizobium meliloti* flagellin antibodies (Robinson *et al.*, 1992) indicated that these two abundant proteins are flagellin subunits (Fig. 2b). These proteins were absent when the cells were cultured in Ca^{2+}-depleted medium. Two smaller proteins, presumably the degraded products of flagellin, were recognized by the flagellin antibodies (Fig. 2b).

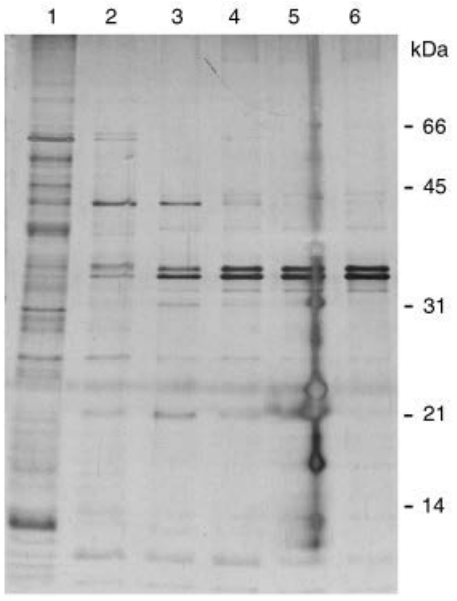

Fig. 1. Effect of calcium on Nops secretion. Extracellular proteins from USDA257 grown in defined GM medium were separated by SDS-PAGE and proteins visualized by silver stain. USDA257 was cultured in GM medium containing 0.01 mM (lane 1), 0.1 mM (lane 2), 0.5 mM (lane 3), 1 mM (lane 4), 2 mM (lane 5) and 3 mM (lane 6) calcium. Cells were collected after 48 h of induction with 1 μM genistein. The sizes of molecular weight markers are indicated on the right.

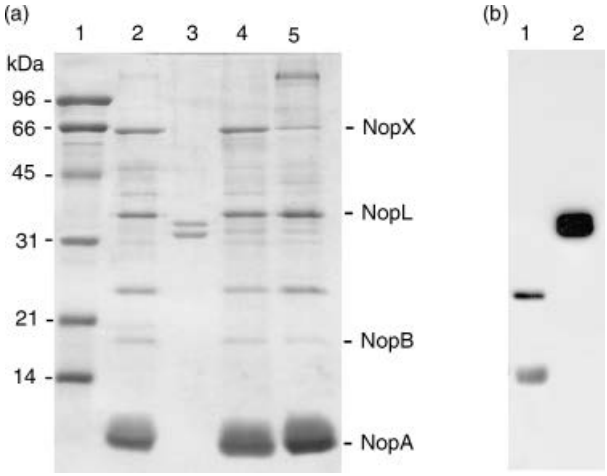

Fig. 2. Effect of divalent cations on Nops and flagellin production. (a) Proteins associated with surface appendages of flavonoid-induced USDA257 grown in YEM media (lane 2) or YEM media supplemented with calcium (lane 3), or magnesium (lane 4), or manganese (lane 5) were separated by SDS-PAGE. Resolved proteins were stained with Coomassie Brilliant Blue. The sizes of molecular weight markers (lane 1) in kDa are also shown. (b) Western blot. Proteins [corresponding to lanes 2 and 3 of (a)] were transferred to nitrocellulose membrane and incubated with *Sinorhizobium meliloti* flagellin antibody. The immunoreactive polypeptides were visualized by the chemiluminescent detection method.

Because USDA257 grown in Ca^{2+}-supplemented YEM broth did not accumulate Nops, it was examined whether calcium was involved in preventing the secretion of Nops. Extracellular proteins were isolated from flavonoid-induced USDA257 cells that were grown in Ca^{2+}-limiting or Ca^{2+}-supplemented media. Polyclonal antibodies raised against NopA, NopB, NopL and NopX were employed in Western blot analysis to detect their presence in the extracellular protein fraction. These proteins were readily detected in USDA257 grown in low calcium (Fig. 3). In contrast, these proteins, with the exception of NopX and NopL, were conspicuously absent in USDA257 grown in calcium-supplemented medium (Fig. 3). The accumulation of NopX and NopL was several-fold lower in cells grown in Ca^{2+}-supplemented media in comparison to cells grown in low calcium (Fig. 3). Because NopA, NopB, NopL and NopP in the extracellular protein fraction could not be detected, it was examined if they accumulated inside the cells. Western blot analysis of total protein fraction from USDA257 grown in Ca^{2+}-supplemented media did not detect the accumulation of these Nops inside the cell (data not shown).

Calcium does not affect the transcription of *nop* genes

Since calcium had a pronounced effect on the production of several Nops, it was examined if this divalent cation had any

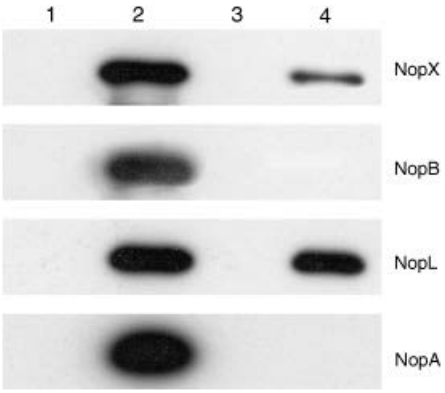

Fig. 3. Immunoblot analysis of Nops secretion by USDA257. Extracellular proteins from USDA257 grown in Ca^{2+}-depleted (lanes 1 and 2) or Ca^{2+}-supplemented (lanes 3 and 4) YEM broth were separated by SDS-PAGE and subjected to Western blot analysis. The nitrocellulose membrane was immunostained with NopX, NopB, NopL and NopA antibodies, respectively. The immunoreactive polypeptides were visualized by the chemiluminescent detection method. Lanes 2 and 4 contain extracellular proteins from genistein-induced USDA257.

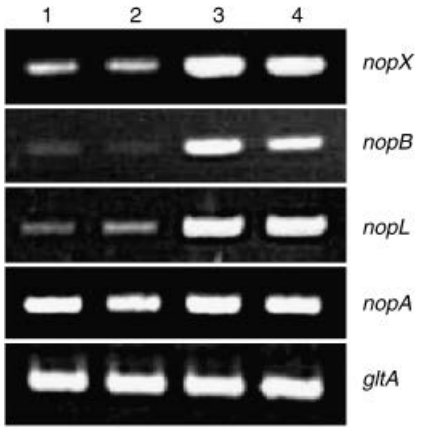

Fig. 4. RT-PCR detection of *nops* mRNA. Primer pairs specific for *nopX*, *nopL*, *nopB* and *nopA* were utilized in the RT-PCR reaction. A 1.2-kb fragment of USD257 citrate synthase (*gltA)*, reverse transcribed under similar conditions, was used as an internal control. Lane 1, USDA257 grown in Ca^{2+}-depleted YEM; lane 2, USDA257 grown in Ca^{2+}-supplemented YEM; lane 3, USDA257 grown in Ca^{2+}-depleted YEM containing 1 μM genistein; lane 4, USDA257 grown in Ca^{2+}-supplemented YEM containing 1 μM genistein.

effect on the transcription of *nop* genes. RT-PCR analysis was performed utilizing RNA isolated from USDA257 cells that were grown in Ca^{2+}-limiting or Ca^{2+}-supplemented media (Fig. 4). RT-PCR analysis indicated high basal-level expression of *nopA* even in the absence of flavonoid induction. Much lower basal-level expression of *nopX*, *nopB* and *nopL* were also seen (Fig. 4). The transcript levels of *nopX*, *nopB*, *nopL* and *nopA* were enhanced by the addition of genistein (Fig. 4). Calcium addition to the growth media, however, had no obvious effect on the accumulation of these *nop* transcripts. This observation suggests that the inhibitory effect of calcium on Nops production occurs at the post-transcriptional level. Since RT-PCR analysis demonstrated a high basal-level expression of *nopA* even in the absence of flavonoids induction, Northern blot analysis was performed to verify this observation. Northern blot analysis confirmed that the transcription of *nopA* occurs even in the absence of flavonoid induction, but the addition of genistein enhanced the transcription of the gene (data not shown).

Discussion

In this study, it was demonstrated that accumulation of several Nops in the extracellular media is drastically curtailed when USDA257 is grown in presence of 1 mM calcium. This cation clearly induces the synthesis of flagella as evidenced by the accumulation of 34 and 36 kDa flagellin subunits. The observation of this study is consistent with the earlier report, which has shown the requirement of calcium for maintaining the rigidity of flagella (Robinson *et al.*, 1992; Kim *et al.*, 2005). A recent study has shown that genistein-induced *B. japonicum* secretes an abundant 32 kDa flagellin subunit to the extracellular media. Since this protein accounts for 80% of the extracellular proteins, it was necessary to delete the flagellin genes to characterize T3SS proteins of *B. japonicum* (Suss *et al.*, 2006). A similar approach may be required to rule out the possibility that an overabundance of flagellins could mask the detection of Nops when USDA257 are grown in calcium-rich media.

It is now well established that Nops play an important role in nodulation as the presence or absence of these proteins can promote or inhibit the process in a host-specific manner (Viprey *et al.*, 1998; Marie *et al.*, 2001, 2003; Krause *et al.*, 2002; Krishnan *et al.*, 2003; Lorio *et al.*, 2004; Deakin *et al.*, 2005). The observation of this study that calcium abolishes the production of Nops by USDA257 may have agronomic significance. Calcium is required for efficient nodulation of soybean and other legumes (Balatti *et al.*, 1991). The two soybean symbionts, *S. fredii* and *Bradyrhizobium* spp., secrete several proteins into the rhizosphere, some of which are mediated by T3SS (Krause *et al.*, 2002; Krishnan *et al.*, 2003). In calcareous soils, rhizobia may be impaired in Nops production leading to either a positive or negative interaction with the legume host. Since USDA257 has an extensive host-range nodulating at least 79 genera of legumes (Pueppke & Broughton, 1999), which grow in different ecological conditions, successful symbiotic association may be mediated by calcium-regulated Nops production.

The expression of *nopX*, *nolL*, *nopB* and *nopA* is not significantly affected by calcium, yet these proteins do not accumulate in the extracellular media. This suggests that calcium interferes with the secretion of these Nops. If this

were true, one would expect the accumulation of Nops inside the cell. However, results from Western blot analysis revealed no such accumulation of these proteins inside the cell. Even though *nop*s mRNA was detected, no protein was found indicating that calcium exerts its influence at the posttranscriptional level. Calcium may activate some regulatory circuit that prevents the translation of the Nops or activate proteases that target the Nops for degradation. It is interesting to note that a NopA mutant of NGR234 is unable to secrete Nops nor does it accumulate the proteins (Deakin *et al*., 2005). It has been suggested that regulatory controls exist that prevent the transcription or translation of Nops in the absence of functional T3SS apparatus (Deakin *et al*., 2005). It is speculated that the accumulation of Nops inside the cell could be deleterious to the rhizobial cells and thus subjected to rapid degradation.

Calcium activated proteases are present in both prokaryotes and eukaryotes (Mellgren, 1987; Gottesman, 2003). Such proteases may also be present in USDA257 and regulate T3SS-dependent Nops production. Even though this study does not provide evidence for the existence of such calcium-activated proteases in USDA257, the involvement of proteases in regulating T3SS of *Yersinia* (Jackson *et al*., 2004), *Salmonella* (Boddicker & Jones, 2004) and *Pseudomonas* (Bretz *et al*., 2002) stress the important role of proteases in T3SS. In *Yersinia*, the expression of T3SS is regulated by ATP-dependent ClpXP and Lon proteases (Gottesman, 2003; Jackson *et al*., 2004). These cytosolic ATP-dependent proteases degrade improperly synthesized proteins as well as regulate the concentration of short-lived regulatory proteins (Gottesman, 2003). Currently, the role of proteases, if any, in the regulated proteolysis of Nops produced by USDA257 is investigated by the authors.

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