Symbiosis-specific expression of *Rhizobium etli* casA encoding a secreted calmodulin-related protein

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Symbiosis between *Rhizobium* and its leguminous host requires elaborate communication between the partners throughout the interaction process. A calmodulin-like protein, termed calysmin, was identified in *Rhizobium etli*; a calmodulin-related protein in a Gram-negative bacterium had not been described previously. Calysmin possesses three repeated homologous domains. Each domain contains two predicted EF-hand Ca\(^{2+}\)-binding motifs. Ca\(^{2+}\)-binding activity of calysmin was demonstrated on purified protein. *R. etli* efficiently secretes calysmin without N-terminal cleavage of the protein. The gene encoding calysmin, *casA*, is exclusively expressed during colonization and infection of *R. etli* with the host. Expression of *casA* is controlled by a repressor protein, termed CasR, belonging to the TetR family of regulatory proteins. Mutation of the *casA* gene affects the development of bacteroids during symbiosis and symbiotic nitrogen fixation.

Although the presence of bacterial proteins with calmodulin-like properties has been reported repeatedly, calmodulin-like genes with authentic EF-hands appear to be very uncommon in prokaryotes (11). The only example so far described is a calmodulin-like protein from the Gram-positive bacterium *Saccharopolyspora erythraea* that contains four EF-hands (12). The biological role for this protein is presently unknown. Here we describe the isolation of a calmodulin-like gene from *Rhizobium etli*, *casA*, that is implicated in symbiosis and regulated by a TetR-type of repressor.

Materials and Methods

**Growth Conditions.** *R. etli* strains were routinely grown in liquid tryptone/yeast extract (TY) or acid minimal salts (AMS) medium at 30°C and maintained on yeast-mannitol agar plates (13). *Escherichia coli* was grown in Luria–Bertani medium at 37°C.

**Screening of a Mutant Expression Library.** The *R. etli* mutant library was constructed as described by Xi et al. (14). Approximately 4,000 mutants were screened for differential induction of the *gusA* gene when the cultures were grown in a microoxic environment with 0.3% oxygen and/or in the presence of nodule extracts. Details on the screening procedure are described by Xi et al. (15).

**Plant Culture and Bacteroid Isolation.** *Phaseolus vulgaris* cv. Limburgse vroege plants were grown in the plant growth room essentially as described by Michiels et al. (16) and analyzed 3 weeks after inoculation. For symbiotic expressions, bacteroids were purified from plant material by differential centrifugation (13).

**Cloning of the casA–casR Gene Region.** Standard methods were used for *in vitro* DNA manipulations (17). Total DNA from the selected mutant strain FAJ1806 was digested with *Xho* I and ligated into the SalI site of pUC18. Inserts containing part of the mTn5*gusA*-pgfp21 transposon were selected. From the cloned fragment, the partial sequence flanking the mTn5*gusA*-pgfp21 insertion was determined, using the *gusA* primer 5′-GATT-TCACGGGTTGGTTCT-3′. To isolate the corresponding wild-type *R. etli* CNPAF512 gene, the sequenced fragment was amplified by PCR using the primers OJM142 (5′-GGCT-GTCGTCATAAGCTTCCGATCTCGATACCG-3′) and OJM143 (5′-CATGAACTTCCACGACACCAGC-3′).
CCATCCC-3'). The resulting 360-bp PCR fragment was used as a probe to hybridize a λ phage EMBL3 gene library of CNPASF512. DNA from a positive plaque was hybridized with the 360-bp digoxigenin-labeled probe. Two positive SalI bands (1.8 kb and 0.7 kb) were cloned in pUC18 and sequenced.

**Construction of Mutants.** To construct *R. etli* casR mutants, the 1.8-kb SalI fragment containing the complete casR gene was first cloned in the SalI site of pUC19, yielding plasmid pFAJ1822. The 2.1-kb spectinomycin resistance (spe)9 cartridge from pHPl45 (18) was removed with HindIII and ligated in the FseI and PshAI sites of pFAJ1822 to obtain plasmids pFAJ1824 (the orientation of the spe9 gene is opposite to casR) and pFAJ1825 (the orientation of the spe9 gene is the same as casR) after blotting of the fragments. The resulting SalI fragment from pFAJ1824 and pFAJ1825 were cloned in the SalI site of the suicide plasmid pQ2008K (19), generating plasmids pFAJ1826 and pFAJ1827, respectively. These plasmids were introduced into strain CNPASF512 and double recombinants were selected as described (13). The casR mutants were FAJ1802 (spe9 gene in FseI site in the same orientation as casR) and FAJ1803 (spe9 gene in PshAI site, opposite orientation to casR).

For the construction of an *R. etli* casA mutant strain, a 2.5-kb fragment containing the casA and casR genes was amplified by PCR using primers OJM161 (5'-GATCTTGCAGGCGCGCTGACG-3') and OJM162 (5'-GATCTTGCAGGCGCGCTGACG-3') and, after digestion with XhoI, ligated into the SalI site of pUC19-ΔE (a pUC19 derivative from which the EcoRI site was removed), yielding plasmid pFAJ1828. Plasmid pFAJ1828 was digested with EcoRI, thereby removing the casA internal EcoRI fragment, and ligated to the 2.2-kb HindIII fragment from pHPl45 (18), containing a kanamycin resistance (kmR) gene, by blunt-end ligation, generating plasmids pFAJ1829 (the orientation of the kmR gene is the same as casA) and plasmid pFAJ1830 (the kmR gene and casA have opposite orientations). The BamHI fragments containing the inserts of pFAJ1829 and pFAJ1830 were cloned into the BamHI site of pQ2008K to obtain plasmids pFAJ1831 and pFAJ1832, respectively. Plasmids pFAJ1831 and pFAJ1832 were used to mutate the wild-type strain CNPASF512, yielding the casA mutant strains FAJ1804 and FAJ1805, respectively. *R. etli* casAR double mutants are described in the section on the construction of casA-gusA and casR-gusA fusions.

**Construction of gusA Fusions.** The *R. etli* casA::gusA strain FAJ1806 carries the insertion of mTn5gusA-pgfp21 between the nucleotides 838 and 839 from the ATG start codon of the casA gene. To construct the *R. etli* casA::mTn5gusA-pgfp21 casR::Ω-Spc strains FAJ1807 and FAJ1808, plasmids pFAJ1826 and pFAJ1827 were introduced into the *R. etli* mutant strain FAJ1806 and double recombinants were selected, generating the casAR double mutant strains FAJ1807 and FAJ1808, respectively.

To construct a genomic casR::gusA fusion, the 4.5-kb BamHI fragment from pWM6 containing a promoterless gusA gene and a kmR cartridge were ligated into the PshAI site of pFAJ1822, yielding plasmid pFAJ1836. The resulting 6.3-kb KpnI fragment from pFAJ1836 was cloned in the SalI site of pQ2000-UC1 by blunt-end ligation, yielding plasmid pFAJ1837. This plasmid was introduced into the *R. etli* wild-type strain CNPASF512 and double recombinants were selected to obtain the casR::gusA mutant strain FAJ1809.

Plasmid-borne P<sub>casA</sub>-gusA (pFAJ1842) and P<sub>casR</sub>-gusA (pFAJ1843) fusions were constructed. For this, the 450-bp casAR intergenic region, amplified by PCR using the primers OJM163 (5'-GATCTTGCAGGCGCGCTGACG-3') and OJM164 (5'-GATCTTGCAGGCGCGCTGACG-3'), was digested with XhoI and ligated into the XhoI site of pUC18NotI (20), yielding plasmid pFAJ1839. The 4.5-kb gusA-kmR Spe9 fragment from pWM6 was inserted into the SalI sites of pFAJ1839 to obtain plasmids pFAJ1840 and pFAJ1841, respectively. The Nor1 fragments containing the P<sub>casA</sub>-gusA and P<sub>casR</sub>-gusA fusions from the respective plasmids were inserted into the BamHI site of pLAFR3 (21) by blunt-end ligation, yielding plasmids pFAJ1842 and pFAJ1843.

**Constructs for Complementation Analysis.** A 1.6-kb fragment containing the complete casA gene sequence but not the casR gene was obtained by PCR using primers OJM162 (5'-GATCTTGCAGGCGCGCTGACG-3') and OJM163 (5'-GATCTTGCAGGCGCGCTGACG-3'). This fragment was digested with XhoI and XhoI and ligated with XhoI/SalI-digested pUC18NotI, yielding plasmid pFAJ1833. To facilitate subsequent cloning in pLAFR3, the 2.1-kb SalI fragment containing the Spe9 gene from pHPl45 was inserted into the SalI site of pFAJ1833. From the resulting plasmid pFAJ1834, the 3.8-kb Nor1 fragment containing the casA gene was cloned into the BamHI site of pLAFR3 by using blunt-end ligation, yielding pFAJ1835. Plasmid pFAJ1838 was obtained by inserting the 4.2-kb BamHI fragment from pFAJ1829 containing the complete casR gene into the BamHI site of pLAFR3.

**Light and Electron Microscopy.** For light microscopic analysis, 3-μm sections of 3-week-old nodules were prepared as described (16).

To obtain transmission electron micrographs, 3-week-old nodules were fixed overnight at 4°C in 2% cold glutaraldehyde solution in 10 mM sodium cacodylate buffer (pH 7.5) and postfixed in osmium tetroxide in the same buffer. Then the sample was block-stained in uranyl acetate (in 10% aqueous acetone) and dehydrated in a graded acetone series, followed by embedding in Araldite. Serial semithin sections were stained with methylene blue and thionin. Thin sections, made with a Reichert Ultracat E microtome, were stained with uranyl acetate and lead citrate in an LKB 2168 Ultrotainer and examined in a Zeiss EM 900 electron microscope.

**Purification of Proteins.** Bacterial cells and culture supernatant were collected from the wild type or the CasA-overproducing casR strain FAJ1802 grown overnight at 30°C in TY medium. Proteins were precipitated from the growth medium supernatant by incubation with trichloroacetic acid (10%, wt/vol) for 2 h on ice and pelleted by centrifugation at 15,000 × g for 20 min. The cell pellets were washed twice with PBS at pH 6.8 and resuspended in the same buffer. The mixture was passed three times through a French press (SLM Instruments, Rochester, NY) at 10,000 psi (69 MPa). Cell debris was removed by centrifugation for 20 min at 10,000 rpm. The proteins were precipitated as described above. Proteins were separated by electrophoresis on SDS/polyacrylamide gels (5% stacking gel, 15% separating gel, 0.1% SDS). After electrophoresis, proteins were transferred to poly(vinylidene difluoride) (PVDF) membranes by electroblotting.

To overexpress calymin in *E. coli*, a 900-bp fragment was amplified by PCR using primers OJM186 (5'-GTCATCTGAGATGACAGACATTTTCCTGTCGAAACATC-3') and OJM187 (5'-GTCATCTGACGACGCGAGACTGG-3') and cloned as a BamHI fragment in the corresponding sites of pBAD/HisA, yielding pFAJ1845. To express the recombinant gene, *E. coli* Top10 cells carrying pFAJ1845 were grown exponentially and subsequently induced for 6 h in the presence of 0.2% arabinose. The recombinant calymin fusion protein carrying the N-terminal polyhistidine tag was purified under denaturing conditions on ProBond as recommended by the manufacturer (Invitrogen).
Caseymin contains two EF-hand helix–loop–helix structural motifs (10). Amino acids in boldface in the CasR sequence represent the conserved helix–turn–helix structure as found in the six putative EF-hand calcium-binding sites of CasA and the helix–turn–helix structure of CasR. The secondary structure predictions are based on the algorithm of Geourjon and Deleage (28). Amino acids in boldface in the CasR sequence show the six putative EF-hand calcium-binding sites of CasA and the helix–turn–helix structure of CasR as found in Tetrater-related proteins (27). The amino acids in the CasR and CasA proteins marked with asterisks are part of predicted α-helices. Underlined amino acids in CasA are similar to EF-hand calcium-binding motifs (10). Amino acids forming putative short β-strands within the loops are in boldface.

**Calcium-Binding Assay.** After electroblotting of the proteins on PVDF membranes, the same membrane was first used in a 45Ca2+ binding assay, then stained with ruthenium red, and finally stained with Coomassie brilliant blue. The 45Ca2+ binding assay was performed essentially as described by Maruyama et al. (24). Ruthenium red staining was carried out as described by Charuk et al. (23). To confirm the identity of the ~45-kDa 45Ca2+-binding band, it was excised (50 μg) from several lanes from PVDF blots and N-terminally sequenced by Edman degradation on a pulsed liquid-phase 477A radiations on a pulsed liquid-phase 477A

**Sequence and structure of the R. etli**

![Diagram of CasA and CasR](image)

**Results**

**Sequence of casA and casR.** R. etli is the nodulating symbiont of P. vulgaris, the common bean plant. To identify new symbiotic genes in this species, R. etli was mutagenized with the miniTn5 transposon derivative mTn5gusA-pgfp21 (14) carrying a promoterless gusA gene suitable for promoter trapping. Expression of the gusA gene in the mutants was assessed under conditions of aerobic or microaerobic growth in the presence or absence of nodule extracts. One of the strains displaying a differential induction pattern was selected for further characterization.

Sequence analysis of a 2442-bp segment containing the region flanking the mTn5gusA-pgfp21 insertion in mutant FAJ1806 indicated the presence of two divergently transcribed genes (Fig. 1A). The casA gene (calmodulin-like symbiosis gene A), which was inactivated in FAJ1806, codes for a protein of 293 amino acids (M, 29,981) that is highly acidic (pI 3.6) (Fig. 1B). This protein, named casymin, is composed of three domains (A, B, and C), containing 57 or 58 amino acids, structurally similar to the two domains of calmodulin, and a fourth nonhomologous N-terminal domain (Fig. 1B). Each of the three domains of casymin contains two EF-hand helix–loop–helix structural motifs (Fig. 1). The 12-residue Ca2+-binding loops I, III, and V of casymin contain the calmodulin hallmark residues at positions 1, 3, 5, 7, 9, and 12. These residues provide one (positions 1, 3, 5, 7, and 9) or two (position 12) oxygen ligands for the coordination of Ca2+ in calmodulin. Loops II, IV, and VI are atypical because of the absence of the conserved glutamic acid bidendate ligand at position 12. The conserved glycine residue at position 6, enabling side-chain and main-chain ligations at positions 5 and 7, respectively, is found in the six Ca2+-binding loops of casymin. Structure predictions indicate the presence of short β-strands of 3 or 4 residues in casymin at positions 7–11 in all loops (Fig. 1A). In calmodulin, two β-strands (residues 7–9 in both loops) from each domain interact and form an antiparallel sheet structure allowing cooperativity of Ca2+ binding. The amino acid sequence is more conserved between alternate loops (I, III, V and II, IV, VI) than between adjacent loops, similar to what is observed in calmodulin. In addition, highly similar linkers connect loops I and II (Fig. 1A) and B and C (22 amino acids between loop II and III) and B to C (22 amino acids between IV and V). Casymin may therefore have arisen by successive duplications and fusions of an ancestral gene coding for one Ca2+-binding domain giving rise to a protein composed of three homologous domains.

Casymin also possesses two proline-rich stretches, between amino acids 148–152 and 226–231, with five and six consecutive proline residues, respectively. These sequences contain the SH3 domain core recognition consensus sequence Pro-Xaa-Xaa-Pro (Xaa is any amino acid) (25). In the case of class I peptides, an arginine residue is found three amino acids N-terminal from the core recognition sequence. Also in calmodulin, arginines are located N-terminal from the core recognition sequence. In addition, highly similar linkers connect domains A to B (20 amino acids between loops II and III) and B to C (22 amino acids between IV and V). Casymin may therefore have arisen by successive duplications and fusions of an ancestral gene coding for one Ca2+-binding domain giving rise to a protein composed of three homologous domains.

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A divergently transcribed gene, casR (R, repressor), is located in the region upstream from casA (Fig. 1A). The casR gene product contains 214 amino acids (M, 24,240) and is homologous to transcriptional repressors belonging to the TetR family of bacterial regulatory proteins [21% identical and 28% conserved residues with TetR(E), accession no. X14035.1 (Fig. 1A)]. Sequence conservation is particularly strong in the helix–turn–
helix structure localized in the N-terminal region of the protein and responsible for DNA binding in the TetR protein (27).

**Symbiotic Phenotype.** To study the role of calsymin during symbiosis with *P. vulgaris*, *R. etli* casA and casR insertional mutants were constructed and tested on plant. Symbiotic acetylene reduction activity (ARA), a measure for nitrogen fixation, was reduced by approximately 70% in nodules infected with the casA mutant strain versus wild type (Fig. 2A). A similar phenotype was observed when multiple copies of the casR gene, supplied on a multicopy plasmid, were present (Fig. 2A), which is in agreement with the postulated role of CasR as a repressor of CasA (see below). The ARA phenotype of a casA mutant could be restored to the wild-type level by complementation. No effect of a casR mutation on ARA was observed (Fig. 2A). Nodule numbers on plants inoculated with *R. etli* wild type or casA or casR mutants were not statistically different.

Light microscopic examination of stained nodule sections suggested a clearly reduced number of bacteroids in nodules infected with casA mutants compared with those infected with wild-type *R. etli* (Fig. 3A and B). This was confirmed by a transmission electron microscopic analysis showing a reduction of the number of bacteroids by approximately 40% in plant cells colonized by the casA mutant (3.3 bacteroids per 10 μm², sample standard deviation 1.1, 3 samples of 750 μm² each analyzed) compared with the wild type (5.7 bacteroids per 10 μm²).

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**Fig. 2.** Symbiotic phenotype of mutants and expression of *R. etli* casA and casR genes. (A) Nitrogen fixation of *P. vulgaris* plants inoculated with the *R. etli* wild-type strain, casA (FAJ1804) and casR (FAJ1802) mutant strains, and their complemented derivatives. Values are the mean ± SD (*n* = 10). The means are indicated above the bars. Plasmids pFAJ1835 and pFAJ1838 carry casA and casR genes, respectively. Similar results were obtained between strains FAJ1802 and FAJ1803 (casR mutants) and between strains FAJ1804 and FAJ1805 (casA mutants). (B and C) Expression of fusions between gusA and the casA (B) and casR (C) genes in free-living aerobic cultures (AMS) medium (13) supplemented with 10 mM sodium succinate, OD595 = 0.1–0.2, read with 100 μl culture in a Versamax microplate reader (Molecular Devices) and during symbiosis. Direct comparison of GUS activities under the two conditions may be difficult. Expressions were determined by using plasmid-borne *PcasA–gusA* (pFAJ1842) and *PcasR–gusA* (pFAJ1843) fusions and chromosomally located casA: gusA (FAJ1806 and FAJ1807) and casR: gusA (FAJ1809) fusions. β-Glucuronidase activities are expressed in Miller units. Values are the mean ± SD (*n* = 3).

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**Fig. 3.** Microscopic analysis of nodules and infection threads formed by *R. etli* wild-type (A, C, and E) and casA mutant FAJ1805 (B, D, F, and G) strains. (A and B) Toluidine blue stainings of 3-μm-thick sections of *P. vulgaris* nodules. (C–F) Transmission electron micrographs of 3-week-old nodules. (G) GusA staining of FAJ1806 bacteria expressing a casA–gusA fusion inside the infection threads. Black arrowhead, plant plasma membrane; white arrowhead, symbiosome membrane; black arrow, infection thread (IT); b, bacteroid; SS, symbiosome space; v, vesicle.

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Expression Patterns. The expression patterns of casA are consistent with a role during symbiosis. In the absence of the plant, expression of chromosomally integrated as well as plasmid-borne fusions between the casA promoter and gusA (PcasA–gusA) in a wild-type or a casA mutant background was repressed under aerobic or microaerobic (0.3% oxygen) conditions in the absence or presence of nodule extracts (Fig. 2B). Inhibition of casA expression under free-living conditions is relieved by the inactivation of casR, indicating that the expression of casA is negatively controlled by CasR (Fig. 2B). At the moment we cannot exclude that the casA gene could be induced under yet-unknown specific free-living growth conditions.

In contrast, plasmid-borne or chromosomal casA–gusA fusions in a wild-type or a casA mutant background are strongly expressed on the root surface of P. vulgaris plants approximately 1 day after inoculation (not shown). The expression is specific for P. vulgaris plants as no induction of the fusions was observed under similar conditions on Medicago sativa or wheat rootlets. GusA activity of PcasA–gusA was maintained in the infection threads (Fig. 3G) and inside the nodules of P. vulgaris (Fig. 2B).

Under free-living conditions, a plasmid-borne casR–gusA fusion is expressed in the wild type but GusA activity is strongly reduced in the casR mutants. Therefore, expression of casR is positively autoregulated permitting efficient repression of casA in the absence of the plant (Fig. 2C). The expression of casR–gusA was also reduced during symbiosis both in the wild type and in the casR mutant. Likely, under these conditions, the activator function of CasR on casR expression is inhibited. Expression of the casR gene occurred independently of the oxygen tension (results not shown).

Plant root expression of casA occurs independently of nod gene regulation as the activity of a PcasA–gusA (pFAJ1842) fusion is not reduced in a pSym-cured R. etli background whereas, under the same conditions, expression of a PnodA–gusA fusion is reduced to background levels (results not shown). Also, no effect of a mutation in fixL, nifA, and fumR, regulators of symbiotic gene expression in R. etli, on casA expression was observed. In addition, expression of the casA–gusA fusion was not induced by a low oxygen tension as it is the case with nif/fix genes (results not shown).

Calysmin Is a Calcium-Binding and Secreted Protein. The Ca\(^{2+}\)-binding activity of calysmin was demonstrated on overexpressed and purified protein from E. coli after polyacrylamide gel electrophoresis (PAGE) and transfer to a nylon membrane, using a \(^{45}\)Ca\(^{2+}\) overlay technique (22) (Fig. 4). Ca\(^{2+}\)-binding activity of calysmin was not abolished/or could be restored after treatment by several protein denaturing treatments, including boiling, trichloroacetic acid precipitation, and denaturing PAGE. Unexpectedly, when overproduced in an R. etli casR mutant, \(^{45}\)Ca\(^{2+}\)-binding activity was detected only in the growth medium supernatant, indicating that the protein was secreted (Fig. 5B). The same result was obtained when a ruthenium red binding assay was used (Fig. 5C). The identity of the secreted \(^{45}\)Ca\(^{2+}\)-binding protein as calysmin was confirmed by N-terminal sequence analysis. The amino acid sequence obtained, TTISATISSSYSY, corresponds to the predicted N terminus of the protein based on the DNA sequence. Calysmin is therefore secreted from R. etli without cleavage of the casA gene product. Calysmin migrates on PAGE with a molecular mass of approximately 45 kDa, slightly higher than the calculated molecular mass. aberrant migration may be caused by the acidity of the protein as noticed previous for other calmodulin-like proteins. Secretion of calysmin is efficient, because the protein could never be detected inside of cultured cells carrying a casR mutation. In agreement with the expression analysis of casA, no calysmin was secreted from R. etli wild-type cultures. In the mutant strain FAJ1808 (casR mutant derived from FAJ1806),...
the miniTn5 insertion is located in codon 280 in the loop of the sixth calcium-binding site of the casA gene and therefore lacks the 13 C-terminal amino acids found in wild-type calysmin. As a result, the truncated protein is still able to bind calcium and is secreted in the growth medium (Fig. 5D). Overproduction of CasA in free-living _R. etli_ cells does not produce any obvious phenotype. Calysmin protein, purified from the casR mutant FAJ1803 culture supernatant, was not able to activate chicken NAD kinase.

**Discussion**

Two divergently transcribed genes, _casA_ and _casR_, were identified in _R. etli_. Transcription of the _casA_ gene is negatively controlled by the gene product of _casR_. The CasR repressor belongs to the TetR family of regulators. In the case of TetR, a helix–turn–helix motif on the N terminus is responsible for protein binding to an operator sequence in the _tetA_ promoter, which inhibits transcription of the latter gene (27). Recognition of the antibiotic metabolite tetracycline by TetR causes dissociation of the repressor–DNA complex and induces transcription of the _tetA_ gene, conferring tetracycline resistance. Other members of this family, including BarA from _Streptomyces virgineae_ and ArpA from _Streptomyces griseus_, also bind to small effector molecules such as the butyrolactone autoregulators IM-2 and the A-factor. The _casA_ gene is expressed on the roots of _P. vulgaris_ plants, in the infection threads, and inside the bacteroids but not under the free-living conditions tested, suggesting that the inducing compound is plant derived. The expression pattern of _casA_ is not altered in a _pSym_-cured _R. etli_ strain and was not affected by mutations in _nif_ and _fix_ regulatory genes. Therefore, _casA_ regulation occurs independently of known regulatory mechanisms of noduleation and nitrogen fixation and constitutes a type of symbiotic regulation in _R. etli_ that has not been described previously.

The _casA_ mutant was affected in the production of a calmodulin-like protein named calysmin. Reports on this type of protein in prokaryotes are very uncommon, and, to our knowledge, calysmin constitutes the first example of a calmodulin-like protein with canonical EF-hand motifs in a Gram-negative bacterium. In other prokaryotes, proteins with calmodulin-like properties have been reported previously (11, 29). However, the amino acid sequences of the corresponding proteins are still unknown. In the Gram-positive bacterium _Saccharopolyspora erythraea_, a 20-kDa calcium-binding protein, named calerythrin, was previously shown to possess a structural organization similar to calmodulin with four potential helix–loop–helix EF-hand motifs (12). More specifically, the protein belongs to the subfamily of eukaryotic sarcoplasmic Ca\(^{2+}\)-binding proteins and might therefore function as an intracellular calcium buffer (30).

Calysmin belongs to the calmodulin superfamily. Calysmin possesses a three-domain structure similar to the organization of calbindin D\(_{28k}\) and calretinin. Pairs of EF hands in each of the domains of calysmin may interact through short \(\beta\)-strands present in the loops and form a compact structure. Proteins belonging to the calmodulin superfamily are often divided into sensor and buffer proteins. The latter class may act as calcium buffers in the cell and control the concentration of free cytosolic calcium. Some sensor proteins change their conformation upon binding Ca\(^{2+}\) and interact directly with target proteins to modulate their activity. It is presently unclear to which class calysmin belongs.

Other calcium-binding rhizobial proteins have been implicated in symbiosis previously (31, 32). Secretion of the calcium-binding NodO protein requires a specialized secretion system homologous with ABC transporters involved in the secretion RTX proteins (32). _N_-terminal amino acid sequence analysis of calysmin isolated from the culture supernatant indicated that, similarly to NodO, calysmin is secreted without cleavage of an _N_-terminal transit peptide. The mechanism by which calysmin is secreted is presently unknown. However, the observation that calysmin possesses an _N_-terminal region of approximately 100 amino acids that is different from the three other homologous Ca\(^{2+}\)-binding domains suggests that determinants for secretion might be located within the _N_-terminal part of the protein. Also, in mutant FAJ1808, a truncated calysmin protein lacking part of the C terminus is still efficiently secreted.

Calcium has been implicated in a number of symbiotic functions, arguing for an important role of this ion in this compartment (33, 34). Complexation of Ca\(^{2+}\) by calysmin could have a direct or indirect effect on Ca\(^{2+}\)-dependent processes in the plant. The identified gene may therefore constitute a valuable tool to study the communication between plant and bacteria at the late stages of symbiosis.

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