Expression of Different Calmodulin Genes in Bean (*Phaseolus vulgaris* L.): Role of Nod Factor on Calmodulin Gene Regulation

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Three calmodulin (PvCaM-1, PvCaM-2, and PvCaM-3) clones were isolated from a *Phaseolus vulgaris* nodule cDNA library. All clones contain the complete coding region and are 62 to 74% homologous within this region. Compared to plant CaM consensus sequences, PvCaM-2 has a novel tyrosine₁₁₈ residue, representing a putative phosphorylation site. Southern analysis suggested that calmodulin is encoded by a gene family. These three CaM clones are expressed mainly in young tissues and meristems. The expression pattern of PvCaM-2 and PvCaM-3 is almost identical but different from that of PvCaM-1, suggesting that PvCaM-1 is a well-defined CaM gene, whereas PvCaM-2 and PvCaM-3 could be alleles. PvCaM clones are expressed early in nodules, and transcript levels increase from nodule primordia to nodule-like structures induced by the Nod factor. Conversely, in roots, Nod factor lowers mRNA levels of all three PvCaM clones, but especially of PvCaM-1. Inhibition of PvCaM-1 expression also is observed when 2,3,5-triiodobenzoic acid is added and is prevented when roots are treated with indole-3-acetic acid, suggesting that PvCaM-1 regulation is related to the Nod factor inhibition of polar auxin transport. These results could suggest that CaM clones do not participate in the early signaling generated by the Nod factor but do participate in early events of nodule formation.

Additional keywords: auxin transport inhibition, lipochitoooligosaccharide, *Rhizobium etli*.

Plant cells are subjected to a variety of environmental stimuli. They often respond by a rapid and transient increase in the concentration of cytosolic calcium (Ca²⁺). These Ca²⁺ signals modulate cellular processes via high-affinity, Ca²⁺-binding proteins, of which calmodulin is one of the best characterized. Most Ca²⁺-binding proteins contain a common structure motif, the EF hand, which is a helix-loop-helix structure that binds a single Ca²⁺ ion (O'Neil and DeGrado 1990). Calmodulin is composed of 148 amino acids (aa) arranged in two globular domains connected with a long, flexible helix. Each globular domain contains a pair of EF hands. The binding of Ca²⁺

drives a conformational change in the protein. In its Ca²⁺-bound state, calmodulin activates numerous target proteins involved in a variety of cellular processes (Ikura et al. 1992; Snedden and Fromm 1998; Zielinski 1998).

In animal systems, over 25 calmodulin targets have been identified, including kinases, receptors, ion channels, and G-proteins (Zhou and Lewis 1995). In plants, different calmodulin-binding proteins have been isolated. Among them are proteins implicated in cellular metabolism (Baum et al. 1996), phosphorylation (Ramachandiran et al. 1997; Watillon et al. 1993), ion transport (Harper et al. 1998; Malmstrom et al. 1997), cytoskeleton function (Durso and Cyr 1994; Narasimhulu et al. 1997), and DNA binding (Poovahiah and Reddy 1993; Snedden and Fromm 1998; Szymanski et al. 1996).

One of the intriguing properties of calmodulin is that it can bind numerous target proteins with no discernible amino acid sequence motif composing a consensus CaM-binding domain. CaM binds with high affinity to peptides ranging from 17 to 25 aa in length with positive charge characteristics, having a basic amphiphilic α -helical structure. Several reports have documented that Ca²⁺ signals are perceived by CaM acting in concert with its target protein (Line and Forsen 1995; Olwin and Storm 1985). Enhanced binding of Ca²⁺ by CaM also is observed in the presence of peptides composing the CaM binding domain of target proteins (Haiech et al. 1991). Independent of the mechanism by which CaM binds target proteins, the complex interplay between Ca²⁺, CaM, and target proteins can account for a significant portion of the diversity attributed to CaM-mediated signal transduction.

In addition, the calmodulin multigene family, composed of several genes, has been described in many plant species. In wheat, seven genes encode the conserved calmodulin TaCaM-I and two other genes encode TaCaM-II, which differs from TaCaM-I in just two conserved amino acid substitution (Yang et al. 1998). In *Arabidopsis* spp., four CaM isoforms that differ from one another by as many as six amino acid substitutions were found (Gawienowski et al. 1993). It also was shown that the mRNAs encoding these CaM proteins are differentially expressed, suggesting a particular role for each isoform. In this respect, CaM mRNAs are known to be developmentally regulated and signal responsive (Botella and Arteca 1994; Braam and Davis 1990; Chen et al. 1994; Ling and Assmann 1992; Schuurings et al. 1996; Stinemetz et al. 1987).

The interaction between legume plants and rhizobia lead to the formation of a new plant organ (the nodule), where nitrogen fixation takes place. After plant inoculation with *Rhizobium* spp., root hair deformation occurs. The root hair tip curls upon itself; within these curled root hairs, rhizobia promote the

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formation of infection threads, which allows the invasion of the bacteria into the cortex of the root and, ultimately, the release of bacteria into membrane-enclosed compartments within the cells of the nodule. The inner cortical cell of the root immediately below the infection thread divided, eventually forming a nodule primordium (Mylona et al. 1995). During the initial step of the interaction, root-secreted flavonoids induce the expression of rhizobial nodulation genes (nod genes). The nod genes encode enzymes involved in the synthesis of lipochitooligosaccharide molecules, also termed Nod factor (Dénarié et al. 1996). Nod factors are excreted by the rhizobia and trigger root hair deformation, preinfection thread formation, cortical cell division, and nodule formation (Lhuissier et al. 2001; Reli et al. 1994). The first event, occurring a few seconds after Nod

factor application, is a Ca^{2+} influx leading to an increase in the cytosolic Ca^{2+} concentration (Cárdenas et al. 1998; Felle et al. 1998), which has been related to tip growth in root hairs (Erhardt et al. 1992, 1996; Lhuissier et al. 2001; Wymer et al. 1997). Together with Ca^{2+} influx, Nod factors promote a reduction in the polar auxin transport, creating a change in plant hormone physiology that precedes the formation of nodule primordia (Hirsch and Fang 1994; Hirsch et al. 1997; Mathesius et al. 1998). As well as Nod factors, direct application of cytokinins or polar auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and N-(1-naphthyl)phthalamic acid induces nodulelike structures on *Macroptillum atropurpureum* (Reli et al. 1993) and *Medicago sativa* roots (Hirsch et al. 1989). In this way, Nod factor, as well as increases in the cytokinin–auxin balance, have

A	
PvCaM-1	MADQLTDEQISEFKEAFSLF DKDGDGCITTKE LGTVMRSLGQNPTAEALQ 49
PvCaM-2	
PvCaM-3	 A
	* * * * *
PvCaM-2	DMINEV DADGNGTIDFPE FLNLMARKMKDTSDEEELKEAFRVE DKDQNGF 99
PvCaM-2	
PvCaM-3	
	* * * * *
PvCaM-3	ISAAE LRHVMTNLGEKLTDEEVDEMIREA DVDGDGQINYE EFVKVMMAK* 148
PvCaM-2	 Y
PvCaM-3	
B	
ALFALFA	MADQLTDEQI SEFKEAFSLF DKDGDGCITT KELGTVMRSE GQNPTAEALQ 49
SOYBEAN	 49
PvCaM-1	 49
PvCaM-2	 49
PvCaM-3	 A 49
RICE	D...A..... 49
Acam-6	D..... 49
CHICKEN	E...A.....T... 49
ALFALFA	DMINEVDADG NGTIDFPEFL NLMARKMKDT DSEEELKEAF RVFDKDQNGP 99
SOYBEAN	 99
PvCaM-1	 99
PvCaM-2	 99
PvCaM-3	 99
RICE	E..... 99
ACaM-6	 99
CHICKEN	TM.....IR...G... 99
ALFALFA	ISAAELRHVM TNLGEKLTDE EVDEMIREAD VDG DGQINYE EFVKVMMAK* 148
SOYBEAN	 148
PvCaM-1	 148
PvCaM-2	 Y 148
PvCaM-3	 148
RICE	 148
ACaM-6	S..... 148
CHICKEN	Y.....I.....V...QM.T. 148

Fig. 1. Alignment of the predicted amino acid sequences of the PvCaM clones. Comparison of amino acid sequences deduced for the three *Phaseolus vulgaris* CaM clones and those of CaM from other plants and animals. **A**, Boxed residues in CaM-2 and CaM-3 isoforms indicate the two amino acid differences compared with the same position at the CaM-1 amino acid sequence. Shaded areas mark the putative EF-hand domains. Asterisks indicate the position of amino acids involved in Ca^{2+} binding. **B**, Comparison of the deduced amino acid sequences of *P. vulgaris* calmodulins with those of calmodulin of alfalfa (Barnett and Long 1990), soybean (SCAM-1) (Hyoung et al. 1995), *Arabidopsis* (AcaM-6) (Gawienowski et al. 1993), rice (Liu et al. 1993), and chicken (Putkey et al. 1985). Asterisks indicate the position of the stop codons. Dots represent identical amino acid residues.

the same effect on the formation of “pseudo-nodules.” Although not produced by plants, Nod factors are in fact “hormonelike” substances.

In this report, we present the characterization of three calmodulin clones isolated from a cDNA library that are differentially expressed in bean tissues and during nodule development. Particularly, PvCaM-1 transcript is lowered by the Nod factor. The results presented here suggest that regulation of PvCaM-1 is due to the polar auxin transport inhibition caused by the Nod factor.

RESULTS

Characterization of calmodulin cDNA clones.

Three cDNA clones, PvCaM-1, PvCaM-2, and PvCaM-3, containing the complete CaM coding region were isolated from a nodule library and studied. Nucleotide sequences of the three *Phaseolus vulgaris* CaM shows that PvCaM-1 has 858 bp and contains a 5' untranslated sequence of 93 bp and its 3' untranslated region is 315 bp long. PvCaM-2 is 852 bp with a 38-bp 5' untranslated region and a 364-bp 3' untranslated region. PvCaM-3 is 822 bp long with a 19-bp 5' untranslated region and a 3' untranslated region of 353 bp. All three cDNA

inserts contain an open reading frame of 444 bp that encodes a 148-aa polypeptide. As with other plant CaMs, the nucleotide sequence of these clones revealed that there is no consensus polyadenylation signal at the 3' end (Botella and Arteca 1994). The observed differences in nucleotide sequence among these three CaM cDNAs were found dispersed throughout the entire coding region as well as within the untranslated region (data not shown). These results suggest that the three CaM cDNA clones could represent three different CaM genes in *P. vulgaris*. The deduced amino acid sequence of PvCaM-1 is identical to that of alfalfa CaM (Barnett and Long 1990); therefore, we used this clone as a reference to compare the amino acid sequences of PvCaM-2, PvCaM-3, and other calmodulins already reported (Fig. 1A and B). The deduced amino acid sequence of PvCaM-2 shows a nonconservative and unique plant tyrosine (Tyr) substitution at position 118. This substitution of a Tyr for an Asp lies between the third and fourth Ca²⁺-binding domain. Two Tyr residues have been found in CaM amino acid sequences reported for different species; however, none of these Tyr residues is at position 118 (Fig. 1B) (Hyoung et al. 1995; Lagace et al. 1983; Marshak et al. 1984; Poovaiah and Reddy 1993; Vanaman 1980). The deduced amino acid sequence of PvCaM-3 contains a nonconservative alanine (Ala) for serine (Ser) substitution at position 10 with respect to the PvCaM-1 and the alfalfa amino acid sequences (Fig. 1B). The presence of Ala at this position is well represented in CaM polypeptides from vertebrates, invertebrates, and unicellular eukaryotes as well as in potato (Takezawa et al. 1995), barley (Zielinski 1987), and rice (Liu et al. 1993).

CaM genomic DNA organization in bean.

To obtain more information on the genomic organization of the three CaM clones, *P. vulgaris* DNA was digested with different restriction enzymes and analyzed by Southern blot hybridization using the PvCaM-1 coding region as a common probe and the specific probes for each CaM clone (Fig. 2). When the PvCaM-1 coding region was used as a probe, at least two different-sized restriction fragments were detected in each one of the DNA digests. Minor bands were also observed in *EcoRI*, *HindIII*, and *SauI* digests but not in the *PstI* digest (Fig. 2A). When specific probes were used to hybridize *HindIII*-digested DNA, the PvCaM-1-specific probe showed a single restriction fragment (5.3 kb) (Fig. 2B, white arrowhead) that was not detected with either PvCaM-2- or PvCaM-3-specific probes. When *HindIII*-digested DNA was hybridized with PvCaM-2- or with PvCaM-3-specific probes, three common fragments of 0.43, 2.3, and 3.5 kb were detected (Fig. 2B, black arrowheads). Another 15.8-kb fragment was detected only when a PvCaM-2-specific probe was used (Fig. 2B, dash arrowhead). These results indicate that the PvCaM-1 clone represents a single member of a CaM gene family and suggest that PvCaM-2 and PvCaM-3 represent either two closely related genes or two alleles.

Further genomic characterization of these CaM clones was carried out by amplification of the intron regions from total DNA, using specific primers. A 1.4-kbp fragment was amplified for PvCaM-1 and a 2.0-kbp fragment was amplified for PvCaM-2 and PvCaM-3. Considering the corresponding position of the specific primers in its cDNA clones, the data obtained indicate that calmodulin gene *PvCaM-1* contains a 940-bp intron and that *PvCaM-2* and *PvCaM-3* introns are 1,212 and 1,375 bp long, respectively (data not shown).

Similar results have been published for rice, where CaM is also coded by a multigene family. Rice CaM-1 and CaM-2 genes contain a single 1,400- and a 300-bp-long intron, respectively (Poovaiah and Reddy 1993).

Fig. 2. Genomic Southern analysis of bean calmodulin gene family. *Phaseolus vulgaris* DNA was digested, size-fractionated on a 0.8% agarose gel, blotted onto a nylon membrane, and probed with **A**, PvCaM-1 entire coding region or **B**, PvCaM-1-, PvCaM-2-, and PvCaM-3-specific probes. Arrowheads indicate the different bands recognized by the PvCaM specific probes. E = *EcoRI*, H = *HindIII*, P = *PstI*, S = *Sau3A*, and ER = *EcoRV*.

Calmodulin protein in bean tissues.

CaM protein levels were determined in the stem, root, and nodule at 12, 15, and 25 days after inoculation. As shown in Figure 3, calmodulin protein is abundant in nodules at 15 and 25 days but is very sparse in stems, roots, and 12-day nodules. Additionally, in these later three tissues, CaM protein electrophoretic migration is slower than that for 15- and 25-day nodules. To explain this difference in electrophoretic mobility, purified CaM from spinach (S-CaM) and from PvCaM-1, PvCaM-2, and PvCaM-3 expressed in *Escherichia coli* were incubated in the presence of and in the absence of Ca^{2+} (eliminated with EGTA). Our results indicate that the lighter protein band corresponds to Ca^{2+} -bound CaM and that the heavier band corresponds to Ca^{2+} -free CaM (Fig. 3). These results indicate, first, that the amount of CaM protein is higher in mature nodules than in young nodules and, second that, considering the low amount of CaM protein in other tissues, CaM could be a nodule-enhanced protein. Previous works indicated that the amount of CaM protein is higher in nodules than in roots (Jablonsky et al. 1991). Two protein bands can be detected in stems and roots (Fig. 3), suggesting that bean calmodulin may be polyubiquitinated in these tissues, as has been reported earlier (Ziengenhagen and Jenissen 1990). Protein blotting was carried out with anti-CaM monoclonal antibodies; therefore, a nonspecific immunoreactivity is unlikely.

CaM expression in bean tissues.

To determine whether these three *P. vulgaris* CaM clones are differentially expressed in plant tissues and during nodule development, RNA isolated from the cotyledons, stem, leaves, roots, root apex, and nodules was hybridized with specific *P. vulgaris* CaM probes. A single band of approximately 1.2 kbp was detected on autoradiograms, corresponding to the predicted size of CaM mRNA. *PvCaM-1* was expressed in young roots, root apex, and cotyledons but was not detected in either stems or leaves (Fig. 4A and B). During nodule development, *PvCaM-1* transcripts reached maximal expression at days 15 and 18 and declined by day 21 (Fig. 4C). *PvCaM-2* and *PvCaM-3* expression pattern was the same in all tissues tested; however, *PvCaM-2* transcripts seemed to be the most abundant. Both transcripts were expressed in almost all tissues tested except in mature stems (Fig. 4). *PvCaM-2* and *PvCaM-3* transcript levels were higher in root apex than in other tissues (Fig. 4A). In nodules, both transcripts declined gradually from day 13 to 21 after inoculation (Fig. 4C). *PvCaM-2* and *PvCaM-3* expression occurred early, during nodule development, because their transcript levels were high at 11 days after inoculation, similar to that seen with early nodulin ENod40. However, ENod40 mRNA levels did not decline, remaining almost constant until day 18 after inoculation (Fig. 4D).

Effect of Nod factor on the expression of PvCaM clones.

As mentioned above, Nod factor promotes nodule formation stimulating cell division on root cortex. To determine whether nodule CaM participates in the initial signaling of nodule formation, CaM transcript levels were examined in roots exposed to Nod factor. As shown in Figure 5, expression of *PvCaM-1* in roots was clearly inhibited by Nod factor after 48 and 96 hr, and the expression of *PvCaM-2* and *PvCaM-3* also was reduced, but to a much lesser extent. Conversely, ENod40 expression was highly increased by the presence of Nod factor (mainly after 48 hr) (Fig. 5). These results suggest that *PvCaM-1* does not participate in the initial events signaled by the Nod factor. A common feature between the *PvCaM* clones and ENod40 is that their expression increased significantly from nodule primordia to nodulelike structure (Fig. 5A and B). However, ENod40 expression was higher than that of *PvCaM* clones in nodule primordia. These data suggest a role of these genes in nodule development.

Polar auxin transport and PvCaM-1 expression.

Experiments were done to explain the Nod factor inhibition of *PvCaM-1* expression. Taking into account that Nod factor reduces auxin transport capacity (Boot et al. 1999; Hirsh et al. 1989; Mathesius et al. 1998) and that an increase in auxin levels increases CaM expression (Yang and Poovaiah 2000), bean roots were cotreated with the Nod factor and indole acetic acid and with a polar auxin transport inhibitor (TIBA) alone or in the presence of indole-3-acetic acid (IAA). Our results demonstrated that the Nod factor, as well as TIBA, inhibits CaM-1 expression (Fig. 6, lanes 2 and 4). This inhibition was prevented when IAA is present (Fig. 6, lanes 3 and 5). These results suggest that the regulation of CaM-1 by the Nod factor is mediated by auxins.

DISCUSSION

Calmodulin cDNA clones and CaM genomic organization in beans.

Three complete CaM clones from a cDNA library derived from 15-day-old root nodules were characterized. The nucleotide sequence comparison at the coding region shows 62 to 74% homology and, at the 3' untranslated region, an identity of 69 to 77%. The differences in nucleotide sequence among the three CaM-cDNAs are distributed throughout the coding regions as well as in the 5' and 3' untranslated regions of the clones (data not shown).

Southern blot analysis (Fig. 2) suggests that nodule CaM cDNAs are derived from different genomic DNA sequences and that calmodulin is encoded by a gene family in beans. These data are in agreement with the finding that CaM is encoded by a gene family in *Arabidopsis* spp. (Gawienowski et al. 1993), po-

Fig. 3. A, Immunoblot detection of calmodulin in different plant tissues. R = root; S = stem; N-12, N-15, and N-25 = nodules 12, 15, and 25 days postinoculation. **B,** Analysis of Ca^{2+} -mediated electrophoretic mobility shifts of purified proteins from PvCaM-1, PvCaM-2, and PvCaM-3 expressed in *Escherichia coli*. Pure spinach calmodulin (S-CaM) was used as control.

tato (Takezawa et al. 1995), and *Vigna radiata* (Botella and Arteca 1994). Hybridization analysis of *Hind*III-digested DNA using PvCaM-specific probes suggests that PvCaM-1 is a calmodulin gene well distinct from *PvCaM-2* and *PvCaM-3*, because both *PvCaM-2*- and *PvCaM-3*-specific probes detected three common fragments that were not detected with *PvCaM-1* and because the *PvCaM-1*-specific probe detected a single fragment that is not detected with either a *PvCaM-2* or a *PvCaM-3* probe. A *PvCaM-2* probe detected a 15.8-kb fragment that is not detected with a *PvCaM-3* probe; therefore, these two clones could represent two closely related genes. Moreover, genomic characterization shows that the *PvCaM-1* gene has an intron smaller than that observed for *PvCaM-2* and *PvCaM-3* (data not shown). Preliminary nucleotide sequences of *PvCaM-2* and *PvCaM-3* introns indicated that they are different (data not shown). However, the expression patterns of *PvCaM-2* and *PvCaM-3* are almost identical in different tissues and during nodule development; therefore, we believe that more studies are needed to ascertain whether *PvCaM-2* and *PvCaM-3* represent two different genes or are alleles.

Calmodulin amino acid sequences.

Regarding the Ala/Ser substitution at position 10, the *PvCaM-3* clone seems to be functionally conservative

(Creighton 1993). However, Tyr at position 118 in the *PvCaM-2* clone is a unique substitution that has not been described in plant CaM. This change lies between the third and fourth Ca^{2+} -binding domains and may have important physiological effects. After Ca^{2+} binding, a transition between two stable conformational states of CaM occurs, affecting the environment around Tyr 138 and trimethyllysine at position 115. This change increases the α -helix content of the protein. These two residues are conserved in plants calmodulin (Roberts and Harmon 1992); therefore, the presence of Tyr 118 close to trimethyllysine 115 and Tyr 138 could be involved in these conformational changes in *PvCaM-2*. Also, Tyr 118 could represent a putative phosphorylation site. The presence of two acidic residues (glutamic acid) at position P+1 and P+2 and a hydrophobic residue (valine) at position P+3 of Tyr 118 in clone *PvCaM-2* (Fig. 1) represents two of the most important features of cytosolic protein tyrosine kinase phosphorylation sites (Zhou and Lewis 1995). More studies need to be done in order to understand the physiological meaning of these amino acid substitutions.

Calmodulin expression in bean tissues.

Northern analysis carried out using *PvCaM*-specific probes (Fig. 4A and B) showed that in all tissue tested, *PvCaM-2* and

Fig. 4. Calmodulin and Enod40 mRNA levels in different tissues of *Phaseolus vulgaris*. Total RNA was separated in formaldehyde/agarose gels, blotted onto a nylon membrane, and hybridized with ^{32}P -labeled *pVcaM-1*-, *pVcaM-2*-, and *pVcaM-3*-specific probes. Transcript levels of CaM in **A**, young tissues: stems (S), leaves (L), roots (R), and root apex (Ra); **B**, mature tissues: cotyledons (C), stems (S), and leaves (L); and **C** and **D**, nodules from 11 to 21 days postinoculation (10 to 15 μg loaded per lane in independent experiments).

PvCaM-3 display a very similar expression pattern that is different from *PvCaM-1*, supporting the idea that *PvCaM-2* and *PvCaM-3* could be alleles and that *PvCaM-1* represents a different gene. In general, the main expression of these three *PvCaM* clones was detected in young tissues and meristems. This expression pattern is similar to that reported for other plant CaM genes (Allan and Trewavas 1985; Dauwalder et al. 1986; Ling and Assman 1992; Yang et al. 1998). It is important to note that, as in other plant species, these three *PvCaM* clones were found to display a very high expression in the root apex, which is considered a perception organ (Lu and Feldman 1997; Stinemetz et al. 1987). *PvCaM-1* expression was not detected in stem and leaves, as reported for the wheat calmodulin SF-1 clone (Yang et al. 1998).

Calmodulin expression during nodule development.

In bean nodules, all three CaM clones were expressed at all the times examined. The higher levels of *PvCaM-2* and *PvCaM-3* transcripts were detected starting on day 11 and of *PvCaM-1* at days 15 and 18 after inoculation. The higher expression of *PvCaM-1* at this time could suggest a metabolic role of this CaM, because increased nitrogenase activity, carbon metabolism, and ammonia assimilation and transport occurs during this period. The presence of noduline 26 (Weaver et al. 1991) and glutamic acid decarboxylase (GAD), which are Ca^{2+} (calmodulin)-regulated enzymes, and the accumulation of γ -Amino-n-butyric acid in nodules (Ling et al. 1994; Satnarayan and Nair 1990) supports this idea. However, our results show a discrepancy between the amount of CaM protein and the transcript levels during nodule development. Protein concentration increased from day 12 to day 21 and up to day 25 (Fig. 3), while all *PvCaM* transcripts decreased by day 21 after inoculation (Fig. 4). This CaM protein accumulation in mature nodules could suggest that the main role of CaM is related to nodule function instead of nodule development. However, specific anti-

bodies raised against each one of the three CaM isoforms need to be obtained in order to define if this CaM protein accumulation is due to all CaM isoforms or to a specific one, allowing us to further study CaM function during symbiosis.

Calmodulin expression in the presence of Nod factor.

PvCaM transcripts also were detected in nodule primordia and, in higher levels, in the nodulelike structure induced by Nod factor. Interestingly, CaM expression in nodule primordia is very low compared with the CaM transcript levels detected in root apex (Fig. 5), suggesting that *PvCaM* genes are differentially expressed in these two types of meristematic tissues. A clear reduction in the expression of *PvCaM-1* and, to a lesser extent, of *PvCaM-2* and *PvCaM-3* was promoted by the Nod factor (Fig. 5). As is shown in Figure 6, reduction of *PvCaM-1* transcript induced by the Nod factor also is observed when roots are treated with TIBA but is prevented when either Nod factor- or TIBA-treated roots are in the presence of IAA. With these results, we can conclude that Nod factor negative regulation of *PvCaM-1* gene expression is mediated by auxin. Also, our results suggest that CaM does not participate in the initial events of the symbiotic process but does participate in early events of nodule formation. More studies need to be done in order to understand if the early Nod factor negative regulation of CaM-1 gene expression is a key factor that allows nodule development.

MATERIALS AND METHODS

Plant material.

P. vulgaris L. (cv. Negro Jamapa) seeds were surface sterilized and germinated for 2 days before inoculation and were sown in pots with vermiculite. Seedlings were inoculated with *Rhizobium leguminosarum* bv. *phaseoli* strain CNPAF-512 (Hungria et al. 1991). Plants were grown and maintained under

Fig. 5. Effect of Nod factors on CaM and Enod40 mRNA levels. **A**, Total mRNA from roots grown in the absence (C) and presence (Nf) of *Rhizobium etli*-purified Nod factor and from nodule primordia (Np) and nodulelike structures (NI) induced by the Nod factor was hybridized with ^{32}P -labeled *PvCaM-1*-, *PvCaM-2*-, and *PvCaM-3*-specific probes. **B**, Nodule primordia (a) and nodulelike (b) structures were completely absent in the control roots (c). LR = lateral root; rRNA-EtBr = ethidium bromide-stained rRNA before transfer to nylon membrane, indicating the relative amount of RNA analyzed.

greenhouse conditions as previously described (Lara et al. 1984). Nodules were harvested from day 11 after *Rhizobium* inoculation onward and were immediately frozen in liquid nitrogen.

Isolation of *P. vulgaris* CaM-cDNA clones.

Using a λ gt-22a cDNA library derived from *P. vulgaris* nodules (provided by Dr. Carroll Vance, University of Minnesota, St. Paul), the CaM coding region probe was generated by polymerase chain reaction (PCR). Primers were designed from CaM consensus sequence in different plant species. The sequences are 5'-dATGCGCGATCAGCTCACCGACGATCAGATC-3' and 3'-dCAAGCAGTTTCAGTACTACCGGTTCACTC-5'. PCR protocol was performed as described by Friedman and associates (1990). Samples were heated to 94°C, kept for 4 min at this temperature, and then subjected to 35 cycles of the following sequence: 1 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C. Subsequently, they were subjected for 5.0 min to 72°C and stored at 4°C until use. Amplified product was separated by agarose gel electrophoresis, purified (GeneClean II Kit; BIO 101, Inc., Vista, CA, U.S.A.), and used as a probe for the λ Zap cDNA library screening.

A λ Zap cDNA library derived from 15-day-old bean nodule mRNA was used to isolate bean calmodulin clones. Plaque hybridization was performed at 42°C. Filters were washed in 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) and in 1 $\times$ SSC, 0.1% SDS at 65°C. The filters were rinsed and exposed to a Kodak X-ray film. Three positive hybridizing clones (PvCaM-1, PvCaM-2, and PvCaM-3) were purified by three rounds of plaque hybridization.

Characterization of *P. vulgaris* CaM-cDNA clones.

cDNA inserts from positively hybridizing phage were excised in the pbluescript SK⁺ plasmid (Stratagene, La Jolla, CA, U.S.A.) and transformed into "SOLR" strain (Stratagene). The pbluescript SK⁺ recombinant plasmid was purified and digested with *Pst*I and *Xho*I restriction enzymes. Inserts were subjected to electrophoresis in 1% agarose gels, blotted to Hybond-N⁺ filters (Amersham Pharmacia Biotech, Buckinghamshire, U.K.), and hybridized with a ³²P-labeled CaM coding region probe. The filter was washed progressively with: 2 $\times$ SSC, 0.1% SDS; 1 $\times$ SSC, 0.1% SDS; and 0.1 $\times$ SSC, 0.1% SDS. All washes were carried out at 65°C. The filter was exposed to Kodak X-ray film at -70°C.

Sequence analysis.

DNA sequencing was performed by the dideoxy chain termination method using Sequenase (ver. 2.0 DNA sequencing

kit; Amersham Pharmacia Biotech) and (³⁵S)-ATP with double stranded plasmid DNA template. Both strands were sequenced using T₇ and T₃ universal primers.

Genomic southern analysis.

Total DNA was isolated from *P. vulgaris* leaves, using the cetyltrimethylammonium bromide procedure as described by Murray and Thompson (1980). Final yield was 250 μ g of total DNA per g of tissue. Total DNA (10 μ g) was electrophoresed, digested with different restriction enzymes, blotted onto a nylon membrane, and then, hybridized using the PvCaM-1 coding region as a common probe and against specific probes for each of the three PvCaM clones. To obtain clone-specific probes, cDNAs oligonucleotide primers were synthesized, enabling the recovery of the 3' untranslated regions by PCR. A common sense primer anchored in the carboxyl terminus of the coding region was used in conjunction with clone-specific antisense primers for each one of the CaM clones. PCR fragments are 258, 369, and 205 bp long for PvCaM-1, PvCaM-2, and PvCaM-3, respectively. These PCR-generated fragments do not cross-hybridize on DNA blots at high stringency (data not shown). Genomic hybridization was carried out at 50°C for 16 hr in a solution containing 0.3 M NaH₂PO₄, pH 7.2, 10 mM EDTA, and 7% (wt/vol) SDS with ³²P-labeled PvCaM-1 and PvCaM-1-, PvCaM-2-, and PvCaM-3-specific probes. The membranes were washed at 55°C with 2 $\times$ SSC, 0.1% SDS, and exposed to Kodak film at -70°C.

CaM introns analysis.

Total DNA (50 ng) was used to perform a genomic PCR analysis using the CaM primers described above. PCR program was as follows: denaturing step at 94°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 2.0 min, with a final extension segment for 5.0 min at 72°C. The reaction was carried out for a total of 35 cycles. Following chloroform extraction, the reaction products were fractionated by electrophoresis in 1% (wt/vol) agarose gel.

Western blot analysis of *P. vulgaris* calmodulin.

Total protein of nodule and other plant tissues was extracted with three volumes (wt/vol) of extraction buffer (50 mM HEPES, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, 5% ethylenglycol, pH 7.8). The homogenate was then centrifuged (20 min, 20,000 $\times$ g) and proteins in the supernatant were solubilized in Laemmli buffer (Laemmli 1970). Samples of 10 μ g of protein were analyzed in 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels.

Fig. 6. Northern blot analysis showing the effect of Nod factor or 2,3,5-triiodobenzoic acid (TIBA), alone or in combination with indole-3-acetic acid (IAA), on the expression of PvCaM-1 gene. Total mRNA from root segments was isolated at 48 hr after each treatment. A ³²P-labeled PvCaM-1 clone-specific probe was used for hybridization. Lane C shows mRNA from roots grown in the absence of the Nod factor. Nf and TIBA indicate the presence of the Nod factor or TIBA, with and without IAA. Hybridization against a 28S-rRNA probe was used as an RNA loading control.

Proteins were transferred to nitrocellulose filter (Amersham) and Western blots were performed using commercial rabbit anti-CaM monoclonal antibodies raised against *Dictyostelium discoideum* (Sigma C-7055, Sigma-Aldrich Co., Milwaukee, WI, U.S.A.) as the primary antibody. A peroxidase-linked mouse antirabbit antibody was used as the secondary antibody and the peroxidase color reaction was developed following Sigma's instruction.

SDS-PAGE mobility shift assay of purified bean CaM.

The coding region of the *P. vulgaris* CaM cDNA clones were subcloned into the expression vector PQE-30 (QIAexpress, QIAGEN, Hilden, Germany) and transformed into *E. coli* cells (M-15). Cultures were grown at 37°C until the optical density at 600 nm reached 0.7 before addition of IPTG to a final concentration of 1 mM. After 3.0 hr in the induction medium, in vitro-expressed CaM was purified by affinity chromatography using Ni-NTA resin (QIAexpress) following the manufacturer's instructions. The eluted protein from the resin was resuspended in 50 mM Tris-HCl, pH 7.5, buffer and purified CaM was analyzed by 15% SDS-PAGE in the presence of either 2 mM CaCl₂ or 2 mM EGTA.

Nod factor assays.

Two-day-old seedlings were placed in assay tubes containing Fahraeus buffer (Fahraeus 1957). After 2.0 hr, roots were treated with purified *R. etli* Nod factor (Cárdenas et al. 1995, 1998) or TIBA, alone or in combination with IAA, all diluted to a final concentration of 1×10^{-6} M. After eliminating the root apex (3 mm), 2-cm-long root segments were harvested at 48 and 96 hr. Nodule primordia and nodulelike structures were dissected independently. All tissues were stored at -70°C until they were analyzed.

RNA isolation and northern hybridizations.

Total RNA was isolated from 250 mg of cotyledons, roots, stems, leaves, nodule primordia, nodulelike structures, and nodules tissues at different times, as indicated, using a guanidinium thiocyanate procedure (RNA aid plus kit, Bio. 101, Inc.) electrophoresed on formaldehyde-agarose gels and blotted onto Hybond-N⁺ filters according to the manufacturer's instructions. Hybridizations were carried out at 50°C for 16 hr in 0.3 M NaH₂PO₄, pH 7.2, 10 mM EDTA, and 7% (wt/vol) SDS with ³²P-labeled PvCaM-1-, PvCaM-2-, and PvCaM-3-specific probes. 28S-rRNA probe was used as an RNA loading control (Colmenero et al. 1997) All washes were carried out at 65°C.

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