CPP1, a DNA-binding protein involved in the expression of a soybean *leghemoglobin c3* gene

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Nodulin genes are specifically expressed in the nitrogen-fixing root nodules. We have identified a novel type of DNA-binding protein (CPP1) interacting with the promoter of the soybean leghemoglobin gene *Gmlbc3*. The DNA-binding domain of CPP1 contains two similar Cys-rich domains with 9 and 10 Cys, respectively. Genes encoding similar domains have been identified in *Arabidopsis thaliana, Caenorhabditis elegans,* the mouse, and human. The domains also have some homology to a Cys-rich region present in some polycomb proteins. The *cpp1* gene is induced late in nodule development and the expression is confined to the distal part of the central infected tissue of the nodule. A constitutively expressed *cpp1* gene reduces the expression of a *Gmlbc3* promoter–*gusA* reporter construct in *Vicia hirsuta* roots. These data therefore suggest that CPP1 might be involved in the regulation of the *leghemoglobin* genes in the symbiotic root nodule.

The symbiosis between legumes and the nitrogen-fixing *Rhizobia* bacteria results in the formation of a new organ (root nodule) on the roots of the legume. In this organ, the bacteria convert dinitrogen to ammonia. In soybean, it takes 12–14 days from the infection of the root until the nodule is fully functional and nitrogen fixation starts (1). During this time, a number of plant genes are specifically activated (2). Some of these genes are expressed shortly after the infection by the bacteria. The products encoded by these genes, early nodulins, are most likely involved in the formation of the nodule structure. After several days, other proteins (late nodulins) appear which primarily are involved in the metabolic activities in the nitrogen-fixing root nodule. The most predominant late nodulins are the leghemoglobins (Lbs) which constitute about 5% of the total protein content in the mature nodule. Lb facilitates oxygen transport to the respiring bacteria within the central infected zone of the nodule. In soybean, there are four sequentially expressed *lb* genes of which the *Gmlbc3* gene is the first one to be activated (1). The *Gmlbc3* gene transcript was detected in root nodules 8 days after infection and the expression remained low until a dramatic increase in the expression occurred about 12–14 days after infection. The high-level expression of the *lb* genes is confined to the cells in the central infected zone of the nodule.

Only a limited number of transcription factors serving a function in root nodule formation and function have been identified. Two MADS-box-containing genes, *nmh5* and *nmh7,* from *Medicago sativa* are expressed in the root nodules and these two putative transcription factors might therefore be involved in the regulation of nodule-expressed genes (3, 4). Two AT-rich sequence motifs in the soybean *Gmlbc3* promoter interact with a nuclear factor NAT2, present in soybean root nodules (5, 6). NAT2-binding activities are also present in *Sesbania rostrata* nodules, roots, and leaves (7). Binding sites for NAT2-like proteins are also present in the *S. rostrata glbc3* promoter and the promoter of the nodule-enhanced *gln-*γ gene from the French bean (7, 8). Functional studies of the NAT2-binding sites in the soybean *Gmlbc3* gene demonstrated that these sites are general *cis*-elements (9). Thus, NAT2 is most likely a general activator of transcription and is not directly involved in the specific activation of the *lb* genes.

To identify DNA-binding proteins regulating the *lb* genes, a soybean nodule λgt11 cDNA expression library was screened by using oligonucleotides covering the proximal promoter region from the soybean *Gmlbc3* gene as probes. One of the isolated cDNA clones encoded a novel DNA-binding protein (CPP1), which binds to the *Gmlbc3* promoter. In the nodule, *cpp1* and *lb* transcripts appear in the same tissue, but in different locations. A plasmid expressing CPP1 was able to repress the expression of a *Gmlbc3*–*gusA* reporter construct in transgenic vetch roots. These data suggest that CPP1 is able to down-regulate the expression of a *lb* gene. CPP1 contains a region similar to a domain present in some polycomb group proteins which are known to suppress gene expression of developmentally important regulatory genes.

Materials and Methods

Fusion Protein Purification and Plasmid Constructs. A cDNA clone, p*cpp1*$_{418-896}$ encoding amino acids 418–896 was isolated from a soybean nodule cDNA expression library in the same way as *Gmndx* (10). A subfragment of p*cpp1*$_{418-896}$, corresponding to amino acids 456–596 was subcloned into the pGEX-5X-3 expression vector (Amersham Pharmacia). The CPP1 peptide was expressed as a glutathione *S*-transferase fusion protein (11) and the glutathione *S*-transferase domain was removed by using factor Xa.

The full-length *Gmlbc3* promotor used for the *trans*-activation experiment was a 2-kb fragment (12). The *gusA* reporter gene was interrupted by the second intron (IV2) of the potato ST-LSl gene (13). The *CaMV* 35S promoter originated from the −829 promoter (14). The coding region of *cpp1* constituted a fragment from 59 bp upstream to the initiator AUG to 243 bp downstream from the stop codon.

The *gfp*–*cpp1* gene fusion was made in pZP211 (15) by fusing the *CaMV* 35S promoter to the full-length *cpp1* cDNA with an in-frame green fluorescent protein (GFP)-encoding fragment modified from mRS-GFP (16). The control plasmid was identical to the latter plasmid except that the *cpp1* sequence was deleted.

Electrophoretic Mobility Shift Assays. A bacterial-expressed CPP1 peptide (amino acids 456–596) was incubated with a ^{32}P-labeled

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Abbreviations: GFP, green fluorescent protein; GUS, β-glucuronidase; *lb*, *leghemoglobin*.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AJ010165 for cpp1).

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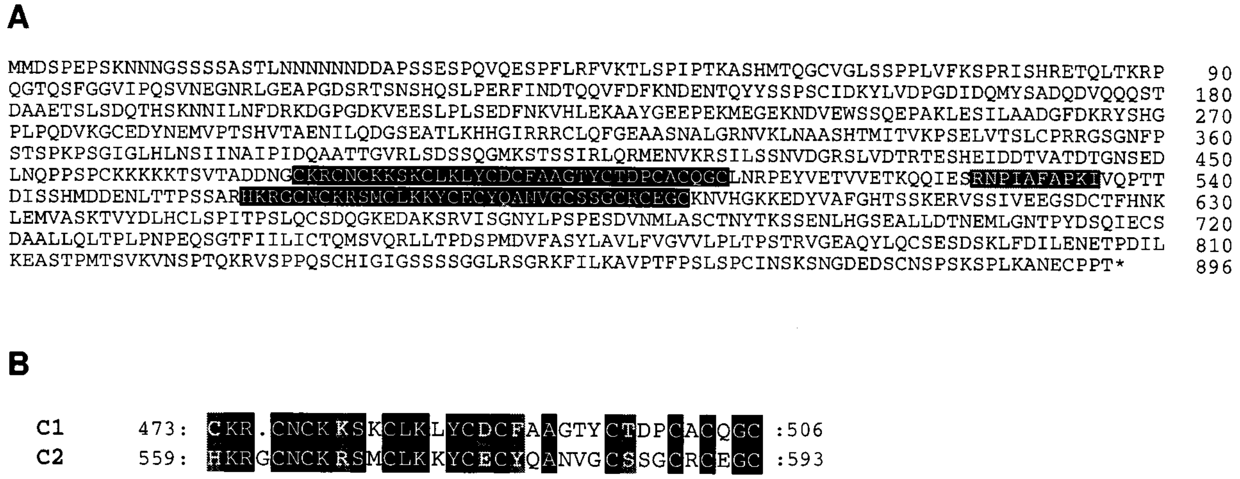

A

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MMDSPEPSKNNNGSSSSASTLNNNNNNDDAPSSESPQVQESPFLRFVKTLSPIPTKASHMTQGCVGLSSPPLVFKSPRISHRETQLTKRP   90
QGTQSFGGVIPQSVNEGNRLGEAPGDSRTSNSHQSLPERFINDTQQVFDFKNDENTQYYSSPSCIDKYLVDPGDIDQMYSADQDVQQQST  180
DAAETSLSDQTHSKNNILNFDRKDGPGDKVEESLPLSEDFNKVHLEKAAYGEEPEKMEGEKNDVEWSSQEPAKLESILAADGFDKRYSHG  270
PLPQDVKGCEDYNEMVPTSHVTAENILQDGSEATLKHHGIRRRCLQFGEAASNALGRNVKLNAASHTMITVKPSELVTSLCPRRGSGNFP  360
STSPKPSGIGLHLNSIINAIPIDQAATTGVRLSDSSQGMKSTSSIRLQRMENVKRSILSSNVDGRSLVDTRTESHEIDDTVATDTGNSED  450
LNQPPSPCKKKKKTSVTADDNGCKRCNCKKSKCLKLYCDCFAAGTYCTDPCACQGCLNRPEYVETVVETKQQIESRNPIAFAPKIVQPTT  540
DISSHMDDENLTTPSSARHKRGCNCKRSMCLKKYCECYQANVGCSSGCRCEGCKNVHGKKEDYVAFGHTSSKERVSSIVEEGSDCTFHNK  630
LEMVASKTVYDLHCLSPITPSLQCSDQGKEDAKSRVISGNYLPSPESDVNMLASCTNYTKSSENLHGSEALLDTNEMLGNTPYDSQIECS  720
DAALLQLTPLPNPEQSGTFIILICTQMSVQRLLTPDSPMDVFASYLAVLFVGVVLPLTPSTRVGEAQYLQCSESDSKLFDILENETPDIL  810
KEASTPMTSVKVNSPTQKRVSPPQSCHIGIGSSSSGGLRSGRKFILKAVPTFPSLSPCINSKSNGDEDSCNSPSKSPLKANECPPT*     896
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B

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C1     473: CKR.CNCKKSKCLKLYCDCFAAGTYCTDPCACQGC :506
C2     559: HKRGCNCKRSMCLKKYCECYQANVGCSSGCRCEGC :593
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Fig. 1. (*A*) Complete aa sequence of CPP1. The two Cys-rich regions are highlighted in black. A conserved aa sequence is highlighted in gray. The numbers refer to the aa numbers starting from the initiator Met. (*B*) Alignment of the two Cys-rich regions (C1 and C2). The positions of the first and last aa are indicated. Identical aa are highlighted in black and similar aa are indicated by a gray background.

Gmlbc3 promoter fragment (−284 to +44) in 20 mM Tris·HCl (pH 7.5)/0.5 mM DTT/0.1% Nonidet P-40/6% glycerol/630 mM KCl, in the presence of 0.5 μg of BSA and 0.5 μg of salmon sperm DNA at 25°C for 60 min. Control DNA fragments: a 368-bp fragment from the pPZP211 vector (GenBank accession no. V10490, nucleotides 8650–0004), and a 188-bp fragment of the CaMV 35S promoter (GenBank accession no. V00141, nucleotides 6,622–6,810). The complexes were separated in a native 4% polyacrylamide gel in 0.1 M Tris-borate buffer (pH 8.2).

RNase Protection. The probe used for the detection of *cpp1* transcripts was obtained from p*cpp1*$_{418-896}$. The ribonuclease protection assay was performed as described (17).

***In Situ* Hybridization.** The plasmid pCPP1$_{418-896}$ was used as a template for runoff transcription. Antisense of *cpp1* was obtained by using a SP6 polymerase after digestion with *Eco*RI. Sense *cpp1* was made by using a T3 polymerase after digestion with *Hin*dIII. The sections were made from 21-day-old soybean nodules. The antisense and sense probes were radioactively labeled with [^{35}S]UTP and degraded to a length of about 150 nt before hybridization (18, 19). Sections were stained with 0.1% toluidine blue and mounted with DPX.

Nuclear Localization of CPP1. A control plasmid expressing only GFP and a plasmid expressing CPP1-GFP was introduced and analyzed in onion epidermal cells (20).

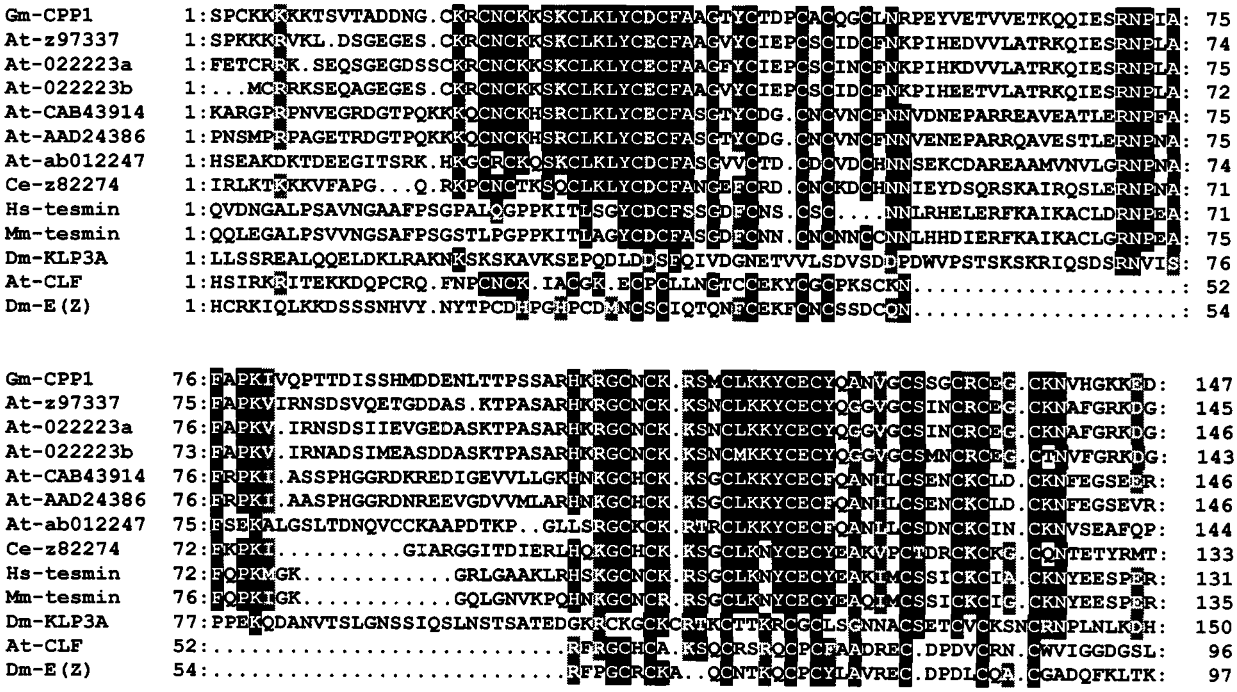
Transformation of *Vicia hirsuta*. The constructs were inserted into the binary vector pPZP211 (15). The vectors were electrotransformed into the *Agrobacterium rhizogenes* strain ARqual, which mediates the generation of transgenic hairy roots (21). To inoculate these roots with *Rhizobium leguminosarum* bv. *viciae*, the wild-type seed and roots were removed from the seedlings and composite plants were subcultured and infected with the bacteria (21). Plant material was cultured at 18°C 16 h light/8 h dark. Inoculated roots harvested 16 days after infection were used for the qualitative histochemical β-glucuronidase assay by using X-gluc and the quantitative fluorometric assay by using MUG as the enzyme substrates (14).

Results

CPP1 Contains Two Cys-Rich Repeats. A *cpp1* cDNA (gmN14) was isolated from a soybean nodule expression library (22). The full-length sequence of *cpp1* was determined from the sequence of the isolated cDNA clone combined with the sequence of a 5′ rapid amplification of cDNA ends product (GenBank accession no. AJ010165). An ORF-encoding 896 aa was identified in *cpp1* (Fig. 1*A*). It is likely that this ORF corresponds to the full-length product encoded by the *cpp1* gene, because an in-frame stop codon is located 27 bases upstream of the potential initiator AUG in the rapid amplification of cDNA ends product.

The encoded aa sequence contains two Cys-rich motifs of 34 and 35 aa located at positions 473–506 (C1) and between 559 and 593 (C2), respectively (Fig. 1*B*). C1 and C2 are 57% identical with the highest degree of similarity in the basic N-terminal region. All nine Cys present in C2 are conserved in C1. C1 has an additional Cys and by introducing a gap of 1 aa, this residue matches a His in C2 (Fig. 1*B*). Six of the Cys form CXC motifs. A homology search in the European Molecular Biology Laboratory database identified six genomic *A. thaliana* sequences on chromosomes 2, 3, and 4, respectively (accession nos. CAB43914, AB012247, AB022223, AAD24386, and Z97337), a genomic sequence from *Caenorhabditis elegans* (accession no. Z82274), *tesmin* genes from mouse and human (accession nos. U86074 and U67176), and a KLP3A-encoding gene from *Drosophila melanogaster* (L19117). In addition, a Cys-rich region present in some polycomb proteins, e.g., CURLY LEAF from *A. thaliana* (accession no. Y10580) and E(z) from *D. melanogaster* (accession no. U00180), showed some similarity to CPP1. An alignment of the CPP1 aa sequence to the indicated sequences showed that the Cys-rich domains are highly conserved in most of the sequences (Fig. 2). The N-terminal part of each Cys-rich motif which consists mainly of basic aa appears to be more conserved than the C-terminal part of the domains. The CPP1 aa sequence, RNPLAFAPK, located between the two Cys-rich domains and a basic region N-terminal to C1, is also conserved between the sequences, except for the polycomb proteins (Fig. 2). In the latter, the region between the Cys motifs appears to be absent. Apart from the conserved regions, no other sequence similarities were detected.

To investigate the DNA-binding properties of CPP1, a peptide containing the two Cys-rich motifs was expressed in *Escherichia coli*. After purifying the protein, an electrophoretic mobility shift assay was performed, by using a probe spanning from −284 to +44 of the *Gmlbc3* promoter. This region contains the important regulatory elements WPE, OSE, and NE (12). Two distinct retarded complexes were observed in the presence of the CPP1



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Gm-CPP1       1:SPCKKKKKTSVTADDNG.CKRCNCKKSKCLKLYCDCFAAGTYCTDPCACQGCLNRPEYVETVVETKQQIESRNPIA: 75
At-z97337     1:SPKKKRVKL.DSGEGES.CKRCNCKKSKCLKLYCECFAAGVYCIEPCSCIDCFNKPIHEDVVLATRKQIESRNPLA: 74
At-022223a    1:FETCRRK.SEQSGEGDSSCKRCNCKKSKCLKLYCECFAAGFYCIEPCSCINCFNKPIHKDVVLATRKQIESRNPLA: 75
At-022223b    1:...MCRRKSEQAGEGES.CKRCNCKKSKCLKLYCECFAAGVYCIEPCSCIDCFNKPIHEETVLATRKQIESRNPLA: 72
At-CAB43914   1:KARGPRPNVEGRDGTPQKKKQCNCKHSRCLKLYCECFASGTYCDG.CNCVNCFNNVDNEPARREAVEATLERNPFA: 75
At-AAD24386   1:PNSMPRPAGETRDGTPQKKKQCNCKHSRCLKLYCECFASGTYCDG.CNCVNCFNNVENEPARRQAVESTLERNPNA: 75
At-ab012247   1:HSEAKDKTDEEGITSRK.HKGCRCKQSKCLKLYCDCFASGVVCTD.CDCVDCHNNSEKCDAREAAMVNVLGRNPNA: 74
Ce-z82274     1:IRLKTKKKVFAPG...Q.RKPCNCTKSQCLKLYCDCFANGEFCRD.CNCKDCHNNIEYDSQRSKAIRQSLERNPNA: 71
Hs-tesmin     1:QVDNGALPSAVNGAAFPSGPALQGPPKITLSGYCDCFSSGDFCNS.CSC....NNLRHELERFKAIKACLDRNPEA: 71
Mm-tesmin     1:QQLEGALPSVVNGSAFPSGSTLPGPPKITLAGYCDCFASGDFCNN.CNCNNCCNNLHHDIERFKAIKACLGRNPEA: 75
Dm-KLP3A      1:LLSSREALQQELDKLRAKNKSKSKAVKSEPQDLDDSEQIVDGNETVVLSDVSDDPDWVPSTSKSKRIQSDSRNVIS: 76
At-CLF        1:HSIRKRITEKKDQPCRQ.FNPCNCK.IACGK.ECPCLLNGTCCEKYCGCPKSCKN......................: 52
Dm-E(Z)       1:HCRKIQLKKDSSSNHVY.NYTPCDHPGHPCDMNCSCIQTQNFCEKFCNCSSDCQN......................: 54

Gm-CPP1      76:FAPKIVQPTTDISSHMDDENLTTPSSARHKRGCNCK.RSMCLKKYCECYQANVGCSSGCRCEG.CKNVHGKKED:  147
At-z97337    75:FAPKVIRNSDSVQETGDDAS.KTPASARHKRGCNCK.KSNCLKKYCECYQGGVGCSINCRCEG.CKNAFGRKDG:  145
At-022223a   76:FAPKV.IRNSDSIIEVGEDASKTPASARHKRGCNCK.KSNCLKKYCECYQGGVGCSINCRCEG.CKNAFGRKDG:  146
At-022223b   73:FAPKV.IRNADSIMEASDDASKTPASARHKRGCNCK.KSNCMKKYCECYQGGVGCSMNCRCEG.CTNVFGRKDG:  143
At-CAB43914  76:FRPKI.ASSPHGGRDKREDIGEVVLLGKHNKGCHCK.KSGCLKKYCECFQANILCSENCKCLD.CKNFEGSEER:  146
At-AAD24386  76:FRPKI.AASPHGGRDNREEVGDVVMLARHNKGCHCK.KSGCLKKYCECFQANILCSENCKCLD.CKNFEGSEVR:  146
At-ab012247  75:FSEKALGSLTDNQVCCKAAPDTKP..GLLSRGCKCK.RTRCLKKYCECFQANLLCSDNCKCIN.CKNVSEAFQP:  144
Ce-z82274    72:FKPKI.........GIARGGITDIERLHQKGCHCK.KSGCLKNYCECYEAKVPCTDRCKCKG.CQNTETYRMT:  133
Hs-tesmin    72:FQPKMGK...........GRLGAAKLRHSKGCNCK.RSGCLKNYCECYEAKIMCSSICKCIA.CKNYEESPER:  131
Mm-tesmin    76:FQPKIGK...........GQLGNVKPQHNKGCNCR.RSGCLKNYCECYEAQIMCSSICKCIG.CKNYEESPER:  135
Dm-KLP3A     77:PPEKQDANVTSLGNSSIQSLNSTSATEDGKRCKGCKCRTKCTTKRCGCLSGNNACSETCVCKSNCRNPLNLKDH:  150
At-CLF       52:...........................RFRGCHCA.KSQCRSRQCPCFAADREC.DPDVCRN.CWVIGGDGSL:   96
Dm-E(Z)      54:...........................RFPGCRCKA..QCNTKQCPCYLAVREC.DPDLCQA.CGADQFKLTK:   97
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Fig. 2. Alignment of the CPP1 aa sequence to the following sequences: Translated genomic DNA sequences from *A. thaliana* and *C. elegans*. The names include the GenBank accession nos. Introns have been excised from the genomic sequences at appropriate positions. Tesmins from human (Hs) and mouse (Mm) and polycomb group proteins from *Drosophila* (Dm) and *Arabidopsis* (At). Identical aa are highlighted in black and similar aa are highlighted in gray.

peptide (Fig. 3, lbc3), whereas control DNA fragments showed no or very little interaction with CPP1 (Fig. 3, pPZP211 and CaMV35S). Thus, the region containing the two Cys-rich motifs, constitutes a DNA-binding domain in CPP1 and the binding appears to require specific DNA motifs.

CPP1 Is Induced Late in Nodule Development. The expression of *cpp1* was studied by RNase protection analysis. Identical amounts of RNA were used for each reaction as measured by an ethidium bromide-stained gel. In root nodules, the *cpp1* transcripts appeared in significant amounts 10 days after the infection, reaching a maximum 3 days later (Fig. 4*A*). Subsequently, the level of the transcripts declines. In root or callus, *cpp1* was expressed at low levels. Thus, the *cpp1* gene is induced late in root nodule development.

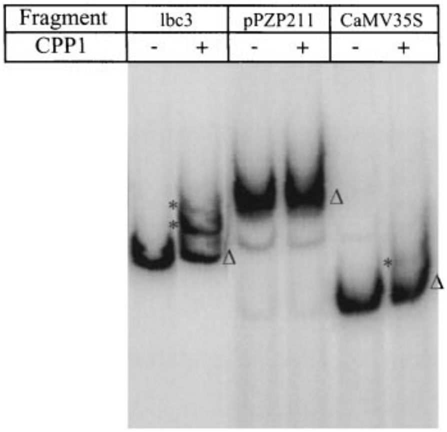

Fig. 3. Binding of a CPP1 subdomain to the *Gmlbc3* promoter. A bacterial-expressed CPP1 peptide (amino acids 456–596) was incubated with a ^{32}P-labeled *Gmlbc3* promoter fragment (lbc3), a DNA fragment from pPZP211 (pPZP211), and a promoter fragment from CaMV35S (CaMV35S), respectively. The complexes were separated in a native 4% polyacrylamide gel. (−) No CPP1 protein. (△) Free fragments and (*) DNA–protein complexes.

The expression of *cpp1* is confined to the infected region of the soybean root nodules as shown by the sensitive epipolarization image of an *in situ* hybridization experiment (Fig. 4*B*). The level of expression is higher in the distal part of the nodule, whereas almost no expression is observed at the base of the nodule. The expression of *Gmlbc3* is similarly restricted to the infected region, but in contrast to *cpp1*, the expression level is low at the tip and high from the center toward the base of the nodule (Fig. 4*D*). The level of *Gmlbc3* expression was very high in comparison to *cpp1* expression and could therefore easily be visualized by bright-field microscopy. In conclusion, the *cpp1* and *Gmlbc3* genes are expressed in the central infected region, but their expression appears to be mutually exclusive in this region.

CPP1 Is Localized in the Nucleus. To investigate the cellular localization of CPP1, the coding region of *cpp1* was fused in-frame to the GFP-coding sequence. The expression of the gene construct was driven by a constitutively expressed *CaMV* 35S promoter. A construct without the *cpp1*-coding region served as a control. The two constructs were delivered into an onion epidermal cell layer by a particle inflow gun, and after an overnight incubation, the cells were inspected by fluorescence microscopy. The control plasmid gave rise to a uniform distribution of the GFP within the cell (Fig. 5 *A* and *B*), whereas the in-frame fusion to *cpp1* resulted in a strictly nuclear localization of the GFP fusion protein (Fig. 5 *C* and *D*). This implies that CPP1 is located in the nucleus.

CPP1 Represses the Expression of a Leghemoglobin Gene in Vetch Roots. CPP1 interacts *in vitro* with a promoter fragment of the *Gmlbc3.* To investigate the function of CPP1, the expression of a *Gmlbc3–gusA* fusion was studied in transgenic *V. hirsuta* roots

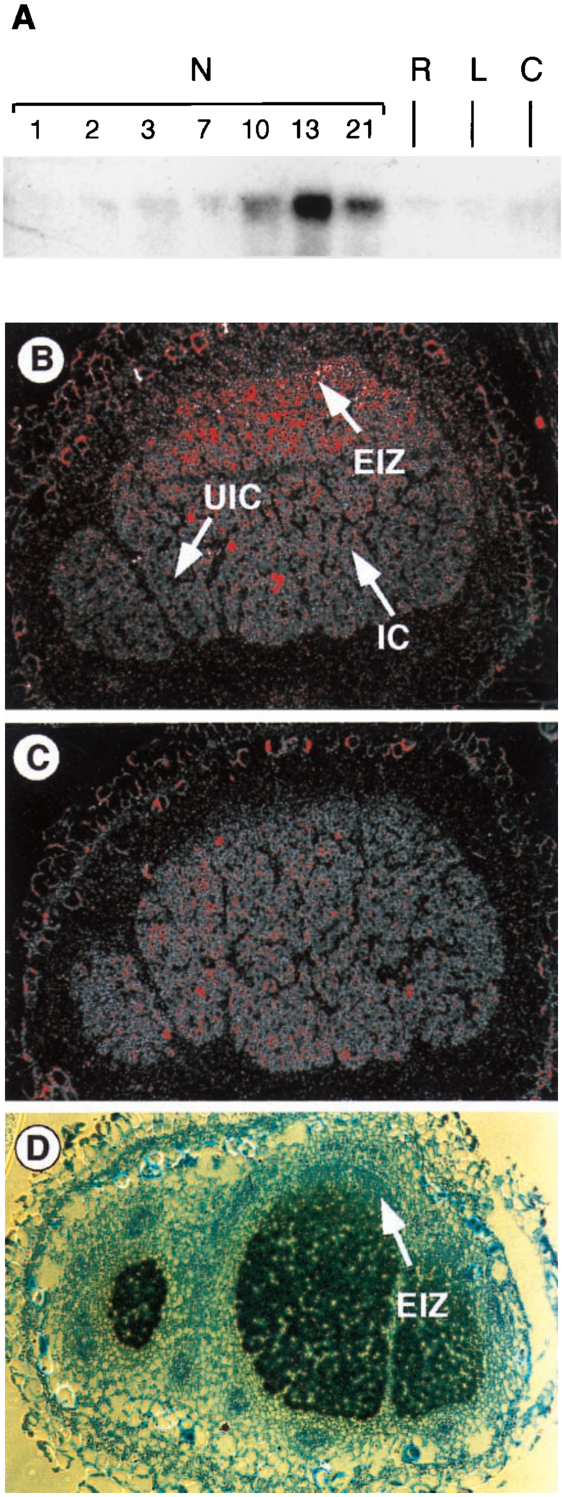

Fig. 4. Expression analysis of *cpp1*. (*A*) RNase protection analysis by using a transcript of *cpp1* corresponding to amino acid numbers 757–811 as a probe. The RNA used in this analysis was isolated from uninfected soybean roots (R), leaves (L), callus (C), and nodules harvested 1–21 days after infection by *Bradyrhizobium japonicus* (N1–N21). (*B*) *In situ* hybridization with *cpp1* antisense, (*C*) *cpp1* sense, and (*D*) *Gmlbc3* antisense transcripts as probes to cross sections of a 13-day-old soybean nodule. Infected cells (IC), uninfected cells (UIC), and early infection zone (EIZ) are indicated. The silver grains were visualized by epipolarization microscopy through a red filter (*B* and *C*) or directly by bright-field microscopy (*D*). The images were adjusted in Adobe PHOTOSHOP to enhance the weak red color.

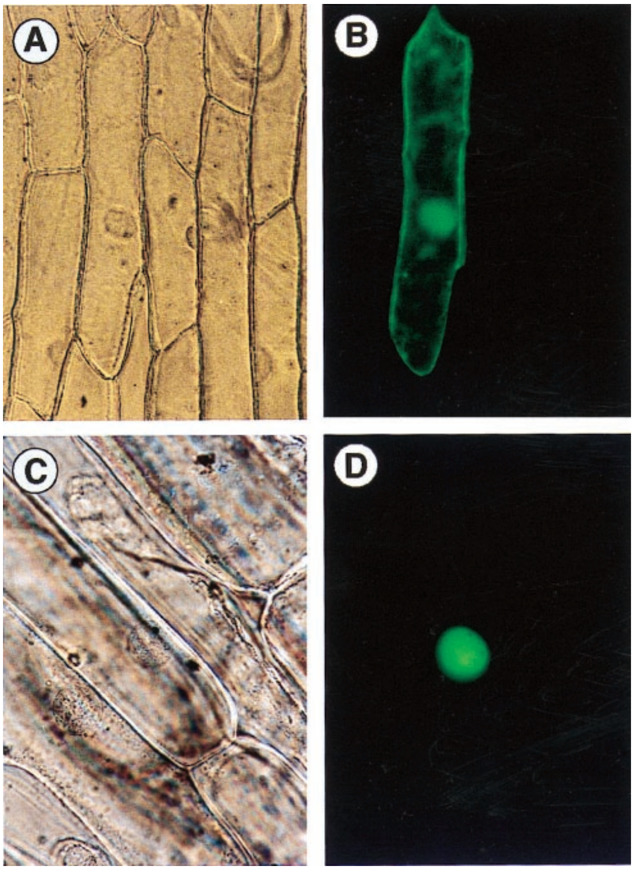

Fig. 5. Nuclear localization of CPP1. (*A*) Onion epidermal cell expressing GFP viewed by bright-field microscopy. (*B*) Same as *A* but viewed by epifluorescence microscopy. (*C*) Onion epidermal cell expressing CPP1–GFP fusion protein viewed by bright-field microscopy. (*D*) Same as *C* but viewed by epifluorescence microscopy.

in the presence of a constitutively expressed *cpp1* gene. The *Gmlbc3* promoter is specifically expressed in *Lotus corniculatus* root nodules (12). A 2-kb *Gmlbc3* promoter was fused to a *gusA* reporter gene. A *CaMV* 35S promoter was placed upstream to the *Gmlbc3–gusA* reporter gene (Fig. 6*E*, lbc3-GUS). The construct was transformed into *V. hirsuta* by using *A. rhizogenes*, and the emerging transgenic hairy roots were inoculated with *Rhizobium leguminosarum* bv. *viciae.* As observed (12), the *Gmlbc3* promoter was active in young and mature nodules (Fig. 6 *C* and *D*). However, β-glucuronidase (GUS) activity was also detected in nodule primordia (Fig. 6 *A* and *B*). In contrast to the expression pattern observed in *L. corniculatus*, a few *V. hirsuta* roots also showed a low level of GUS activity in the vascular tissue. To regulate the expression of the *Gmlbc3* promoter, the coding region of *cpp1* was fused to the *CaMV* 35S promoter upstream to the *Gmlbc3–gusA* reporter gene. The orientation of the two chimeric genes are shown in Fig. 6*E*. After transformation into *V. hirsuta,* 40 independent roots with the control construct were obtained in addition to 23 roots with the *cpp1*-coding region containing construct. To quantify GUS expression from the constructs, visible nodules were dissected from the roots and GUS activity was determined by a fluorometric assay for each individual plant of the dissected nodules and the remaining roots which contained also small nodules and nodule primordia. The fluorometric values related to the amount of protein in the extracts as determined by Coomassie blue staining (23) are shown in Fig. 6*F*. A number of plants had very low GUS activities (below 0.01 μmol/h per mg protein) probably because

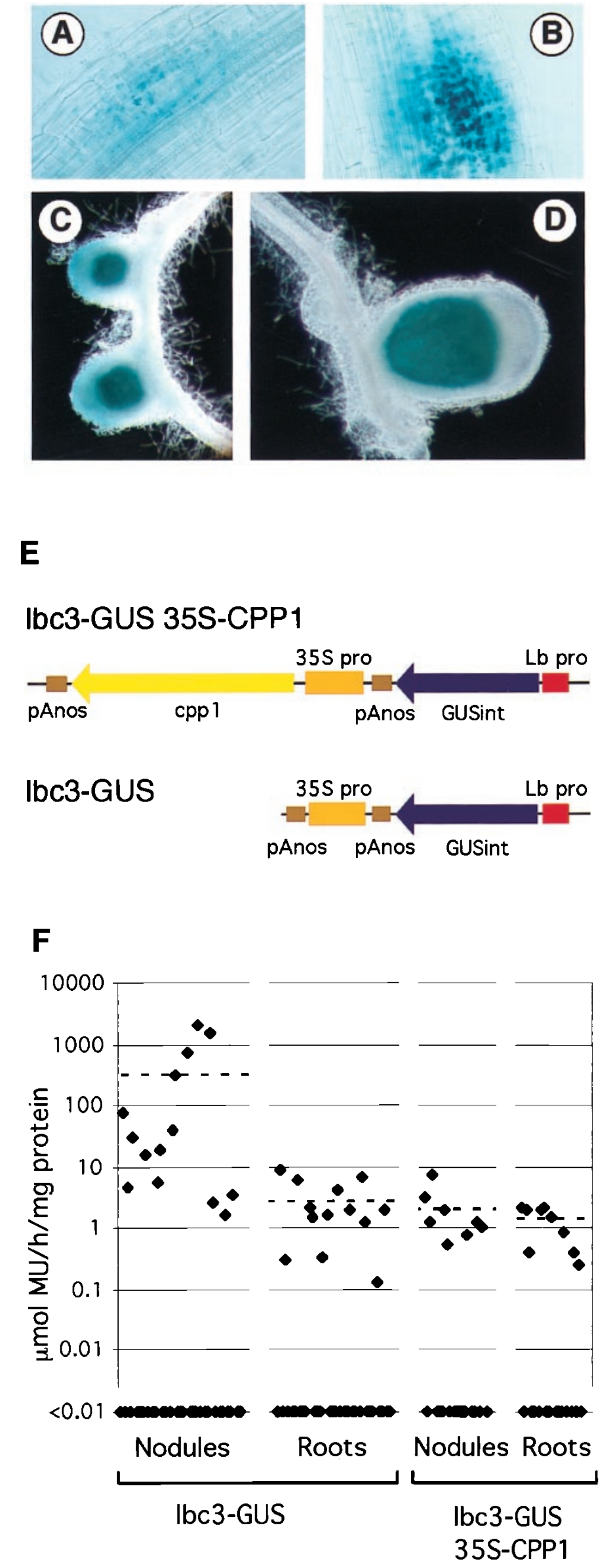

Fig. 6. Regulation of a *Gmlbc3* promoter in *V. hirsuta* roots by CPP1. (*A–D*) Histochemical GUS staining of *V. hirsuta* nodule primordia, roots, and nodules. Different developmental stages of nodules from plants transformed with a *Gmlbc3* promoter fused to *gusA*: (*A* and *B*) nodule primordia; (*C*) young nodules; and (*D*) a mature nodule. (*E*) Schematic presentation of the lbc3–GUS and lbc3–GUS 35S-CPP1 constructs. (*F*) Fluorometric measurement of GUS activity expressed from a *Gmlbc3* promoter in the absence of CPP1 (lbc3–GUS) or in the presence of CPP1 (lbc3–GUS 35S-CPP1). The GUS activities determined in nodules and roots for each individual plant are indicated by diamonds on a logarithmic scale. Dashed lines indicate the mean values.

the plants only contained the T-DNA from the Ri-plasmid. These plants were excluded when calculating the mean values. The mean values of the control plants were 118 μmol MU/h per mg protein in nodules and 1.2 units in the root fraction. However, when the CPP1 was present, the expression in nodules was reduced more than 100-fold to a mean value of 0.7 units. The expression in the roots was only slightly reduced to a mean value of 0.5 units. A lbc3-GUS 35S-CPP1 construct in which the lbc-GUS was inverted gave essentially the same results (data not shown). These data show that the presence of the constitutively expressed *cpp1* gene leads to a more than a 100-fold reduction in expression of the *Gmlbc3* promoter in nodules. This indicates that CPP1 functions as a repressor of the soybean *Gmlbc3* gene.

Discussion

CPP1 Contains a Cys-Rich DNA-Binding Domain. Here, we report the identification and characterization of a DNA-binding protein CPP1 which consists of 896 aa with two similar Cys-rich domains, C1 and C2. The amino acid sequences of the two motifs are very similar in the N-terminal basic region, whereas only the Cys residues are conserved in the C-terminal region. Six *A. thaliana* genomic sequences and a sequence from *C. elegans* showed a high similarity to the two Cys-rich domains of CPP1. All but one Cys residue are conserved between all these sequences and also noncysteine residues are conserved in the N-terminal part of both C1 and C2. The sequence alignment also revealed a basic stretch of aa located N-terminal to C1 in several of the peptides and a highly conserved short aa sequence (RNP$\overset{\text{I}}{\underset{\text{N}}{\text{L}}}$AFAPK) between C1 and C2. The sequences outside these conserved regions do not show any significant similarities. All of the identified *A. thaliana* and *C. elegans* sequences contain both C1 and C2, as well as the RNP$\overset{\text{I}}{\underset{\text{N}}{\text{L}}}$AFAPK sequence. This region is able to bind DNA and we name this novel DNA-binding domain CRC (*C*1-*R*NP$\overset{\text{I}}{\underset{\text{N}}{\text{L}}}$AFAPK-*C*2).

A family of human and murine genes (*tesmins)* also encodes a region showing high similarity to the Cys-rich region of CPP1, including the conserved RNP$\overset{\text{I}}{\underset{\text{N}}{\text{L}}}$AFAPK sequence (24). However, the C1 region in the tesmins is truncated in the conserved N-terminal part. The tesmins are believed to play a role in the early events of male germ cell differentiation, because in mice, the transcripts are restricted to spermatocytes undergoing meiosis.

A region in the kinesin-like protein KLP3a contains nine Cys in a pattern similar to one found in the C2 motif of CPP1 (25). Similarity is also observed to a few noncysteine residues in C2, as well as to three conserved residues in the RNP$\overset{\text{I}}{\underset{\text{N}}{\text{L}}}$AFAPK sequence. No region similar to C1 is present in KLP3a. Mutations in the *KLP3A* gene cause male and female sterility in *Drosophila melanogaster*. The female sterility is probably caused by an arrest of the male and female pronuclei preventing them from coming together. An additional effect of a *KLP3A* mutation is aberrations in meiosis and mitosis indicating a role of KLP3A in spindle function (25). Thus, both KLP3A and tesmins appear to control the development of the reproductive organs. However, no molecular information is available about the functions of tesmin and KLP3A.

Some polycomb group proteins like Enhancer of Zeste from *Drosophila* and CURLY LEAF from *A. thaliana* also contain a region with similarity to C1 and C2 (26, 27). In CURLY LEAF, the positions of 8 of the 10 Cys are conserved in C1 and similarly 7 of 9 Cys are conserved in C2. However, in these polycomb proteins, the C1 and C2 regions are located adjacent to each other in contrast to CPP1 in which approximately 60 aa separate C1 and C2. Polycomb group gene products are responsible for the maintenance of a silent stage of repressed developmentally important regulatory genes including the homeotic genes (28). This is normally achieved through an interaction with DNA-binding proteins which bind to chromatin in the regions of the target genes. However, one example of a DNA-binding polycomb group protein has been reported, namely Pleiohomeotic (PHO) from *Drosophila* (29). PHO contains a DNA-binding zinc-finger domain similar to the one present in the mammalian YY1 transcription factor (30).

CPP1 Is a Potential Repressor of Leghemoglobin Gene Expression. Repression of a minimal *Gmlbc3* promoter in vetch root nodules by a constitutively expressed *cpp1* suggests that CPP1 is involved in the expression of *lb* genes in the nodule. Nevertheless, it still cannot entirely be ruled out that a high level of CPP1 is toxic and we therefore select for integration events in relatively silent regions when *cpp1* is present on the construct, resulting in a lower expression level of the *gusA* gene. However, the reduced expression of *gusA* in nodules is not observed in the corresponding roots. Furthermore, overexpressing CPP1 in transgenic *Lotus japonicus* plants does not impair proper root or nodule development (data not shown). For these reasons, we consider it highly unlikely that the observed results are caused by a toxic effect of CPP1.

In all legumes analyzed so far, the expression of the *lb* genes is restricted to the central infected part of the nodule and the amount of *lb* transcripts increases until the onset of nitrogen fixation (1, 2, 31). *Gmlbc3* transcripts were not detected at the periphery of the infected zone whereas *cpp1* transcripts showed the highest level of expression in this region. This observation supports the hypothesis that CPP1 is a repressor of *lb* gene expression or alternatively maintains a repressed state of the *lb* genes in these cells, similar to the function of the polycomb group proteins. Until recently, it was believed that cells in the central infected zone of a mature determinate nodule were all at the same developmental stage. However, an analysis of the expression of symbiotic bacterial genes in *Phaseolus vulgaris* nodules indicated that the cells at the periphery of the infected zone contain bacteria which are not differentiated into the nitrogen-fixing form (32). The authors therefore suggest that there are developmental zones in the determinate nodule with the less differentiated cells located at the periphery. Recent expression studies of a homeobox gene, *Gmndx,* also suggest that the soybean nodule has a zone with less differentiated cells (10). These cells are smaller and are not highly packed with bacteria as compared with large cells in the center. It is therefore possible that, in contrast to the *lb* genes, *cpp1* is expressed in cells which are not fully differentiated.

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