ANALYSIS OF *Vigna unguiculata* LEGHEMOGLOBIN GENES

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ABSTRACT - A gene coding for the cowpea (*Vigna unguiculata* L) leghemoglobin II (LbII) was previously cloned and sequenced. This work concerns the gene and expression analysis of cowpea LbII. Southern blot analysis using the cowpea LbII gene as probe showed that at least two copies of the leghemoglobin gene exist in cowpea. A gene coding for the cowpea leghemoglobin I (LbI) was cloned and sequenced using PCR and primers for the LbII gene. Sequence analysis showed that the cowpea LbI and LbII genes are remarkably similar. Expression of leghemoglobin genes was detected in cowpea nodules, but not in roots, stems or leaves using RNA-PCR and primers for cowpea LbII. The same method, but combined with Southern and Western blotting, was used to analyze the expression of leghemoglobin genes in cowpea nodules as a function of time. Leghemoglobin transcripts were detected in young (white) nodules at levels that were comparable to levels found in mature (pink) nodules. In contrast, leghemoglobin transcripts were not detected in senescent nodules, although leghemoglobin proteins were still detected.

**Additional index terms:** Cowpea, nodules.

**INTRODUCTION**

Leghemoglobin (*Lb*) is a heme protein that reversibly binds *O*₂ and that is very abundant in root nodules of leguminous plants (Appleby, 1974). Nodules are organs that develop on plant roots or stems after the infection by soil bacteria of the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (collectively known as rhizobia). After a complex series of events (Mylona et al., 1995), rhizobia differentiate into bacteroids which synthesize the enzymatic complex nitrogenase that fixes atmospheric nitrogen. High *O*₂ concentrations are deleterious for nitrogenase activity and expression of nitrogenase genes (Shah & Brill, 1977), however *O*₂ concentration in nodules is low because of the existence of a gas diffusion barrier (Hunt & Layzell, 1993). Nitrogen fixation is an energy-demanding process and a constant supply of *O*₂ to the bacteroidal oxidases must be maintained. Because the high affinity of leghemoglobin for *O*₂ and of its high concentration in nodules, it was postulated that leghemoglobin functions by facilitating the...
diffusion of \( \text{O}_2 \) to the bacteroids (Appleby, 1984; Appleby, 1992), thus leghemoglobin is essential for nitrogen fixation but its role in this process is indirect.

Several different isoleghemoglobins exist in nodules that result from the expression of individual leghemoglobin genes and the post-translational modification (i.e. acetylation) of pre-existing leghemoglobins (Bogusz et al., 1987). Ratios of leghemoglobin subcomponents vary with nodule age (Fuchsman & Appleby, 1979; Bisseling et al., 1980), however the physiological significance of this variation is presently unknown. Dakora et al. (1991) detected three isoleghemoglobins (\( \text{LbI, LbII and LbIII} \)) in cowpea nodules, and showed that \( \text{LbII} \) is the major, and \( \text{LbI and LbIII} \) are the minor components. Recently, a gene coding for the cowpea \( \text{LbII} \) was cloned and sequenced, and the corresponding cDNA was expressed in \( \text{Escherichia coli} \) to generate recombinant \( \text{LbII} \) (Arredondo-Peter et al., 1997), however very little is known about the cowpea \( \text{leghemoglobin} \) genes. This work reports the gene and expression analysis of cowpea \( \text{LbII} \) gene. Results showed that at least two copies of the leghemoglobin gene exist in cowpeas and that their expression is similar to other \( \text{leghemoglobin} \) genes.

**MATERIALS AND METHODS**

Plant growth, total DNA and RNA Isolation, and Sequencing of the DNA. Cowpea (\( \text{Vigna unguiculata} \) var. California No. 5) seeds were germinated in wet paper towels for about 5 days. Cowpea seedlings were inoculated with \( \text{Bradyrhizobium japonicum} \) USDA3456 and grown in the greenhouse as described previously (Sarath et al., 1986). Total DNA was isolated from 3 g of cowpea seedlings using the CTAB method (Doyle & Doyle, 1990). Total RNA was isolated from cowpea organs using the RNA-REagent (Biogentex) following the manufacturer’s protocol, quantitated assuming 1 \( \text{A}_260 = 40\text{mg of RNA/L} \), and checked by denaturing gel electrophoresis (Ausubel et al., 1995). DNA manipulations were performed following standard methods (Sambrook et al., 1989). PCR fragments were separated by electrophoresis, extracted from the gel using Geneclean Kit (BIO 101, Vista, CA) and cloned into the pCR II vector (Invitrogen) as described previously (Arredondo-Peter et al., 1997). The cloned fragments were sequenced in both directions using the dideoxy method (Sanger et al., 1977) at the DNA Sequencing Facility of the University of Nebraska-Lincoln.

Southern Blot Analysis. The clone corresponding to the cowpea \( \text{LbII} \) gene (Arredondo-Peter et al., 1997) was PCR-labeled by incorporating dig-11-dUTP (Boehringer) as described by Lu et al. (1993) using cowpea \( \text{LbII} \) sense (5’-ATGGTTGCTTTCTCTGAC 3’) and antisense (5’-CTAATATGCTTTTTTAAT-AGCTGC 3’) primers. Conditions for amplification were 35 cycles at 55°C/1 min for annealing, 72°C/1 min for extension and 95°C/1 min for denaturation. After gel electrophoresis and staining with ethidium bromide, the labeled probe was purified from the 1.6% agarose gel slices using the Geneclean kit (Bio 101e).

Total cowpea DNA (~ 15\( \mu \text{g} \)) was digested using 30 U of \( \text{BamHI, EcoRI, HaellI, HindIII or XhoI} \) restriction enzymes. The samples were electrophoresed in a 1% agarose gel and transferred to a nylon membrane (N+, Amersham). Southern blots were performed at high stringency using standard procedures (Sambrook et al., 1989). Membranes were hybridized overnight at 50°C, and then washed at 65°C in 2X SSC/0.1% SDS for 5 min (twice), and 0.5X SSC/0.1 % SDS for 15 min (twice). Membranes were incubated in the NBT/x-Phosphate mix of the Genius kit (Boehringer) to develop color.

Gene Expression in Organs and as a Function of Time in Cowpea Plants. Gene expression was examined in cowpea nodules, roots, stems and leaves that were collected six weeks after plant inoculation. Gene expression as a function of time was done on nodules harvested at 20, 29, 41, 54 and 67 days after plant inoculation. In each case, total RNA was isolated and 1 mg was used as a template for RNA-PCR (Wang et al., 1989). A RNA-PCR kit (Cetus) was used for the reverse transcription and amplification of transcripts following the manufacturer’s protocol. Oligonucleotides corresponding to cowpea \( \text{LbII} \) sequences were used as primers (above) and PCR components and concentrations were: 1 \( \mu \text{M} \) of each sense and antisense primer, 200 \( \mu \text{M} \) of each dNTP, 500 \( \mu \text{M} \) MgCl\(_2\) and 0.5 U of \( \text{Taq DNA polymerase} \) (GIBCO BRL) in a final volume of 100 \( \mu \text{L} \) of the PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl\(_2\), 0.001% gelatin). Amplification was carried out for 35 cycles at 55°C/1 min for annealing, 72°C/1 min for extension and 95°C/1.5 min for denaturation. At the end of the 35 cycles one additional annealing and extension step was done at 55°C/1 min and 72°C/5 min, respectively. Samples were extracted with chloroform and 25 \( \mu \text{L} \) aliquots were electrophoresed in a 1.6% agarose gel. Amplification products were detected in gels after staining with ethidium bromide. In order to achieve a higher specificity, the PCR fragments were transferred to nylon membranes for Southern blotting using the (dig) labeled cowpea \( \text{LbII} \) probe as described above. Levels of leghemoglobin proteins in nodules were determined by Western blots using anti-soybean leghemoglobin antibodies as described by Sarath & Wagner (1989).
RESULTS AND DISCUSSION

The cowpea LbII gene was cloned and sequenced recently by Arredondo-Peter et al. (1997) who showed that it codes for a predicted protein of 145 amino acids in length. The sequence for the predicted cowpea LbII is highly similar to bean leghemoglobins and contains the same conserved residues as in other plant hemoglobins (Hbs) (Arredondo-Peter & Escamilla, 1991), such as the distal (H62) and proximal (H93) histidines, proline P39, and phenylalanines F45, F67 and F103 (Figure 1).

Leghemoglobin subcomponents and gene families have been detected in many legumes. For instance, soybean nodules have eight isoleghemoglobins, but only major forms (Lba, Lbc1, Lbc2 and Lbc3) are encoded by individual genes (Brisson & Verma, 1982; Lee et al., 1983), with the minor leghemoglobins (Lbb and Lbd5) resulting from post-translational modification of major leghemoglobins. Truncated and pseudolehemoglobin genes also exist in soybeans (Brisson & Verma, 1982). Southern blot analysis of total cowpea DNA, using the cowpea LbII gene as probe, showed two hybridizing fragments in DNAs digested with EcoRI (3 and 5 Kbp) and Haell (6 and 10 kbp), and a complex banding pattern with HindIII (Figure 2). Computer-generated restriction maps showed that restriction sites for HindIII exist in cowpea LbII at sites 242 and 529, thus explaining the complex hybridization pattern observed for HindIII digestions. In contrast, restriction sites for EcoRI and Haell were not detected in cowpea LbII, thus indicating that at least two copies of the leghemoglobin gene exist in cowpeas.

To clone a second copy of the cowpea leghemoglobin gene, DNA fragments of approximately 3 or 5 kb in length were isolated from the agarose gel after digestion with EcoRI. These fragments were named 3k and 5k, respectively, and were used as templates for PCR with the cowpea LbII primers (above). PCR products of ~660 bp were amplified from

FIGURE - Predicted cowpea lbII sequence and alignment with bean leghemoglobins. Asterisks show the most conserved residues. Distal and proximal His (H62 and H93, respectively) are shown in bold. Bean leghemoglobin sequences were obtained from the Genbank data base (accession numbers are indicated in square brackets): SoybnLba, soybean Lba [J01299]; SoybnLb1, soybean Lbc1 [J01303]; SoybnLb2, soybean Lbc2 [J01301]; SoybnLb3, soybean Lbc3 [J01302]; Psotelb, winged bean (Psophocarpus tetragonolobus) Lb [P27199]; Phavulb, common bean (Phaseolus vulgaris) Lb [K03152].

FIGURE 2. Southern blot analysis of cowpea total DNA digested with restriction enzymes and hybridized with the (dig) labeled cowpea LbII probe at high stringency. Molecular sizes are shown for the hybridizing fragments in the EcoRI- and Haell-digestions.
the 3k and 5k fragments, and sequencing of the DNA revealed that the \textit{leghemoglobin} gene isolated from the 5k fragment corresponded to the cowpea \textit{LbII} gene, and that the \textit{leghemoglobin} gene isolated from the 3k fragment was slightly different from cowpea \textit{LbII}, although the predicted protein was identical to the partial sequence of cowpea \textit{LbI} (Sarath et al., 1990); therefore, the 3k-derived copy was named cowpea \textit{LbI} gene. Sequencing of several 5k- and 3k-derived clones showed that copies of cowpea \textit{lbI} gene exist in both the 5k and 3k fragments, and that \textit{cowpeaLbII} exists only in the 5k fragment.

Comparison of the sequences showed that the \textit{lbI} and \textit{LbII} genes are quite similar with only six substitutions in the first exon, two substitutions in the first intron, and one insertion/deletion in the second intron (Figure 3). The predicted sequences for cowpea leghemoglobin\textit{I} and \textit{LbII} proteins were also very similar and only two substitutions, at positions 10 and 22, were detected.

Symbiotic and non-symbiotic \textit{hemoglobin} genes are known to exist and to be expressed in plant organs (Appleby, 1992). Symbiotic \textit{hemoglobin} genes are specifically expressed in nodules of legume and non-legume species. Non-symbiotic hemoglobin transcripts have been detected in diverse organs of dicots (Bogusz et al., 1988; Anderson et al., 1996), and in the seed aleurone and microaerobic organs of monocots (Taylor et al., 1994). Two different methods were used in this work to determine if leghemoglobin transcripts were localized in various cowpea organs. Using RNA-PCR of total RNA from different cowpea organs with cowpea \textit{LbII} primers, leghemoglobin transcripts (~400 bp in length) were detected in nodules, but not in roots, stems or leaves (Figure 4). Identical results were obtained by Southern blotting the RNA-PCR products with the (dig) labeled cowpea \textit{LbII} probe (results not shown).

Leghemoglobin is considered a late nodulin in that it is synthesized after nodule formation at the onset of symbiosis.
nitrogen fixation, when the rhizobia are fully differentiated into bacteroids (Legocki & Verma, 1980; van Kammen, 1984). At this stage of development, root nodules are characteristically pink and are considered mature nodules. RNA-PCR experiments with cowpea nodules of various ages showed that considerable amounts of leghemoglobin transcripts were detected in young (white) nodules, at 20 days after inoculation, and that the levels were comparable to those observed in mature nodules, at 29 days after inoculation (Figure 5a and b). Western blot analysis of the same samples showed that very low levels of leghemoglobin protein exist in young nodules (Figure 5c). This suggests that the cowpea leghemoglobin genes are expressed at high levels early in nodule development, resulting in the accumulation of leghemoglobin transcripts. Much higher levels of leghemoglobin protein were detected in nodules at 29 days after inoculation, so it is likely that leghemoglobin transcripts are massively translated between 20 and 29 days after inoculation. In contrast, leghemoglobin transcripts were not detected in senescent nodules, at 67 days after inoculation (Figure 5a and b), indicating that in cowpeas the leghemoglobin genes are turned off between flowering, at 54 days after inoculation, and pod filling, at 67 days after inoculation, although leghemoglobin proteins were still detected in nodules at 67 days after inoculation (Figure 5c).

**CONCLUSIONS**

Cowpea leghemoglobin genes are highly similar to leghemoglobins from other beans, such as those from soybeans and common beans. Southern blot analysis indicated that at least two copies of the leghemoglobin gene exist in cowpeas (Figure 2), and because the intensity of the hybridizing bands was different, it is likely that different numbers of leghemoglobin copies exist in the strong and weak hybridizing bands.

Cowpea leghemoglobinI and LbII genes were detected in the 5k fragment, and only the cowpea LbI
gene was detected in the 3k fragment. This indicates that at least two gene duplications occurred in the evolution of cowpea leghemoglobin genes: one duplication generated the Lbl copies that are localized in the 3k and 5k fragments, and another duplication generated the cowpea Lbl and LblI genes. Genes and deduced Lbl and LblI proteins are remarkably similar, suggesting that leghemoglobins were highly conserved in the evolution of cowpeas or, alternatively, that the cowpea leghemoglobin genes duplicated recently.

As with all of the known leghemoglobin genes, expression of cowpea leghemoglobins was detected only in nodules, but not in other plant organs. Cowpea leghemoglobin genes were expressed early in nodule development (Figure 5), and levels of leghemoglobin transcripts in young (white) nodules were comparable to those found in mature nodules, although the levels of leghemoglobin protein were low. Transcripts must accumulate before proteins can be synthesized, however, the high amounts of leghemoglobin transcripts and low abundance of leghemoglobin proteins in young cowpea nodules indicated that the translation of leghemoglobin transcripts does not occur gradually but rather quickly between 20 and 29 days after inoculation. Figure 5 shows that the leghemoglobin genes are turned off in senescing nodules, after plant flowering, but leghemoglobin proteins were still detected in these nodules however it is unknown if these leghemoglobins in aging nodules are functioning proteins.

REFERENCES


SHAH, V.K. & BRILL, W.J. Isolation of an iron-molybdenum cofactor from nitrogenase.

ERRATA

- p. 144. In the subsection "Gene Expression in Organs and as a Function of Time in Cowpea Plants", line 8, it is stated that "...and 1 mg was used as a template...". This statement should read "...and 1 µg was used as a template...".

- p. 146 and 148. "Anderson" in ref. Anderson et al., 1996, should spell as "Andersson".

- Legend of figure 5, line 9. Reads "days after inoculation, days after plant inoculation", and should read "dain, days after plant inoculation".