

# The *Vicia faba* Leghemoglobin Gene *VfLb29* Is Induced in Root Nodules and in Roots Colonized by the Arbuscular Mycorrhizal Fungus *Glomus fasciculatum*

Martin Frühling,<sup>1</sup> H  l  ne Roussel,<sup>2</sup> Vivienne Gianinazzi-Pearson,<sup>2</sup> Alfred P  hler,<sup>1</sup> and Andreas M. Perlick<sup>1</sup>

<sup>1</sup>Universit  t Bielefeld, Lehrstuhl f  r Genetik, Postfach 100131, D-33501 Bielefeld, Germany; <sup>2</sup>Station de G  n  tique et d'Am  lioration des Plantes, INRA, BV 1540, F-21034 Dijon c  dex, France

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**To investigate similarities between symbiotic interactions of broad bean (*Vicia faba*) with rhizobia and mycorrhizal fungi, plant gene expression induced by both microsymbionts was compared. We demonstrated the exclusive expression of 19 broad bean genes, including *VfENOD2*, *VfENOD5*, *VfENOD12* and three different leghemoglobin genes, in root nodules. In contrast, the leghemoglobin gene *VfLb29* was found to be induced not only in root nodules, but also in broad bean roots colonized by the mycorrhizal fungus *Glomus fasciculatum*. In uninfected roots, none of the 20 nodulin transcripts investigated was detectable. *VfLb29* has an unusually low sequence homology with all other broad bean leghemoglobins as well as with leghemoglobins from other legumes. It can be regarded as a novel kind of leghemoglobin gene not described until now and the induction of which is common to symbiotic interactions of broad bean with both *Rhizobium* and a mycorrhizal fungus.**

The majority of legume species can establish symbiotic associations with two important groups of rhizosphere microorganisms, bacteria of the genus *Rhizobium* and arbuscular mycorrhizal fungi of the order Glomales. The *Rhizobium*-legume interaction leads to symbiotic nitrogen fixation carried out by differentiated bacteria within a specialized plant organ, the root nodule (Brewin 1991). Root colonization by arbuscular mycorrhizal fungi increases plant biomass and enhances vigour and ecological fitness of both organisms (Harley and Smith 1983). Closer examination of root nodule formation induced by rhizobia and root colonization by arbuscular mycorrhizal fungi reveals several similarities of these two interactions. In both cases the target tissue for the symbiont is derived from the root cortex. During penetration of root tissue, the microsymbionts are separated from the host cells by plant-derived cell wall material (VandenBosch et al. 1989; Perotto et al. 1989). The invasion and colonization of the plant cells is

linked to proliferation of the microsymbiont and its development into pleomorphic intracellular forms, the bacteroids in nodules and the arbuscules in mycorrhiza. Both bacteroids and arbuscules are separated from the plant cytoplasm by a host-derived perisymbiotic membrane (Mellor 1989). The peribacteroid membrane (in nodules) and the periarbuscular membrane (in mycorrhiza) are suggested to be extended interfaces between the symbiotic partners where bidirectional exchange of metabolites takes place (Smith and Smith 1990).

The development of an effective, nitrogen-fixing symbiosis in root nodules is determined by genes from both rhizobia and their host plants. In recent years, the bacterial genetics of nodule formation and nitrogen fixation have been extensively studied (Fisher and Long 1992; Fischer 1994). An increasing number of plant genes specifically expressed in nodules, termed nodulin genes (van Kammen 1984), have been identified and subdivided into early and late nodulin genes according to the time point of their expression (Brewin 1991; Verma et al. 1992). Early nodulin genes (ENODs) are associated with organogenesis and bacterial invasion of the root nodule. In general, late nodulin gene (NOD) products are thought to be involved in nodule function and include the oxygen transporter leghemoglobin, enzymes of carbon and nitrogen metabolism, proteins located in the peribacteroid interface as well as proteins of unknown function (Delauney and Verma 1988).

In contrast, analysis of the molecular mechanisms involved in arbuscular mycorrhiza are still in their infancy mainly due to recalcitrance of the fungal partner to pure culture (Gianinazzi-Pearson et al. 1994). However, there are evidences indicating some molecular mechanisms may be common to symbiotic *Rhizobium* interactions. Firstly, identification of mycorrhiza-resistant (*myc*<sup>-</sup>) phenotypes among non-nodulating (*nod*<sup>-</sup>) genotypes of different legumes (Duc et al. 1989; Weeden et al. 1990; Bradbury et al. 1991; Gianinazzi-Pearson et al. 1991) has demonstrated that some steps in recognition or infection processes are controlled by common plant genetic determinants. On the other hand, the existence of *myc*<sup>+</sup> *nod*<sup>-</sup> plant mutants (Duc et al. 1989) demonstrates the involvement of different plant genes in the two types of symbiosis. Secondly, transcripts coding for some polypeptides that are immunologically cross-reactive with nodule-specific plant proteins (nodulins) have been detected in mycorrhizal roots

Corresponding author: A. M. Perlick: Telefax: +49 (0)521 106 5625, Telephone: +49 (0)521 106 5607

The sequence data reported will appear in the EMBL database under the accession numbers Z54157 (*VfLbB*), Z54158 (*VfLbK*), Z54159 (*VfLb49*) and Z54160 (*VfLb29*), respectively.

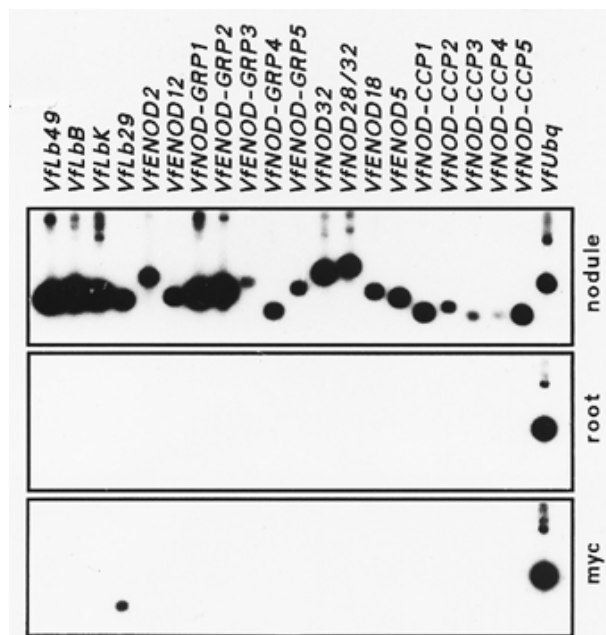
(Wyss et al. 1990). Furthermore, monoclonal antibodies raised against components of the infection thread or the peribacteroid membrane in nodules localize antigenic sites in the periarbuscular interface (Gianinazzi-Pearson et al. 1990; Perotto et al. 1994; Gollotte et al. 1995). However, activation of nodulin genes induced during rhizobial infection or nodule formation has not yet been reported in mycorrhizal roots.

The aim of the present work was to obtain more information about the extent to which similarities and differences exist between arbuscular mycorrhiza and nodule symbioses. For this, we analyzed 20 broad bean (*Vicia faba*) nodulin genes for their induction in nodules and in broad bean roots colonized by the arbuscular mycorrhizal fungus *Glomus fasciculatum*. We report on the identification of one leghemoglobin transcript common to nodules and mycorrhizal roots. This transcript is compared with other broad bean leghemoglobin transcripts which are undetectable in mycorrhizal roots and implications of the findings are discussed.

## RESULTS

### Members of a nodule-specific broad bean cDNA library encode 20 different nodulins.

To investigate the nodule-specific gene expression in broad bean, we have constructed a nodule-specific cDNA library of approximately 700 independent cDNAs (Perlick and Pühler 1993). Following cross-hybridization experiments and sequence analysis, this library has been subdivided into 44 clone groups representing different genes activated in the root nodule. Twenty of these groups (Table 1) specify transcript sequences expressed in the nodule but not in uninfected roots (Fig. 1) and therefore code for nodulins. Searches of the GenBank database has revealed homologies for nearly all the broad bean nodulin transcripts (Table 1). In addition to four



**Fig. 1.** Expression of broad bean nodulin transcripts in nodules, uninfected and mycorrhizal roots. *Eco*RI digested cDNA clones representing transcript sequences which code for broad bean nodulins (see Table 1) and for ubiquitin (VfUbq) were separated on agarose gels and blotted onto nylon membranes. Hybridizations were carried out with radioactively-labeled cDNA probes synthesized from poly(A)<sup>+</sup> RNA isolated either from nodules, uninfected roots or roots colonized by *Glomus fasciculatum* (myc). The times of exposure for root and myc autoradiographs were adjusted according to the ubiquitin hybridization signals.

**Table 1.** Broad bean nodulin genes analyzed

Broad bean gene	cDNA clone sequenced <sup>a</sup>	Clone group <sup>b</sup>	Homologous genes or gene products	References
<b>Leghemoglobins</b>				
<i>VjLbB</i>	150-1	VfNDS-B	Leghemoglobins	Perlick and Pühler 1993
<i>VjLbK</i>	111-51	VfNDS-K	Leghemoglobins	Perlick and Pühler 1993
<i>VjLb49</i>	1b84	VfLb	Leghemoglobins	Perlick and Pühler 1993
<i>VjLb29</i>	226-1	VfNDS-X29	Leghemoglobins	This study
<b>Proline- and glycine-rich nodulins</b>				
<i>VjENOD2</i>	107-68	VfNDS-H	ENOD2	Perlick and Pühler 1993
<i>VjENOD12</i>	114-96	VfNDS-X7	ENOD12	Perlick and Pühler 1993
<i>VjNOD-GRP1</i>	2-7-3	VfNDS-F	GRPs	Perlick and Pühler 1993
<i>VjENOD-GRP2</i>	103-53	VfNDS-G	GRPs	Perlick and Pühler 1993
<i>VjENOD-GRP3</i>	125-71	VfNDS-X14	GRPs	Küster et al. 1995
<i>VjNOD-GRP4</i>	163-2	VfNDS-X20	GRPs	This study
<i>VjENOD-GRP5</i>	168-2	VfNDS-X22	GRPs	This study
<b>Other nodulins</b>				
<i>VjNOD32</i>	1-1-2	VfNDS-A	Narbonin, chitinases	Perlick et al. 1996
<i>VjNOD28/32</i>	6-1-27	VfNDS-L	<i>MsNOD25</i>	Küster et al. 1994
<i>VjENOD18</i>	70-1	VfNDS-X9		Perlick and Pühler 1993
<i>VjENOD5</i>	118-24	VfNDS-X11	<i>PsENOD5</i>	Perlick and Pühler 1993
<b>Nodulins with conserved cysteine clusters</b>				
<i>VjNOD-CCP1</i>	182-1	VfNDS-J	<i>PsENOD3</i> and <i>14</i> , <i>PsNOD6</i>	Perlick and Pühler 1993
<i>VjNOD-CCP2</i>	121-79	VfNDS-X12	<i>PsENOD3</i> and <i>14</i> , <i>PsNOD6</i>	Perlick and Pühler 1993
<i>VjNOD-CCP3</i>	167-1	VfNDS-X19	<i>PsENOD3</i> and <i>14</i> , <i>PsNOD6</i>	This study
<i>VjNOD-CCP4</i>	197-1	VfNDS-X25	<i>PsENOD3</i> and <i>14</i> , <i>PsNOD6</i>	This study
<i>VjNOD-CCP5</i>	301-1	VfNDS-X30	<i>PsENOD3</i> and <i>14</i> , <i>PsNOD6</i>	This study

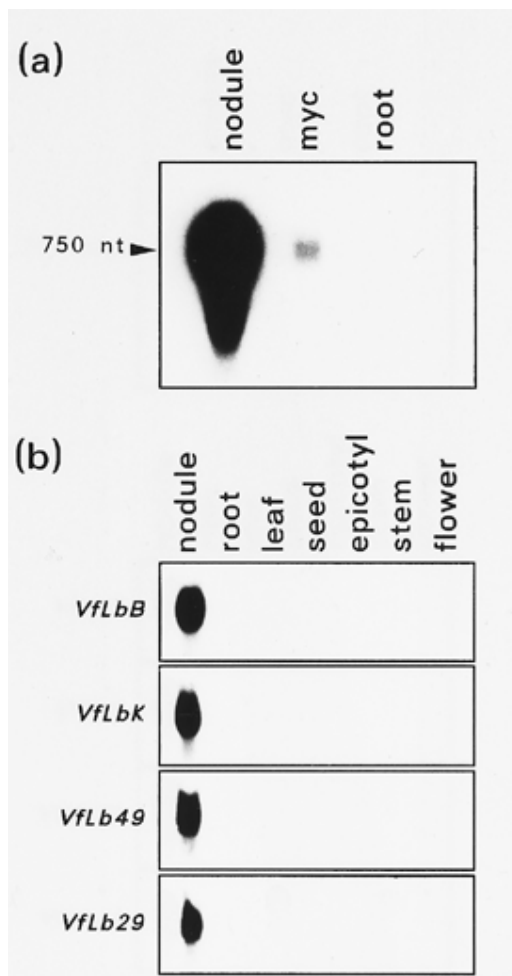
<sup>a</sup> and used for cDNA-cDNA hybridizations (Fig. 1).

<sup>b</sup> in accordance to Perlick and Pühler (1993).

different leghemoglobins, transcripts homologous to the *PsENOD2* (van de Wiel et al. 1990), *PsENOD5* (Scheres et al. 1990) and *PsENOD12* genes (Scheres et al. 1990) were isolated (Perlick and Pühler 1993). We also detected the recently described *VfENOD-GRP3* (Küster et al. 1995), one of at least five nodule-specifically expressed broad bean genes encoding glycine-rich proteins (GRPs), *VfNOD28/32* the homologue of *MsNOD25* (Küster et al. 1994) and *VfNOD32* encoding a narbonin-like nodulin with homologies to chitinases (Perlick et al. 1996). We further identified five groups of clones representing different members of a small gene family coding for nodulins with conserved cysteine clusters (CCPs) similar to the pea nodulin genes *PsENOD3*, *PsENOD14* (Scheres et al. 1990), and *PsNOD6* (Kardailsky et al. 1993).

#### Detection of leghemoglobin *VfLb29* transcripts in mycorrhizal roots.

To investigate whether the identified nodulin genes are also induced in arbuscular mycorrhiza, we carried out stringent



**Fig. 2.** Expression of broad bean leghemoglobin genes in different tissues. **A**, A Northern blot containing total RNA from nodules (20 µg), mycorrhizal roots (myc; 40 µg) and uninfected roots (40 µg) was hybridized against a *VfLb29* probe. The size of hybridizing transcripts calculated using RNA distance markers is indicated. **B**, Northern blots containing 30 µg of total RNA from nodules, uninfected roots, leaves, seeds, epicotyls, stems and flowers, hybridized against different leghemoglobin probes. Abbreviation: nt, nucleotides.

cDNA-cDNA hybridizations. This technique enables the expression analysis of several genes in a selected tissue just in one experiment. Twenty cDNA sequences representing the different nodule-specifically expressed broad bean genes (Table 1) were hybridized with radioactively labeled cDNA probes. As a control, a broad bean transcript sequence coding for ubiquitin was included (Fig. 1). The probes were synthesized from poly(A)<sup>+</sup> RNA isolated either from broad bean root nodules, uninfected roots or roots colonized by the mycorrhizal fungus *G. fasciculatum*. The mycorrhizal infection was in the exponential phase of development within the roots, characterized by active proliferation of the intraradical mycelium (29.7% colonised cortex) with intercellular hyphae and formation of intracellular haustorial arbuscules in parenchyma cells of 27.2% of the root system.

As expected, all cDNA sequences were detected by the nodule probe. The root cDNA probe exclusively hybridized with the ubiquitin cDNA indicating that this tissue was sterile with respect to *Rhizobium*. These results correspond to Northern blot hybridizations (Fig. 2). In comparison to the root probe, the mycorrhizal probe additionally hybridized with one out of the four different leghemoglobin transcript sequences, *VfLb29*. Even after overexposure and by analyzing several filters with independent cDNA probes the observed hybridization patterns were always the same (data not shown).

However, Northern blot hybridizations were carried out in order to confirm the *VfLb29* expression in mycorrhizal roots. The *VfLb29* probe detected a transcript in nodules and in lower amounts in mycorrhizal roots. The hybridizing transcripts were of the same size in both tissues (Fig. 2A). Analysis of nodules, uninfected roots, leaves, seeds, epicotyls, stems, and flowers showed expression of the leghemoglobin genes *VfLbB*, *VfLbK*, *VfLb49* and *VfLb29* only in the nodule tissue (Fig. 2B).

Since the knowledge of fungal gene expression in planta is limited, it is possible that the hybridization of the *VfLb29* cDNA with RNA isolated from mycorrhizal broad bean roots (Fig. 1 and Fig. 2A) was due to cross-hybridization with homologous transcripts of fungal origin. This was ruled out by the isolation and sequencing of a 139-bp *VfLb29* RACE fragment, which was amplified from mycorrhizal broad bean roots. This fragment was identical to the 5'-end of the *VfLb29* transcript sequence presented in Figure 3, indicating the plant origin of the hybridizing transcripts and confirming the presence of this plant leghemoglobin transcript in mycorrhizal roots.

#### *VfLb29* is an unusual member of the broad bean leghemoglobin family.

Isolated cDNA clones representing the four different leghemoglobin groups were subjected to restriction mapping. To

**Table 2.** Comparison of four broad bean leghemoglobins<sup>a</sup>

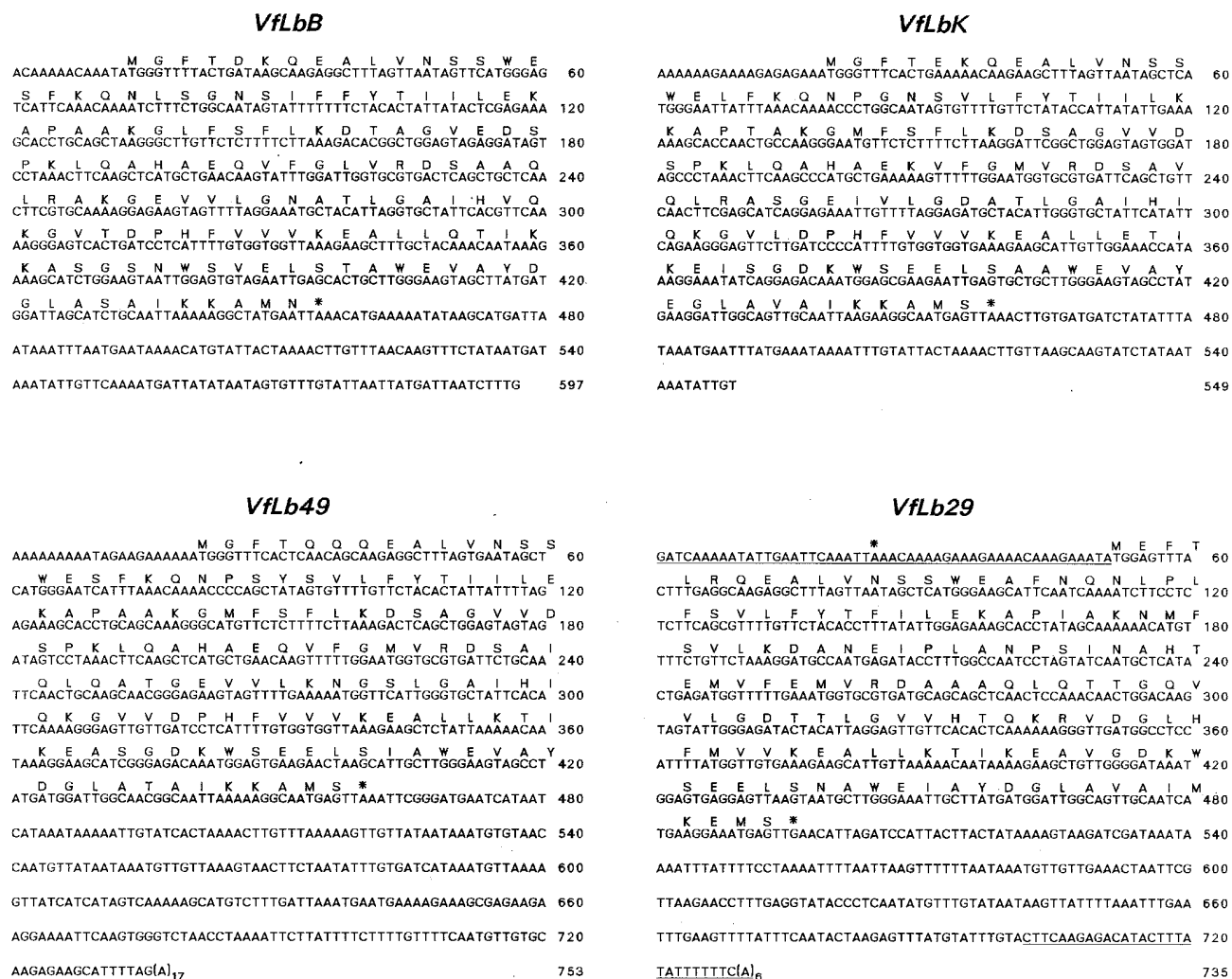
Broad bean leghemoglobin	Protein size in		Calculated pI	Amino acid sequence identity to (%)			
	aa	kDa		VfLbK	VfLbK	VfLb49	VfLb29
VfLbB	147	15.8	7.7	/	80.3	81.0	61.5
VfLbK	146	15.9	5.9	80.3	/	84.9	65.5
VfLb49	146	15.9	5.9	81.0	84.9	/	67.6
VfLb29	148	16.5	4.7	61.5	65.5	67.6	/

<sup>a</sup> Abbreviations: aa, amino acids; kDa, kilodaltons; pI, isoelectric point.

determine the complete coding regions the longest cDNAs 150-1 (*VfLbB*), 111-51 (*VfLbK*), lb49 (*VfLb49*) and 257-1 (*VfLb29*) were sequenced (Fig. 3). Based on sequence similarities to leghemoglobins from other legumes, the *VfLbB*, *VfLbK* and *VfLb49* clones were identified as full-length cDNAs. Since the clone 257-1 did not extend to the 5'-untranslated transcript region the RACE-PCR technique was used to complete the cDNA. The *VfLb29* RACE fragment amplified from root nodule RNA was 219 bp in length, overlapping 160 identical base pairs of the corresponding cDNA clone 257-1. Whereas the sequence of lb49 was terminated by a poly(A) tail, the other cDNAs lacked this feature. This absence was also observed in further sequenced leghemoglobin clones of the cDNA library and could be explained by an incomplete second-strand cDNA synthesis. One possible poly(A) site could be identified with another *VfLb29* clone 226-1, which was identical for 459 base pairs with the cDNA clone 257-1. The parts of the *VfLb29* sequence completed by

RACE fragments and the cDNA clone 226-1 are underlined in Figure 3.

The molecular masses of the specified leghemoglobins range from 15.8 kDa for the *VfLbB* up to 16.5 kDa for the acidic *VfLb29* (Table 2). An alignment of the deduced amino acid sequences is shown in Figure 4A. Whereas the *VfLbB*, *VfLbK*, and *VfLb49* sequences showed an identity of over 80% between each other, the *VfLb29* sequence differed from these leghemoglobins by 32 to 38% at the amino acid level. Database searches revealed no higher homologies to *VfLb29* for other (leg)hemoglobins. Figure 4B presents an alignment dendrogram based on amino acid identities which reflects the relationship between plant (leg)hemoglobins and the outstanding position of *VfLb29* among the broad bean leghemoglobins. However, residues conserved between known plant and animal hemoglobins (Arredondo-Peter and Escamilla 1991) were also found to be conserved in all broad bean leghemoglobins (marked in Fig. 4). On the other hand, three of



**Fig. 3.** Nucleotide and deduced amino acid sequence of broad bean leghemoglobin cDNAs. The nucleotide sequences of the cDNA clones 150-1 (encoding *VfLbB*), 111-51 (encoding *VfLbK*), lb49 (encoding *VfLb49*) and 257-1 (encoding *VfLb29*) are presented. The *VfLb29* 5'-sequence completed by RACE-PCR and the 3'-untranslated sequence completed by the cDNA clone 226-1 are underlined. Deduced amino acid sequences are indicated above the nucleotide sequences. The length of poly(A) tails is indicated. Stop codons are marked by asterisks.

the amino acid residues conserved among leghemoglobins (Arredondo-Peter and Escamilla 1991) changed in the VfLb29 protein sequence. Two of these exchanges are conservative (Ile-60 and Thr-64 replace Leu and Ala, respectively), whereas Lys in position 144 changed to Met.

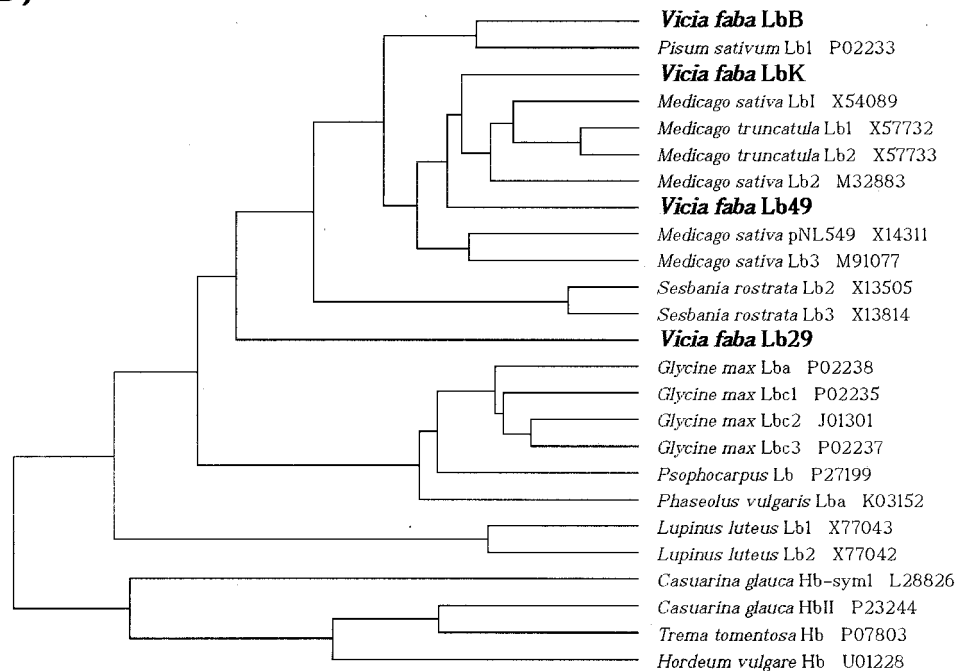
## DISCUSSION

The expression of 20 different broad bean genes encoding early and late nodulins was analyzed in broad bean roots colonized by the arbuscular mycorrhizal fungus *G. fasciculatum*. Only the leghemoglobin gene *VfLb29* was found to be induced

A)

VfLbB	MGFTDKQEALVNSSWESFKQNL SGNS I FFYTI I LEKAPAAKGLF	FSFLKDTAG		
VfLbK	MGFTEKQEALVNSSWELFKQNP -GNSVL FYTI I LKKA	P	TAKGMF	
VfLb49	MGFTQQQEALVNSSWESFKQNP -SYSVL FYTI I LEKAPAAKGM	F	FSFLKDSAG	
VfLb29	MEFTLRQEALVNSSWEAFNQNL PL FSVL FYTF I LEKAP	I	AKNME	
	* * * * *		* * * * *	
VfLbB	VEDS-PKLOAHAEQVFG	LVRD	SAAQ	
VfLbK	VVDS-PKLOAHAEKVFG	MVRD	SAVQ	
VfLb49	VVDS-PKLOAHAEQVFG	MVRD	SAIQ	
VfLb29	IPLANPSI NAHTEMVF	FEMVRD	AAAQ	
	* * * * *		* * * * *	
VfLbB	FVVVKEAL LQT I KKASGSNWSVEL	STAW	EVAYDGLASA I KKAMN	147 aa
VfLbK	FVVVKEAL LET I KE I SGDKWSEEL	SAW	EVAYEGLAVA I KKAMS	146 aa
VfLb49	FVVVKEAL LKT I KEASGDKWSEEL	SIW	EVAYDGLATA I KKAMS	146 aa
VfLb29	FVVVKEAL LKT I KEAVGDKWSEEL	SNW	E I AYDGLAVA I MKEMS	148 aa
	* * * * *		* * * * *	

B)



**Fig. 4.** Sequence comparison of plant (leg)hemoglobins. **A**, Alignment of the deduced amino acid sequences of VfLbB, VfLbK, VfLb49, and VfLb29. Identical residues in all four sequences are indicated by asterisks. Residues conserved between plant and animal hemoglobins are boxed. **B**, Unrooted dendrogram of the alignment from 25 plant (leg)hemoglobin protein sequences. The database accession numbers are indicated after the (leg)hemoglobin names. Abbreviations: aa, amino acids; Lb, leghemoglobin; Hb, hemoglobin.

in mycorrhizal roots, whereas all other nodulin transcripts tested, including other leghemoglobin transcripts, could not be detected. In particular, we were unable to show a mycorrhiza-induced expression of the early nodulin genes *VfENOD5* and *VfENOD12*. These results are in accordance with preliminary findings for pea where *PsENOD5* and *PsENOD12* transcripts (Scheres et al. 1990) could not be detected during arbuscular mycorrhizal infection by in situ hybridizations (Gollotte et al. 1993). To our knowledge homologues of the other broadbean nodulin genes tested have not been analyzed for their expression in mycorrhizal roots until now. It cannot be excluded that the absence of detection of early nodulin gene expression may have been linked to a low frequency of early infection stages (appressoria and first intercellular hyphae) which were present in less than 3% of the root system. This will have favored the detection of later infection events. However, the present results suggest that the large majority of the broad bean nodulins analyzed seem to play no role during the exponential phase of development within the arbuscular mycorrhiza symbiosis. On the other hand, the results of Wyss et al. (1990) indicate the presence of at least five different nodulins in mycorrhizal soybean roots, one of which may be related to nodulin 26. Therefore the existence of yet unidentified broad bean genes specifically activated in both symbiotic tissues cannot be excluded.

Northern blot experiments confirmed that the leghemoglobins VfLbB, VfLbK, and VfLb49 are exclusively expressed in root nodules whereas VfLb29 is additionally induced in mycorrhizal roots. The different signal intensities for nodules and mycorrhizal roots do not necessarily indicate a low expression level of *VfLb29* during mycorrhiza, since this gene might be expressed only in a limited number of cells within the mycorrhizal roots. The role of leghemoglobin as an oxygen carrier and facilitator of oxygen diffusion in legume nodules has been well documented (Appleby 1984; Layzell and Hunt 1990). In recent years, plant hemoglobins have also been identified in several non-legumes where they were found to be expressed in different tissues (e.g., Landsmann et al. 1986; Bogusz et al. 1988; Christensen et al. 1991; Taylor et al. 1994). Alternative functions, e.g., as oxygen sensors, have been suggested for nonsymbiotic plant hemoglobins (Appleby et al. 1988), and it has recently been reported that a barley hemoglobin gene is induced by hypoxic conditions (Taylor et al. 1994) supporting the oxygen transport role. Therefore, a possible function of the leghemoglobin in mycorrhizal roots could be to assure an oxygen supply for respiration of the microsymbiont, as in root nodules. Localization of the leghemoglobin *VfLb29* gene products in root cells infected by arbuscular mycorrhizal fungi by in situ hybridization or immunocytochemistry would lead support to this hypothesis. However, to demonstrate the symbiotic-specific nature of *VfLb29* expression in mycorrhiza, it will be necessary to prove that the *VfLb29* gene is induced by the fungus itself and not within the framework of an anaerobic response in infected roots.

Considering its unique expression pattern it is interesting to note that the leghemoglobin VfLb29 is characterized by a relative low level of sequence identity compared to the other broad bean leghemoglobins. Whereas the sequence divergency between VfLbB, VfLbK, and VfLb49 at the amino acid level does not exceed 20%, the VfLb29 sequence differs from these leghemoglobins by 32 to 38%. The outstanding characteristics

of VfLb29 within the broad bean leghemoglobin family resemble the differences between symbiotic and nonsymbiotic hemoglobins in the non-legume plant *Casuarina glauca*. The *CaHb-sym1* gene is exclusively expressed in nodules, whereas the *CaHbII* transcript could be detected in a number of different tissues, where oxygen supply is likely to be limited (Christensen et al. 1991; Jacobsen-Lyon et al. 1995). These hemoglobins show an amino acid identity of only 53%, indicating their distant relation. In contrast to *CaHbII*, in nodulated plants *VfLb29* is exclusively expressed in nodules but it is also induced in roots when colonized by a mycorrhizal fungus. Moreover, the *VfLb29* gene product, as is typical for symbiotic hemoglobins, lacks the N-terminal amino acid extension which is present in nonsymbiotic hemoglobins. The existence and role of leghemoglobins in tissues other than nodules have been speculated earlier (Appleby et al. 1988; Bogusz et al. 1988) and were supported by experiments of Jacobsen-Lyon et al. (1995). The authors demonstrated that the *Casuarina* hemoglobin gene *CaHbII* maintains its nonsymbiotic expression pattern even in the legume *Lotus corniculatus*.

In conclusion, *VfLb29* appears to represent a new class of leghemoglobin genes not identified until now which are inducible in different symbiotic associations, whereas *VfLbB*, *VfLbK*, and *VfLb49* belong to the well-established group of nodule-specific leghemoglobin genes. Taking into account the ancestral nature of arbuscular mycorrhiza (Remy et al. 1994), it has been speculated that some plant processes involved in nodulation may have evolved from those already established for mycorrhiza (Gianinazzi-Pearson et al. 1994; Gollotte et al. 1995). The identification of the mycorrhiza induced broad bean leghemoglobin VfLb29 lends support to this hypothesis. Further investigations are necessary to determine whether mycorrhiza-induced (leg)hemoglobin genes exist in other legumes or even non-legumes.

## MATERIALS AND METHODS

### Biological material and cDNA libraries.

Broad bean plants (*Vicia faba* L. cv. Kleine Thüringer) were grown in the greenhouse in sterile clay granules (Seramis) using surface-sterilized seeds (saturated Ca-hypochlorite, 30 min). Nodulated plants were obtained by inoculation of 2 day-old seedlings with *Rhizobium leguminosarum* bv. *viciae* VF39 (Priefer 1989) and mycorrhizal plants by inoculation at transplanting into sterile soil (clay loam, pH 7.5, 23 ppm Olsen P) with *Glomus fasciculatum* (Thaxter sensu Gerdeman) Gerd and Trappe amend Walker and Koske (isolate LPA 7), using surface sterilized (1% Chloramine T, 2 min) leek root inoculum placed in the planting hole. Uninoculated plants served as controls. To avoid contamination from *Rhizobium*, mycorrhizal and control plants were grown in individual Sunbags (Sigma) in a constant environment chamber (16 h day, 330  $\mu\text{mol s}^{-1} \text{m}^{-2}$ , 19/22°C). Flowers and seeds were analyzed from field-grown plants. The nodule-specific cDNA library was constructed in  $\lambda\text{gt}11$  from poly(A)<sup>+</sup> mRNA isolated from root nodules of *Vicia faba* L. cv. Kleine Thüringer (Perlick and Pühler 1993).

### Isolation of nucleic acids and recombinant DNA techniques.

Isolation of recombinant  $\lambda\text{gt}11$  DNA was carried out using standard protocols (Sambrook et al. 1989). Plasmid DNA was

isolated from *E. coli* JM83 using the "Plasmid Midi Kit" (Qiagen) according to the manufacturer's instructions. Probe DNA was extracted from agarose gels using the "QIAEX Gel Extraction Kit" (Qiagen). For Northern blotting, total RNA was isolated from nodules (32 days after sowing), mycorrhizal roots (25 days after sowing), uninfected roots (25 and 32 days after sowing), leaves (32 days after sowing), seeds (90 days after sowing), epicotyls (8 days after sowing), stems (12 days after sowing) and flowers (60 days after sowing) of broad beans using standard protocols (de Vries et al. 1982). Mycorrhiza colonization was estimated microscopically from random root samples taken prior to extraction using the method described by Trouvelot et al. (1986). Poly(A)<sup>+</sup> RNA fractions were isolated by one cycle of oligo(dT)-cellulose chromatography.

The "5'-AmpliFINDER RACE KIT" (Clontech) was used to obtain cDNA clones encoding the N-terminal amino acids of VfLb29. The cDNA synthesis was carried out according to the manufacturer's instructions using the VfLb29 specific primer 5'-TCACTCCATTTATCCCAACAGC-3'. Amplification of VfLb29 5' cDNA fragments was performed during 35 cycles of 45-s denaturation at 94°C, 45-s primer annealing at 54°C and 2 min elongation at 72°C using nested VfLb29 specific 3' primers (5'-GGTATCTCATTGGCATCCTTTAG-3' and 5'-TAGAACAAAACGCTGAAGA-3').

To generate overlapping sequencing clones, exonuclease III digestions were carried out using the "Double Stranded Nested Deletion Kit" (Pharmacia) according to the manufacturer's instructions. All other in vitro DNA manipulations were carried out using standard protocols (Sambrook et al. 1989).

#### DNA sequencing and sequence analysis.

Sequencing reactions have been carried out according to Zimmermann et al. (1990) using the "AutoRead Sequencing Kit" (Pharmacia). Sequencing gels were run on the "A.L.F. DNA Sequencer" (Pharmacia) using sequencing gel mixes of standard composition (Biozym Diagnostik GmbH). All sequences reported here were determined from both strands. Nucleic acid sequences were read using the "A.L.F. MANAGER V2.6" software (Pharmacia) and analyzed using the programme "ANALYSEQ" (Staden 1986) and "LFASTA" (based on Pearson and Lipman 1988). Multiple sequence alignments were carried out using the program "CLUSTAL" (Higgins and Sharp 1988) from the PC/Gene software package (IntelliGenetics, release 6.80).

#### Northern blot hybridizations.

Northern blotting and hybridizations were carried out as described previously (Perlick and Pühler 1993). About 50 ng of probe DNA isolated from agarose gels were labeled with 50 µCi of α<sup>32</sup>P-dATP according to Feinberg and Vogelstein (1983). Stringent washes were carried out at room temperature using 2 × SSC, 0.1% (w/v) SDS (5 min) and at 68°C using 0.2 × SSC, 0.1% (w/v) SDS (twice for 30 min each).

#### cDNA-cDNA hybridizations.

For cDNA-cDNA hybridizations, 0.2 µg of EcoRI digested DNA from different cDNA clones was separated electrophoretically and blotted onto Hybond-N nylon membranes (Amersham) using standard protocols (Sambrook et al. 1989).

First-strand cDNA probes were synthesized from 1 µg of poly(A)<sup>+</sup> RNA according to Gerard (1988) in an assay buffer containing 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 12 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM dCTP, dGTP and dTTP each, 100 µCi (α<sup>32</sup>P) dATP, unlabeled dATP to a final concentration of 11.5 µM, 1 µg oligo(dT)<sub>12-18</sub> and 200 units SUPER Script reverse transcriptase RNase H<sup>-</sup> (Gibco BRL) at 37°C for 60 min. RNA was hydrolyzed by 5 min incubation at 95°C with 1 vol 0.3 M NaOH, 30 mM EDTA. The resulting single-stranded cDNA probe was neutralized with 0.5 vol 1 M Tris-HCl pH 8.0 and used immediately for hybridization. Filters were hybridized in a solution containing 50 mM Na phosphate pH 7.0, 5 × SSC, 0.1% (w/v) lauroylsarcosine, 2% (w/v) blocking reagent (Boehringer), 7% (w/v) SDS and 50% (v/v) formamide for 48 h at 42°C. Stringent washes were carried out as described for Northern blot hybridizations.

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