The Promoter of the *Vicia faba* L. Leghemoglobin Gene *VfLb29* Is Specifically Activated in the Infected Cells of Root Nodules and in the Arbuscule-Containing Cells of Mycorrhizal Roots from Different Legume and Nonlegume Plants

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The *VfLb29* leghemoglobin gene promoter was polymerase chain reaction-amplified from a *Vicia faba* genomic library and was fused to the *gusA* coding region. Expression of the chimeric gene was analyzed in transgenic hairy roots of the legumes *V. faba*, *V. hirsuta*, and *Medicago truncatula* as well as in transgenic *Nicotiana tabacum* plants. The *VfLb29* promoter was found to be specifically active not only in the infected cells of the nitrogen-fixing zone of root nodules but also in arbuscule-containing cells of transgenic *V. faba* and *M. truncatula* roots colonized by the endomycorrhizal fungus *Glomus intraradices*. In addition to these two legumes, specific expression in arbuscule-containing cells was also observed in the nonlegume *N. tabacum*. All studies were done in comparison to the *V. faba* leghemoglobin gene promoter *VfLb3* that as *VfLb29* was expressed in the infected cells of root nodules but showed no activity in endomycorrhiza. An activation of the *VfLb29* promoter due to hypoxia in metabolically active tissues was excluded. The conserved activation in arbuscule-containing cells of legumes and the nonlegume *N. tabacum* suggests a conserved trigger for this promoter in legume and nonlegume endomycorrhiza symbioses.

Additional keywords: root transformation.

The symbiotic interaction of N₂-fixing soil bacteria of the family Rhizobiaceae and leguminous plants is the best studied system of plant-microbe interactions. By contrast, the molecular research concerning mycorrhiza symbioses is still at an early stage. Both symbioses have to be looked upon as ecological and economical factors of great importance (Jeffries 1987). In contrast to the taxonomically very restricted N₂-fixing symbiosis, the majority of higher plants are able to establish symbioses with arbuscular mycorrhizal (AM) fungi of the order Glomales. Mycorrhization offers several benefits to the host plant, including improved nutrition, greater drought resistance, and protection from pathogens (Harley and Smith 1983).

Closer examination of both root nodule formation induced by rhizobia and root colonization by arbuscular mycorrhizal

fungi reveals several similarities of these two interactions. Both symbioses are characterized by a bidirectional exchange of nutrients with carbon components flowing to the microsym-biont and inorganic nutrients moving to the plant. In both cases, the target tissue for the microsym-biont is the root cortex. Within colonized root cells, the microsym-bionts are separated from the host cytoplasm by specific plant-derived membranes and cell wall material (Gianinazzi-Pearson 1996). For the AM symbiosis, the main site of exchange between plant and fungus is the arbuscule, a highly branched structure that is formed by the fungus within inner cortical cells of the root (Harrison 1999). Arbuscules are transient structures, since they develop and subsequently degenerate within 6 to 10 days (Alexander et al. 1989; Dickson and Smith 2001). In case of the N₂-fixing symbiosis, the exchange occurs in the rhizobia-infected cells of the nitrogen-fixing zone III of the root nodule (Provorov et al. 2002). Due to the obvious similarities between these two sym-biotic interactions and the existence of mutations resulting both in *nod*[−] and *myc*[−] phenotypes, it was supposed that the two symbioses share a common genetic basis (Duc et al. 1989; Gianinazzi-Pearson et al. 1997).

Genes particularly involved in the N₂-fixing symbiosis have been subdivided into early and late nodulin genes, depending on the onset of their expression during nodule development. Early nodulin genes are expressed after mutual recognition between the plant and the rhizobia and before the start of nitrogen fixation. They are supposed to play a role in the infection process and in nodule organogenesis. Early nodulin genes such as *MtEnod11*, *MtEnod12* (Journet et al. 2001), *MsEnod40*, *MsEnod2* (VanRhijn et al. 1997), *PsEnod5*, and *PsEnod12* (Albrecht et al. 1998) have already been shown to be induced not only during nodulation but also in legume root tissues colonized by mycorrhizal fungi.

The expression of late-nodulin genes is activated around the onset of nitrogen fixation, and these gene products are involved in nodule functioning, which is mainly the metabolic exchange between plant and bacteroid. The most prominent and most thoroughly investigated late nodulins are the leghemoglobins (Lb), which are expressed in the infected cells just before and during nitrogen fixation. These oxygen-binding heme proteins are responsible for supporting the flux of oxygen to the nitrogen-fixing bacteroids. Apart from these symbiotic hemoglobins, several nonsymbiotic hemoglobin genes have been found

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in many legume and nonlegume plant species in recent years (Andersson et al. 1996; Seregelyes et al. 2000; Trevaskis et al. 1997). These genes are expressed in several plant tissues, and many of them were shown to be inducible by hypoxia, suggesting that they are somehow involved in the oxygen supply under this stress condition. However, an active transport of oxygen in this case is not likely due to the oxygen binding kinetics of these proteins (Hill 1998; Hunt et al. 2001). The most recently discovered type of plant hemoglobins are the 2-on-2 hemoglobins that share common structural characteristics with bacterial hemoglobins. The 2-on-2 hemoglobin gene *glb3* from *Arabidopsis thaliana* was found to be expressed throughout the plant, and it was shown that this kind of hemoglobin exists in many plant species (Watts et al. 2001). Remarkably, a 2-on-2 hemoglobin gene from *M. truncatula* was recently shown to be specifically induced in the root nodule (Fedorova et al. 2002).

The leghemoglobin gene *VfLb29* from *V. faba* is the first late-nodulin gene that has already been shown to be transcriptionally activated not only in the root nodule but also in roots colonized by an endomycorrhizal fungus. Interestingly, *VfLb29* exhibits a significantly low sequence homology to other *V. faba* leghemoglobin genes as well as to leghemoglobin genes from other legumes (Frühling et al. 1997). In this study, we report on further characterizations of this unusual leghemoglobin gene. By the use of promoter-*gus* fusions in transgenic plant tissues, we were able to demonstrate a specific expression in infected cells of legume root nodules as well as in legume and nonlegume root tissues colonized by the endomycorrhizal fungus *Glomus intraradices*. To our knowledge, this is the first legume gene that is specifically activated both in infected cells of the root nodule and in arbuscule-containing cells of the root cortex.

RESULTS

The promoter sequences of *VfLb29* and *VfLb3* contain motifs characteristic of symbiotic hemoglobin gene promoters.

A 1,261-bp *VfLb29* promoter fragment (accession number AJ564166) and a 2,003-bp *VfLb3* promoter fragment (acces-

sion number AJ001752) were isolated by polymerase chain reaction amplification from an adapter-ligated genomic broad bean library and by screening a genomic library of broad bean, respectively. The promoter fragments were sequenced and subsequently compared by multiple alignments to other symbiotic and nonsymbiotic hemoglobin gene promoters from various species. These database searches revealed that both the *VfLb29* and the *VfLb3* promoter shows characteristic similarities to symbiotic hemoglobin promoters. Common with other known leghemoglobin promoters, the *VfLb29* and *VfLb3* promoters exhibit the two consensus sequence motifs 'AAAGAT' and 'CTCTT' (Fig. 1). These motifs are characteristic elements of leghemoglobin and other nodulin gene promoters with respect to their expression in the infected cells of the root nodule tissue. These two motifs are part of the 'organ-specific element' (OSE) sequences originally identified in the soybean leghemoglobin *lbc3* promoter reported by Stougaard and associates (1987). By contrast, the sequence motif 'GAAGAG', characteristic of nonsymbiotic hemoglobin gene promoters, is not present in any of the two leghemoglobin promoter sequences isolated. Thus, on the nucleotide level, the *VfLb29* promoter shows typical characteristics of a symbiotic leghemoglobin promoter.

The *VfLb29* and *VfLb3* promoters are active in infected cells of *V. faba* and *M. truncatula* root nodules.

In order to study the expression properties of both *Lb* promoters during symbiotic nitrogen fixation, chimeric *pLb29-gusAint* and *pLb3-gusAint* constructs were inserted into the T-DNA (transfer-DNA) of the root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* ARqual (Quandt et al. 1993). In case of the *VfLb29* promoter, two fragments ranging from -1,261 to -31 and -987 to -31 were tested in all approaches. Since both fragments led to comparable patterns of gene expression (data not shown), the results presented below are based on the -987 to -31 *pLb29-gusAint* fusion. For the study in the homologous legume *V. faba* and the heterologous legume *M. truncatula*, an induction of transgenic roots was performed by an improved method in open pots, based on the protocol de-

<i>Vicia faba</i> ,	<i>VfLb29</i>	TATTATTTTAAAGATAA TTGTCTCTTAGC AATGACAATGGC CATCTCTA -152
<u>Symbiotic hemoglobins:</u>		
<i>Vicia faba</i> ,	<i>VfLb3</i>	GTCTTTTTTAAAGATTTC TTGTCTTTTAAATAATGCCAATGGT CATTTCCTA -154
<i>Medicago truncatula</i> ,	<i>MtLb1</i> ^a	TAGTTTTTGAAGATTTT TTGTCTCTT AATACTACAATGGT CACCTCCA -112
<i>Medicago truncatula</i> ,	<i>MtLb2</i> ^a	AAGTTTTTAAATGATTA TTGTCTCTT AATAATGTCAACAGC CATTTCCTA -115
<i>Sesbania rostrata</i> ,	<i>SrgLb3</i> ^b	TAAATTTTTAAAGATTA TTGTCTCTT AATAATGTCAATGGC CACCCTTC -165
<i>Glycine max</i> ,	<i>GmLbc3</i> ^c	AAGTTTTTGAAGATGA TTGTCTCTTCACCATACCAATTGAT CACCCTCC -104
<i>Glycine max</i> ,	<i>GmLbc1</i> ^c	AGGATTTTGAAGATCA TTGGCTCTTCGTCATGCCGATTGA CACCCTCC -91
<i>Glycine max</i> ,	<i>GmLbc2</i> ^c	AAGATTTTGAAGATCATTGGCTCTTCATCATGCCGATTGA CACCCTCC -91
<i>Casuarina glauca</i> (non-legume),	<i>CgsymHb</i> ^d	ACTTCAATCCCAAGATGTCCTCTCTCTTATTGATATTGAACAACAACAAAGAT -284
<u>Nonsymbiotic hemoglobins:</u>		
<i>Trema tomentosa</i> ,	<i>TremaHb</i> ^e	AAAAAACCCCAAGGAG ATGG CTCTCCAGTACCCT GAAGAGTTACATCTTA -85
<i>Casuarina glauca</i> ,	<i>CgHb</i> ^e	CAATTGACCCAAAGAA ATGG CTTTC GACCCACGAAGAGCCGGAGCTAT -177
<i>Parasponia andersonii</i> ,	<i>PaHb</i> ^e	TAAAAAACCCAAAGAT ATGG CTCCCCAATACCCT GAAGAGTTACACACGA -77
<i>Glycine max</i> (legume),	<i>GmHb</i> ^f	TAAGCCACACAAATGGGAATGA CTCTTCATTACA ATGAAGGGCCAACCTTTCA -133

Fig. 1. Alignment of promoter sequences from different symbiotic and nonsymbiotic hemoglobin genes. The consensus sequence motifs of organ-specific elements characteristic of the promoters activated in infected cells of root nodules and the common motif of the nonsymbiotic hemoglobin promoters are highlighted. The positions of the motifs are marked by boxes. Numbers indicate the distance from the translation initiator ATG (*VfLb29*, *VfLb3*, *CgsymHb*, *CgHb*) or the distance from the transcription initiation site (*MtLb1*, *MtLb2*, *SrgLb3*, *GmLbc1*, *GmLbc2*, *GmLbc3*, *TremaHb*, *PaHb*, *GmHb*). References indicated by superscript letters: a, Gallusci et al. 1991; b, Metz et al. 1988; c, Stougaard et al. 1987; d, Jacobsen-Lyon et al 1995; e, Christensen et al. 1991; and f, Andersson et al. 1996.

scribed by Quandt and associates (1993). This improved method was originally developed for the induction of hairy roots on *V. faba*, since this species is difficult to cultivate under sterile conditions, due to its size. However, this method also proved to be a simple and effective way to induce hairy roots on other legume species, such as *M. truncatula* (discussed below).

After the induction of hairy roots, the plants were inoculated with *Rhizobium leguminosarum* VF39 (Priefer 1989) and *Sinorhizobium meliloti* 1021 (Casse et al. 1979). After four weeks, *V. faba* and *M. truncatula* plants transformed with either construct were histochemically analyzed for β -glucuronidase (GUS) activity in roots and different stages of nodule development. Both *Lb* promoter-*gus* fusions showed a strong GUS activity in the infected cells of the nitrogen-fixing zone of mature nodules and only a low level of GUS activity in the interzone II-III of mature nodules (Fig. 2A, B, and C). These results were obtained both in the homologous legume *V. faba* (Fig. 2A and B) and the heterologous legume *M. truncatula* (Fig. 2C and D). Figure 2D documents in more detail the restriction of GUS activity to the infected cells of *M. truncatula* root nodules. GUS activity during earlier stages of nodule development, as described for the leghemoglobin gene *VsLb1* (Heidstra et al. 1997), was not observed (Fig. 3A, arrow). The expression patterns of *pLb29-gusAint* and *pLb3-gusAint* can be looked upon as an activity that is typical of leghemoglobin promoters.

The *VfLb29* promoter shows activation in arbuscule-containing root cells of *V. faba*, *V. hirsuta*, and *M. truncatula* plants colonized by the mycorrhizal fungus *G. intraradices*.

Since Northern hybridizations demonstrated that *VfLb29* transcripts are not only present in root nodules but also in root tissues colonized by the endomycorrhizal fungus *G. fasciculatum* (Frühling et al. 1997), our main interest was to examine in detail the activity of the *VfLb29* promoter during this symbiotic interaction. We made use of *pVfLb29-gusAint* and *pVfLb3-gusAint* transgenic hairy roots of *V. faba*, *V. hirsuta*, and *M. truncatula*, obtained as mentioned before. After root transformation, the plants were inoculated with the arbuscular mycorrhizal fungus *G. intraradices*. Approximately four weeks after inoculation, colonized roots were harvested for histological GUS-staining and were examined by light microscopy. In the case of roots transformed with the *pVfLb3-gusAint* construct, no GUS-staining above an occasional unspecific background was observed. By contrast, the examination of transgenic hairy roots carrying the *pVfLb29-gusAint* fusion revealed inner cortical cells with intense and specific *gus* expression (Fig. 3A, B, C, and D). For a closer look at these inner cortical cells, additional semithin sections of about 80 μ m were performed (Fig. 3A and B). To confirm that the *VfLb29* promoter is activated in root segments colonized by the fungus, the GUS-stained roots and semithin sections were subsequently treated with chlorazole black E (CBE), which visualizes fungal structures within the root tissue. The double-staining revealed that both the *gus* expression and the CBE-staining were mainly restricted to arbuscule-containing cells (Fig. 3F). Neither root tips, nodule primordia, nor other root tissues showed GUS activity (Fig. 3A, arrow, and C). In some cases, a weak GUS-staining was also observed in closely surrounding cells. This might reflect different stages of arbuscule development (Fig. 3E, arrows). As a control, wild-type roots were also examined, and no *gus* expression was observed in cells containing an arbuscule (data not shown). As in the case of the nodule symbiosis, the expression patterns observed were very similar in *V. faba*, *V. hirsuta*, and *M. truncatula*, and a low variability was observed in a large number of independent hairy roots of all species. Thus,

the presence of *VfLb29* transcripts in mycorrhizal roots of *V. faba* shown by Frühling and associates (1997) was now extended to the cellular level by the localization of specific promoter activity in arbuscule-containing cells.

The *VfLb29* promoter is active in root cells of the nonlegume *N. tabacum* that contain an arbuscule.

Symbiotic *Lb* were supposed to be specialized proteins with a specific function in the root nodule (Appleby 1984). The nodulation-independent activation of a leghemoglobin gene during the mycorrhiza symbiosis, as shown here for *VfLb29*, led to the question whether the *VfLb29* promoter might also be activated under mycorrhization in a nonlegume incapable of forming nodules. To answer this question, *pVfLb29-gus* transgenic tobacco plants were generated by *A. tumefaciens*-mediated transformation. Again, the *pVfLb3-gusAint* construct was used as a control. For investigations of promoter activities, the plants obtained were inoculated with *G. intraradices* spores and were grown in pots comparable to the treatment of the legume plants mentioned before. Following GUS-staining, the *VfLb29* promoter in *N. tabacum* showed the same specific activation in arbuscule-containing cells (Fig. 3F) that had been observed before in the case of *V. faba*, *V. hirsuta*, and *M. truncatula*. This was again verified by additional CBE-staining (data not shown). In accordance to the results in legumes, tobacco wild-type and *pVfLb3-gusAint* mycorrhizal roots showed no GUS-staining (data not shown).

Fig. 2. Histochemical localization of β -glucuronidase (GUS) activity in 3-week-old nitrogen-fixing nodules of transgenic hairy roots from *Vicia faba* and *Medicago truncatula* expressing *pVfLb29-gusAint* or *pVfLb3-gusAint*. Activity of **A**, *pVfLb29-gusAint* and of **B**, *pVfLb3-gusAint* shown in a longitudinal semithin section (80 μ m) of a *V. faba* root nodule, and activity of **C**, *pVfLb29-gusAint* shown in a longitudinal semithin section (80 μ m) of two *M. truncatula* root nodules. GUS activity is located in the infected cells. **D**, Close-up of the activity of *pVfLb29-gusAint* in a semithin section (80 μ m) of a *M. truncatula* root nodule. oc = outer cortex, u = uninfected cells, i = infected cells, and intZ = interzone II-III. Bars = 1 mm.

Fig. 3. Histochemical localization of β -glucuronidase (GUS) activity during mycorrhiza symbiosis in pVfLb29-gusAint transgenic hairy roots from *Vicia faba*, *V. hirsuta*, and *Medicago truncatula* and roots of transgenic *Nicotiana tabacum* expressing pVfLb29-gusAint. **A** and **B**, Semithin section (100 μ m) of a *V. faba* root showing GUS activity restricted to arbuscule-containing cells. The arrow in **A** indicates a nodule primordium without GUS activity. **C**, Whole root segment of *V. hirsuta* showing GUS activity in arbuscule-containing cells and the tip of the same root without GUS-staining. **D**, Whole root segment of *M. truncatula* showing GUS activity in arbuscule-containing cells. The arrow indicates an extraradical hyphae. **E**, Closeup of arbuscule-containing cells of *M. truncatula*. Arrows indicate cells that either show accumulating or disappearing GUS activity. **F**, Detail of an arbuscule-containing cell of *M. truncatula* showing GUS activity (blue) after double staining with chlorazole black E for visualization of fungal structures (black). **G**, Root segment of *N. tabacum* showing GUS activity in arbuscule-containing cells. **H**, pVfLb29-gusAint transgenic *N. tabacum* shows no GUS activity after hypoxic treatment. **I**, pGapC4-gusAint transgenic *N. tabacum* (control) shows GUS activity after hypoxic treatment. Both plantlets (**H** and **I**) were treated in the same container at the same time.

The *VfLb29* promoter shows no activity under hypoxic conditions.

Since Lb are involved in the oxygen supply of bacteroids during nitrogen fixation and since there is evidence that non-symbiotic hemoglobins are induced in plant tissues under hypoxic conditions (Hunt et al. 2001), a possible activation of the *VfLb29* promoter by hypoxia was tested. Both transgenic *N. tabacum* lines carrying the p*VfLb29-gusAint* and the p*VfLb3-gusAint* constructs, respectively, were used for that purpose. As a positive control, transgenic *N. tabacum* plants expressing the *gusAint* gene under the control of a glycolytic *GapC4* promoter from maize (Kohler et al. 1996) were used. The p*GapC4-gusAint* fusion provides ubiquitous *gus* expression under hypoxic conditions and was, therefore, a suitable control to study the expression behavior of p*VfLb29-gusAint* in tobacco. The p*VfLb29-gusAint* and p*VfLb3-gusAint* tobacco plants as well as the p*GapC4-gusAint* control plants were incubated in a container together with a hypoxic reagent for 48 h. After this treatment, the plants were analyzed histochemically for GUS activity in comparison with the untreated counterparts. The hypoxic-treated p*GapC4-gusAint* control plants showed strong *gus* expression in all parts of the plant (Fig. 4A), whereas no activity was observed in the untreated counterparts, verifying that hypoxia was established successfully. In

the case of the p*VfLb29-gus* (Fig. 4B) and p*VfLb3-gus* (data not shown) tobacco plants, however, no induction in either treated or untreated plants was visible in all plant tissues. This result shows that hypoxia signals alone are not sufficient to trigger expression of the *VfLb29* gene. It has to be noted that apart from arbuscule-containing cells, no GUS activity was observed in any tissue of the transgenic *N. tabacum* plants (Fig. 3H; data not shown), indicating that the promoter activity is specific for root-symbiotic interactions, at least in this model system.

DISCUSSION

In this report, we extended the characterization of the *V. faba* leghemoglobin gene *VfLb29* that was previously shown to be induced not only in *V. faba* root nodules but also in *V. faba* roots colonized by an endomycorrhizal fungus (Frühling et al. 1997). In accordance with the identification of an OSE-like motif characteristic of symbiotic leghemoglobin genes (Andersson et al. 1997; Stougaard et al. 1987), we observed *gus* expression in the infected cells of p*VfLb29-gusAint* transgenic root nodules of *V. faba* and *M. truncatula*, allowing us to conclude that *VfLb29* exhibits the characteristic cellular expression pattern of a typical leghemoglobin gene. However, in contrast to other Lb, *VfLb29* is activated not only in the infected cells of root nodules but also in arbuscule-containing root cells of the legumes *V. faba*, *V. hirsuta*, and *M. truncatula*. Since Lb are proteins typical of nodulating plants, it is interesting that the activation of the *VfLb29* promoter in cells carrying an arbuscule is conserved even in the nonlegume *Nicotiana tabacum*. This finding suggests that there is a common trigger for this promoter in arbuscule-containing root cells of different plant species, which probably relates to the common evolutionary origin of the mycorrhiza symbiosis.

For other nodulin genes activated during both symbiotic interactions, only the *gus* expression pattern of p*MtEnod11-gusAint* has been presented so far (Journet et al. 2001). For this early nodulin gene, the strongest GUS activity was observed in very early stages of nodule development. In the mature nodule, GUS activity was restricted to the distal end of the nodule, in contrast to p*VfLb29*, which showed activation restricted to the infected cells. The observations concerning the activity of p*MtEnod11* during the mycorrhiza symbiosis correlate to some extent with p*VfLb29*, since both promoters show activation in cells containing arbuscules. However, in contrast to p*VfLb29*, p*MtEnod11* is also active in early stages of infection with mycorrhizal fungi (Chabaud et al. 2002), and it is additionally activated in some nonsymbiotic tissues (Journet et al. 2001). In case of *VfLb29*, no activity was observed in nonsymbiotic tissues, e.g., the root tip, as described in the case of *Lbc3* (Franche et al. 1998), or in earlier stages of rhizobial symbioses, as found for the leghemoglobin gene *VsLb1* (Heidstra et al. 1997). Apart from arbuscule-containing cells, GUS-staining was not only absent from roots but also from any other tissues of the transgenic p*VfLb29-gus* *N. tabacum* plants, suggesting an exclusive induction of the *VfLb29* promoter in arbuscule-containing cells in the heterologous system tobacco. Although Northern hybridizations did not reveal *VfLb29* expression except for *V. faba* mycorrhizal roots and root nodules (Frühling et al. 1997), a local expression of the *VfLb29* gene in legume tissues outside the root cannot be excluded completely. Nevertheless, it is a true symbiosis-specific gene with respect to the root area. Since arbuscule-containing cells can be looked upon as analogs of the infected cells in the nodule, p*VfLb29* is the first promoter shown to be specifically active in the infected cells of both symbiotic interactions. Functional analyses of *VfLb29* promoter deletions could elucidate if the same sequence motif is responsible for the activation in infected cells

Fig. 4. Induction of hairy roots on *Medicago truncatula* in open pots. **A**, Plant 10 days after infection with *Agrobacterium rhizogenes*. The arrow indicates a swelling formed by callus cells at the inoculation site. **B**, The inoculation site is covered by a layer of vermiculite/sand. **C**, Examination of the development of hairy roots. **D**, Four-week-old plant with nodulated hairy roots. Arrows indicate hairy roots that have developed from the inoculation site, an arrow head marks the wild-type roots. At this stadium, wild-type root can be cut off to exclusively inoculate with the appropriate microsymbionts.

of nodules and mycorrhizal roots. A similar specific activity in cells containing an arbuscule as shown here for the *VfLb29* promoter has, so far, only been shown for promoters of two phosphate transporter genes, *Mtp4* of *M. truncatula* (Harrison et al. 2002) and *Spt3* of potato (Rausch et al. 2001). Transgenic roots carrying promoter-*gusAint*-fusions of these phosphate transporter genes showed *gus* expression patterns restricted to cells containing arbuscules, as shown for p*VfLb29-gusAint*. A sequence motif, which is part of the mycorrhiza and resistance-related element reported by Rausch and associates (2001) for the *Spt3* promoter, was not present in the promoter sequence of *VfLb29*. However, multiple sequence alignments of the three promoters revealed conserved sequence motifs (data not shown) that might be involved in their activity mediating specific expression in cells carrying an arbuscule. In combination with analyses using promoter deletions, these putative motifs could lead to the identification of common specific sequence motifs concerning the activation of plant promoters in arbuscule-containing cells.

By investigating the GUS activity of transgenic *N. tabacum* plants harboring a promoter-*gus* fusion of *VfLb29* under hypoxia, we were able to exclude an induction of *VfLb29* within the frame of a hypoxic response, as shown for nonsymbiotic hemoglobin genes (Hunt et al. 2001). This implies that, although the *VfLb29* protein might be involved in the oxygen supply as it is known for other Lb (Appleby 1984), the *VfLb29* gene is apparently not exclusively induced by this trigger.

The role of Lb to ensure the oxygen supply of microsymbionts in the root nodule has been well documented (Appleby 1984; Layzell and Hunt 1990). Thus, if we interpret arbuscule-containing cells as analogs of the infected cells in the root nodule, the most obvious role for a Lb in this context is to supply oxygen for the microsymbiont or to prevent the cell from running out of oxygen. The *VfLb29* gene, however, is outstanding amongst the other *V. faba* leghemoglobin genes because its deduced amino acid sequence differs from these by 32 to 38% (Frühling et al. 1997). Nevertheless, based on its expression pattern and the organ-specific element in the promoter sequence, this hemoglobin gene was classified as a leghemoglobin gene. This classification, however, does not exclude the possibility that the *VfLb29* protein exhibits similarities to the nonsymbiotic hemoglobins concerning the oxygen-binding kinetics. The affinity of nonsymbiotic hemoglobins for oxygen is much higher compared with Lb, resulting in a very slow release of the bound molecule (Duff et al. 1997). Only little is known about the role of the nonsymbiotic hemoglobins so far, but they are supposed to have several functions in different tissues (Arredondo-Peter et al. 1998). Expression in stressed tissues seems to be their common characteristic, and a role for the maintenance of metabolism under oxygen-limited conditions in these tissues is most likely (Arredondo-Peter et al. 1998; Hill 1998). Considering this, the function of *VfLb29* could also be related to support metabolism under oxygen-limiting conditions.

However, several other potential functions and multiple roles in different tissues can be postulated for plant Lb, e.g., the ability to bind nitric oxide (NO). NO, which is highly reactive and toxic, is produced in plant tissues due to various factors, and its role in plants is currently a point of discussion (Herouart 2002; Wojtaszek 2000). The synthesis of NO in the root nodule and its inhibitory effect on nitrogenase activity has already been demonstrated (Trinchant 1982). For animals, the reaction of NO with blood hemoglobin is considered to be the major route of detoxification (Wenmalm et al. 1992), which might be the same also for plants (Dordas et al. 2003). Thus, it seems to be possible that a hemoglobin protein could also be involved in the detoxification of nitric oxide that is produced in the frame

of the altered metabolism in the infected cells of nodules and mycorrhizal roots. Furthermore, NO is known to be involved in plant defense response against pathogens. Application of exogenous NO to plant tissues proved to trigger programmed cell death and defense gene induction. In accordance with that, NO scavengers were able to suppress defense response (Delledonne et al. 1998; Durner et al. 1998). Considering that plant defense response has to be suppressed in symbiotic interactions, a heme-protein could possibly act as a NO scavenger and contribute to the defense suppression process, both in the root nodule and in cells containing an arbuscules. In a recent publication, Uchiumi and associates (2002) also come to a similar conclusion. It should be noted that there is evidence that processes concerning the defense suppression take place specifically in cells containing an arbuscule, since an induction of several genes related to defense suppression was already shown to occur exclusively in these cells (Blee and Anderson 1996; Bonanomi et al. 2001; Salzer et al. 1999).

The hypotheses mentioned above show that there are several plausible explanations for the induction of a Lb gene in the context of plant-microbe symbioses. Nevertheless, it remains to be evaluated if this protein is present in meaningful amounts in the infected cells of root nodules and in the arbuscule-containing root cells of legumes. Since the possible function of *VfLb29* in these cells depends on its binding properties for oxygen, further investigations concerning the biochemical characteristics of *VfLb29* need to be carried out to elucidate its function in the nodule and mycorrhiza symbiosis.

MATERIALS AND METHODS

Plant material and microbial strains.

For the induction of hairy roots *V. faba* cv. Kleine Thueringer (University of Bielefeld, Germany), *V. hirsuta* (University of Bielefeld, Germany) and *M. truncatula* cv. Jemalong A17 (T. Huguet, INRA, Toulouse, France) were used. Leaf disks of *N. tabacum* cv. SAM (University of Bielefeld, Germany) were used for generating p*VfLb29* and p*VfLb3* transgenic tobacco, performed according to standard procedures (Rosahl et al. 1987). Transgenic tobacco harboring the hypoxic-inducible *GapC4* promoter-*gus* fusion (Kohler et al. 1996) was provided by R. Hehl (Technical University Braunschweig, Germany).

A. rhizogenes Arqua1 (Quandt et al. 1993) and *A. tumefaciens* EHA 105 (Hood et al. 1993) were used for the induction of hairy roots and for the tobacco leaf-disk transformation, respectively. Nodules were induced on 12- to 14-day-old transgenic hairy roots of *V. faba* by *R. leguminosarum* VF39 (Priefer 1989) and, in the case of *M. truncatula*, by *S. meliloti* 1021 (Casse et al. 1979). Inoculations with *G. intraradices* were carried out with a commercially available inoculum based on aseptic liquid medium containing spores (Premier Tech Biotechnologies, Rivière-de-Loup, Québec, Canada).

Construction of the *VfLb29* and *VfLb3* promoter-*gusAint* fusions.

The *VfLb3* promoter (–2003 to –1 in relation to the ATG) was inserted as a *Clal/EcoRI* fragment in the plasmid pGUS-INT (Küster et al. 1995), and the p*VfLb3-gusAint* fusion was subsequently cloned as a *SpeI* fragment in plasmid pIV2 (Küster et al. 1995). This plasmid was used to integrate the fusion into the T-DNA of the Ri plasmid of *A. rhizogenes* Arqua1 (Quandt et al. 1993) by single cross-over. Two fragments of the *VfLb29* promoter (1,261 to –31 and –87 to –31 in relation to the ATG) were inserted as an *EcoRI/SalI* fragment in plasmid pGUS-INT, and these p*VfLb29-gusAint* fusions were subsequently cloned as a *SalI* fragment in plasmid pIV2. The resulting plasmids were used to integrate the fusions into the T-DNA

of the Ri plasmid of *A. rhizogenes* Arqual (Quandt *et al.*, 1993) by single cross-over. The *A. tumefaciens*-mediated transformation of *N. tabacum* was carried out using derivatives of the binary vector pBIN19 carrying the *Lb* promoter-*gusAint* fusions. In the case of the pVfLb3-*gusAint* fusion, a *SpeI* fragment was cloned into the *XbaI* site of pBIN19, whereas in the case of the pVfLb29-*gusAint* fusions, *SalI* fragments were cloned into the *SalI* site of BIN19.

Plant growth conditions and induction of transgenic hairy roots

on *V. faba*, *V. hirsuta*, and *M. truncatula*.

The following protocol for the induction of hairy roots in open pots was originally developed for *V. faba*, since this species is difficult to cultivate under sterile conditions, due to its size. However, this method also proved to be a simple and efficient way to induce hairy roots on other legume species, such as *Pisum sativum* (data not shown), *V. hirsuta*, and most remarkable, *M. truncatula*. It could thus be an interesting alternative to the well-established in vitro method for inducing hairy roots on *M. truncatula* described by Boisson-Dernier and associates (2001).

Scarification of *M. truncatula* and *V. hirsuta* seeds with H₂SO₄ was carried out as described by Quandt and associates (1993). Scarification of *V. faba* seeds is not necessary. *M. truncatula* and *V. faba* seeds were spread out for germination in pots with humid vermiculite/perlite (1:1) and were incubated in the dark at 24°C for about 48 h. Afterwards, they have to be kept to 4°C for at least three days to make the tissue of the seedlings more susceptible to inoculation with agrobacteria. Seedlings were then planted into small pots with vermiculite/perlite (1:1) fertilized with 1/2 strength Hoagland's solution (Arnon and Hoagland 1940). The plants were now inoculated with *A. rhizogenes* carrying the *Lb* promoter-*gusAint* fusions integrated into the T-DNA. An inoculation with *A. rhizogenes* grown in liquid media is not possible, since this would lead to rotting symptoms on the inoculation site. Thus, the bacteria were grown on petri dishes with the appropriate antibiotics for 24 h at 30°C. The agrobacteria were scraped off the petri dish and were resolved in 1 ml of sterile PS buffer. The *V. faba* and *V. hirsuta* seedlings were inoculated by an injection three times parallel to the vascular bundle of the epicotyl, as described for *V. hirsuta* by Quandt and associates (1993). In the case of *M. truncatula*, the injection was performed at the white part of the hypocotyl. The plantlets were then set under a plastic cover for about two days in the growth chamber at 20°C. After about 10 days, small swellings formed by callus cells became visible at the inoculation site (Fig. 4A, arrow). These parts were covered by a layer of vermiculite/sand (1:1) (Fig. 4B), and the temperature was set up to 22°C. In the following time, the plants developed hairy roots, which could be examined by careful removal of the vermiculite/sand (Fig. 4C). After hairy roots developed, all roots that did not emerge from the infection site (Fig. 4D, arrows indicate hairy roots, arrow head wild-type roots) were removed, and the plants were planted into bigger pots for inoculation with appropriate microbes. Apart from the exceptions mentioned before, all plants used for this study were grown in the growth chamber under a 16-h light (22°C) and 8-h dark (18°C) regime.

Typical frequencies of hairy root formation at the inoculation site were about 70% with about 75% of the roots expressing the transgene (data for *M. truncatula*).

Histochemical analysis of transgenic tissues.

Histochemical assays for GUS activity were performed as described by Jefferson *et al.* (1987). The GUS substrate solution contained 2 mM X-Gluc (5-bromo-4-chloro-3-indolyl- β -D-

glucuronide cyclohexylammonium salt; Biosynth, Switzerland), 2 mM potassium ferricyanide, 100 mM Tris, pH 7.0, 50 mM sodium chloride, pH 7.0, and 0.1% Tween-20. Roots were vacuum-infiltrated prior to incubation in the dark at 37°C for 1 to 24 h. GUS assays of hairy roots expressing promoter-*gusAint* fusions were performed four weeks after nodulation and 25 days after inoculation with *G. intraradices*.

For staining of fungal structures, roots were first cleared with 10% KOH (wt/vol, potassium hydroxide dissolved in water) for 10 min at 90°C and were subsequently assayed by 0.03% wt/vol CBE in a lactoglycerol solution (1:1:1, lactic acid/glycerol/water) for 4 h at 90°C, as described by Brundrett and associates (1984). Prior to observation, the roots were de-stained with 50% vol/vol glycerol for several days.

Before the GUS-staining of mycorrhizal roots and root nodules, semithin sections (80 to 120 μ m) were prepared with a microtome (Leica VT/000S; Wetzlar, Germany). Examination of tissues was carried out by light microscopy (Olympus BH-2; Hamburg, Germany), and documentation was done using a digital camera (Olympus C-2000Z).

Stress treatment.

For hypoxic induction, plants were incubated in an air-tight glass container (Merck; Darmstadt, Germany), together with Anaerocult A (Merck) for 48 h. The establishment of a hypoxic atmosphere was verified by Anaerotest stripes (Merck).

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