



RESEARCH PAPER

# The promoter of the leghaemoglobin gene *VfLb29*: functional analysis and identification of modules necessary for its activation in the infected cells of root nodules and in the arbuscule-containing cells of mycorrhizal roots

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## Abstract

In this study the further characterization of the *Vicia faba* leghaemoglobin promoter *pVfLb29* is presented that was previously shown to be specifically active in the infected cells of root nodules and in arbuscule-containing cells of mycorrhizal roots. Using promoter studies in transgenic hairy roots of the *Pisum sativum* mutant *RisNod24*, disabled in the formation of functional arbuscules, *VfLb29* promoter activity is assigned to later stages of arbuscule development. In order to narrow down the regions containing *cis*-acting elements of *pVfLb29*, the activity of five *VfLb29* promoter deletions (–797/–31 to –175/–31 in relation to the start codon) fused to the *gusAint* coding region were tested in transgenic *V. hirsuta* hairy roots. The results specify a promoter region ranging from position –410 to –326 (85 bp) as necessary for *gus* expression in arbuscule-containing cells, whereas this segment is not involved in the nodule-specific activity. Sequence analysis of the *pVfLb29* fragment –410/–326 (85 bp) revealed sequence motifs previously shown to be *cis*-acting elements of diverse promoters. To investigate the autonomous function of *pVfLb29* regions for activation in arbuscule-containing cells, different regions of *pVfLb29* from positions –410 to –198 were used to

prepare chimeric promoter constructs for *trans*-activation studies. These fragments alone did not activate the mycorrhiza inactive promoter of the *Vicia faba* leghaemoglobin gene *VfLb3*, showing that the activation of *pVfLb29* in arbuscule-containing cells is governed by a complex regulatory system that requires at least two modules located between position –410 and –31 of the *VfLb29* gene.

Key words: Arbuscules, leghaemoglobin promoter, mycorrhizal roots, root nodules, *Vicia faba*.

## Introduction

The majority of legume species can establish symbiotic associations with bacteria of the genus *Rhizobium* and arbuscular mycorrhizal fungi of the order *Glomeromycota* (Schüssler *et al.*, 2001). The *Rhizobium*–legume interaction leads to symbiotic nitrogen fixation carried out by differentiated bacteria within a specialized plant organ, the root nodule (Brewin, 1991). The arbuscular mycorrhiza (AM) symbiosis offers several benefits to the host plant, including improved nutrition (mainly phosphorus), enhanced drought resistance, and protection from pathogens (Smith and Read, 1997; Brundrett, 2002). Due to these facts both symbioses have to be looked upon as factors of great importance both

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for nature ecosystems and for agriculture (Provorov *et al.*, 2002).

For the N<sub>2</sub>-fixing symbiosis, nutrient exchange occurs in the rhizobia-infected cells of the nitrogen-fixing zone III of root nodules (Provorov *et al.*, 2002). In the case of the AM symbiosis, the major site of exchange between plant and fungus is the arbuscule, a highly branched structure that is formed by the fungus within the inner cortical cells of the root. In addition, the intercellular hyphae are also discussed as sites for the transfer of nutrients (Harrison, 1999). Arbuscules are transient structures since they develop and subsequently degenerate within 6–10 d (Alexander *et al.*, 1989; Dickson and Smith, 2001). Due to the obvious similarities between both symbiotic interactions and the existence of mutations resulting both in nod<sup>-</sup> and myc<sup>-</sup> phenotypes, a common genetic basis of the two symbioses was supposed (Duc *et al.*, 1989; Gianinazzi-Pearson, 1997). It was recently shown that both microsymbionts induce a common signalling cascade during initiation of root nodules and AM (Cullimore and Denarie, 2003), and several nodulin genes such as *MtEnod11*, *MtEnod12* (Journet *et al.*, 2001), *MsEnod40*, *MsEnod2* (van Rhijn *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 arbuscular mycorrhizal fungi.

The most abundant and best-characterized nodule-specific proteins are the leghaemoglobins (Lb), which are expressed in the infected cells just prior to the onset of nitrogen fixation. These oxygen-binding haem proteins are supposed to be responsible for supporting the flux of oxygen to the nitrogen-fixing bacteroids (Appleby, 1984). One member of this family, the *Vicia faba* Lb gene *VfLb29*, was shown to be expressed in both symbiotic interactions (Frühling *et al.*, 1997) and, recently, the promoter of this gene was found to be active not only in the infected cells of root nodules but also in arbuscule-containing cells of mycorrhizal roots (Vieweg *et al.*, 2004). A similar highly specific activity in arbuscule-containing cells, as shown for the *VfLb29* promoter (Vieweg *et al.*, 2004), has also been demonstrated for the promoters of two phosphate transporter genes, *MtPt4* of *M. truncatula* (Harrison *et al.*, 2002) and *Spt3* of potato (Rausch *et al.*, 2001).

Specific *cis*-acting elements, which play a role in gene regulation, have traditionally been identified by carrying out deletion analyses of the promoter regions of diverse plant genes. In particular, the structural properties of several nodule-specific promoters were studied that way (Stougaard *et al.*, 1990; Rodriguez-Llorente *et al.*, 2003; Nakawaga *et al.*, 2003). These analyses led to the identification of several regulatory elements, for example, enhancers, organ- or cell-specific elements, and strong positive elements in addition to core promoter motifs. Common with other known leghaemoglobin promoters, p*VfLb29* exhibits the two consensus sequence motifs 'AAAGAT' and 'CTCTT'

between positions -193 and -175 (Vieweg *et al.*, 2004). These two motifs were shown to be necessary for promoter activity in the infected cells of root nodules and are part of the organ-specific element (OSE) originally identified in the soybean leghaemoglobin *lbc3* promoter reported by Stougaard *et al.* (1990).

In order to identify regulatory elements in the *VfLb29* promoter that are required for its activity in both arbuscule-containing cells of mycorrhizal roots and the infected cells of root nodules, promoter deletion and cross-activation studies in transgenic mycorrhizal roots and nodules of the vetch *V. hirsuta* were carried out. As far as is known, this is the first approach to identify minimal regulatory regions mediating activity of promoters in arbuscule-containing cells of mycorrhizal roots.

## Materials and methods

### Plant material and microbial strains

Seeds of the *P. sativum* mutant RisNod24 (Engvild, 1987; Sagan *et al.*, 1994) and the corresponding wild-type 'Finale' were supplied by Gerard Duc (INRA Dijon, France). *V. hirsuta* seeds were obtained from John Chambers Ltd., London. *A. rhizogenes* strain Arqua1 (Quandt *et al.*, 1993) was used for the induction of hairy roots. Nodules were induced on 12–14-d-old transgenic hairy roots of *V. hirsuta* and *P. sativum* wild-type by *R. leguminosarum* strain VF39 (Priefer, 1989). 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 promoter-gusAint fusions

The 5' promoter deletions of *VfLb29* were amplified by PCR, and their sequences were subsequently confirmed. The amplified regions -534/-31, -410/-31, and -325/-31 were cloned as *XhoI/EcoRI* fragment, whereas the -797/-31 region was cloned as *Clal/EcoRI* and the -175/-31 region as the blunt/*EcoRI* fragment into plasmid pGUS-INT (Küster *et al.*, 1995). The resulting p*VfLb29-gusAint* fusions were subsequently cloned as *SpeI* fragments (filled in using Klenow polymerase) into the *SmaI* site of the binary vector pRedRoot (Limpens *et al.*, 2004), and the resulting binary vectors were transformed into *A. rhizogenes* Arqua1 (Quandt *et al.*, 1993).

The p*VfLb29* fragments for the construction of the chimeric promoter-gusAint fusions were isolated by PCR and cloned as *SmaI/BglII* in front of the *VfLb3* (-356/-1) promoter-gusAint fusion. The resulting chimeric promoter-gusAint fusions *VfLb3+(-410/-326)*, *+(-410/-245)*, and *+(-410/-198)* were subsequently cloned as *SpeI* fragments (filled in using Klenow polymerase) into the *SmaI* site of the binary Vector pRedRoot (Limpens *et al.*, 2003), and the resulting binary vectors were transformed into *A. rhizogenes* Arqua1 (Quandt *et al.*, 1993).

### Induction of transgenic hairy roots and conditions of plant growth

The induction of transgenic roots was performed by a method in open pots as described by Vieweg *et al.* (2004). Plants were fertilized weekly with half-strength Hoagland's solution (Arnon and Hoagland, 1940) containing 20 µM phosphate and grown in pots with sterilized clay granulate (Seramis, Masterfoods GmbH, Verden, Germany). All

plants were grown in growth chambers under a photoperiod of 16 h at 22 °C with a decrease to 18 °C during the dark phase. The relative humidity was set to 70%.

### Histochemical analysis of transgenic tissues

The results presented in this study are based on the analysis of a representative number of independent hairy roots for each approach.

Prior to the histochemical analysis of root tissues, transgenic roots were selected by using the binary vector pRedRoot (Limpens *et al.*, 2004) that provides constitutive *dsRed* gene expression in order to ensure the identification of transgenic hairy roots by *dsRed* fluorescence. Examination of fluorescence was carried out using a fluorescence stereomicroscope (Leica MZ FL III, Wetzlar, Germany).

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- $\beta$ -D-glucuronide cyclohexylammonium salt; Biosynth, Switzerland), 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, 100 mM TRIS pH 7.0, 50 mM sodium chloride pH 7.0, and 0.1% (v/v) Tween-20. Roots were first vacuum-infiltrated prior to incubation in the dark at 37 °C for 1–24 h. GUS assays of hairy roots expressing promoter-*gusAint* fusions were performed 4 weeks after nodulation and 25 d after inoculation with *G. intraradices*. For staining of fungal structures, roots were first cleared with 10% KOH (w/v potassium hydroxide dissolved in water) for 10 min at 90 °C and were subsequently assayed using 0.03% (w/v) Chlorazol black E in a lactoglycerol solution (1:1:1 by vol. lactic acid, glycerol, and water) for 4 h at 90 °C as described by Brundrett *et al.* (1984). Prior to observation, roots were destained with 50% (v/v) glycerol for several days. For GUS staining of root nodules, semi-thin sections of 80–120  $\mu$ 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, Hamburg, Germany).

## Results and discussion

### The activity of the *VfLb29* promoter is confined to cells containing fully developed arbuscules

The activity of the *VfLb29* promoter (Fig. 1) was analysed in transgenic hairy roots induced on the *P. sativum* mutant

RisNod24 (Engvild, 1987; Sagan *et al.*, 1994). This mutant is not only unable to develop nodules, but is also disabled in the formation of late developmental stages of mycorrhiza symbiosis (Lapopin *et al.*, 1999), since only the formation of deformed and unbranched arbuscules that lack a complete function is admitted by the plant (Fig. 2E). The activity of the *VfLb29* promoter (–996/–31 region, GenBank accession number AJ564166) in the mutant background was analysed in order to give information on the start of *VfLb29* expression during the development of arbuscules within a cell. The root transformations on the *P. sativum* RisNod24 mutant and the corresponding wild-type were performed as described in Vieweg *et al.* (2004).

In transgenic roots induced on the *P. sativum* wild-type (cv. Finale), p*VfLb29* shows activity in the infected cells of root nodules (Fig. 2A) and in the arbuscule-containing cells of mycorrhizal roots (Fig. 2B). To verify the presence of the mycorrhizal fungus *G. intraradices*, the GUS-stained roots were subsequently treated with Chlorazole Black E (CBE) which visualizes fungal structures within the root tissue (Fig. 2C). This result is in complete accordance to the *VfLb29* promoter activity in other legumes such as *V. faba*, *V. hirsuta*, and *M. truncatula* (Vieweg *et al.*, 2004).

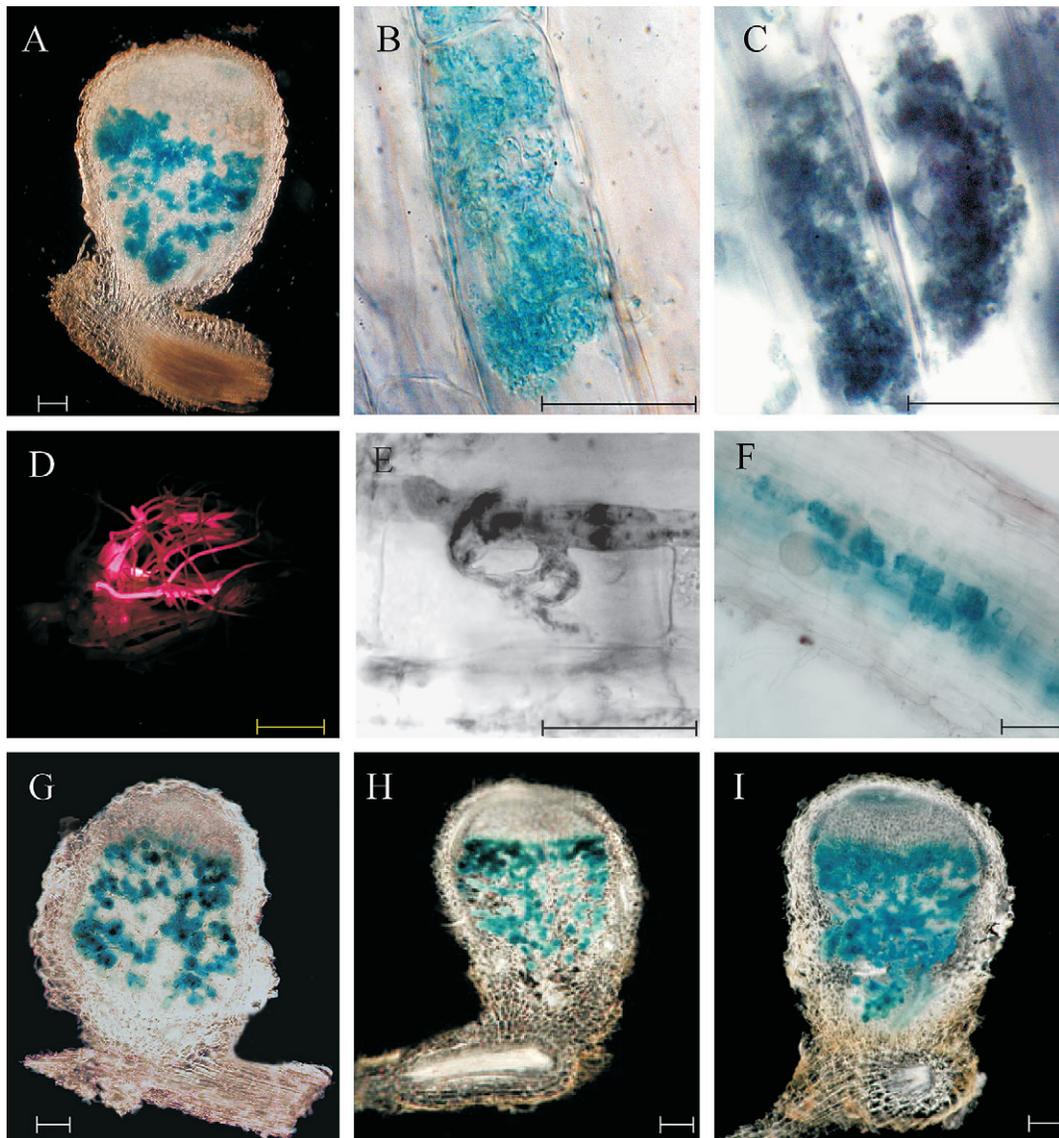
In the case of the *P. sativum* RisNod24 mutant, no GUS staining was observed in cells containing the characteristically deformed arbuscules, as verified by subsequent treatment with CBE (Fig. 2E). In the case of no detectable GUS activity concerning the p*VfLb29-gusAint* transgenic roots of the mutant, it was of importance to verify the transgenic nature of the roots studied. This was ensured using the binary vector pRedRoot (Limpens *et al.*, 2004) that provides constitutive *dsRed* gene expression in order to enable the identification of transgenic hairy roots by *dsRed* fluorescence (Fig. 2D). Arbuscules are transient structures which have a life span of 6–10 d including the stages of arbuscule formation, metabolic activity, and subsequent degeneration (Alexander *et al.*, 1989; Dickson and Smith, 2001). It is obvious that these different developmental steps

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-996      ATTCTAACCAACCCCTTAAGCAACCAATGTTGAAGGACTCAAAGGGGTATTAGGAATGAGAAGCTTAGGAAATC
-920 TAAATTAGAAAGTAATTTTAGTGGTTGTTTCAAAGTATTGCATGTGTGACTTGTAAACCAAGCAAAATGCCATAACCTGACTGTT
-835 ATATTTAGTTTGTGGTTATGGTTTTTCCAACATATAAGCACGCGTCTCTCTCTCACTCTCTCAATCTCTCTCAGTCTCTCT
-750 CTATGTAATTTGTATTCTGTATTCAATTAATTCATTTTTCTCTCAAATTAATCTTTTATCTTTCTTTAATTTTTCAATTCGTA
-665 TAAACTTAAAAAGTCAACTTACCTAATATTGAGTATGCTAAAAGTTAAATTAACCTTTGTATTAGTAGTTATTAATGCAAAGTTA
-580 GCAATTTGTAACCAAAAATATGCATTGTTATCGAAATCAACTCCAACCGCGGAATCAATACAAAGACAAACCTCAACCTAACAA
-495 GCATTCCTAAGATAGTGGATCAACTTTCAAGTCCAAAAGGAAAATAAAAAGGTCGGTTTATTTGAGAATTTTTATCAGTATTA
-410 GGACGTGCAGTAGCAATTACCTCTAAATTTATTAAATGTTTTTGCATTATCTTTTAAATATCTAAAAGTAGTTTTGGTTCATTA
-325 GCTTAGAAAATCCCATCTATTCAAAGTATATTATTTGATAAATTTATCTCTTAAAAATTAAGTAGACAAAGTTTGTATTTCATT
-240 TTTTAAATTTATTTTTTAATTAATTAATTTTATGGTTATTATTTTAAAGATAAATTGTCTCTTAGCAATGACAATGCCATCT
-155 CTAATCAAAATACAATATATTTCTTAAATAAACCAATTACAAGAACTCTTAATTTTTCACTCACCAACCTTCTATATAAGCATT
-70  TGATGAGCCTAAAGCTATAGCATCAAAAATATTGAAATTCAAATTAACAAAAGAAATATAACAAAAGAAATATG

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**Fig. 1.** Sequence of the *VfLb29* promoter. The region –410/–326 (85 bp) necessary for activity in the arbuscule-containing cells is highlighted and indicated by a dotted line. The two parts of the palindromic motif are marked by arrows. The sequence of the SPE (lower strand) is underlined and marked in italics. Consensus sequence motifs of the OSE are highlighted and in bold. The putative TATA-box is marked by a box. A triangle indicates the cloning site of all p*VfLb29* loss-of-function derivatives to the *gusAint* coding region.



**Fig. 2.** Examination of transgenic root nodules and mycorrhizal roots transformed with different pVfLb29-gusAint derivatives. Black and white bars=0.1 mm; yellow bar=1 cm. (A) Histochemical localization of GUS activity in a nodule of transgenic hairy roots from the *P. sativum* wild type expressing pVfLb29-gusAint (-996/-31). GUS activity is located in the infected cells. (B) Close-up of an arbuscule-containing cell of the *P. sativum* wild type expressing pVfLb29-gusAint (-996/-31). (C) Detail of two arbuscule-containing cells of the *P. sativum* wild type, after double staining with CBE for visualization of fungal structures (black). (D) Transgenic hairy roots from the *P. sativum* mutant RisNod24 transformed with pVfLb29-gusAint (-996/-31), showing dsRed fluorescence. (E) Detail of a GUS negative pVfLb29-gusAint (-996/-31) transgenic root cell of the *P. sativum* mutant containing a characteristically deformed arbuscule, after double staining with CBE for visualization of fungal structures (black). (F) Root segment of *V. hirsuta* showing pVfLb29-gusAint (deletion -410/-31) activity in arbuscule-containing cells. (G) Root nodule of *V. hirsuta* showing pVfLb29-gusAint (deletion -410/-31) activity in the infected cells. (H) pVfLb29-gusAint (deletion -326/-31, mycorrhiza inactive) transgenic root nodule of *V. hirsuta* showing activity in the infected cells. (I) Chimeric construct pLb3+(-410/-198)-gusAint (mycorrhiza inactive) transgenic root nodule of *V. hirsuta* showing activity in the infected cells.

require a sophisticated system of differential gene expression and hence regulation of promoter activity. The results presented here suggest that the factor(s) activating pVfLb29 in arbuscule-containing cells are not related to the induction of arbuscule formation or the pure presence of fungal tissues within the root cell, but rather to a fully developed, probably metabolically active arbuscule. It has previously been speculated that a possible function for VfLb29 concerning the detoxification of nitric oxide to avoid cell

death and defence gene induction in symbiotic interactions (Vieweg *et al.*, 2004). It should be noted that there is evidence that processes concerning defence suppression take place specifically in cells containing arbuscules, since an induction of several genes related to defence suppression was already shown to occur exclusively in these cells (Bonanomi *et al.*, 2001; Blee and Anderson, 1996; Salzer *et al.*, 1999). Against this background, it is possible that metabolically active arbuscules produce substances or

provoke processes that directly or indirectly induce a cascade of reactions leading to plant defence suppression, and these processes might include factors activating the *VfLb29* promoter.

#### Analysis of *VfLb29* promoter deletions in transgenic hairy roots of *V. hirsuta*

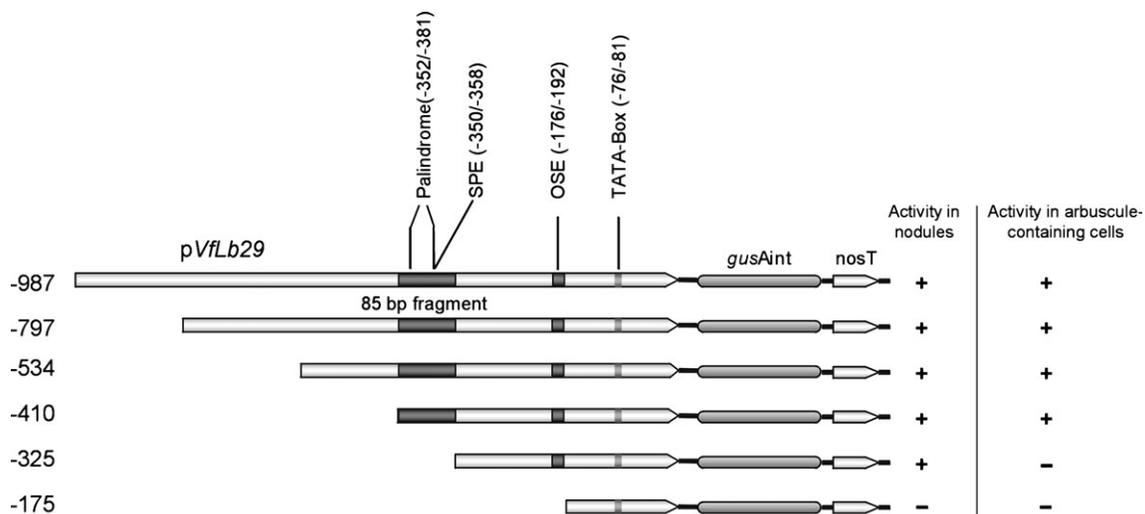
In order to define and characterize regulatory regions of the *VfLb29* promoter responsible for the activation in the infected cells of root nodules and the arbuscule-containing cells of mycorrhizal roots, 5' promoter deletions were constructed of different lengths fused to the *gusAint* coding region (Fig. 3) for their use in a loss-of-function approach. The induction of transgenic roots with these constructs was performed as described by Vieweg *et al.* (2004) on the legume species *V. hirsuta*, a close relative to *V. faba*. As for *P. sativum*, verification of the transgenic nature of the roots was ensured by the use of the binary vector pRedRoot. After induction of hairy roots, the *V. hirsuta* plants were inoculated separately with *Rhizobium leguminosarum* bv. *viciae* and with the arbuscular mycorrhizal fungus *G. intraradices*, respectively. After 4 weeks, transgenic roots were identified by dsRed fluorescence and analysed histochemically for  $\beta$ -glucuronidase (GUS) activity in root nodules and mycorrhizal roots. To verify the presence of arbuscules, GUS-stained roots were subsequently treated with CBE.

The GUS staining of transgenic roots and nodules transformed with the constructs  $-797/-31$ ,  $-534/-31$ , and  $-410/-31$  showed promoter activity of the same pattern as detected for the entire promoter, both in the infected cells of root nodules shown in Fig. 2G ( $-410/-31$  fusion) and in arbuscule-containing cells shown in Fig. 2F ( $-410/-31$  fusion). The next shorter deletion  $-325/-31$ , however, still

showed unaltered activity in root nodules (Fig. 2H) but no activity in cells containing arbuscules (data not shown, Fig. 3). This result suggests that *cis*-acting elements involved in the activity of the *VfLb29* promoter in arbuscule-containing cells are located in the sequence between the  $-410/-31$  and the  $-325/-31$  deletion (Figs 1, 3), clearly demonstrating that the activity of the *VfLb29* promoter in both symbioses is based on different regulatory regions. This result further implies that although there are obvious analogies between the infected cells of root nodules and the arbuscule-containing cells of mycorrhizal roots, at least one additional trigger is responsible for the activation of p*VfLb29* in AM tissues. The shortest deletion  $-175/-31$  shows neither GUS activity in nodules nor in mycorrhizal roots (data not shown, Fig. 3). This construct lacks the consensus motifs of the organ-specific element (OSE) (Figs 1, 3) which was previously shown to be an essential element of leghaemoglobins and other nodulin gene promoters with respect to their expression in the infected cells of root nodules (Stougaard *et al.*, 1990). Therefore, the lack of promoter activity of the  $-175/-31$  construct in the infected cells of root nodules is probably due to the absence of the OSE consensus sequence.

#### The *VfLb29* promoter contains putative *cis*-acting elements between position $-410$ bp and $-326$ bp

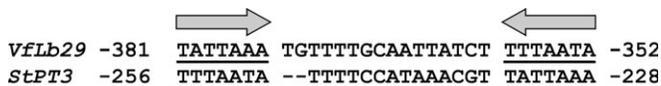
The deletion analysis of the *VfLb29* promoter in *V. hirsuta* suggests the presence of *cis*-acting elements essential for *VfLb29* expression in arbuscule-containing cells between position  $-410$  and  $-326$ . At the nucleotide level, it was possible to identify several motifs within these 85 bp (Fig. 1) that represent putative *cis*-acting elements possibly involved in the activation of p*VfLb29*. From position  $-350/-358$  a sequence motif AATATTAAA was found



**Fig. 3.** p*VfLb29* deletion constructs fused to the *gusAint* reporter gene used for a loss-of-function analysis of p*VfLb29*. The activation patterns in root nodules and arbuscule-containing cells are indicated for each construct.

(Fig. 1, lower strand) which is also present in the strong positive element (SPE) of the soybean *lbc3* promoter (Stougaard *et al.*, 1990) and which was also discovered recently in the soybean phosphoenolpyruvate carboxylase *GmPEPC7* promoter as a part of a regulatory element (Nakagawa *et al.*, 2003).

Within the 85 bp fragment (−410/−326) of the *VfLb29* promoter, a palindromic sequence motif of 14 bp between position −381 and −351 (Figs 1, 4) that might represent a putative regulatory element was further identified. Interestingly, the left part of this palindromic motif overlaps to some extent with the SPE-like element (Fig. 1). Cross-activation studies of the *GmPEPC7* promoter also identified a 14 bp palindromic sequence as part of a switch region involved in its regulatory function (Nakagawa *et al.*, 2003). Alignments with promoters that are also specifically active in arbuscule-containing cells revealed a palindromic sequence motif at position −253 and −226 relative to the start codon in the promoter of the potato phosphate transporter gene *StPt3* (Rausch *et al.*, 2001) (Fig. 4). Although the two inverse sequences of the palindrome are exchanged, the position and sequence of this 14 bp palindromic element are similar to that of p*VfLb29* (Fig. 4). That finding can be considered as a hint that these palindromic sequences exhibit a *cis*-acting function for the activation in cells containing arbuscules and that the regulation of these promoters might be based on similar regulatory mechanisms.

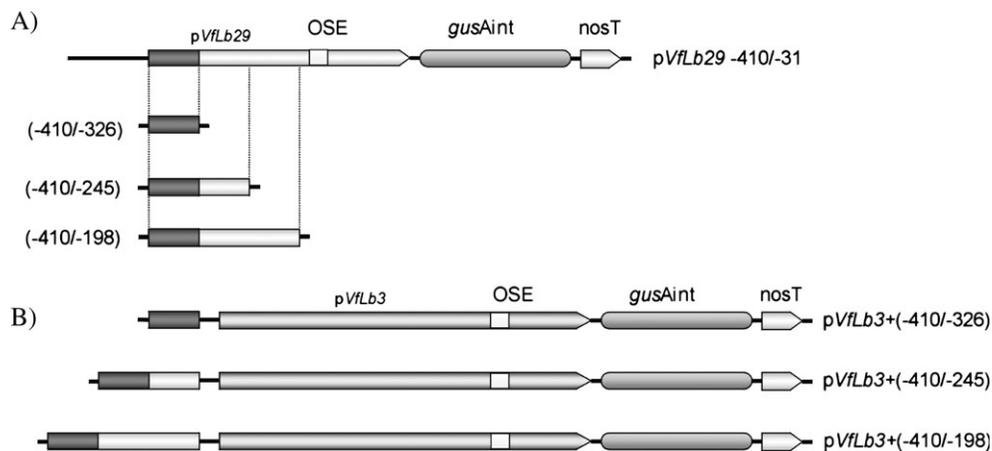


**Fig. 4.** Comparison of the palindromic motif in the *VfLb29* promoter region −410/−326 (85 bp) to a palindrome identified in the arbuscule-containing cell specific promoter of the potato phosphate transporter gene *StPt3* (Rausch *et al.*, 2001). Palindromic motifs are underlined and marked by arrows.

#### Cross-activation studies indicate a modular structure of the *VfLb29* promoter

To investigate the autonomous function of the 85 bp (−410/−326) p*VfLb29* region (Fig. 1) in terms of promoter activation in arbuscule-containing cells, chimeric promoter constructs were prepared. In order to show a cross-activation effect of the sequence from −410 to −326 (85 bp) on a promoter that is not active in arbuscule-containing-cells, a −356/−1 promoter fragment of the *VfLb3* gene encoding a leghaemoglobin that is expressed in root nodules but not in mycorrhizal roots was used as a target (Vieweg *et al.*, 2004). The 85 bp p*VfLb29* fragment (−410/−326) was amplified by PCR and cloned in front of a *VfLb3* (−356/−1) promoter *gusAint* fusion (Fig. 5). As there was the possibility that regulatory elements interact with promoter regions located downstream from position −326, two additional fragments (−410/−245; −410/−198) were prepared which were also fused to the *Lb3* (−356/−1) promoter fragment (Fig. 5). Verification of the transgenic character of the roots was ensured by dsRed fluorescence. After the induction of transgenic roots on *V. hirsuta*, plants were inoculated with the AM fungus *G. intraradices*.

The analysis of roots transformed with the chimeric promoter fusions p*Lb3*+(−410/−326), p*Lb3*+(−410/−245), and p*Lb3*+(−410/−198) revealed no activation effect in arbuscule-containing cells. The verification of the presence of arbuscules was shown by CBE staining, and the general activity of the chimeric promoter-fusions was confirmed by the p*VfLb3* specific expression pattern in root nodules (Fig. 2I, construct p*Lb3*+(−410/−198)). These results indicate that the activation of p*VfLb29* in arbuscule-containing cells probably requires two regulatory modules being located from position −410 to −198 and from position −197 to −31. Within the −410/−198 module, at least one important regulatory element is located in the −410/−326 region. Although this region is not



**Fig. 5.** Constructs used to assess *trans*-activation of the mycorrhiza-inactive promoter of the *V. faba* leghaemoglobin gene *VfLb3* in arbuscule-containing-cells. (A) Regions of the *VfLb29* promoter selected for *trans*-activation studies. (B) Chimeric constructs used to study the *trans*-activation potential of three different regions of p*VfLb29* on the promoter of the *VfLb3* gene.

autonomous in mediating expression in arbuscule-containing cells, the loss-of-function approach shows that its presence is nevertheless essential to activate *VfLb29* expression in arbuscule-containing cells.

## Conclusions and perspectives

The purpose of this study was the further characterization of the *VfLb29* promoter, which was previously shown to be active not only in the infected cells of root nodules but also in arbuscule-containing cells of mycorrhizal roots (Vieweg *et al.*, 2004). We were able to present functional and structural features of the *VfLb29* promoter and narrowed down the minimal regulatory region that mediates activity in arbuscule-containing cells of mycorrhizal roots. As far as is known, this is the first investigation of a promoter that is active in arbuscule-containing cells of mycorrhizal roots using a loss-of-function approach. These results demonstrated that *VfLb29* expression in arbuscule-containing cells is mediated by a complex regulatory system. Further characterizations of the structural features and putative *cis*-acting elements of the *VfLb29* promoter presented in this study might contribute to an elucidation of the still unexplored mechanisms that regulate gene expression in cells containing arbuscules.

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