Analysis of genes encoding modular nodulins from *Vicia hirsuta* and *Vicia faba*

Helge Küster *, Alfred Pühler, Andreas M. Perlick

Biologie VI (Genetik), Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Germany

Received 10 February 1999; received in revised form 21 April 1999; accepted 26 April 1999

Abstract

The expression of genes encoding modular nodulins was analysed in the *Vicia hirsuta* and the *Vicia faba* system. From *V. hirsuta* root nodules, we isolated two families of transcript sequences designated VhNOD28:32-A and VhNOD28:32-B encoding nodulins homologous to the *V. faba* nodulins Nvf-28/32 and the *Medicago sativa* nodulin-25. The modular proteins encoded by the *V. hirsuta* transcript sequences consisted of N- and C-terminal unique modules flanking two types of repetitive modules. Specific repetitive modules were deleted from individual VhNOD28:32 transcripts. Southern hybridizations indicated the presence of either a small VhNOD28:32 gene family or a single copy gene comprising at least two alleles. VhNOD28:32 transcripts were expressed specifically in root nodules, where they were localized in the nitrogen-fixing zone III. In the *Vicia faba* system, we isolated the promoter of two alleles of the VfNOD28:32 gene by PCR-based DNA walking. The promoter sequences were similar to the promoter of the *M. sativa* nodulin-25 gene and contained an organ-specific element at position – 68 to – 55 from the transcriptional start. An analysis of transgenic *V. hirsuta* root nodules expressing the gusAint reporter gene under the control of the two VfNOD28:32 promoters demonstrated that both promoters were active in the central region of transgenic *V. hirsuta* nodules. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hairy roots; Late nodulin; PCR-based DNA walking; Peribacteroid space; Tissue-print

1. Introduction

Soil bacteria belonging to the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* induce the formation of novel plant organs designated root nodules on the roots of legume plants [2,21,35]. In mature nodules, nitrogen fixation is carried out by the microsymbiont. The initiation of nodule formation requires specific signal exchanges of both symbiotic partners, which in turn lead to the induction of specific bacterial and plant genes [6,8,30]. Initially, symbiotic bacteria enter root nodules via infection threads [11]. After their release from these structures, the bacteria differentiate into bacteroids enclosed by a specifically modified plant-derived membrane, the peribacteroid membrane [17,21,37]. Bacteroids surrounded by the peribacteroid space and the peribacteroid membrane are regarded as transient symbiotic organelles termed symbiosomes [18]. Up to now, a number of plant genes expressed exclusively in root nodules were identified [5]. The encoded gene products were designated nodulins [22,34] and were divided into early and late nodulins according to their timing of synthesis. Whereas early nodulins are mainly structural proteins involved in nodule organogenesis or infection [4,29], late nodulins comprise leghemoglobins, enzymes of nodule carbon and nitrogen metabolism and peribacteroid membrane proteins [20].

To investigate organ-specific gene expression in broad bean (*Vicia faba* L.) nodules, we constructed a nodule-specific cDNA library by differential hybridization [23,24]. Sequence analysis of cDNAs from clone group VfNDS-L (*V. faba* nod-
ule differential screening, group L) of this library revealed that these cDNAs encode six different nodulins designated Nvf-28/32 [13,16]. The corresponding transcripts were termed VfNOD28/32. The Nvf-28/32 nodulins were composed of two types of repetitive sequence modules flanked by unique N- and C-termini. All repetitive modules and the N-terminal module corresponded to single exons of the VfNOD28/32 gene, whereas the C-terminal module was encoded by two exons [16]. Interestingly, specific repetitive modules were deleted from each of the six Nvf-28/32 nodulins resulting in individual modular structures for these proteins. The deletion of repetitive modules is most likely caused by alternative splicing [16]. Both the repetitive and the two unique modules were significantly homologous to sequence parts of the late nodulin Nms-25 from Medicago sativa [12,36], which was suggested to be localized in the peribacteroid space. Due to the late expression of the VfNOD28/32 gene in nodule development and to the localization of VfNOD28/32 transcripts in the central region of root nodules, Nvf-28/32 nodulins were proposed to be associated with the nitrogen-fixing stage [16]. Since Nvf-28/32 nodulins displayed no homologies to proteins of known function, their role in nodule organogenesis remained obscure. 

So far, an analysis of promoters from genes encoding modular nodulins was not reported. In order to complete the expression analysis of VfNOD28/32, we isolated promoter sequences of this gene and analysed their activity in transgenic hairy roots of V. hirsuta. To verify the observed expression properties of the V. faba VfNOD28/32 promoter in the heterologous system V. hirsuta, we isolated VhNOD28/32 cDNAs and characterized the expression properties of the corresponding V. hirsuta gene encoding modular nodulins. Here we demonstrate that both the endogenous VhNOD28/32 gene and the heterologous VfNOD28/32 promoters are active in the central region of V. hirsuta root nodules.

2. Methods

2.1. Reverse transcription-polymerase chain reaction (RT-PCR) experiments, isolation of nucleic acids and in vitro techniques

V. hirsuta root nodule polyA⁺ RNA was isolated using the Dynabeads® mRNA DIRECT Kit (Dynal) according to the manufacturers instructions. First-strand cDNA was synthesized from approximately 2 µg of polyA⁺ RNA immobilized on 250 µg of Dynabeads®-280 Oligo(dT)₂₅ using SuperScript™ II RNase H⁻ reverse transcriptase (Gibco-BRL) in a buffer recommended by the manufacturer. The immobilized cDNA was resuspended in 200 µl of 1 × TE buffer (10 mM Tris–Cl pH 8.0, 1 mM EDTA). Subsequently, 20 µl of this mixture were suspended in 44 µl of 1 × Taq buffer (Pharmacia). PCR-products were amplified from this template using the degenerate primers N1 (GAATTCAATATGMYTTBKTCTTTGG) and C1 (GAATTCATATGAAGGR-TAYTGGT) derived from the N- and C-module sequences of MsNOD25 [12] and VfNOD28/32 transcripts [16], respectively. For subsequent cloning, EcoRI restriction sites were introduced at the 5’ termini of both primers. PCR reactions were performed in a buffer containing 250 µM dNTPs, 100 pmol of each primer and 5 U of Taq DNA polymerase (Pharmacia). Thirty-five of the following PCR-cycles were performed: 94°C, 1 min; 50°C, 1 min; 72°C, 1 min. Following a final extension of 10 min at 72°C, PCR-products were purified using the QIAquick PCR Purification Kit (QIAGEN) and cloned directly into plasmid pXT [7] using A/T overhangs or into plasmids pK18 [26] using the terminal EcoRI restriction sites introduced with the PCR-primers and internal EcoRI sites of the PCR-products.

For cloning purposes, pUK21 [38] and pSVB30 [1] were employed. Isolation of recombinant plasmid DNA was carried out using the QIAGEN Plasmid Mini Kit (QIAGEN) as recommended. Other in vitro techniques were carried out using standard protocols [28].

2.2. PCR-based DNA walking to isolate V. faba promoter sequences

Promoter sequences of the VfNOD28/32 gene were isolated by PCR-based DNA walking using genomic V. faba DNA prepared according to [25] in conjunction with the PromoterFinder Construction Kit (Clontech) and the Advantage Genomic PCR Kit containing Tth DNA polymerase (Clontech). Both kits were used according to the manufacturers instructions. For PCR-amplifications, the
gene-specific primer VFP2 (ACATTTTTGGAGCATTCACATAATCTA) binding to exon2 and the nested gene-specific primer VFP1 (AGAAGAGATAATAAGAGAAACACCAAGAAG) binding to exon1 of the VfNOD28:32 gene [16] were used. PCR products obtained were cloned into plasmid pXT [7].

2.3. Automatic sequencing, sequence analysis and primer extension

All sequencing reactions were carried out from double-stranded plasmid DNA templates using the Thermo Sequenase pre-mixed cycle sequencing kit (Pharmacia). Sequencing gels were run on the ALFexpress DNA Sequencer from Pharmacia using sequencing gel mixes of standard composition. All sequences reported here were determined from both strands. Nucleic acid and the derived amino acid sequences were analysed using the ALF Manager V3.0 software (Pharmacia) and the Staden software package [32]. Primer extension was carried out according to [15] using a Cy5-labeled primer (ATAAGAGAAACACCAAGAA) corresponding to bp 51 to 33 on the first exon of the VfNOD28:32 gene [16]. Annealing to total root nodule RNA was carried out for 4 h at 44°C.

2.4. Southern hybridization

V. hirsuta genomic DNA was prepared according to [25]. A sample consisting of 20 µg of this DNA was digested with appropriate enzymes, separated electrophoretically and blotted onto positively charged Nylon membranes (Boehringer). DNA was fixed by baking at 120°C for 30 min and was hybridized for 24 h in DIG Easy Hyb hybridization solution (Boehringer) against probes labeled with DIG-dUTP using the PCR DIG Probe Synthesis Kit and standard M13 sequencing primers (Boehringer). Filters were washed according to [10] with a final wash in 0.5 × SSC, 0.1%(w/v) SDS at 65°C. The detection of hybridizing bands was carried out using the DIG Luminescent Detection Kit (Boehringer) as recommended.

2.5. Northern and tissue-print hybridizations

Total RNA was isolated using the RNeasy Plant Total RNA Kit (QIAGEN GmbH, Hilden, Germany) as recommended. RNA gels were run as described [23] using 15 µg of total RNA per lane. Hybridization was carried out against DIG-labeled antisense riboprobes using the DIG RNA Labeling Kit (Boehringer Mannheim, Mannheim, Germany). The detection of hybridizing bands was carried out using the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, Mannheim, Germany) as recommended by the manufacturer. Tissue-print hybridizations were carried out as described [14] using DIG-labeled antisense riboprobes.

2.6. Construction of fusions of promoters to the gusAint reporter gene

The −2268/−2 and −2351/−2 regions of the p28/32-A and p28/32-B promoters were PCR-amplified using primers incorporating a HindIII site for cloning purposes immediately downstream of the −2 position. The promoters amplified were cloned as SmaI/HindIII fragments in front of the gusAint gene in plasmid pGUS-INT [15]. The resulting fusions of the p28/32-A and p28/32-B promoters to the gusAint gene were subcloned into plasmid pIV2 [15]. The pIV2 derivatives obtained were subsequently integrated into the TL DNA of Agrobacterium rhizogenes ARqua1 by a single crossover event. The A. rhizogenes strains AR-A and AR-B obtained contained the p28/32-A-gusAint and p28/32-B-gusAint fusion, respectively. Both strains were verified by Southern blotting for the length and correct integration of the promoter–gusAint fusion.

2.7. Plant material, generation and analysis of transgenic roots and nodules

V. faba L. cv. Kleine Thüringer plants were grown and nodulated as described [16]. V. hirsuta seeds were obtained from John Chambers Wild Flower Seeds, Kettering, UK. Seeds were surface-sterilized and plants were grown in petri dishes in a growth chamber as described in [27]. For the generation of transgenic V. hirsuta hairy roots according to the procedure described in [27], the AR-A and AR-B derivatives of A. rhizogenes ARqua1 [27] described above were used. Wild type roots and transgenic hairy roots were nodulated with Rhizobium leguminosarum bv. viciae Vh5eSm [27]. Histochemical analyses of hairy roots expressing fusions of VfNOD28/32 promoters to the
gusAINT reporter gene [33] were performed as described [15].

3. Results and discussion

3.1. The VhNOD28/32-A and VhNOD28/32-B transcript families encode V. hirsuta nodulins homologous to the V. faba modular nodulins Vf-28/32

To study the promoter of the V. faba VfNOD28/32 gene in the model system V. hirsuta we first analysed the expression of the endogenous gene encoding modular nodulins. For this purpose, we isolated VfNOD28/32-homologous transcript sequences from V. hirsuta nodules. Using the sequences of VfNOD28/32 [13,16] and MsNOD25 [12] transcripts, we designed primers covering regions of particularly strong homologies in the N- and C-terminal regions of these transcripts. These primers were used to amplify VhNOD28/32 sequences by RT-PCR using V. hirsuta nodule mRNA as a template. In these experiments, two predominant products of 1150 and 950 bp were obtained. In addition, bands of lower intensity were detected between 300 and 400 bp (data not shown). Sequence analysis of cloned PCR-products revealed the presence of two different, but highly homologous subfamilies of

---

Fig. 1. Comparison of Nvh-28/32-A10 and Nvh-28/32-B40 sequences. The deduced amino acid sequences of cDNAs VhNOD28/32-A10 and VhNOD28/32-B40 are compared. Dashes denote the presence of internal deletions, identical positions are marked by vertical lines. Amino acid sequences corresponding to the primers used for RT-PCR are underlined. In case of the Nvh-28/32-B40 sequence, the use of degenerate primers led to the incorporation of a stop codon, which is marked by an asterisk. The extent of unique and repetitive sequence modules (Figs. 2 and 3) is indicated by shaded arrows as follows: black arrow: N-module, white arrow: C-module, dark grey arrow: repeat 1 type module, light grey arrow: repeat 2 type module. The sequences VhNOD28/32-A10 and VhNOD28/32-B40 can be obtained from the EMBL database using the accession numbers AJ237840 and AJ237841, respectively.
Fig. 2. Comparison of Nvh-28/32-A10 and Nvh-28/32-B40 sequences to the modules of the broad bean nodulins Nvf-28/32. Nvh-28/32-A10 (A) and Nvh-28/32-B40 sequences (B) are compared to the N- and C-terminal modules of the modular nodulin Nvf-32a and to the consensus sequence of repeat 1 and repeat 2 modules of the modular nodulins Nvf-28/32 [13]. Amino acids identical in the V. faba and the V. hirsuta sequences are printed in bold type. Amino acid sequences corresponding to the primers used for RT-PCR are underlined. The extent of modules is indicated according to the shadowing defined in the legend of Fig. 1.

VhNOD28/32 transcripts designated VhNOD28/32-A and VhNOD28/32-B. Fig. 1 shows a sequence comparison of the deduced amino acid sequences from cDNAs VhNOD28/32-A10 and VhNOD28/32-B40 representing the longest cDNAs amplified for each family. In addition, a sequence comparison of these sequences to the sequence modules of the V. faba modular nodulins Nvf28/32 [13] was carried out (Fig. 2). These comparisons show that modular nodulins homologous to the V. faba Nvf28/32 nodulins are encoded by the V. hirsuta VhNOD28/32-A10 and VhNOD28/32-B40 transcripts and that both types of repetitive sequence modules identified in the modular nodulins of V. faba [13,16] are present within the V. hirsuta sequences.

3.2. Specific sequence modules are deleted from different VhNOD28/32 transcripts

In V. faba, six different VfNOD28/32 transcripts can be distinguished by specific internal deletions removing complete exons each encoding repetitive sequence modules [16]. In Fig. 3, the modular structures of different PCR-products belonging either to the VhNOD28/32-A or the VhNOD28/32-B transcript family are compared. This comparison shows that the VhNOD28/32-A sequences -A10 and -A4 can be distinguished by one repeat 1 sequence module missing in the VhNOD28/32-A4 cDNA, whereas in V. faba, several repeat 1 sequence modules were found to be deleted from individual transcripts [16]. It therefore appears that the modular structure within VhNOD28/32 sequences is more uniform than the modular structure within V. faba sequences. On the other hand, the VhNOD28/32-A10 and VhNOD28/32-B40 cDNAs can be distinguished not only by sequence differences (Fig. 1), but also by the deletion of six repetitive modules in the VhNOD28/32-B40 transcript and by the deletion...
of one repetitive module in the VhNOD28/32-A10 transcript. In contrast to the situation in *V. faba*, these deletions also remove repeat 2 type modules (Figs. 1 and 3). It is possible that these differences are generated by alternative splicing, as was proposed for the VfNOD28/32 transcripts [16]. Alternatively, the VhNOD28/32-A and -B transcript families might represent two different alleles or isogenes. The two VhNOD28/32 cDNAs -A1 and -B1 are derived from short transcripts encoding only two repetitive sequence modules (-A1) or no repetitive modules at all (-B1, Fig. 3). We assume that these sequences correspond to the RT-PCR products of 300–400 bp mentioned above. These cDNAs might represent alternatively spliced transcripts, too. On the other hand, since almost the complete coding sequences are removed, a splicing event leading to the VhNOD28/32 cDNAs -A1 and -B1 would probably give rise to cDNAs encoding non-functional proteins.

3.3. *VhNOD*28/32 transcripts are encoded by a single-copy gene or a small gene family

To determine, if the presence of two VhNOD28/32 transcript families is due to the existence of a gene family, genomic Southern hybridizations were performed using VhNOD28/32-A10 and VhNOD28/32-B40 probes. These should cross-hybridize due to an overall sequence homology of 93%. In case of the *Eco*RI and *Hind*III digests performed, at least three bands should be detected, since two *Eco*RI and *Hind*III sites are present on the VhNOD28/32-B40 cDNA. One of each sites is missing on cDNA VhNOD28/32-A10. The VhNOD28/32-A10 probe detects three bands in *Eco*RI and five bands in *Hind*III digests of genomic *V. hirsuta* DNA. Hybridizations using VhNOD28/32-B40 probes detect the same set of bands (data not shown). These results are consistent with either a low copy number of the VhNOD28/32 gene or a single-copy gene with two alleles represented by the VhNOD28/32-A and -B cDNA families, respectively. The latter situation would resemble the situation in *M. sativa* and *V. faba*, where the homologous MsNOD25 and VfNOD28/32 genes are present in single copy [12,13]. Since in contrast to MsNOD25, at least two different alleles of the VfNOD28/32 gene exist [16], the existence of different alleles might be a common phenomenon for *Vicia* genes encoding modular nodulins.

3.4. *VhNOD*28/32 transcripts are exclusively present in the central region of root nodules

To determine the expression pattern of the VhNOD28/32 gene, Northern hybridizations were performed. These experiments revealed the exclusive expression of the VhNOD28/32 gene in root nodules. No hybridizing bands were detected in uninfected roots and in shoots containing both stem and leaf tissues (Fig. 4). The size of hybridizing bands of 1.3 and 1.1 kb is in accordance with the size of the two major RT-PCR products observed, if the 5′ and 3′ regions missing in the RT-PCR products are taken into account. Even after prolonged exposure, no RNAs of 0.3–0.4 kb were detected, which might reflect the lower sensitivity of Northern hybridizations in comparison to RT-PCR experiments.

To determine the site of expression of the VhNOD28/32 gene in root nodules, tissue-print hybridizations were performed (Fig. 4). In mature *V. hirsuta* nodules, these experiments detected VhNOD28/32 transcripts in the nitrogen-fixing...
Fig. 4. Detection of VhNOD28/32 transcripts in different tissues and within root nodules. In A, the result of a Northern hybridization of V. hirsuta total RNA isolated from nodules, uninfected roots and shoots against VhNOD28/32-A10 antisense riboprobe is shown. The size of hybridizing bands is indicated. In B, the result of a tissue-print hybridization of a V. hirsuta nodule section against a mixture of -A10 and -B40 antisense riboprobes is presented, which has to be compared to the corresponding nodule section stained for starch (C). VhNOD28/32 transcripts were detected in the nitrogen-fixing zone III. As is typical for Vicia nodules, the distal part of the nitrogen-fixing zone III and the senescence zone contain large amounts of starch. Abbreviations: kb, kilo basepairs; II, prefixing zone; III, nitrogen-fixing zone; N, root nodules; R, uninfected roots; S, shoots.

zone III corresponding to the region of leghemoglobin synthesis, whereas in the nitrogen-fixing zone III of older nodules with a large senescence zone IV, VhNOD28/32 transcripts were only detected in the very proximal part of the nitrogen-fixing zone III (data not shown). No signals significantly above the background level were detected in peripheral nodule tissues and in the root tissue. Together, these data demonstrate a strictly nodule-specific expression of the VhNOD28/32 gene, which is in accordance to the expression properties of the homologous genes in M. sativa [12] and V. faba [13,16]. The data presented demonstrate that V. hirsuta is a suitable model system to study the activity of the promoter of the broad bean VfNOD28/32 gene.

3.5. The promoter of the VfNOD28/32 gene is similar to the promoter of the M. sativa nodulin-25 gene in the region close to the transcriptional start site

We previously localized transcripts of the V. faba VfNOD28/32 gene in the central region of broad bean root nodules [13,16]. Since attempts to obtain clones from a λEMBL3 library covering the VfNOD28/32 locus failed, genomic VfNOD28/32 sequences were isolated by PCR experiments [16]. To study the molecular basis for the strictly nodule-specific expression of VfNOD28/32, we now isolated the putative VfNOD28/32 promoter by PCR-based DNA-walking [31]. Using this approach, we amplified two different putative VfNOD28/32 promoter fragments of approximately 2.3 and 2.4 kb designated p28/32-A and p28/32-B (Fig. 5). Relevant parts from the sequence of these fragments are presented in Fig. 5. Interestingly, the two fragments only differed by a deletion of 83 bp in p28/32-A. This difference cannot be regarded as a PCR-artefact, since a number of independent PCR-fragments covering this region were isolated which all either contained or lacked this particular region (data not shown). Although VfNOD28/32 is a single-copy gene [13], our PCR-amplifications of the VfNOD28/32 coding region demonstrated that at least two different VfNOD28/32 alleles exist [16]. This is in accordance to the fact that the broad bean population used in our experiments is not an inbred line. It is therefore possible that the p28/32-A and p28/32-B promoters correspond to different alleles of the VfNOD28/32 gene.

By primer extension experiments, we mapped the transcriptional start site of VfNOD28/32 (Fig. 6). Since two major adjacent primer extension products were identified, the G corresponding to the largest primer extension product was defined as +1 position. In a distance of 17 bp upstream from this transcriptional start site, a putative TATA box was identified (Fig. 5), which conforms
to the TATA box consensus sequence defined in [9]. In the region upstream of the transcriptional start, a putative promoter of 2268 bp (p28/32-A) or 2351 bp (p28/32-B) was present (Fig. 5). In this region, a high content of AT nucleotides of 67.7 or 68%, respectively, was found, which is typical of eukaryotic promoter sequences [9]. At position −68/−55, the sequence element TAA-

Fig. 5. Strategy for the amplification of the VfNOD28/32 promoter and sequence of relevant parts of the promoter and 5′ region of the VfNOD28/32 gene in comparison to the promoter of the MsNOD25 gene. In A, a sketch of the 5′ region of the VfNOD28/32 gene is shown. The adapters ligated to the NaeI-cleaved genomic V. faba DNA are indicated as light grey boxes. The positions of the adapter primer AP1 and the nested adapter primer AP2 as well as the gene-specific primers VFP1 and VFP2 used for PCR-amplifications are indicated. The position of the OSE is indicated by a black and the 83 bp deletion present in p28/32-A is denoted by a dark grey box. In B, relevant parts of the p28/32-B promoter are shown. The 83 bp segment missing in p28/32-A is indicated by light grey shadowing, the putative OSE is shown inverted. The positions of the TATA box and the transcriptional start site (1) are indicated. The N-terminal sequence of the deduced amino acid sequence is given above the exon1 sequence. The position of the gene-specific primer VFP1 is underlined. In C, the sequence of the 216/36 region of the p28/32-B promoter up to the start codon is shown in comparison to the corresponding part of the homologous MsNOD25 gene [36]. The complete sequence of the 28/32-B promoter can be retrieved from the EMBL database using the accession number AJ237842. Abbreviations: E1, exon1; E2, exon2; kb, kilobasepairs.
Fig. 6. Mapping of the transcriptional start site of the VfNOD28/32 gene by primer extension. The transcriptional start site of the VfNOD28/32 gene was determined by primer extension using a Cy5-labeled oligonucleotide. Products obtained by primer extension are shown. The length of the two predominant primer extension products was determined by comparison to a cycle sequencing reaction using the same oligonucleotide (data not shown). The region of the VfNOD28/32 sequence containing the transcriptional start sites identified is shown, the two major start sites are indicated. The G corresponding to the largest primer extension product was defined as +1 position.

GATTGACTCTT was identified displaying significant similarities to the organ-specific-elements (OSE) or nodule-infected cell expression elements (NICE) from leghemoglobin gene promoters [3,19]. Five out of six positions of the commencing nodulin consensus sequence AAAGAT and the complete terminal nodulin consensus sequence CTCTTof the putative OSE of the VfNOD28/32 promoter were conserved. Interestingly, the putative OSE is much closer to the transcriptional start site than the OSEs from leghemoglobin gene promoters, which are typically found 140–200 bp upstream from this position [3]. No significant homologies to other characterized parts of nodulin gene promoters were found. A comparison of the putative VfNOD28/32 promoter sequences to the promoter of the homologous MsNOD25 gene revealed that in addition to a high degree of sequence similarity, the positions of the OSE, the TATA-box and the transcriptional start site were either identical or very similar (Fig. 5) indicating that the fragments isolated by PCR-based DNA walking indeed represented VfNOD28/32 promoter sequences.

3.6. The promoter of the VfNOD28/32 gene is active in the central region of transgenic V. hirsuta nodules

To analyse if both VfNOD28/32 promoter fragments isolated are active and to determine if their expression properties differ, we constructed fusions of the complete p28/32-A and p28/32-B promoters into the gusAint reporter gene [33] and introduced these fusions into the TL DNA of A. rhizogenes ARqua1 [27]. The resulting A. rhizogenes strains AR-A and AR-B were used to induce transgenic hairy roots on V. hirsuta. Transgenic nodules induced on these hairy roots were analysed for the expression characteristics of the p28/32-A and p28/32-B promoters. In Fig. 7, sections of representative transgenic root nodules stained for GUS activity are shown. These experiments demonstrate that both VfNOD28/32 promoters isolated are active in the central region of transgenic V. hirsuta nodules, with a maximal activity in the proximal part of the nitrogen-fixing zone III. This expression pattern is in accordance to the localization of VfNOD28/32 transcripts in V. faba nodules. No significant differences between the expression properties of the p28/32-A and p28/32-B promoters were observed indicating not only that both VfNOD28/32 promoters stem from active alleles but also that the 83 bp lacking in p28/32-A are dispensable for promoter activity. The fact that the expression pattern of the endogenous VhNOD28/32 gene is similar to the activity of the heterologous VfNOD28/32 promoter demonstrates that both the V. faba and the V. hirsuta gene encoding modular nodulins are regulated in a comparable way making the vetch V. hirsuta a suitable model system for a detailed analysis of the broad bean VfNOD28/32 promoter.
Fig. 7. Activity of the p28/32-A and p28/32-B promoter in transgenic V. hirsuta root nodules. In A and B, longitudinal sections of representative transgenic V. hirsuta nodules expressing fusions of the p28/32-A or the p28/32-B promoters, respectively, to the gusAint reporter gene is shown. GUS activity can be detected in the proximal part of the nitrogen-fixing zone III.

Acknowledgements

This work was supported by Deutsche Forschungsgemeinschaft grants Pu28/20-1 and Pu28/20-2 and by Universität Bielefeld grant MODNOD.

References


