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ORIGINAL ARTICLE

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The promoter of a rice glycine-rich protein gene, *Osgrp-2*, confers vascular-specific expression in transgenic plants

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Abstract The genomic sequence of a rice (*Oryza sativa* L.) glycine-rich protein (GRP) gene, designated *Osgrp-2*, has been previously determined (GenBank U40708). Primer extension analysis indicated that transcription starts 47 bp upstream of the translation start codon. To gain an insight into the transcriptional regulation of this gene, the 2,401-bp promoter sequence and a series of its 5′ deletions were transcriptionally fused to the β-glucuronidase (GUS) gene. GUS activity was subsequently assayed in a transient expression system of tobacco (*Nicotiana tabacum* L.) protoplasts, which revealed the presence of a positive regulatory region (–2290 to –1406) and two negative regulatory regions (–2401 to –2291 and –1405 to –1022) in the *Osgrp-2* promoter for the promoter activity. The positive regulatory region displayed an enhancer-like activity when fused to the cauliflower mosaic virus (CaMV) 35S minimal promoter (–89 to +6) to drive GUS expression and assayed on tobacco leaves by the *Agrobacterium*-mediated transient expression technique (agroinfiltration). Histochemical staining for GUS activity on transgenic tobacco plants has further indicated a preferential expression in vascular tissues of stems and leaves conferred by the positive regulatory region. A 1,023-bp fragment of the *Osgrp-2* promoter (–1021 to +2) fused with *GUS* was transformed into tobacco and proved to be capable of conferring vascular-specific expression. Further 5′ and 3′ deletion analysis of the 1,023-bp promoter revealed that a 99-bp fragment located from –497 to –399 contained *cis*-elements responsible for vascular-specific expression.

Keywords Agroinfiltration · Glycine-rich protein · *Nicotiana* · *Oryza* · Promoter–GUS fusion · Vascular-specific gene expression

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Abbreviations CaMV: cauliflower mosaic virus · GRP: glycine-rich protein · GUS: β-glucuronidase · 35S: CaMV 35S promoter · X-gluc: 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid · *nos*: nopaline synthase gene

Introduction

Glycine-rich proteins (GRPs) of plants are a class of simple-structured proteins containing glycine-rich domains, which are likely to form glycine loops (Steinert et al. 1991) or β-pleated sheets (Ringli et al. 2001). The sequence repeats in the glycine-rich domains can be summarized by the formula G_n-X (GGGX, GGXXXGG, GXGX or GGX), where X stands for any amino acid, including glycine. The glycine contents of GRPs are variable, from 20 to 70%.

Plant GRPs were first described for pumpkin and petunia (Condit and Meagher 1986). Since then, a wealth of new GRPs have been identified in a variety of monocotyledons and dicotyledons, including bean, *Arabidopsis*, tobacco, carrot, maize and rice (for a recent review, see Sachetto-Martins et al. 2000). Generally, GRPs can be subdivided into two categories based on consensus sequences frequently observed in their structures: GRPs with a putative N-terminal signal peptide and GRPs with RNA-binding consensus sequences.

Although the biological functions of plant GRPs are still obscure, the wide spread in plant species implies their diverse and essential roles. The GRPs with a signal peptide in the mature protein region are generally believed to be located in the cell wall or the cell wall/membrane interface, suggesting their role as structural components of extra-cellular matrices (Showalter 1993; Cassab 1998). They may also function as linkers interacting with other extra- or inter-cellular proteins (Wyatt and Carpita 1993). For instance, the ligand function of *At*GRP3 was recently proven by its interaction with the extracellular domain of cell wall-associated kinases (Park et al. 2001). Surprisingly, it has been proven that some cell wall-located GRPs are not synthesized in the

observed cells per se, but are created elsewhere and then transported over distances and insolubilized there (Ryser and Keller 1992; Condit 1993; Sakuta and Satoh 2000), indicating still new functions such as repairing.

Study of the regulation of the expression of GRP genes can help to unravel their possible biological functions. The expression of the signal peptide-containing GRPs shows a large diversity of responses to various stimuli, including wounding, cold, flooding, hormone and infection by pathogens (Cornels et al. 2000; Sachetto-Martins et al. 2000; Ringli et al. 2001). This suggests that they were participating in important metabolite regulation such as plant defense-related processes. In addition, in situ hybridization and/or immunolocalization studies of GRP expression indicate that it can be highly tissue- or organ-specific: vascular-specific in most cases (Keller et al. 1988; Cheng et al. 1996) or root-specific (Bergeron et al. 1994; Matsuyama et al. 1999). And a rice GRP gene (*Osgrp-1*) was found to be closely associated with cell elongation/expansion during plant development (Xu et al. 1995).

The transcriptional regulation of plant GRP gene expression can be studied initially and representatively by promoter analysis. In contrast to a wealth of information gained in the past 10 years regarding expression modulation and expression pattern of a number of plant GRP genes, only a few plant GRP promoters have been studied in detail. Among genes that are predominantly expressed in vascular tissues, the promoter of the bean GRP1.8 gene (*Pvgrp1.8*) could serve as a prototype and the best-studied example. Through analysis of promoter–β-glucuronidase (*GUS*) fusions in transgenic tobacco plants, at least five sequence stretches have been found in the promoter region –293 to –36 to be responsible and acting combinatorially for the correct regulation of gene expression (Keller and Baumgartner 1991; Keller and Heierli 1994). A negative regulatory element restricts the expression of the reporter gene to vascular tissue whereas the other four sequence elements positively determine the expression in vascular tissues, stems and roots, respectively. A transcriptional factor has been isolated from tomato (Torres-Schumann et al. 1996), which binds to a 9-bp sequence in the negative regulatory element. This minimal binding site was proven to be sufficient to confer promoter activity in the vascular tissue when fused in the triplicate form to the minimal promoter sequence (Ringli and Keller 1998). For *Osgrp-1*, which encodes a protein homologous to *Pv*GRP1.8 (Lei and Wu 1991), the histochemical analysis of promoter–GUS fusion expression patterns in transgenic rice plants indicated that the *Osgrp-1* promoter was highly active in vascular tissues (Xu et al. 1995). However, detailed promoter deletion analysis for *cis*-elements responsible for the expression pattern has not been performed.

We have isolated a cDNA encoding a GRP from the cDNA library of rice yellow stunt virus (RYSV)-infected rice (*O. sativa* cv. Aijiaonante) using the differential screening strategy (Fang et al. 1991). The overall structure of this rice GRP is similar to the rice cell wall structural GRP encoded by *Osgrp-1* (Lei and Wu 1991) in that both consist of a putative signal peptide and a GGGX motif in the glycine-rich region, although the deduced amino acid sequences of the two GRPs and the 5′ and 3′ untranslated regions of their coding genes are different. We thus named this virus-inducible GRP gene *Osgrp-2*. Further, Northern blot analysis indicated that expression of *Osgrp-2* is also developmentally regulated as well as induced by mechanical injury (unpublished data). The preliminary data of tissue-printing experiments suggested that *Os*GRP-2 is preferentially localized in vascular tissue of the rice leaf sheath (data not shown), exhibiting an expression pattern similar to GRPs in bean and petunia (Condit 1993; Keller et al. 1988). A corresponding genomic clone was isolated from a genomic library with a labeled cDNA fragment. As part of a study on transcriptional regulation of *Osgrp-2* expression we present in this report an analysis of the *Osgrp-2* promoter using GUS as a reporter. The sequence regions responsible for the promoter activity were examined in tobacco transient expression systems and the sequence elements capable of conferring vascular-specific expression were identified through analysis of transgenic tobacco plants.

Materials and methods

Plant materials

The rice (*Oryza sativa* L. ssp. *Indica* cv. Aijiaonante) seedlings were planted in the greenhouse at 28 °C. Tobacco (*Nicotiana tabacum* L. cv. BY-2) suspension cells (provided by Prof. Nam-Hai Chua, Rockefeller University) were maintained in liquid medium on a gyratory shaker (140 rpm) at 25 °C in the dark and subcultured once a week. Tobacco plants (*N. tabacum* cv. K326) used in stable transformation were grown in vitro on half-strength Murashige and Skoog (MS) medium (Sigma) at 28 °C with a photoperiod of 16 h light and 8 h darkness. Tobacco plants (*N. tabacum* cv. Xanthi nc) for agroinfiltration were grown in a greenhouse.

Primer extension

To determine the transcription start point, total RNA was isolated using a hot-phenol method (De Vries et al. 1991) from rice seedlings that had been wounded by crushing followed by incubation on water-saturated paper in darkness for 12 h. About 40 μg of total rice RNA was annealed to a 5′-^{32}P-labeled extension primer (5′-CTAAGGAGGACAAGGATGGC-3′, complementary to a stretch in the putative signal-peptide coding region) in RT buffer (Promega) plus 5 mM $MgCl_2$ for 30 min at 42 °C. Four dNTPs (0.5 mM each) and AMV reverse transcriptase (10 units) were added and the reaction was incubated for another 60 min at 42 °C. The RNA was degraded by incubation for 20 min at 42 °C in the presence of NaOH (0.3 mM). After neutralization with HCl, the reaction mix was phenol-extracted and ethanol-precipitated. The extension products were analyzed on a 6% polyacrylamide sequencing gel. A sequencing reaction of a subclone of pBS5111 using the extension primer was loaded in parallel onto the same gel as a reference.

Promoter–GUS fusions

The pBS5111 (a pBlueScript KS derivative) harbors the 3.1-kb *Sal*I fragment of an *Osgrp-2* genomic clone of *O. sativa* cv. Aijiaonante. This 3.1-kb sequence spans from –2401 relative to the transcription

start to the 138th nucleotide downstream of the termination codon of the *Os*GRP-2 coding region. The full-length *Osgrp-2* promoter was obtained from pBS5111 by PCR amplification using the T3 universal primer flanking the 5′ end of the promoter and a synthetic primer F2 (5′-CTGGGATCCGTGGTTGGAGTGAAGGGGAG-3′, the *Bam*HI site added next to the + 2 nucleotide is underlined). The *Pst*I–*Bam*HI and *Hin*dIII–*Bam*HI fragments of the PCR products were ligated into the corresponding sites of pBI221 (Clontech), generating pG1–GUS (–2401/ + 2, the full-length promoter) and pG2–GUS (–2290/ + 2), respectively. Deletions pG4–GUS (–1794/ + 2) and pG9–GUS (–183/ + 2) were constructed following the same strategy: promoter fragments were amplified from pBS5111 using primer F2 in combination with either primer F3 (5′-TTGTCGACCCTAG-TTATGATACGCTC-3′, an added *Sal*I site is underlined) or F4 (5′-TTAGTCGACAACAGCTACTGGCACTC–3′, an added *Sal*I site is underlined) and cloned in pG1–GUS as *Sal*I–*Bam*HI fragments to replace the –2401/ + 2 fragment.

Deletions pG5–GUS (–1405/ + 2) and pG7–GUS (–924/ + 2) were constructed by digesting pG1–GUS with *Sal*I/*Asp*718 and *Sal*I/*Xba*I respectively, end-filling with Klenow DNA polymerase, and self-ligation. To construct the deletions pG3–GUS (–2036/ + 2), pG6–GUS (–1021/ + 2) and pG8–GUS (–569/ + 2), pG1–GUS was partially digested with *Spe*I and then completely digested with *Sal*I, followed by end-filling and self-ligation. To construct the internal deletion pG2Δ–GUS (Δ–1404/–1021), the fragment –2290/–1401 was excised from pG2–GUS with *Hin*dIII and *Asp*718 (filled-in) and cloned into the *Hin*dIII/*Spe*I (filled-in) sites of pG6–GUS.

To construct the deletions pG8a–GUS (–497/ + 2) and pG8c–GUS (–294/ + 2), the promoter sequences were first amplified from pG6–GUS using a primer corresponding to –497/–465 (5′-ATAAGCTTAAGCACGTCAATGTATGC-3′, the introduced *Hin*dIII site is underlined) or a primer corresponding to –294/–268 (5′-ATAAGCTTAAAAGCAAAAGTAAACACAC-3′, the introduced *Hin*dIII site is underlined) in combination with the 3′ primer GusR (5′-CACGGGTTGGGGTTTCTACAGG-3′, complementary to a 22-nucleotide sequence 9 bp downstream of the translation start codon of *GUS*) and then inserted into pBI221 as *Hin*dIII–*Bam*HI fragments at corresponding restriction sites. The deletion pG8b–GUS (–398/ + 2) was constructed by cloning the *Eag*I–*Bam*HI fragment of G8a into the *Pst*I(blunted)/*Bam*HI sites of pBI221.

For construction of the chimeric promoter comprising the –2290/–1401 fragment and the cauliflower mosaic virus (CaMV) 35S minimal promoter, the 35S-89 promoter was first PCR-amplified from pBI221 using GusR as the 3′ primer and P-89 (5′-AAGCTGCAGTCTCCACTGACGTAAGGGATG-3′, the introduced *Pst*I site is underlined) as the 5′ primer. The PCR product was digested with *Pst*I and *Bam*HI and then ligated into pBI221 to replace the full 35S promoter, creating pBI-89. The –2290/–1401 fragment excised from pG2–GUS with *Hin*dIII and *Asp*718 (filled-in) was then cloned into the blunted *Pst*I site of pBI-89. The orientation and duplication of the –2290/–1401 fragment were checked by digestion with *Spe*I and *Bam*HI. The resultant chimeric promoters, designated as Ef/-89, Er/-89 and Eff/-89, were used in agroinfiltration experiments.

pBI-89 was also used in constructing 3′ deletions of the G6 promoter fragment fused with the 35S-89 promoter. To construct pD6P–GUS (the –1021/–238 fragment fused to 35S-89), the promoter sequence was first amplified from pG6–GUS using the primer PreH (5′-CTTCCGGCTCGTATGTTGTGTG-3′, homologous to a stretch in pBI221 78 bp upstream of the *Hin*dIII site) and a primer complementary to the promoter sequence –259/–238 (5′-AAGCTGCAGTTAGAATACTGATTTATTG-3′, the introduced *Pst*I site is underlined). The PCR product was then inserted into pBI-89 as a *Hin*dIII–*Pst*I fragment. The construct pD6E–GUS (the –1021/–394 fragment fused to 35S-89) was created by cloning the *Hin*dIII–*Eag*I (filled in) fragment of D6P into the *Hin*dIII/*Pst*I (blunted) sites of pBI-89.

Protoplast transfection

The tobacco BY-2 suspension cells pre-washed with 0.4 M mannitol were digested for 60 min at 37 °C with 1% cellulase RS and 0.1% pectinase Y-23 in 0.4 M mannitol. The resulted protoplasts were filtered through steel mesh (100 μm and 60 μm successively), washed three times with W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM sucrose, pH 5.9) and resuspended in MaMg (0.4 M mannitol, 15 mM $MgCl_2$, pH 5.6) at a concentration of 2×10^6 protoplasts per ml. To 300 μl of protoplasts, 15 μg of a reference plasmid, which included an expressing cassette 35S promoter–luciferase gene–*nos* terminator, and 20 μg of the test plasmid in 20 μl H_2O were added, and mixed with 300 μl of 40% polyethylene glycol (PEG), 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, followed by incubation for 15 min. After washing with W5, protoplasts were incubated in K3 medium (Nagy and Maliga 1976) for 24 h at 28 °C in the dark. The protoplasts were washed with PBS (0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4 and 0.024% KH_2PO_4, pH 7.4) and collected by centrifugation at 120 *g* for 3 min. Protoplasts were then resuspended in 0.3 ml of Cell Lysis Buffer (Promega) and sonicated.

The supernatant of the lysed protoplasts was used for analysis of enzymatic activities. Twenty microliters of cell lysate was used for GUS assay as described below. To determine the luciferase activity, 5 μl of the same sample was mixed with 50 μl of LUC substrate (Promega) and total light emission in 10 s was measured using a liquid scintillation counter. The relative GUS activities of different constructs were calculated by normalization of measured GUS activities against their luciferase activities.

Agroinfiltration

For agroinfiltration analysis, a binary vector pINT121 containing *GUS* with an intron of the ST-LS1 gene was first constructed by replacing the *GUS* gene in pBI121 (Clontech) with a *Bam*HI–*Sac*I fragment of p35SGUSINT (CAMBIA TG0069). The chimeric promoters, Ef/-89, Er/-89 and Eff/-89, were then separately inserted into pINT121 as *Hin*dIII/*Bam*HI fragments to replace the original 35S promoter, resulting in pEf/-89, pEr/-89 and pEff/-89, respectively. These binary vectors were used for subsequent agroinfiltration analysis as well as for *Agrobacterium*-mediated plant transformation.

Agroinfiltration was assayed essentially as described by Yang et al. (2000). *Agrobacterium tumefaciens* strain LBA 4404 containing each individual construct was incubated in 2 ml LB medium supplemented with appropriate antibiotics (kanamycin and streptomycin) and then inoculated (1%) into 25 ml LB with 10 mM Mes, 20 μM acetosyringone as well as the antibiotics. The culture was grown overnight to log phase ($OD_{600}\approx0.8$) at 28 °C, centrifuged and resuspended in MMA solution (10 mM Mes, 10 mM $MgCl_2$, 100 μM acetosyringone) to a final OD_{600} of 1.5. One hundred microliters of the bacterial suspension per spot was infiltrated into intercellular spaces of near fully expanded leaves using a 1-ml plastic syringe. Typically, up to ten infiltrating spots separated by veins could be arranged in a single leaf of tobacco (*N. tabacum* cv. Xanthi nc). After agroinfiltration, the treated plants were maintained in the greenhouse for 2–6 days before GUS assay of the infiltrated areas.

Plant transformation

All the transformation constructs, except those containing the chimeric promoters comprising the –2290/–1401 fragment and the 35S minimal promoter, were made by cloning the *Hin*dIII–*Eco*RI fragments of the corresponding promoter–*GUS* fusions into the binary pBI121 vector to replace the 35S promoter–GUS–*nos* terminator cassette.

Individual binary vectors were mobilized into *A. tumefaciens* LBA 4404 by electroporation. Leaf discs of tobacco (*N. tabacum* cv. K326) were transformed as described by Horsch et al. (1985). Transformed plants were selected on MS medium containing 200 μg/ml kanamycin and 500 μg/ml carbenicillin. The presence of the *Osgrp-2* promoter and GUS gene in rooted plantlets was verified by PCR using primers PreH and GusR before plantlets were transferred to soil to be grown in the greenhouse.

Fluorometric quantification of GUS activity and histochemical GUS staining

GUS activity was assayed essentially as described by Jefferson et al. (1987) with leaf extracts standardized by measuring the protein concentration with the Bradford reagent (BioRad). The plant tissue was ground using a pestle and mortar in 200 µl Passive Lysis Buffer (Promega). After centrifugation for 10 min (12,000 *g*) at 4 °C, 10 µl of supernatant was mixed with 250 µl of Passive Lysis Buffer containing 1 mM 4-methyl-umbelliferyl glucuronide (MUG) and then the mixture incubated at 37 °C. After 30 min and 90 min, respectively, 100 µl of the reaction was added to 900 µl of 0.2 M Na_2CO_3 and the fluorometric values were determined using a DyNAQuant 200 Fluorometer (Hoefer, San Francisco, Calif., USA). The GUS activity is expressed as pmol of 4-methyl-umbelliferone (MU) per mg protein per min.

GUS activity was histochemically detected according to Jefferson et al. (1987) with minor modifications. Hand-cut sections or the whole tissues were incubated in a solution of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) in 50 mM sodium phosphate (pH 7.0) for 1–12 h at 37 °C. Photosynthetic tissues were cleared of chlorophyll by incubation in 70% ethanol. The samples, sometimes stained with ruthenium red to reveal lignins, were observed and photographed with a stereomicroscope (Olympus, BX51) or an anatomy microscope (Olympus, SZX9).

Results

Determination of the transcription start of the *Os*GRP-2 gene

Total RNA extracted from wounded rice seedlings was used for primer extension to determine the transcription initiation site of *Osgrp-2*. Previous Northern blot analysis had indicated that the expression level of *Osgrp-2* in seedlings was very low, but could be enhanced by approx. 20-fold after mechanical wounding (unpublished data). The primer annealed to the rice RNA was extended to a 94-bp cDNA fragment, placing the transcription start site at an A residue (numbered +1, shown in Fig. 1). As observed in most of the plant genes analyzed (Joshi 1987), the transcription start site was flanked by pyrimidine bases. The result was also confirmed by a 5′-RACE (rapid amplification of cDNA ends) analysis using the RNA extracted from the rice seedlings (data not shown). The sequenced 3.1-kb fragment in pBS5111 therefore contains 2,401 bp upstream from the transcription start, with a typical TATA box located at –32 to –25 and a CAAT box located at –161 to –151 (Fig. 2).

Deletion analysis of the promoter in the protoplast system reveals a positive regulatory region and two negative regulatory regions

In order to localize promoter regions potentially involved in the transcriptional control of the *Osgrp-2*, the full-length promoter (G1) and a series of its 5′ deletions (named G2 to G9) were transcriptionally fused to the GUS reporter gene (Fig. 3). Each construct was co-transfected into tobacco protoplasts with a plasmid carrying the luciferase gene as an internal control for transfection and transient gene expression.

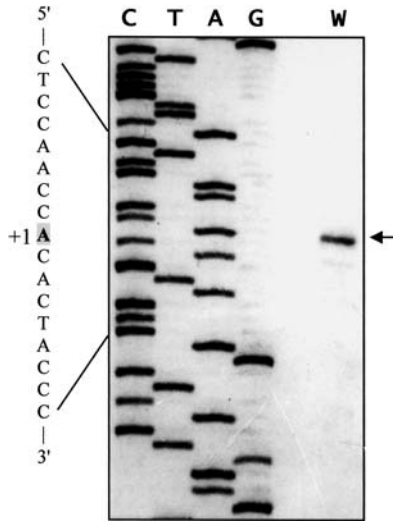
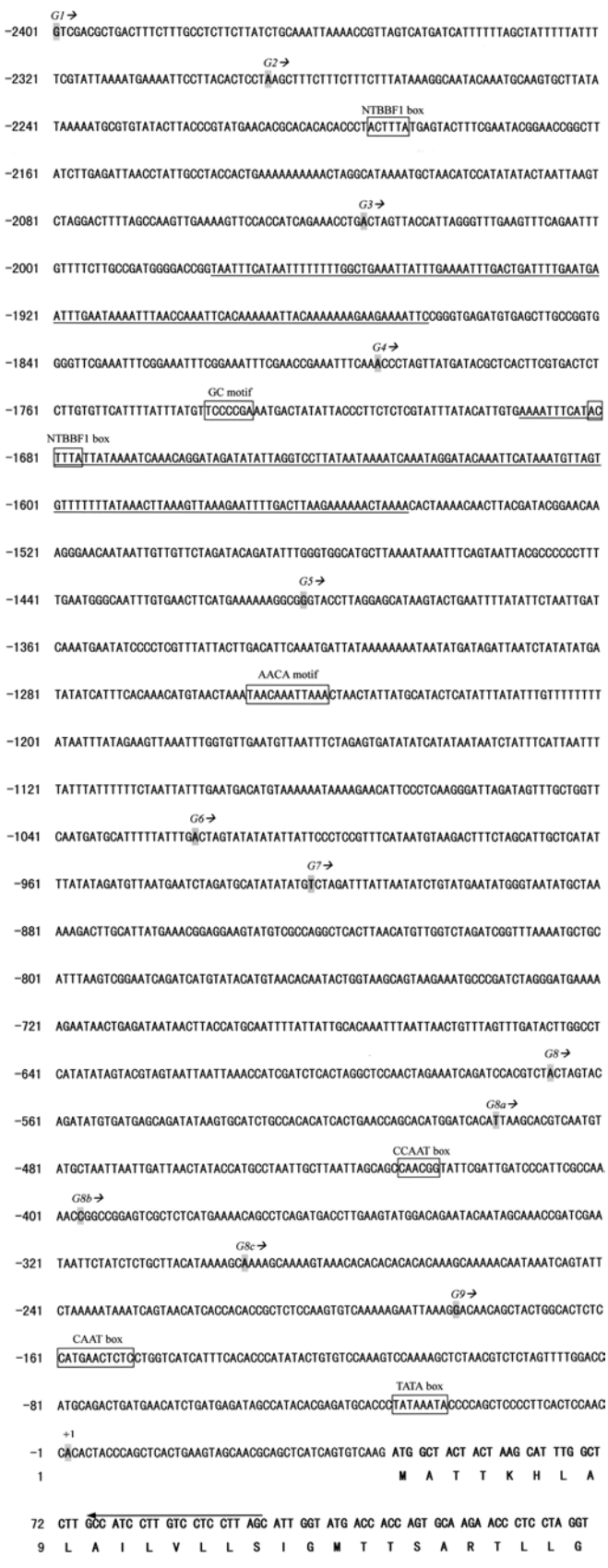


Fig. 1 Determination of the transcription start site of the rice (*Oryza sativa*) *Os*GRP-2 gene. Total RNA of wounded rice seedlings (*W*) was used for primer extension. A sequencing reaction of a pBS5111 subclone encompassing the transcription start by using the same labeled extension primer was separated on the same gel. The primer extension product is marked with an *arrowhead*. The sequence around the transcription start (+1) is indicated

The results of expression of promoter–GUS fusions are shown in Fig. 3, in which the GUS activity level of each construct was normalized to that of the G2 deletion which displayed the highest GUS activity (100%). The full-length promoter G1 exhibited an activity of about 60% of G2, indicating the presence of a negative regulatory element(s) for the promoter activity between –2401 and –2291. A progressive decline of GUS activity was observed in constructs from pG2–GUS to pG5–GUS, implying that a positive regulatory element(s) is likely to be located within the –2290/–1406 region. However, further 5′ deletion to –1021 (pG6–GUS) resulted in an increase in GUS activity of 50% over pG5–GUS, indicating the presence in the region –1405 to –1022 of another negative regulatory element(s) for the promoter activity. A gradual decrease in GUS activity was noted when the promoter was truncated from –1021 to –183 (G6 to G9).

To confirm the function of the –1405/–1022 fragment in the context of the full-length promoter, an internal deletion construct (pG2Δ–GUS) was made, in which the G2 fragment was deprived of the –1400/–1021 region (Fig. 3). The GUS activity of pG2Δ–GUS assayed in the same tobacco protoplast transient expression system demonstrated that removal of this region resulted in an increased expression (approx. 1.3-fold) when compared with pG2–GUS (Fig. 3). This observation is in agreement with that from the 5′-deletion analysis, thus confirming that the –1405/–1022 fragment has a negative regulatory function.



◀

Fig. 2 Nucleotide sequence of the 5′ flanking region and N-terminal coding region of the rice *Os*GRP-2 gene. Numbering is relative to the transcription start (shown by *shading* and labeled +1) as determined by primer extension. The putative TATA and CAAT boxes are shown. The *Osgrp-2* coding region starting from a ATG is shown in *bold*. The location of the extension primer is marked with an *overline arrow*. Deletion end points corresponding to G1–G9 as well as G8a, G8b and G8c are also shown by *shading*. The A/T rich stretches are *underlined*. The NTBBF1 boxes, GC motif, AACA motif and CCAAT box are shown

Activation of a 35S minimal promoter by the positive regulatory fragment

To test whether the –2290/–1406 fragment can activate a heterologous promoter as an enhancer, it was fused in both orientations as a monomer or a dimer upstream of a CaMV 35S minimal promoter (–89 to +6). This portion of the 35S promoter has a normal TATA box and an ASF1 transcription factor-binding site (Fang et al. 1989), but shows a very low level of promoter activity in tobacco leaves when it was fused to the GUS gene and assayed either fluorometrically or by histochemical staining (Keller and Heierli 1994). The chimeric promoters and the 35S-89 promoter (as a control) were transcriptionally fused to the GUS gene with an intron (Fig. 4A) to make sure that GUS activities observed in agroinfiltration experiments are due to the expression of the constructs by the plant cell machinery rather than by agrobacteria. The agrobacteria harboring different constructs were pressure-infiltrated into different areas separated by veins of a single tobacco leaf. The GUS expression level of each construct was quantitatively analyzed in 10 independent leaves. Although there were considerable variations in absolute GUS activities for a specific construct among the tested leaves, the GUS activity ratios between different constructs in each leaf were quite consistent. Evaluation of chimeric promoters was then based on the average GUS activity ratios of relevant chimeric promoter–GUS fusion constructs to the 35S-89–GUS construct derived from the data of 10 infiltrated leaves.

The results demonstrated that the 35S minimal promoter was activated in all the three chimeric promoters (Ef/-89, Er/-89 and Eff/-89) by 4- to 9-fold (pairwise Student's *t*-test, $P < 0.05$) (Fig. 4). The activation rate observed with the reverse-oriented G2/G5 fragment (4-fold) was lower than that with the forward-oriented one (9-fold), and duplication of the fragment in forward orientation did not further increase the activation rate (Fig. 4B). Nevertheless, the 889-bp promoter sequence (–2290/–1401) has an enhancer-like property, as it was active in both orientations. We also tested the enhancing activity of this stretch of sequence on a further truncated 35S minimal promoter (–49 to +6) in both orientations and in duplicated forms. No significant activation in any of the chimeric promoters was observed (data not shown). It is likely that the *as-1* element located in the 35S-89 promoter might be critical for the G2/G5 fragment to exert the enhancer function.

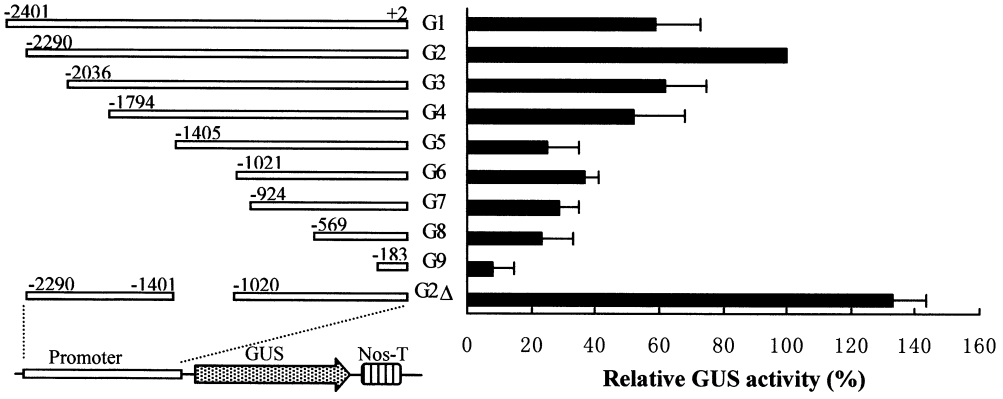


Fig. 3 Deletion analysis of the *Osgrp-2* promoter in the transient expression system of tobacco (*Nicotiana tabacum*) protoplasts. On the *left*, diagrams of 5′ terminal deletions and an internal deletion are presented. The promoter fragments are presented as thin white boxes and the numbers indicate the end points of each deletion. The construct designations are noted in the *middle* of the figure. At the *bottom* is the schematic representation of the constructs. All the constructs include a GUS coding region before a *nos* terminator. GUS activities of different constructs relative to that of pG2-GUS are shown on the *right* as black bars. Values represent the mean and SE from six independent experiments for each construct

Osgrp-2 promoter confers vascular-specific GUS expression in the transgenic tobacco plants

The spatial expression pattern of the *Osgrp-2* promoter was first examined in transgenic tobacco plants through a study of the 1,023-bp promoter fragment (G6, from –1021 to +2). The G6 promoter was fused with the GUS gene and introduced into tobacco via *Agrobacterium*-mediated transformation. Transgenic lines harboring the construct pBI121 were also created for comparison. The in vitro-grown primary transformants were first verified by a PCR analysis before being transferred into a greenhouse. Segregation of kanamycin resistance in the progeny of self-pollinated tobacco transformants, together with Southern blot analysis (data not shown), indicated that a single transgene locus is present in most of the transgenic lines.

The organ distribution of G6–GUS expression was examined in greenhouse-grown primary transformants. The levels of GUS activity quantitively determined in different vegetative organs of several tobacco plants indicated that the G6 promoter is more active in stems and petioles than in leaves, and no activity was found in roots. This result is consistent with the steady-state *Os*GRP-2 mRNA levels in corresponding rice organs previously observed by Northern blot analysis (data not shown).

The tissue-specific expression pattern directed by the G6 promoter was further determined by the histochemical GUS staining of thin sections derived from the transgenic tobacco plants. A vascular-specific activity in stems and leaves was observed in 4 transformants that showed the highest GUS activities among 39 independent lines. Blue staining was exclusively located in the phloem tissues (mainly in the adaxial phloem tissues) as shown in sections of main leaf vein and the stems (Fig. 5A, B). Not surprisingly, no GUS staining was detected in roots, which is in agreement with the results for organ specificities of the G6 promoter activity described above. In contrast, the 35S promoter directed GUS expression in all tissues examined (data not shown).

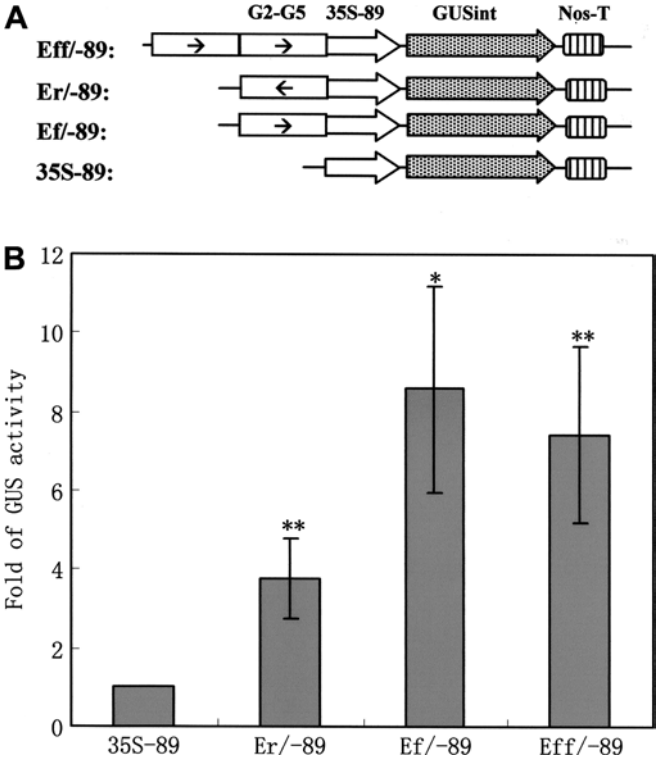


Fig. 4A, B Activation of the 35S-89 minimal promoter by the –2290/–1401 fragment. **A** Structure of the constructs. Fragments are not drawn to scale. Construct names are given in *bold* on the *left*. *G2–G5*, the enhancer-like fragment in forward or reverse direction or in duplicated form; *35S-89*, CaMV 35S minimal promoter (–89 to +6); *GUSint*, GUS gene with an intron; *Nos-T*, *nos* terminator. **B** Activation of 35S minimal promoter by the G2–G5 fragments. The GUS activity of each construct was determined quantitatively in *Agrobacterium*-infiltrated leaves and normalized to that of the 35S-89 for each leaf. Values represent the mean ± SE derived from 10 individual leaves for each construct. Asterisks indicate that values are significantly different from 35S-89 at $P < 0.05$ (*) or at $P < 0.01$(**) as statistically analyzed using a pairwise Student's *t*-test

The –2290/–1401 fragment, which showed an enhancer-like activity in agroinfiltration experiments, was

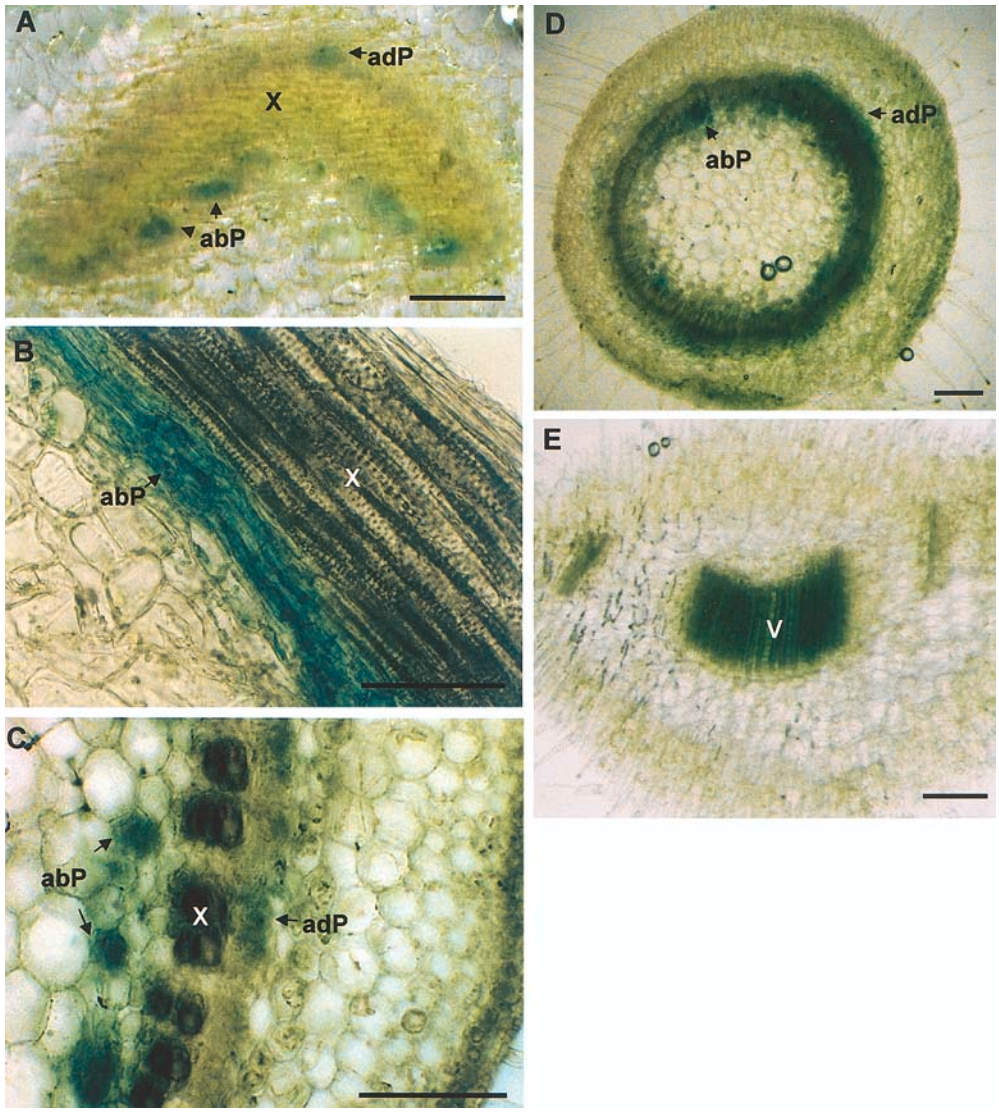


Fig. 5A–E Histochemical localization of GUS activity in representative transgenic tobacco plants containing promoter::GUS constructs. The GUS expression patterns directed by promoters G6 (**A, B**), D6E (**C**), Er/-89 (**D**), and Eff/-89 (**E**) can be recognized by blue indigo dye deposits. The hand-cut cross-sections of petioles (**A, E**), cross-sections of young stems (**C, D**) and longitudinal sections of adult stems (**B**) were stained with X-gluc overnight, decolorized with 70% ethanol and observed using a stereo-microscope or an anatomy microscope. *abP* Abaxial phloem, *adP* adaxial phloem, *V* vascular bundle, *X* xylem. Bars = 50 μm

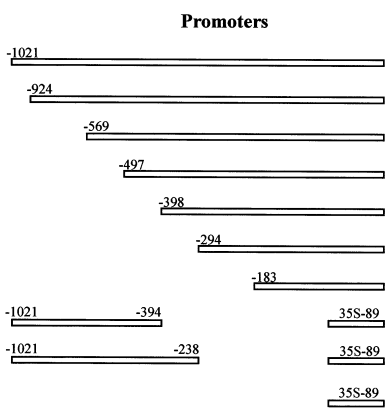
also analyzed for its tissue-specific activity. The binary vectors pEf/-89, pEr/-89, pEff/-89 and p35S-89 were separately transformed into tobacco plants and GUS activity was assayed histochemically. The transformants harboring p35S-89 displayed no detectable GUS activity, as expected, whereas vascular-preferential staining was found in plants transformed with pEr/-89 and pEff/-89 (Fig. 5D, E). For an unknown reason, no detectable blue staining was found in five of the pEf/-89 transformants.

Taken together, these data suggest that the *Osgrp-2* promoter contains redundant elements which determine the vascular tissue-specificity of the promoter activity.

Delineation of a sequence domain in the G6 promoter responsible for vascular-specific expression

To identify the *cis*-acting elements responsible for vascular-specific expression within the G6 promoter, a series of its 5′ deletions, namely G7, G8, G8a, G8b, G8c and G9 (Fig. 6), were transcriptionally fused to the GUS gene. In addition, to perform the gain-of-function analysis, two 3′ deletions of G6 (D6E and D6P, Fig. 6) were placed upstream of the 35S-89 promoter followed by the GUS coding region. These binary constructs were then introduced into tobacco and 10–34 independent transgenic lines for each construct were generated.

For each promoter–GUS fusion construct, transgenic plants that displayed high fluorometric GUS activities were chosen for analysis of tissue specificity of GUS expression. Hand-cut sections of stems and petioles were histochemically stained, and the results indicated that the transformants harboring deletions G7, G8 and G8a exhibited essentially the same patterns of blue staining as that observed with the G6 promoter constructs (Fig. 6). The detected GUS staining was specially localized to the vascular tissues of the petioles and stems. However, further deletion of sequences downstream of –497 (5′-end of G8a deletion) led to such a critical decrease in GUS activity that no blue staining could be detected in any of the transgenic plants (16 for each of G8b and



Promoter	GUS Activity	VS
G6	++	yes
G7	++	yes
G8	++	yes
G8a	+	yes
G8b	-	no
G8c	-	no
G9	-	no
D6E	++	yes
D6P	++	yes
35S-89	-	No

Fig. 6 Identification of the sequence domain responsible for the vascular-specific expression of the G6 promoter. The promoters used to construct GUS fusions are shown on the *left*. The numbers indicate the deletion end points relative to the transcription start site of *Osgrp-2*. Stems and petioles of several transgenic plants bearing each promoter–GUS fusion were sectioned and histochemically stained for GUS activity. Relative intensities of GUS staining in vascular tissues of those plants which showed GUS activity are denoted by + +, high or +, low. No detectable staining in any of the transgenic plants examined is denoted by –. *VS* Vascular-specific expression

G8c, 17 for G9). These results demonstrated that the 99-bp fragment between –497 and –399 was responsible for the ability of the G6 promoter to confer detectable and vascular-specific expression in transgenic tobacco plants. In addition, the chimeric promoters D6P and D6E also directed GUS expression specifically in vascular tissues (for D6E see Fig. 5C). These data collectively suggested that the –497/–399 fragment harbors *cis*-elements responsible for the vascular-specific expression of the G6 promoter.

Discussion

As a first step towards the identification of *cis*-elements responsible for the expression pattern directed by the rice *Osgrp-2* promoter, a heterologous transient expression system of tobacco protoplasts was used in the 5′ and internal deletion analysis of the promoter. According to the data for the deletion analyses, the *Osgrp-2* promoter downstream of –2290 could be roughly dissected into three parts relevant to its transcriptional activity: a 5′-distal region spanning from –2290 to –1406 and a 5′-proximal region downstream of –1021, both of a positive regulatory function, and, in between, a negative regulatory region. Another negative regulatory region was identified between –2401 and –2291 and it is possible that more regulatory elements may exist upstream of –2401, which is beyond the scope of this study.

Of possible relevance to the negative control function of the fragment –1405/–1022 is the presence of an AACA motif (tAACAaattaaa, boxed in Fig. 2), which has been reported to be involved in the negative control of endosperm-specific expression of the rice glutelin gene (Yoshihara et al. 1996). Such negative control regions have also been found in several vascular-specific promoters such as NRE in *Pvgrp1.8* (Keller and Baumgartner 1991), AC elements in bean phenylalanine ammonia-lyase (PAL) and eucalyptus cinnamoyl CoA reductase (CCR) genes (Hatton et al. 1995 , 1996; Lacombe et al. 2000), and the –244/–174 region in the parsley 4-coumarate:CoA ligase (4-CL) gene (Hauffe et al. 1993). Deletion of these negative regulatory elements resulted in ectopic activity of the promoters in cell types other than vascular cells. It remains to be determined whether the negative control region of the *Osgrp-2* promoter also acts co-ordinately with positive regulatory elements to confer a vascular-specific expression pattern.

As to the 5′-distal region (–2290/–1406), the agroinfiltration analysis has indicated that this fragment has an enhancer-like activity when fused in either orientation upstream of a 35S-89 promoter, activating the minimal promoter remarkably. Of particular interest is the presence of two stretches of A/T-rich sequences (underlined in Fig. 2): the first one is 111-bp long and its A/T content is 83%, the second one is a little longer (144 bp) with an A/T content of 82%. A/T-rich sequences have been reported to act as quantitative, non-tissue-specific enhancer elements in transgenic plants (Sandhu et al. 1998). In addition, there is a GC motif (TCCCCGA, boxed in Fig. 2) in this region. This motif has been shown to be responsible for the enhancer-like function in the maize *gpc4* promoter (Manjunath and Sachs 1997). However, the 889-bp enhancer-like region of the *Osgrp-2* gene failed to activate the 35S-49 minimal promoter which lacks the *as-1* element present in the 35S-89 promoter, underlying the importance of some kind of interaction between the putative enhancer *cis* element(s) and the *as-1* element.

The spatial expression patterns of the chimeric promoters with the enhancer-like region placed before the 35S-89 promoter were further investigated in transgenic tobacco plants. The histochemical staining results for transformants harboring pEff/-89 and pEr/-89 revealed a distinctive tissue-specific expression pattern: detectable blue staining was limited exclusively to the vascular tissues. Although further efforts to locate the tissue-specific *cis*-elements have not been made yet, two NTBBF1 motifs (ACTTTA, boxed in Fig. 2), one of which overlaps with the second A/T-rich stretch, may explain, at least in part, the vascular-specific expression pattern, as the NTBBF1 motif, a candidate binding site of Dof proteins, has been found to be responsible for tissue-specific expression in tobacco (Baumann et al. 1999). To our surprise, no GUS staining was found in any tissues tested in any of the 13 transformants of the pEf/-89 construct. It is likely that the low GUS activities of the individual plants are below the threshold of the histochemical detection. In fact, GUS staining can be detected in only a small fraction of fluorometrically GUS-positive transformants harboring the former two constructs.

The 5′ proximal part of the *Osgrp-2* promoter (G6) also displayed vascular-specific activity, as revealed in transgenic tobacco plants harboring the G6-driven GUS gene, thereby indicating the presence of functionally redundant vascular-specific *cis*-elements in the *Osgrp-2* promoter. A similar expression pattern directed by the G6 promoter was also observed in transgenic rice (unpublished data). Collectively, the G6 promoter can confer vascular-specific expression in both homologous monocot plants and heterologous dicot plants, as most investigated vascular-specific promoters do. Thus, the expression pattern conferred by the *Osgrp-2* promoter is reminiscent of those reported earlier for several cell wall-associated plant GRP genes such as *Pvgrp1.8* and *Ptgrp*s (Keller et al. 1988; Cheng et al. 1996), implying their similar biological functions in maintenance of cell wall structure and repair of injuries.

Several truncated versions of the G6 promoter were created and tested in transgenic tobacco plants to define the vascular-specific *cis*-element(s). The 5′ deletions up to G8a retained the same spatial expression pattern, but further deletions caused complete loss of any detectable blue staining, implying the importance of the 99-bp sequence between the G8a end point (–497) and the G8b end point (–398) at least in the context of the G8a promoter. On the other hand, gain-of-function analysis revealed that the promoter fragments D6E and D6P, both containing the 99-bp sequence, can confer a distinctive vascular-specific expression pattern on the 35S-89 minimal promoter. Thus, the 99-bp region seems to have a dual function, enhancing expression in vascular tissues and suppressing expression in other tissues. The region may contain more than one *cis*-element or a single *cis*-element binding to *trans*-acting factors with positive and vascular-specific functions, as in the case of the *vs*-1 element in the *Pvgrp1.8* promoter (Ringli and Keller 1998) and the –159/–84 region in the CoYMV promoter (Medberry and Olszewski 1993). A search for *cis*-elements on the PlantCare website revealed a CCAAT box in this region. It is a Myb-binding site and has been reported to be involved in tissue-specific expression in transgenic tobacco (expressed exclusively in meristematic tissue and conductive tissue associated with vascular bundles) (Wissenbach et al. 1993).

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