Structure, expression pattern and chromosomal localization of the rice *Osgrp-2* gene

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Abstract Glycine-rich proteins (GRPs) belong to a kind of important structural proteins of plant cell walls. The expression of GRP genes is regulated spatially and developmentally as well as by various environmental stresses, thus providing a good model for the study of plant gene expression. We obtained the genomic sequence of a new GRP gene (*Osgrp-2*) from a rice genomic library. The transcription start site of *Osgrp-2* was determined by 5'-rapid amplification of cDNA ends (RACE) and a 2.4-kb promoter sequence was thus delimited. The spatial and developmental expression pattern as well as the wound-inducible character of *Osgrp-2* in rice plants was analyzed in detail. Furthermore, the gene was mapped onto rice chromosome 10 by analysis of restriction fragment length polymorphism (RFLP).

Keywords: ***Oryza sativa*, GRP, gene structure, expression pattern, chromosomal localization.**

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The plant cell walls comprise various enzymes and several kinds of structural proteins. In addition to the structural roles, the structural cell wall proteins also function in altering the physical properties of cell walls as cells grow, divide and differentiate, and in repairing of cell walls after infection or wounding[1,2]. Plant structural cell wall proteins may be divided into four main classes: extensins, proline-rich proteins (PRPs), arabinogalactan proteins (AGPs) and glycine-rich proteins (GRPs). They are hydroxyproline-rich glycoproteins except for GRPs which are rarely glycosylated[3]. GRPs, extensins and PRPs are located in different cell types of plant organs, forming a three-dimensional defense system[4].

Plant cell wall-associated GRPs were first characterized in pumpkin and petunia[5]. Since then, a wealth of new GRPs were identified in almost all species of higher plants investigated, including bean, tobacco, *Arabidopsis*, carrot, tomato, maize, barley, wheat and rice[6]. The widespread of GRPs in higher plants suggests they are involved in essential physiological functions. An N-terminal signal peptide sequence can be found in the precursors of most GRPs, implying their location in the cell wall and apoplastic space. The mature proteins of GRPs are characterized

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by high content of glycine, up to 70% of all amino acid residues. The glycine-rich regions in GRPs often follow the repetitive motif $(Gly\text{-}X)_n$ where X is often glycine, but can also be another amino acid such as Ala, Ser, Phe or Tyr[7]. In plant cell walls, the repetitive nature of the glycine-rich domains is likely to allow the formation of a highly flexible conformation (glycine loop) or an antiparallel β-pleated sheet structure. These structures would make GRPs good candidates for protein-protein interactions or to provide the tensility and the elasticity of cell walls required during developmental processes.

The expression of a number of plant GRP genes has been shown to be developmentally regulated and influenced by a variety of environmental factors such as wounding, pathogen infection, hormone treatment and low temperature[7]. The more detailed studies on bean *grp1.8* and petunia *ptgrp1* have further revealed their tissue- and cell type-specific expression patterns, dominantly in vascular tissue[8,9]. Therefore, the GRP genes provide useful tools to study plant gene expression.

Two GRP genes have been isolated from rice up to now. *Osgrp-1* was reported to produce two different transcripts, each has distinctive developmental specificity[10]. In transgenic rice plants, the expression of the GUS gene directed by the *Osgrp-1* promoter was found exclusively in cells closely related to cell extension and differentiation in roots, stems and leaves[11]. We previously isolated a cDNA clone coding for a GRP from the rice (*Oryza sativa* cv. Aijiaonante) infected by rice yellow stunt virus (RYSV)[12]. We designated this virus-inducible GRP gene *Osgrp-2* because its coding region and the 5′ and 3′ untranslated regions are different from those of *Osgrp-1*. We report here the cloning and analysis of the genomic sequence of *Osgrp-2*, its expression pattern in rice plants and its chromosomal localization. We also determined the transcription start site of *Osgrp-2*. Analysis of the *Osgrp-2* promoter was reported elsewhere[13].

1 Materials and methods

1.1 Isolation of rice genomic DNA

Etiolated seedlings of rice (*Oryza sativa* cv. Aijiaonante) were ground into fine powder in liquid nitrogen. The crushed material was then transferred to a 50-mL centrifuge tube, mixed with 15 mL of extraction buffer (500 mmol/L NaCl, 1.25% SDS, 100 mmol/L Tris pH 8.0, 5 mmol/L EDTA) and incubated at 65℃ for 30 min. Five milliliters of 5 mol/L KAc was then added, followed by an incubation on ice for 20 min. After the addition of equal volume of chloroform and a centrifugation (12000 *g*) for 15 min at 4℃, the supernatant was mixed with 0.6 volume of isopropyl alcohol. The resulting fiber-like DNA was rinsed with 70% ethanol, dried in the air before it was dissolved in double-distilled water.

1.2 Construction and screening of the rice genomic library

The rice genomic DNA was partially digested with *Sau*3A and fragments of 9—23 kb in length were recovered from an 0.4% agarose gel by electroelution[14]. The recovered DNA frag-

ments were partially filled-in using the Klenow enzyme in a reaction with only dGTP and dATP as substrates and ligated into the partially filled-in λFix II/*Xho* I vector (Stratagene, USA). The *in vitro* phage packaging was performed using Gigapack II Gold Packaging Extract (Stratagene) according to the manufacturer's instructions, resulting in a phage library of the rice genome.

A ^{32}P-labeled probe prepared from a cDNA fragment of OsGRP-2 (fig. 1) using the random primer labeling method[15] was used to screen the genomic library. Several rounds of plaque hybridization were performed to identify the positive clones. Primers specific to the coding region of *Osgrp-2* (PA: 5′-CTCATCAGTGTCAAGATGGC-3′ and PB: 5′-AACGAGCTTAGTGGTG-TCCG-3′) (fig. 2) were used in PCR amplifications to confirm the selected clones.

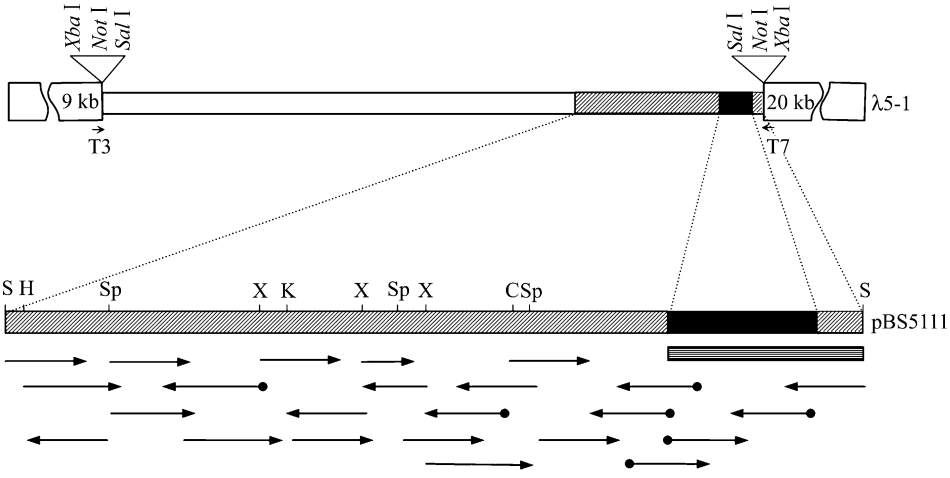

Fig. 1. Cloning, subclonings and sequence determination of *Osgrp-2*. The black box stands for the coding region of *Osgrp-2*, the boxes with slants stand for the upstream and downstream noncoding regions. The probe used in screening the genomic library is shown as a box filled with horizontal lines. The arrows represent sequencing reactions with the universal primer T3 or T7, whereas the arrows with filled circles represent sequencing reactions with specific primers. Abbreviations of restriction enzymes: C, *Cla* I; H, *Hin*d III; K, *Kpn* I; S, *Sal* I; Sp, *Spe* I; X, *Xba* I.

1.3 Cloning and sequencing of the *Osgrp-2* gene

The phage clones isolated from the rice genomic library were digested with a variety of restriction enzymes followed by DNA gel blot analysis[15] using the probe described above. The *Osgrp-2*-containing fragments were cloned into the relevant sites of pBlueScriptII SK-, followed by further subclonings to facilitate the nucleotide sequence determination using the universal primers T3 and T7. Several primers specific to the known sequences were also used in sequencing to assure that each nucleotide was determined by sequencing both DNA strands or one strand for at least twice (fig. 2).

1.4 5′RACE

5′RACE was performed using a kit (Gibco-BRL) following the manufacturer's instructions. mRNA was isolated from aerial tissues of rice seedlings, and the specific primer PB was used in the reverse transcription to obtain the cDNA before a poly-dC tail was added to its 3′ end. The

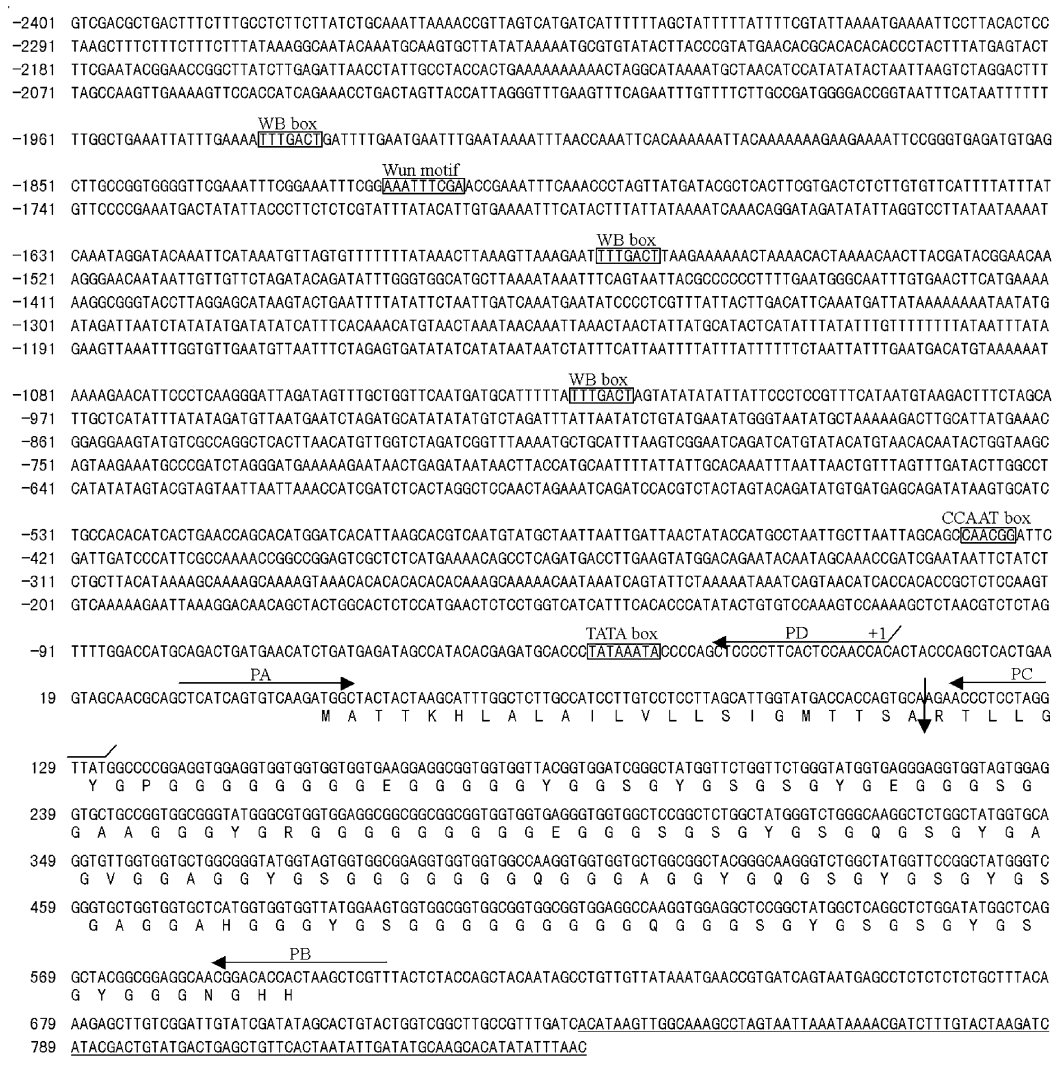

Fig. 2. The nucleotide sequence of *Osgrp-2*. Numbering is relative to the transcription start (+1). The predicted TATA box, WB boxes, CCAAT box and Wun-motif within the 5′ upstream region are boxed. The deduced amino acid sequence is shown below the nucleotide sequence and the vertical arrow stands for the putative cleavage site of the signal peptidase. The nucleotide sequence at the 3′end previously determined in the cDNA clone is underlined. Primers used in probe preparation and transcription start site determination (PA, PB, PC and PD) are shown as horizontal arrows above the nucleotide sequence.

first amplification was performed using the specific primer PB and an anchor primer AAP (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) provided in the kit. The products were used as a template in the second round of PCR with another anchor primer UAP (5′-CUACUACUACUAGGCCACGCGTCGACTAGTAC-3′) and one of the two specific nest primers (PC: 5′-ACGGATCCATAACCTAGGAGGGT-3′ and PD: 5′-CTGGGATCCGTGGTTGGAGTGAAGGGGAG-3′). Finally, the PCR products were cloned into pBlueScriptII SK- for sequence determination.

1.5 RNA gel blot analysis

The ^{32}P-labeled probe was prepared by the PCR method[16], using the cDNA of OsGRP-2 as a template and PA and PB as primers. Total rice RNA was isolated using a hot phenol method[17] from the leaves at five different stages (seedling, shooting, tillering, jointing and booting) and from four different organs (root, stem, ear and leaf) at the booting stage, respectively. Total RNA was also isolated from the rice seedlings at different time points after the wounding treatment (cutting or crushing). Fifteen micrograms of each RNA was resolved in an 1% agarose gel containing formaldehyde followed by blotting and hybridization[15].

1.6 Chromosomal mapping of *Osgrp-2*

The genomic DNAs of two parental lines, ZYQ8 (*indica* type) and JX17 (*japonica* type)[18], were digested completely with different restriction enzymes respectively, transferred to membranes, and subjected to DNA gel blot hybridization using the promoter fragment (–568/+1) of *Osgrp-2* as a probe. The restriction enzyme that resulted in the greatest length polymorphism of restriction fragments was used to digest the genomic DNAs isolated from 127 lines of the DH population. The blot hybridization was performed as described above. Linkage analysis was conducted on a Macintosh II computer using the MAPMAKER/exp 3.0 software[19]: TWO POINT and GROUP commands were carried out successively to obtain the linkage group containing *Osgrp-2*. The multi-point analysis was then performed using the command COMPARE; and finally, the KOSAMBI function was applied to convert the recombinant values to map units (cM).

2 Results

2.1 Construction and screening of the rice genomic library

A rice (*Oryza sativa* cv. Aijiaonante) genomic library was constructed by inserting partially digested rice DNA fragments into the λFix II/*Xho* I vector followed by *in vitro* packaging. The resulting library contained 3.5×10^5 independent recombinant phages and was screened with a probe based on the cDNA sequence of OsGRP-2 (fig. 1). Six phage clones were identified through three rounds of plaque hybridization and further confirmed by PCR amplification using primers (PA and PB) specific to the coding region of *Osgrp-2* and DNA gel blot analysis. Two positive clones, λ5-1 and λ8-1, were selected for further study.

2.2 Cloning and sequence analysis of *Osgrp-2*

λ5-1 and λ8-1 displayed the same DNA fragment pattern when digested with several restriction enzymes and hybridized to an OsGRP-2 cDNA probe. A 3-kb fragment of λ5-1 containing *Osgrp-2* was cloned in pBlueScriptII SK- (resulting in pBS5111) and several subclones were made (fig. 1) for sequence determination. The sequenced DNA fragments comprised a 552-bp coding region, a 2442-bp 5′ upstream region and a 138-bp 3′ untranslated region. Together with the 111 bps of the 3′ noncoding region previously sequenced in the cDNA clone but not present in λ5-1, an overall sequence of 3249 bps of *Osgrp-2* was elucidated. This sequence has been de-

posited in GenBank under the accession No. U40708.

The genomic sequence of the coding region of *Osgrp-2* is identical to the corresponding cDNA sequence, indicating the absence of any intron in this region. The predicted sequence of the OsGRP-2 precursor comprises 183 amino acid residues, the first 23 residues of which are the characteristic of the signal peptide sequence found in eukaryotes[20]. According to the analysis by SignalP WWW Prediction Server V1.1, the putative cleavage site of the signal peptidase is between Ala_{23} and Arg_{24}. The following 160-amino acid mature protein is hydrophilic (analyzed with DNAStar Protean V5.0) and rich in glycine (64.4%). In addition, 42% of other amino acids are Ala or Ser. Such an amino acid composition is very favorable for forming β-sheet secondary structures. Fifteen G-Y-G motifs are observed in the peptide, where the tyrosines are likely to be involved in intramolecular or the intermolecular covalent cross-links of iso-dityrosines, as in the case of extensins[21].

2.3 Determination of the transcription start site

In order to identify the transcription start site of *Osgrp-2*, 5′RACE analysis was performed on the RNA of rice seedlings. After reverse transcription, the cDNA was dC-tailed and PCR-amplified using the primers AAP and PB, and the product was used as a template in the second round of PCR using UAP and one of the two specific nested primers. An approximately 150-bp product was obtained in the nested PCR reaction using a primer (PC) specific to the coding region of *Osgrp-2*. The sequence of this product was subsequently determined (fig. 3) and compared with the genomic sequence of *Osgrp-2* (fig. 2), placing the transcription start site at an A residue (numbered +1, shown in fig. 3) flanked by two Cs. The transcription start was 47 bp upstream of ATG and 25 bp downstream of a putative TATA box, which is a common case observed in most of the plant genes analyzed[22]. However, when primer PD, whose sequence overlaps the transcription start, was used in the nested PCR, no conclusive product was obtained, indicating the absence of another upstream transcription start site, which was found in the case of *Osgrp-1*. The location of the transcription start site was also confirmed by a primer extension analysis of RNA extracted from wounded rice seedlings (data not shown).

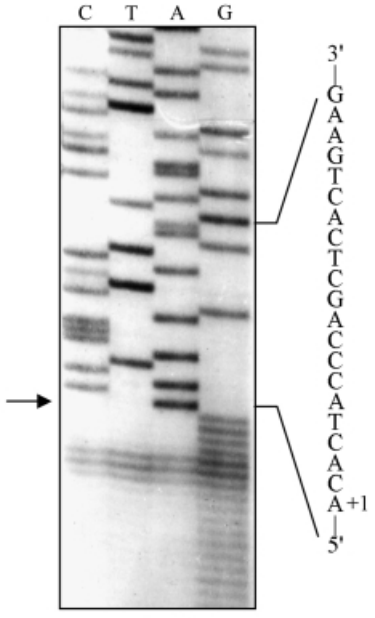

Fig. 3. Determination of the transcription start site of *Osgrp-2*. The sequencing ladders of the 5′RACE product are shown. The 5′RACE was performed using the total RNA of the rice seedlings. The sequence around the transcription start site (shown as +1 and by an arrowhead) is listed on the right.

2.4 The expression pattern of *Osgrp-2* in rice

Total RNA was extracted from the leaves at different developmental stages or from different

organs at the booting stage. Each RNA was subjected to gel blot analysis with the *Osgrp-2* coding region as a probe. As shown in fig. 4(a), (b), a specific signal of 0.9 kb corresponding to the size of *Osgrp-2* transcript was detected in most lanes. The intensity of the hybridization signals revealed that the expression level of *Osgrp-2* was inconsiderable at the seedling stage, became significant at the shooting stage, reached the highest at the jointing stage and began to drop at the booting stage. Based on the quantification of the hybridization signals using a Phosphor Imager, the expression level of *Osgrp-2* at the jointing stage was about threefold higher than that at the shooting stage (data not shown). Among the different organs at the booting stage, *Osgrp-2* displayed the highest expression level in leaves, lower level in stems and ears and undetectable level in roots. These results indicate that the expression of *Osgrp-2* in rice plants is spatially and developmentally regulated.

In order to study the wound inducible expression of *Osgrp-2*, two different wounding treatments (cutting and crushing) were applied to the rice seedlings which showed no detectable expression of *Osgrp-2*. RNAs were extracted from wounded seedlings at certain time points after wounding as well as from the unwounded seedlings. The subsequent RNA gel blot hybridization using the above-mentioned probe showed that the specific 0.9-kb signal could be detected only in

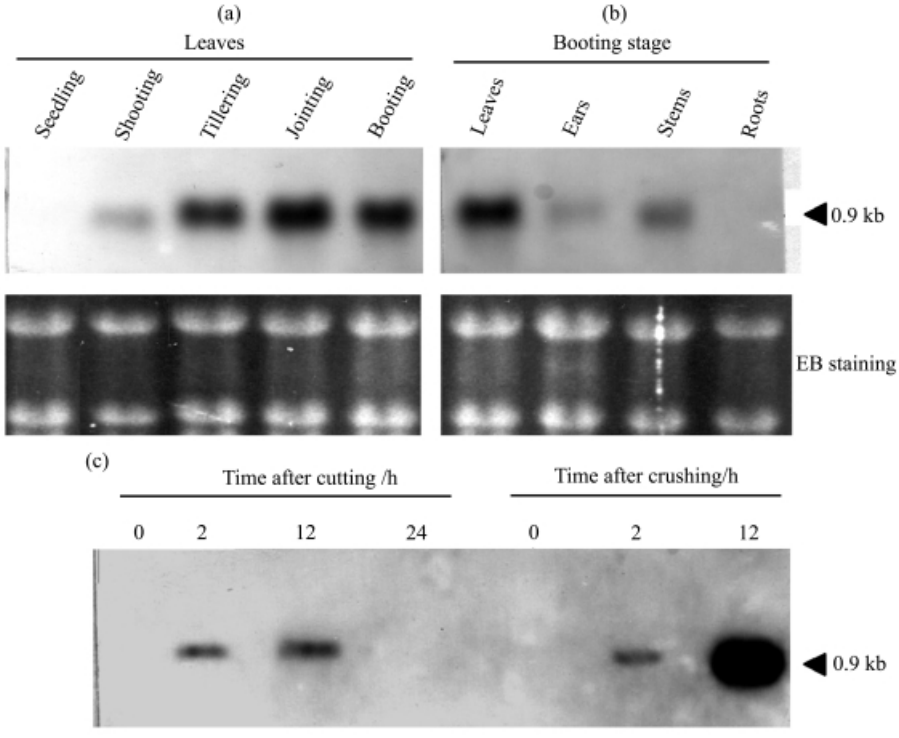

Fig. 4. RNA gel blot analysis for the expression pattern of *Osgrp-2*. (a) The gel blot of leaf RNAs at different developmental stages. (b) The gel blot of RNAs from different organs at the booting stage. (c) The gel blot of RNAs extracted from the seedlings at different time points after and before wounding. Equal loading of RNA in each lane (15 μg) is indicated by the EB staining in (a) and (b). Equal amount of RNA (15 μg) loaded onto each lane in (c) was also verified by EB staining (data not shown). The specific bands corresponding to the *Osgrp-2* transcript are shown by arrowheads.

wounded samples (fig. 4(c)). The induced expression by the cutting treatment was evident 2 h after wounding and was high after 12 h. The expression level induced by the crushing treatment was even higher than that by the cutting treatment. In the cutting treatment, the induced expression of *Osgrp-2* stopped 24 h after wounding.

2.5 Chromosomal localization of *Osgrp-2*

A previous Southern analysis on the *Hin*d III digested rice (*Oryza sativa* cv. Aijiaonante) genomic DNA using a probe based on the cDNA of OsGRP-2 has indicated that there is only one copy of *Osgrp-2* in the rice genome (data not shown). Among the seven restriction enzymes (*Bam*H I, *Bgl* I, *Dra* I, *Eco*R I, *Hin*d III, *Sca* I and *Xba* I) used to digest the genomic DNAs of ZYQ8 and JX17 (the parental lines), *Hin*d III resulted in the most significant RFLP when Southern analyses were performed using a probe derived from the promoter fragment (–568/+1) of *Osgrp-2* (fig. 5(a)). Genomic DNAs extracted from 127 lines of the DH population of the parental

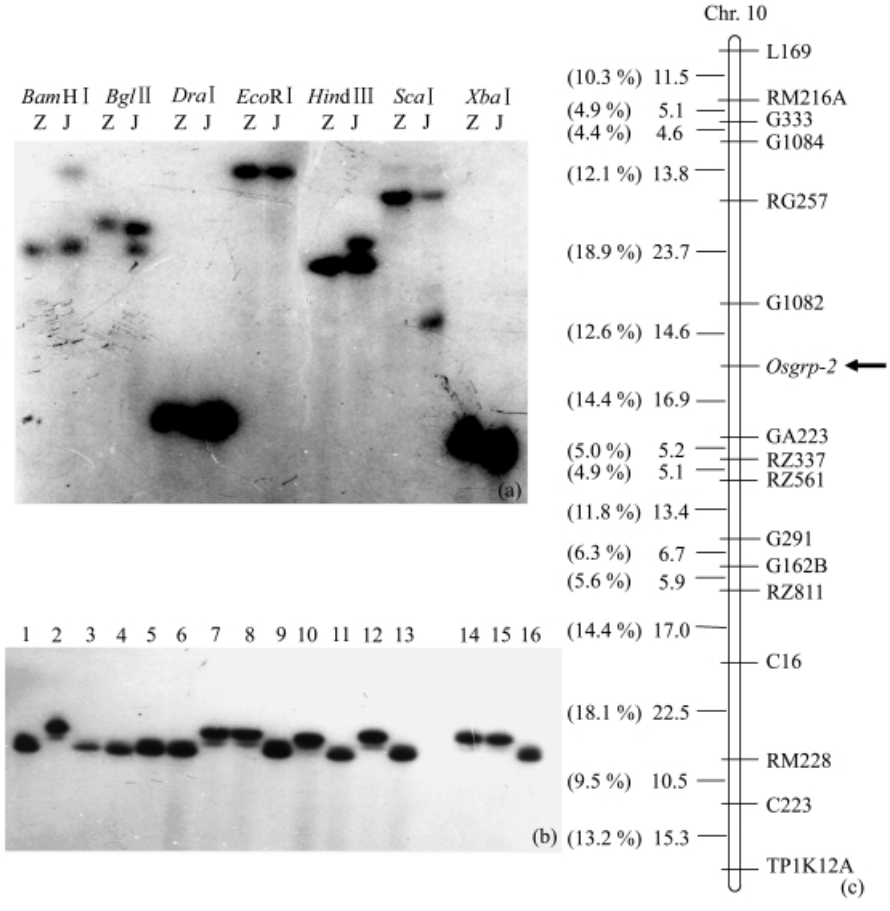

Fig. 5. Chromosomal localization of *Osgrp-2* in rice. (a) The RFLP analysis using the *Osgrp-2* promoter fragment as a probe. Z is for ZYQ8 and J for JX17. (b) Southern analysis of *Hin*d III-digested DNAs extracted from different lines of the DH population. Results of 16 lines are shown. (c) The localization of *Osgrp-2* on the rice chromosome 10. Recombinant values (%) and genetic map distances (cM) are listed on the left; RFLP markers are indicated on the right. *Osgrp-2* is shown by an arrow on the map.

lines, therefore, were digested with *Hin*d III followed by Southern blotting analysis for the chromosomal localization of *Osgrp-2* (fig. 5(b)). On the basis of the segregation of the polymorphic bands, *Osgrp-2* was mapped between two known RFLP markers (G1082 and GA223) on rice chromosome 10 (fig. 5(c)). The map distance was 14.6 centimorgans between G1082 and *Osgrp-2* and 16.9 centimorgans between *Osgrp-2* and GA223.

3 Discussions

The existence of a consensus sequence for signal peptide at the N-terminal of most GRPs indicates that they are located in the cell wall. Up to now, however, the experimental evidence of their cell wall localization has come from studies of only a few GRPs (pvGRP1.8 and ptGRP-1)[7]. We have reported here the cloning of a novel rice GRP gene, *Osgrp-2*. A characteristic signal peptide sequence was identified in the putative precursor protein of this GRP, suggesting the secretion and localization of OsGRP-2 to the cell wall. To confirm the secretary function of the signal peptide sequence, it was translationally fused to GUS and immunolocalization of GUS was conducted in the corresponding transgenic rice and tobacco plants. The subcellular distribution of GUS was assayed by immunogold-labeling method. The gold particles indicative of GUS were observed mainly in the cell wall or in the intercellular spaces between cell walls (unpublished data), thus confirming our speculation.

The biofunctions of the cell wall-related GRPs have been predicted mainly from their tissue localization, expression pattern and protein structures[7]. Apart from their structural function in the cell wall and extracellular matrix[23] , some GRPs have been reported to serve as transductors of extracellular signals through interaction with other macro-molecules[24], and some other GRPs have been demonstrated to be involved in the repairing process of cell wall during development and after wounding[25]. Analogous to those GRPs with many Tyr residues (such as pvGRP1.8, atGRP-3 and OsGRP-1), OsGRP-2, which harbors 15 tyrosine residues, might form a three-dimensional network structure in the cell wall via iso-dityrosine cross-links[21], thus increasing the intensity and elasticity of the cell wall. Taken together with the spatial and developmental expression pattern and the induction by wounding and virus invasion, we propose that *Osgrp-2* probably participates in the strengthening and repairing process of the cell wall after the aerial organs of rice were wounded or infected.

Two cell wall-related GRP genes (*Osgrp-1* and *Osgrp-2*) have been isolated from rice so far. The putative mature proteins encoded by the two genes are homologous in the sequence, and both of them display hydrophilicity and harbor more than ten Tyr residues. Nevertheless, the two genes are completely different in the sequence of the non-coding regions and in the number of transcripts. The spatial and developmental expression patterns of the two genes are also different: *Osgrp-1* is actively expressed in cell types closely associated with cell differentiation and elongation[11], whereas *Osgrp-2* is mainly expressed in leaves and stems with the highest level observed at the jointing stage. According to the analyses above, it is likely that the two GRP genes belong to

the same gene family and their products may be involved in similar biofunctions in different cell types and at different developmental stages.

Since the sequences of the coding regions of plant GRP genes are generally homologous[6], we used a specific probe derived from the promoter sequence in the chromosomal localization of the OsGRP-2 gene. This is the first report of the genomic mapping of plant GRP genes.

Detailed studies of the expression patterns of GRP genes revealed tissue specificities and developmental regulation. Expression of GRP genes has also been shown to be inducible by various external influences. *Cis*-elements responsible for tissue-specific expression have been found in the bean *grp*1.8[7]. In this study, the virus-inducible *Osgrp-2* was shown to be induced by wounding and displayed both spatial and developmental expression pattern. A search in relevant databases (PLACE and PlantCare) has revealed several binding-motifs for transcription factors in its promoter region, e.g., Wun-motif related with wound-induced gene expression, WB boxes related with pathogen induction[26] and CCAAT box responsible for tissue-specific expression[27](fig. 2). Further study on the *Osgrp-2* promoter will contribute to the elucidation of the regulation mechanism of its expression.

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