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Structure of a rice β-glucanase gene regulated by ethylene, cytokinin, wounding, salicylic acid and fungal elicitors

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Abstract

A rice β-glucanase gene was sequenced and its expression analyzed at the level of mRNA accumulation. This gene (*Gns1*) is expressed at relatively low levels in germinating seeds, shoots, leaves, panicles and callus, but it is expressed at higher levels in roots. Expression in the roots appears to be constitutive. Shoots express *Gns1* at much higher levels when treated with ethylene, cytokinin, salicylic acid, and fungal elicitors derived from the pathogen *Sclerotium oryzae* or from the non-pathogen *Saccharomyces cereviseae*. Shoots also express *Gns1* at higher levels in response to wounding. Expression in the shoots is not significantly affected by auxin, gibberellic acid or abscisic acid. The β-glucanase shows 82% amino acid similarity to the barley 1,3;1,4-β-D-glucanases, and from hybridization studies it is the β-glucanase gene in the rice genome closest to the barley 1,3;1,4-β-glucanase EI gene. The mature peptide has a calculated molecular mass of 32 kDa. The gene has a large 3145 bp intron in the codon for the 25th amino acid of the signal peptide. The gene exhibits a very strong codon bias of 99% G + C in the third position of the codon in the mature peptide coding region, but only 61% G + C in the signal peptide region.

Abbreviations: ABA, abscisic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; EtBr, ethidium bromide; GA, gibberellic acid; n.p., nucleotide position in gene sequence; PCR, polymerase chain reaction; 1 × SSPE, 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA pH 7.4.

Introduction

Plant β-glucanases possessing either endo-1,3-β-glucanase (EC 3.2.1.39) or endo-1,3;1,4-β-glucanase (EC 3.2.1.73) activities belong to a diverse family of structurally related enzymes. The 1,3-β-glucanases have been described in both monocots and dicots, but the 1,3;1,4-β-glucanases have thus far been found only in monocots. The β-glucanases are either involved in or associated with many physiological processes, such as cereal germination [19, 48], hypocotyl and coleoptile

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X58877.

growth [16, 22], regulation of phloem transport and callose mobilization [1, 54], flower development [39, 41], pollen tube growth [43], fruit maturation [18], and defense against pathogens (see references below). Members of this gene family are expressed in many tissues and regulated by a variety of hormones, stresses, and other chemical agents.

Much attention has been devoted to the role of β-glucanase in plant defense, which thus far has been studied almost exclusively in dicots. The 1,3-β-glucanases, especially in conjunction with chitinase, are capable of hydrolyzing fungal cell walls *in vitro* [34], and since both of these enzymes are co-induced in response to fungal attack [32, 53], their function is believed to be the inhibition of fungal infection. Defense 1,3-β-glucanase expression is also induced by fungal elicitors [26, 33], which can be derived from both pathogens and non-pathogens [5]. Some have proposed that β-glucanase may activate the expression of itself and other defense genes through the production of β-glucan elicitors [35]. Defense-related β-glucanases are also induced by wounding [34, 39].

The *Nicotiana* defense 1,3-β-glucanases are divided into acid and basic pI isozyme subfamilies. The acidic 1,3-β-glucanases are expressed at low levels in healthy plants, and their expression is induced by pathogen attack, salicylic acid and cytokinin [35, 36, 51]. The acidic 1,3-β-glucanases are secreted into the intercellular spaces where they are believed to degrade fungal hyphae tips [51]. The basic 1,3-β-glucanase subfamily is vacuole-targeted and undergoes substantial post-translational modifications [45, 51]. The basic 1,3-β-glucanases are induced by infection, salicylic acid and ethylene [36, 40]. Cytokinin and auxin together repress mRNA expression of a basic 1,3-β-glucanase gene in *Nicotiana* tissue culture [9, 12, 38]. *Nicotiana* basic 1,3-β-glucanase(s) are constitutively expressed in healthy plants in a gradient that increases from the top to the bottom of the plant, with the highest expression occurring in the roots [7, 11, 36]. The basic 1,3-β-glucanases expressed in roots and flowers may provide a constitutive defense against pathogens [7, 39].

A number of dicot defense-related 1,3-β-glucanase gene sequences have recently been reported [7, 15, 29, 40]. All these genes contain an intron (ranging from 341 to 974 bp) in the signal peptide coding region. One gene for a basic 1,3-β-glucanase has two introns [9]. A cDNA sequence for a barley 1,3-β-glucanase of uncertain physiological function has been reported [19]. In addition to 1,3-β-glucanase genes, barley germination 1,3;1,4-β-glucanase genes have recently been sequenced [30, 47]. They have extremely G + C-rich coding regions, and a single large 2.5 kb intron in the same position in the signal peptide coding region.

We have chosen to investigate the β-glucanase genes because of their potential importance in general defense and stress responses as well as in normal development. Although there is considerable information on this gene/enzyme system in dicots, our understanding of the structure and function of these genes in monocots is less well developed. Rice is particularly well suited to the study of such a diverse gene family because its small genome size [4] greatly facilitates the isolation of all the members of the family. We were also encouraged to pursue this study in rice by the report of high levels of β-glucanase activity in the germinating caryopses of rice [49]. In this report we describe the structure and regulation of a rice β-glucanase gene we designate *Gns1*. This gene has high structural similarity to the barley germination 1,3;1,4-β-glucanases genes, but it exhibits a very different gene expression pattern. Possible physiological roles for *Gns1* are discussed.

Materials and methods

Genomic library and screening

A λEMBL3-*Sau* 3AI genomic library was constructed using rice total genomic DNA isolated from 14-day-old etiolated rice leaves and has been described in detail elsewhere [21]. Approximately 200 000 plaques from the unamplified library were screened. A 30 base oligonucleotide probe was end-labeled by T4-polynucleotide ki-

nase and ^{32}P-γ-ATP [31]. This oligonucleotide (5′-GGGGTGTGCTACGGCATGAGCGCC-AACAAC–3′) corresponds to nucleotides 22 to 51 in the N-terminal coding region for the barley 1,3;1,4-β-glucanase EII cDNA [13, 30]. This region is conserved among the β-glucanases, particularly the 1,3;1,4-β-glucanases. Of the eight clones isolated, clone λOSg4 hybridized the most strongly to the oligonucleotide probe and was chosen for further analysis. The β-glucanase gene on λOSg4 was named *Gns1*.

Southern blotting

Southern blots were prepared essentially as described [21] using total rice genomic DNA isolated from 10-day-old etiolated rice shoots by the CTAB-chloroform/octanol procedure [44]. The blots were probed with either a 1016 bp λOSg4 *Hin*d III (n.p. 4427)-*Nsi* I (n.p. 5442) fragment or a 1138 bp *Hinf* I-*Nde* I λHV29 fragment [30] labeled by the random hexamer method to 10^9 cpm/μg [10], and added to prehybridization solution to 5×10^6 cpm/ml. The blots were hybridized for 12 h at 42 °C, and then washed 3 times in 0.2 × SSPE, 0.5% SDS at 65 °C for 1 h. The hybridization and wash conditions were calculated to be T_m −32 °C and T_m −20 °C, respectively [2]. The blots exposed X-Omat X-ray film (Kodak) for 40 h at −70 °C with an intensifying screen.

DNA sequencing and analysis

Various λOSg4 cloned insert fragments representing both strands of the 6.6 kb to be sequenced, were subcloned into M13mp18, M13mp19 or pBluescript ks$^-$ sequencing vectors. The subcloned fragments were: *Hin*d III-*Xba* I (n.p. 1–2727) in pBluescripts ks$^-$, *Hin*d III (2593–3240) M13mp19, *Xba* I (2727–3954) M13mp19, *Pst* I (3810–4673) M13mp19, *Hin*d III (4464–2.7 kb 3′) M13mp19, *Sst* I-*Kpn* I (4600–5160) M13mp18, *Sal* I/*Xho* I (5186–6138) M13mp18, and *Sph* I (5961–6647) M13mp18. Exonuclease III deletions were produced as described [17], and sequencing was performed by the dideoxy chain termination method using 5′-(α-^{35}S-thio)-dATP and buffer gradient gels [6]. Deoxy-7-deazaguanosine triphosphate was used to alleviate compressions [37]. The contiguous DNA and amino acid sequence was assembled and analyzed with the aid of MacVector computer software (IBI, New Haven, CT).

Plant tissues and treatments

The rice variety used in this study was M202 (*Oryza sativa* L. ssp. *japonica*), a medium-grained semi-dwarf. Rice seeds (caryopses) were surface-sterilized in 50% commercial bleach for 30 min and then washed with sterile H_2O. Germinated seeds were produced by incubating about 100 seeds in the dark at 30 °C for either 2, 4 or 6 d in glass beakers on H_2O-moistened Whatmann 3MM filter paper. Seeds were collected and emergent root and shoot tissues were removed. Seeds and any other of the tissues to be described below, were frozen in liquid nitrogen and stored at −70 °C until RNA extraction. For root tissue, about 250 seeds were incubated in the dark at 30 °C for 7 d on H_2O-moistened filter paper in sterilized pyrex trays. After 7 d the roots, which averaged 5–7 cm, were treated with various hormones and other agents by spraying the plantlets with 50 ml aerosol of the following solutions: GA 1 μM; ABA 10 μM; GA + ABA; auxin (2,4-D) 10 μM; *Sclerotium oryzae* fungal elicitor (see below); or ethylene (ethephon treatment, see below). After 48 h the roots were excised and frozen.

Young shoots were grown on vermiculite beds in the dark for 5 d at 30 °C. After 5 d the shoots, which were 7–9 cm tall, were treated for 48 h with 50 ml of aerosol containing the following hormones and agents. Ethylene treatment was performed using ethephon (Ethrel, Amchem Products, Inc.; 21.6% 2-chloroethyl phosphonic acid) which decomposes to release ethylene. Ethephon was applied at two concentrations: low ethylene, 50 ml of 1 mg/ml Ethrel (10.8 mg ethephon); and

high ethylene, 50 ml of 100 mg/ml Ethrel (216 mg ethephon). The ethephon solutions were applied to the plants as aerosols in a 160 l incubator. For the salicylic acid treatment, 50 ml of 10 mM salicylic acid was applied as an aerosol. The other hormones were applied as 50 ml aerosols in the following concentrations: GA 1 μM; cytokinin (kinetin) 10 μM; auxin (2,4-D) 10 μM; ABA 10 μM.

The pathogenic fungal elicitor was prepared from sclerotia of the ascomycete *Magnaporthe salvinii* (= *Sclerotium oryzae*), the fungal pathogen responsible for rice stem rot [27]. Sclerotia (0.25 g) were suspended in 50 ml of H_2O, sonicated for 5 min, autoclaved for 20 min, sonicated again, passed through a 0.2 μM Nalgene filter, diluted 10-fold, and then applied to the plants as 50 ml of aerosol. The yeast elicitor was prepared in the same way from 0.1 g of *Saccharomyces cerevisiae* cell paste which had been grown in YEPD medium. To inflict wounding, 6.5 d shoots were punctured and bruised along their entire length between two blocks of coarse sandpaper (grit size 36; 3M, Inc.) and then harvested 12 h later.

RNA isolation and northern blotting

Total RNA from the above tissues and treatments was isolated, and northern blots were done essentially as described [20]. Mature leaf RNA was isolated as described [50]. Callus mRNA was isolated as previously described [46]. RNA was isolated from panicles 10 d after heading as previously described [20]. The λOSg4 *Hin*d III-*Nsi* I fragment was labeled to 10^9 cpm/μg using ^{32}P-α-dCTP and the random hexamer method [10]. The blots were hybridized with prehybridization solution plus 5×10^6 cpm/ml of probe for 12 h at 42 °C. The blots were washed in 0.1 × SSPE, 0.5% SDS, for 5 times, 45 min each, at 71 °C in a water bath. For the rRNA control probing, a 3.6 kb *Bam* HI fragment from the wheat rDNA clone pTA250.2 [3] was labeled and probed according to the same protocol, except that the blots were hybridized at 10^4 cpm/ml, washed at 0.5 × SSPE, 0.5% SDS for 3 times at 60 °C. Autoradiographs were scanned with the aid of the Bio-Image integration computer (Milligen/Biosearch, Ann Arbor, MI). The level of *Gns1* expression was normalized to the level of ribosomal RNA.

PCR amplification of mRNA and cDNA cloning

Two oligonucleotides were synthesized with a 'Cyclone Plus' DNA synthesizer (Milligen/Biosearch). The first oligonucleotide, which is the complement of the 30 base oligonucleotide primer used to isolate the genomic clones, is 5′-GTTGTTGGCGCTCATGCCGTAGCACAC-CCC-3′, and corresponds to n.p. 4550 to 4521 on the *Gns1* gene sequence in the mature peptide coding region (Fig. 3). The second primer is a 23 base oligonucleotide, 5′-GGAATTCAGAGAG-GTTTTGAGAG-3′, which corresponds to n.p. 1263 to 1285 on the gene sequence in the nontranslated leader. An *Eco* RI site (GAATTC) was introduced by converting the G at n.p. 1269 to a C in the second primer. The PCR protocol was similar to that described previously [20]. The reaction was run for 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 60 °C, and DNA synthesis for 3 min at 72 °C. The reaction products were electrophoresed and visualized in 3% agarose/TAE EtBr-stained gels. The PCR cDNA product was blunt-ended and the excess primer was removed by S1-nuclease treatment, cut with *Eco* RI, and cloned into M13mp19 cleaved with *Eco* RI and *Sma* I. Eight clones were identified by IPTG/X-GAL blue/white selection and comigration of their cloned insert with that of the PCR product. The inserts were sequenced as described above.

Results

Genomic southern blot

The β-glucanase clone λOSg4 hybridized more strongly to the oligonucleotide constructed from the conserved sequence encoding the N-terminal of the mature barley 1,3;1,4-β-glucanase than the

other seven clones we isolated from the library. A partial restriction map of the 15 kb insert of λOSg4 is presented in Fig. 1. A rice genomic Southern blot containing DNA cleaved with six restriction enzymes was probed with a *Hin*d III-*Nsi* I fragment encoding the mature peptide of *Gns1* (Fig. 2A). The *Hin*d III site is 92 bp above the codons for the putative junction of the signal and mature peptides, and the *Nsi* I site is 4 bp below the stop codon. The most intense band(s) in each lane corresponded in number and size to those predicted from the restriction map for λOSg4 with the exception of the *Sal* I lane. The two bands seen in the *Sal* I and *Kpn* I lanes were due to the presence of sites for these enzymes in the middle of the coding region. The bands in the *Sal* I lane were larger than predicted by the restriction map due to incomplete digestion with this enzyme. We frequently observe this with *Sal* I and it may be due to partial methylation at some sites. Another set of fainter bands was visible which corresponded to one or perhaps two other genes. However, there was no other rice gene of roughly equal hybridization intensity to *Gns1*, even though the blots were processed under mildly stringent conditions (T_m ca. 20 °C). The same rice genomic blot was rigorously stripped and then probed with the barley 1,3;1,4-β-glucanase EI fragment (Fig. 2B). The bands showing the greatest intensity matched those representing the *Gns1* gene. This indicates that the *Gns1* gene is, by cross-hybridization, the closest gene in the rice genome to the barley 1,3;1,4-β-glucanase EI gene.

Gene sequence

The *Gns1* gene sequence is presented in Fig. 3. The general structure of the gene was revealed by comparison to other β-glucanase genes. There are 6614 bp of gene sequence, with 1158 bp of 5′ flank, 129 bp of nontranslated leader, 73 bp of signal peptide coding region in exon 1, 3145 bp of intron, 933 bp of exon 2, and 1176 bp 3′ to the translation termination signal. The single large intron is located between the first and second nucleotide of the 25th codon in the signal peptide coding region. A putative polyadenylation signal site, AATATA [24], was identified at 307 to 312 bp 3′ of the stop codon.

The G + C profile of the gene showed a strong G + C preference (68.8%) in the peptide coding region. The mature peptide and signal peptide coding regions were 69.7% G + C and 58.3% G + C, respectively. In the mature peptide coding region the G + C preference in the third position is 98.7%, with just 4 codons out of 306 codons having an A or T in the third position. In the signal peptide region, however, the G + C preference in the codon third position was just 60.7%. The intron, 5′ and 3′ flanks were 36.4%, 41.2% and 42.0% G + C, respectively.

The 3145 bp intron was an unusually large plant intron, and the largest β-glucanase intron reported to date. There did not appear to be any open reading frames of significant length within the intron. With the exception of two small regions of significant similarity, there was low overall similarity between the introns of *Gns1* and the barley β-glucanase EI gene [30]. The sequence in the rice intron at 1248 to 1275 bp 3′ of the 5′ splice

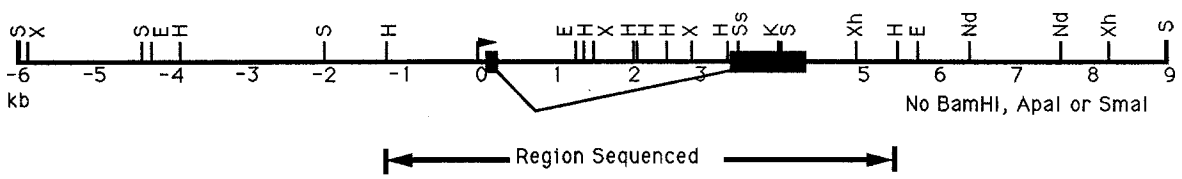

Fig. 1. Partial restriction map of the insert of the rice β-glucanase genomic clone λOSg4. The 15 kb insert was mapped with nine restriction enzymes: S (*Sal* I), X (*Xba* I), E (*Eco* RI), H (*Hin*d III), Ss (*Sst* I), K (*Kpn* I), Xh (*Xho* I) and Nd (*Nde* I). The bold segments denote peptide coding region. The arrows indicate the region sequenced.

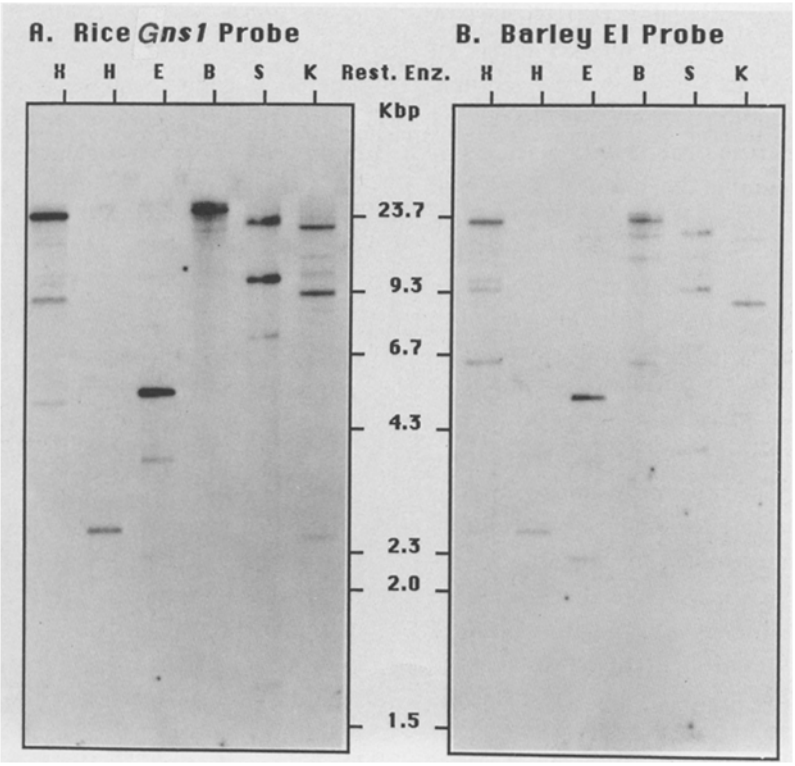

Fig. 2. Rice genomic Southern blot probed with either λOSg4 insert (A) or the barley 1,3; 1,4-β-glucanase EI fragment (B). Six restriction enzymes were used: X (*Xba* I), H (*Hind* III), E (*Eco* RI), B (*Bam* HI), S (*Sal* I) and K (*Kpn* I).

site was 79% similar to a region in the barley intron at 992 to 1019 bp 3′ of the 5′ splice site. There was also a region of 87% similarity at positions 1901–1930 bp and 1545–1575 bp 3′ of the 5′ splice sites in the rice and barley introns, respectively.

The 1196 bp of 5′ flank was 41% G + C; however, the 300 bp proximal to the start site were 52.7% G + C, whereas the distal 896 bp were 37.5% G + C. A computer-generated similarity matrix between the rice *Gns1* and the barley EI gene revealed some similarity in the 5′ flanks for the two genes, especially in the proximal 300 bp (data not shown). The transcription start site has been tentatively identified by comparison to that of the barley 1,3;1,4-β-glucanase EI gene [30]. There was a well-defined TATA box (TATATAA at −32 to −26), which was identical in sequence and location to that in the barley EI gene. The most striking similarity in the 5′ flank between *Gns1* and the barley EI gene was a 36 bp stretch from −274 to −239 in the rice gene and −208 to −172 in the barley gene which was identical in 33 of 36 positions. The conserved sequence was ACAACAACCTGTGTACCGGATAAC--CTG-CCACCA. A pyrimidine-rich region at −326 to −277 flanks a TATCCAT element at −300, an element which has been found in the barley EI gene [30] and in many monocot α-amylase genes [21].

The sequence between −107 and −72 bp contained several regions which were similar to motifs found in other plant promoters. The sequence at −107 to −102 (GTGGATTG) is identical to an enhancer core which occurs in the CaMV 35S promoter at the same position (−105 to −98) [14]. At position −102 to −86 in *Gns1* there was an 8 bp perfect direct repeat, which was also an inverted repeat with three mismatches. In the middle of this repeat at −97 to −93 there was a CAAT box (GGACAATT), although another potential CAAT box (GGTCAATT) is at −165 to

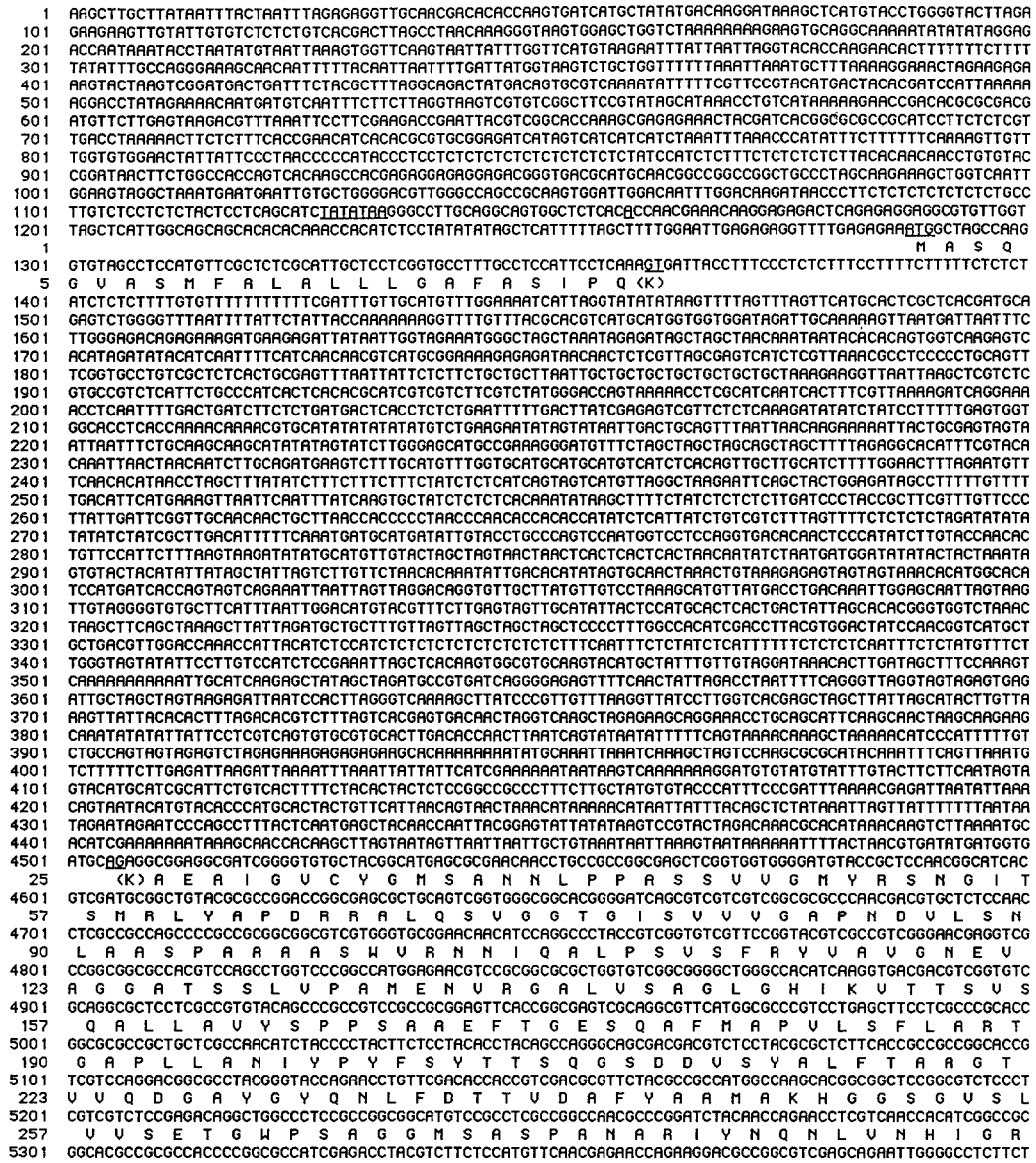

Fig. 3. The nucleotide sequene of the *Gns1* gene and the deduced amino acid sequence of the encoded GNS1 β-glucanase. Underlined in the diagram are the TATA box TATATAA, the putative transcription start site CACACCA, the translation start site ATG, the intron borders GTGATT and TGCAG, the translation stop codon TGA, and the polyadenylation signal AATATA.

−157. At −86 to −72 there was a dyad symmetry element (AGA-AA---TT-TCT) related in sequence and position to dyad elements found in the promoters of tobacco PR-la [42], the soybean heat shock [8], and *Drosophila* heat shock genes [25]. The sequence, ACGGGTGACGCA, at −213 to −202 in the *Gns1* gene matched 11 of 12 positions to a region in the CaMV 35S promoter at −75 to −63 which is thought to be responsible for mediating gene expression in roots [28].

Protein structure

The deduced GNS1 amino acid sequence is also presented in Fig. 3. The total peptide was 334 amino acids, with 28 amino acids in the signal peptide and 306 in the mature peptide. The total peptide was calculated to be 34.7 kDa, whereas the mature peptide was calculated to be 31.9 kDa. The predicted amino-terminal sequence of the mature peptide, Ile-Gly-Val- (n.p. 4518 to 4526), was the same as that found in other monocot β-glucanases [19, 55]. GNS1 contained no N-glycosylation site (Asn-X-Ser/Thr). In addition, there was only one cysteine residue at amino acid position 32. The signal peptide was strongly hydrophobic, but the usual positively charged amino acid near the N-terminus of the signal peptide was not present. There was no C-terminal extension like that of the dicot vacuolar basic pI 1,3-β-glucanases [45]. The pI of GNS1 was calculated to be 6.2.

The GNS1 peptide had 82% and 79% amino acid similarity to the barley germination 1,3;1,4-β-glucanases EI and EII, respectively [30, 47]. The nucleotide similarity was also about 80% in the peptide coding region. The GNS1 peptide had relatively low similarity (53%) to the barley 1,3-β-glucanase GII isozyme [19]. The GNS1 peptide had 46% amino acid similarity to the tobacco acidic 1,3-β-glucanases, and about 53% similarity to the *Nicotiana* vacuolar basic 1,3-β-glucanases [7, 15, 29]. However, there was a region of 10 amino acids at position 173–182 in the mature peptide of GNS1 which showed no similarity to the 1,3;1,4-β-glucanases and yet was 40–50% similar to the dicot basic 1,3-β-glucanases.

Gene expression

A general survey was conducted of the tissues and conditions in which *Gns1* is expressed. The RNA level for *Gns1* in various untreated tissues is presented in Fig. 4 and Table 1. Roots showed the highest expression of *Gns1* among these tissues. Root expression of *Gns1* was analyzed further by treating roots with a variety of potential regulators. These treatments, however, did not greatly affect the relative RNA accumulation (Fig. 5 and Table 1). The expression of *Gns1* in

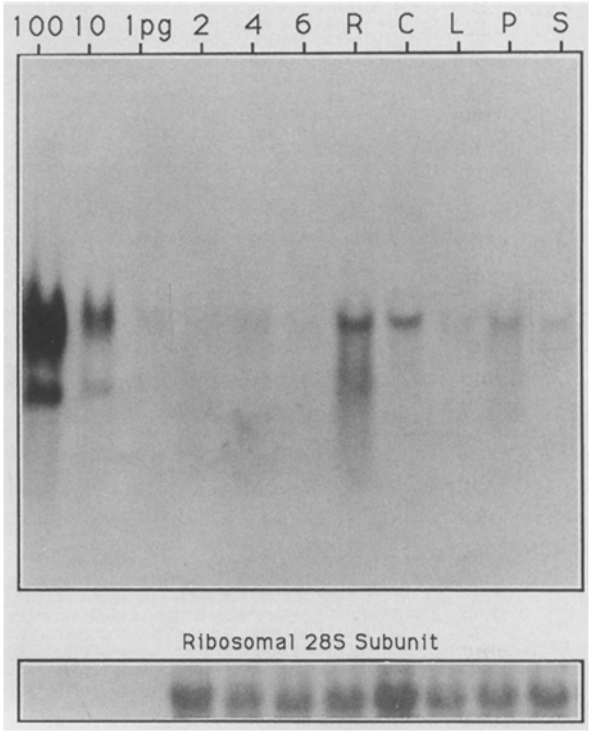

Fig. 4. Northern blot analysis of *Gns1* expression in various untreated rice tissues. The tissues were: 2, 4 and 6 (2, 4 and 6 day germinated seeds, respectively), R (roots), C (callus), L (mature leaves), P (panicles), and S (young shoots). The controls were 100, 10 and 1 pg of the *Hind* III-*Nsi* I 1013 bp *Gns1*-coding region fragment. The narrow panel at the bottom is the ribosomal probing control which demonstrates equality of loading.

Table 1. Relative *Gns1* expression in various tissues, and in roots and shoots in response to hormone, elicitor, and wounding treatments.

Tissue/ treatment	Relative expression	Tissue/ treatment	Relative expression
Various tissues (Fig. 4)		Ethylene	1.4
4 d Germ. seed	0.3	Salicylic acid	1.9
4 d Germ. seed	0.3		
6 d Germ. seed	1.0	*Shoot treatments* (Fig. 6)	
Roots	5.8	Control	1.0[c]
Callus	1.5	GA	1.6
Mature leaves	1.1	ABA	0.3
Panicles	1.5	Auxin	1.3
Young shoorts	1.0[a]	Auxin + kinetin	8.0
		Kinetin	14
Root treatments (Fig. 5)		Ethylene (low)	3.0
Control	1.0[b]	Ethylene (high)	18
GA	1.1	Wounding	58
GA + ABA	2.6	*S. oryzae* elicitor	23
ABA	1.6	Yeast elicitor	10
Auxin	2.6	Salicylic acid	6.7

[a] The level of expression in young shoots was chosen as the reference.

[b, c] The levels of expression in untreated roots and in untreated shoots were chosen as references for their respective northern blots.

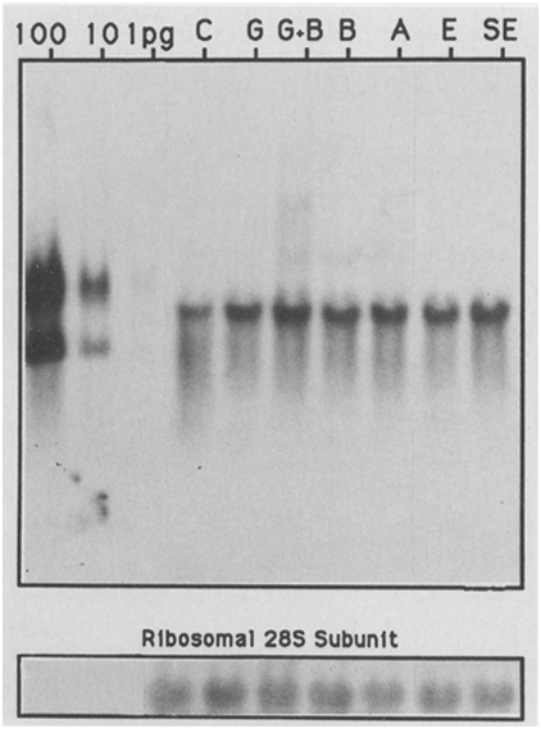

Fig. 5. Northern blot analysis of *Gns1* expression in rice roots. The treatments were: C (control, H_2O), G (GA, 1 μM), B (ABA, 10 μM), A (auxin-2,4-D, 10 μM), E (ethylene-ethephon), and SE (*Sclerotium oryzae* fungal elicitor).

shoots in response to various hormones, elicitors, and wounding was also investigated. Expression in the shoots was substantially induced by ethylene, salicylic acid, cytokinin, fungal elicitors, and wounding, but not by auxin, gibberellic acid or abscisic acid (Fig. 6 and Table 1). Despite its high, 80%, similarity to the *Gns1* probe, the barley EI coding region probe did not hybridize to the RNA on these northern blots under these conditions (data not shown).

PCR amplification of RNA and cDNA cloning

In order to confirm *Gns1* expression in these tissues and the location of the intron, cDNAs representing *Gns1* were PCR-amplified, cloned and sequenced. RNA samples from the following tissues and treatments were used: roots, callus, panicles, and shoots (control, ethylene, fungal elicitor-*S. oryzae* and salicylic acid). The expected PCR-amplified product was a 142 bp fragment encoding the signal peptide and the N-terminal region of the mature peptide. All of these samples yielded a fragment of the expected size was visible on EtBr-stained gels (Fig. 7). In agreement with the relative mRNA levels on the northern blots, the control shoots yielded less PCR product than the induced shoots. Controls using genomic DNA, or total RNA without reverse transcriptase, failed to produce a PCR product. The PCR products from shoots (control, salicylic acid and fungal elicitor) were cloned into M13mp19. A total of eight cDNAs, with representatives from each of these three samples, were sequenced, and all eight cDNAs had sequences identical to the *Gns1* genomic sequence (Fig. 7).

Discussion

We have isolated and sequenced a rice β-glucanase gene named *Gns1*. This gene is similar in

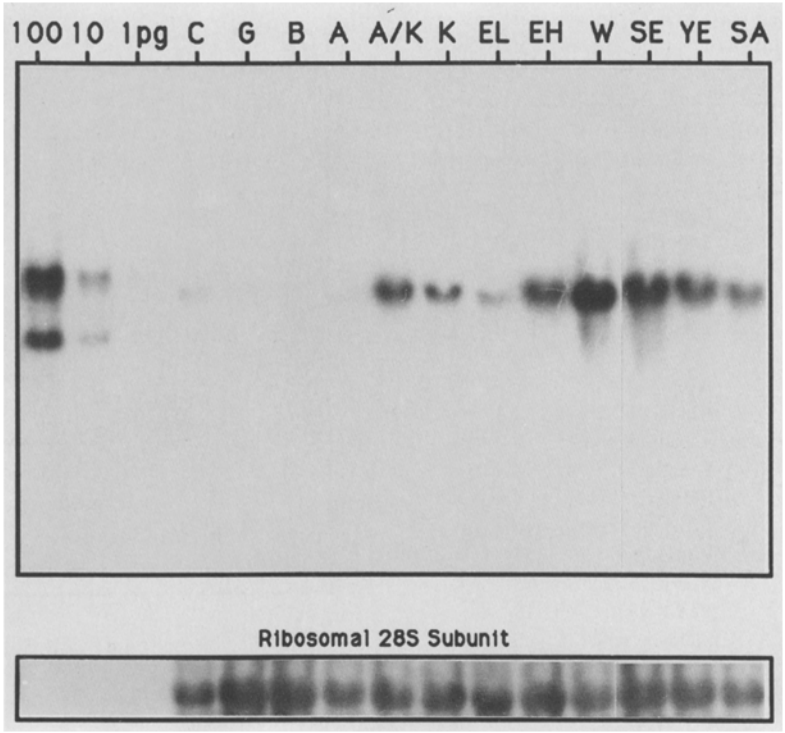

Fig. 6. Northern blot analysis of *Gns1* expression in rice shoots. The treatments were: C (control, H_2O), G (GA, 1 μM), B (ABA, 10 μM), A (auxin-2,4-D, 10 μM), K (cytokinin-kinetin, 10 μM), EL, EH (ethylene-ethephon; L, low, 1 mg/ml; H, high, 100 mg/ml), W (wounding), SE (*Sclerotium oryzae* fungal elicitor), YE (yeast fungal elicitor), and SA (salicylic acid, 10 mM).

structure to other β-glucanase genes in both monocots and dicots in terms of the open reading frame encoding a protein of ca. 32 kDa, the structural similarity of that protein, and the intron in the signal peptide coding region (see Introduction). The extreme G + C codon bias and length of the intron, however, indicate that *Gns1* is most similar to the monocot β-glucanase gene and cDNA sequences [19, 30, 47]. In addition, amino acid alignments and percent similarities demonstrate that GNS1 is most similar to the barley 1,3;1,4-β-glucanases [13, 30, 47]. The 5′ flank of *Gns1* has several interesting regions with marked structural similarity to 5′ flanking regions in the barley EI gene, the cauliflower mosaic virus 35S gene, and other plant genes. These regions may mediate *Gns1* transcriptional regulation, but more experimentation is needed to verify any such role.

This general survey of *Gns1* expression by northern blot analysis indicates that it is consti-

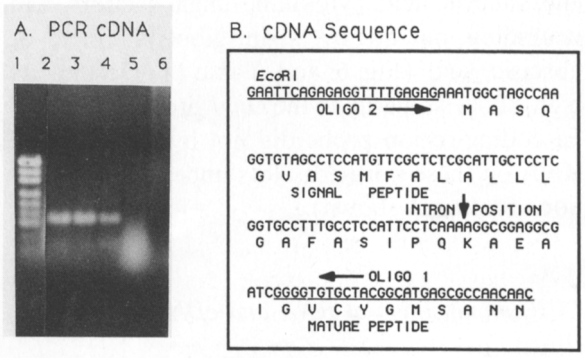

Fig. 7. RNA-PCR amplification of the *Gns1* cDNA and its sequence. *A.* Agarose gel electrophoresis of three PCR products: pUC19 × *Msp* 1 markers (Lane 1), salicylic acid shoot treatment RNA (Lane 2), *S. oryzae* fungal elicitor shoot treatment RNA (Lane 3), control untreated shoot RNA (Lane 4), *S. oryzae* fungal elicitor shoot treatment RNA without reverse transcriptase control (Lane 5), and rice genomic DNA (Lane 6). *B.* PCR-amplified cDNA sequence, its amino acid sequence, and the position of the intron.

tutively expressed in roots and induced in shoots by cytokinin, ethylene, wounding, fungal elicitors, and salicylic acid. The physiological role of *Gns1* is not yet known. Nonetheless, this pattern of expression is consistent with the expression of dicot defense-related β-glucanases (see Introduction). The structural similarity of GNS1 to the 1,3;1,4-β-glucanases suggests that GNS1 is a 1,3;1,4-β-glucanase. All dicot defense β-glucanases identified to date have been 1,3-β-glucanases, which argues against GNS1 being involved in defense. However, since the structure of monocot defense β-glucanases and the substrate specificity of GNS1 are not yet known, further study on Gns1 and other monocot β-glucanases is needed to clarify this issue.

Recently the barley 1,3;1,4-β-glucanase EI gene has been proposed to be involved in aerenchyma formation [47]. Beta-glucanase could play a role in aerenchyma formation and in shoot elongation by degrading cell wall β-glucan and thereby promoting cell separation and turgor-mediated cell expansion. In rice, aerenchyma formation in roots and elongation of shoots in submerged conditions is promoted by ethylene [23]. The expression of *Gns1* is also promoted by ethylene and conditions which have been reported elsewhere to induce ethylene production, such as wounding and cytokinin [52, 56]. Despite these relationships, however, more needs to be known about the similarities and differences in the regulation of β-glucanases involved in defense, aerenchyma formation, shoot elongation, and other physiological processes, before the role of *Gns1* can be definitively inferred from the gene expression pattern.

The expression of *Gns1* in germinating rice seeds was very low, despite the fact that by Southern blot analysis *Gns1* was the rice gene closest to the barley germination 1,3;1,4-β-glucanase genes. These 1,3;1,4-β-glucanases are expressed at high levels in germinating barley seeds, and the expression of at least one isozyme is induced by gibberellic acid [48]. Germinating rice seeds have been reported to possess high levels of 1,3;1,4-β-glucanase activity, but the enzyme responsible for this activity has little antigenic cross-reactivity to barley 1,3;1,4-β-glucanase [49]. This prompted speculation that the rice 1,3;1,4-β-glucanase(s) responsible for the high enzymatic activity is structurally distinct from the barley 1,3;1,4-β-glucanases [49]. The results presented here support this idea.

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