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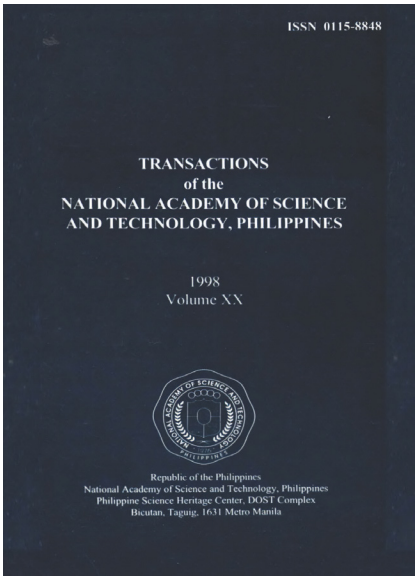

Cloning and Characterization of Five β-Glucanase Genes in Rice

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CLONING AND CHARACTERIZATION OF FIVE β-GLUCANASE GENES IN RICE

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ABSTRACT

β-glucanases play key roles in the defense response of plants and in important physiological processes such as seed germination. As a step toward engineering better resistance and germination, we have cloned five β-glucanase genes in rice and characterized their structure and expression. *Gns5* and *Gns6* are tandemly arranged within a 6-kb region in the rice genome while *Gns7, Gns8,* and *Gns9* are at least 8 kb apart from each other and from *Gns5* and *Gns6. Gns5* encodes a mature peptide of 304 aa, with an estimated pKI of 4.2. *Gns6* species a mature peptide of 307 aa, with an estimated pKI of 4.6. *Gns7* encodes a mature peptide of 311 aa, with an estimated pKI of 5.4. Gns 8 specifies a mature peptide of at least 313 aa, with an estimated pKI of 4.6. *Gns9* encodes a mature peptide of 322 aa, with an estimated pKI of 9.9. The *Gns5* and *Gns6* isozymes likely have a β-1,3-glucanase activity, while *Gns7, Gns8*, and *Gns9* isozymes may have either a β-1,3-glucanase and/or a β-1, 3; 1,4-glucanase activity or a novel substrate-specificity. *Gns5* showed maximal expression in root and mature leaf, *Gns6* in root and germinated seed, *Gns7* in germinated seed, *Gns8* in root, and *Gns9* in calli and root. In young leaf, exogenous salicylic acid strongly induced *Gns5, Gns6*, and *Gns7;* and exogenous GA, auxin, ethylene, and ABA strongly induced *Gns7* and *Gns9.*

Key words: ABA, auxin, β-glucanase, calli, coleoptile, ethylene, GA, PR protein, seed germination

1. INTRODUCTION

Plants produce enzymes that hydrolyze cell wall polysaccharides, including endo-1, 3-β-glucanases (E.C. 3.2.1.39) and endo-1,3;1,4-b-glucanases (E.C. 3.2.1.73). Although these isozymes have distinct enzyme activities, the 3-dimensional structures of the proteins and homology in the DNA sequences of the genes that encode them indicate that these β-glucanases represent a single gene family

(Henrissat and Bairoch, 1993; Høj and Ficher, 1995). Diagnostic amino acids at certain positions within their protein sequences distinguish the 1,3-β- and 1,3;1,4-β-glucanase Subfamilies (Varghese et al., 1994). The 1,3-β-glucanases are ubiquitous in the plant kingdom, with the 1,3;1,4-β-glucanases appearing as a recently-evolved subfamily found only in the grasses. The 1,3-β-glucanases catalyze the hydrolysis of 1,3-β-glucosyl linkages of cell wall polymers found in plant (1,3-β-glucan=callose) and fungal (1,3;1,6-β-glucan = laminarin) cell walls. The 1,3;1,4-β-glucanases hydrolyze the 1,4-β-glucosyl linkages adjacent to 1,3-β-glucosyl linkages found uniquely in the 1,3;1,4-β-glucans of grass plant cell walls.

The 1,3-β-glucanases function in plant defense and in plant development. Certain 1,3-β-glucanases are among the pathogenesis-related (PR) proteins induced when plants are infected by microbial pathogens. The 1,3-β-glucanase genes are also among the systemic-acquired resistance (SAR) genes that are expressed in parallel with the onset of an induced resistant state. The 1,3-β-glucanases act in recognition of the invading fungus by releasing elicitors through limited hydrolysis of the fungal cell wall and in killing the fungus by extensive hydrolysis of the cell wall and cell lysis (Kombrink and Somssich, 1995). The 1,3-β-glucanases also act in microsporogenesis where breakdown of a callose-rich cell wall is required for pollen maturation (McCormick, 1993; Scott, 1991).

The 1,3;1,4-β-glucanases function in cereal plant development. During seed germination, 1,3;1,4-β-glucanases are secreted into the endosperm where they degrade the cell walls and facilitate entry of other hydrolases that mobilize the stored reserve materials (Briggs, 1992). Production of the 1,3;1,4-β-glucanase enzyme and breakdown of 1,3;1,4-β-glucans is also a prominent feature in cell walls of growing tissues throughout the cereal plant (Inouhe et al., 1997). A role for 1,3;1,4-β-glucanases in the control of cereal plant growth and tissue elongation has been proposed (Hoson, 1993). A limitation in our knowledge of the β-glucanases is that the enzymology of the coleoptile system has been studied primarily in maize (Inouhe and Nevins, 1991) while the majority of the β-glucanase molecular biology in the cereals has been done in barley and rice. The only 1,3;1,4-β-glucanase genes known in barley, the EI and EII genes, are not expressed in elongating coleoptiles or young leaves (Slakeski and Fincher, 1992). The 1,3;1,4-β-glucanase *Gns1* gene of rice had only negligible expression in germinating seeds and young shoots (Simmons et al., 1992). Thus, the regulatory properties of these genes suggest that they do not encode the endoglucanase enzymes that are found in the elongating coleoptile.

The endo-1,3-β-glucanase gene family in plants has undergone a great adaptive radiation during evolution to serve the multiple functions described above. Many studies have demonstrated that each β-glucanase gene is distinct in its gene regulation (Slakeski and Fincher, 1992; Cabello et al., 1994; Memelink et al., 1990; Garcia-Garcia et al., 1994) and in the substrate specificity of the isozyme that it encodes (Stintzi et al., 1993; Hrmova et al., 1995; Wong and Maclachlan, 1979). This indicates that each glucanase gene plays a distinct physiological role

within the plant. Only by cloning and characterizing the complete β-glucanase gene family within the plant species under study can we fully understand the functions served by this gene family. We previously reported the cloning and analysis of four β-glucanase genes in rice (Simmons et al., 1992; Romero et al., 1997). Here we characterize five additional rice β-glucanase genes.

2. MATERIALS AND METHODS

2.1 Genomic library screening

A genomic library constructed in lEMBL3-Sau3AI from rice (cultivar M202) was screened using a 513-bp NarI fragment of EI (Litts et al., 1990; Slakeshi et al., 1990) and a 2.5 kb EcoRI-SalI fragment of lHv34 (Rodriguez et al., 1997) which contains a barley β-glucanase gene of Subfamily 3 (Table 2). Sixty positive clones were selected from six genome equivalents of phage clones (Romero et al., 1997; Simmons et al., 1992).

The genomic clones were classified by restriction analysis of PCR products. Oligo primers for polymerase chain reaction (PCR) were synthesized based on conserved domains in three barley genes and one rice β-glucanase gene (Romero et al., 1997). Foward primer, #528 (5' AATTCGGCGTGTGCTACGGCATGA 3') anneals at position 13 to 33 in Gins1 exon 2 (Simmons et al., 1992). Reverse primer, #529 (3'TACAAGTTGCTCTTGGTCTTCTTAA 5'), anneals at position 838 to 858 in Gns1 exon 2. Ten ng DNA template was amplified in a 50 μL reaction as described previously (Foolad et al., 1993). PCR was performed for 30 cycles of two min melting at 96°C, one min annealing at 45°C, and two min extension at 72°C. PCR products were digested with restriction enzymes (HincII, HinfI, MboI, NcoI, or MboI) and separated on a 6% polyacrylamide gel.

2.2 DNA sequence analysis

DNA sequencing was performed using primer-walking strategy and dideoxy chemistry. Template DNA was prepared using Prep8 columns (Qiagen). Automated sequencing was performed on an ABI Automated Sequencer Model 377. Wisconsin Package Version 8 (Genetics Computer Group (GCG), Madison, Wisconsin) and MacVector software (International Biotechnologies, Inc.) were used for analysis of DNA sequence data.

2.3 Southern and northern hybridization

Southern and dots blots were prepared with nylon membrane (Hybond-N+; Amersham) according to the manufacturer's instruction. Northern blots were made on nylon membrane using the Northern Max kit (Ambion). Total RNA was isolated from 1 g tissue as described (Huang et al., 1990), or from 0.1 g of tissue using an RNeasy kit (Qiagen). RNA quality was tested by electrophoresis on a 1%

agarose gel followed by ethidium bromide staining to visualize the integrity of the 28S and 18S rRNA bands.

Gene-specific probes were made from fragments at or near the 3' ends of the genes either by PCR amplification or by isolation of restriction fragments (Fig. 1). DNA fragments were ^{32}P-labeled to at least 108 cpm specific activity using a Rediprime kit (Amersham), purified through a P10 gel column (BioRad) and used at 106 cpm/mL in prehybridization/hybridization solution (Ambion). Oligonucleotides probes were ^{32}P-endlabeled by T7 polynucleotide kinase. Prehybridization was performed for 30 min, and hybridization overnight, both at 42°C. The blots were washed twice in 2% SDS, 0.1% SSC for 15 min and twice in 0.1% SDS, 0.1% SSC for 1 h, and exposed to x-ray film (X-Omat; Kodak) at -70°C for 1-4 days. The autoradiographs were scanned on Scantastic (Epson) into Adobe Photoshop 3.5 and densitometry was carried out using MacBas 2.0 (Fuji).

Production of root and shoot material, application of treatment to shoot, and harvesting of rice tissues for northern hybridization analysis were as described (Simmons et al., 1992; Romero et al., 1997). Two to five cm long coleoptiles were collected from seeds grown 5-cm deep in vermiculite for 3 days in the dark.

Transcript quantification for each gene was carried out by co-hybridizing duplicate RNA dot blots of the high expressing tissues/treatments (e.g., germinated seed, root, +GA or +salicylic acid) with dot blots of dilution series of clone DNA with the gene-specific probe (data not shown). The transcript level was determined by interpolating the RNA hybridization signal from the DNA standard curve, and normalized against the intensity of methylene blue staining to correct for loading variation. The signal ratio on the northern was then factored in to estimate the transcript level in the low expressing tissues/treatments. The data were corrected for differences in fragment length and specific radioactivity among probes, thus allowing comparison of transcript abundance between genes in each tissue or treatment.

3. RESULTS

3.1 Gene isolation and DNA sequence analysis

Sixty putative β-glucanase gene-containing genomic clones were previously classified by PCR using glucanase oligos #528 and #529 (Romero et al., 1997). Twenty-eight of the 60 clones gave a PCR product comigrating on the electrophoretic gel with the 778-bp product expected for glucanase-containing clones. The PCR products were then digested with restriction enzymes. The restriction digest patterns for the PCR products allowed the clones to be separated into seven classes. Gene clones from three of these classes were described previously (Romero et al., 1997). Restriction mapping and Southern hybridization identified two genes, Gns5 and Gns6, linked in tandem (Fig. 1). Gns7, Gns8 and Gns9 were identified in separate clones.

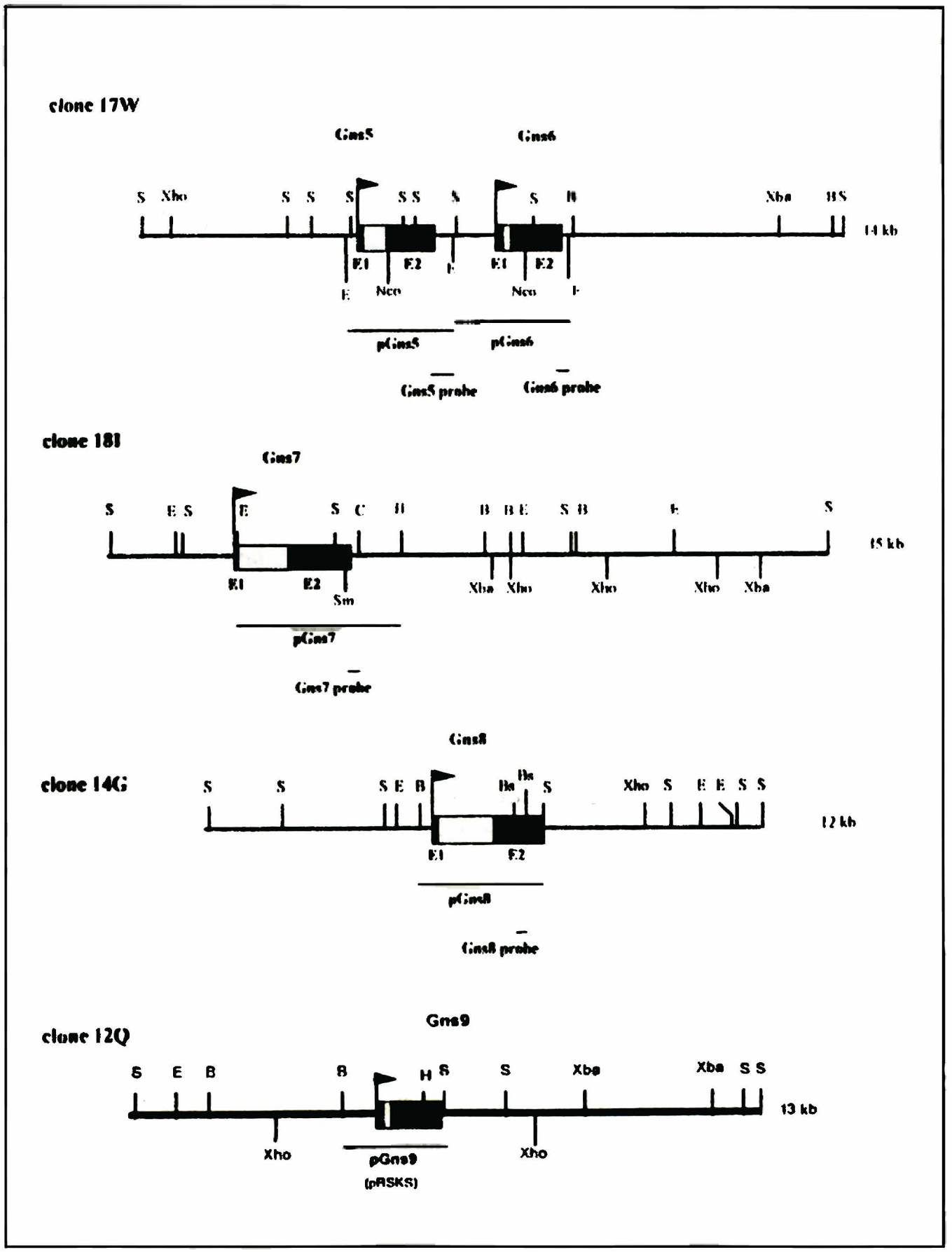

Figure 1. Partial restriction enzyme map of genomic clones showing the physical arrangement and organization of Gns5, Gns6, Gns7, Gns8, and Gns9. Arrow indicates direction of transcription. S-SalI, B-BamHI, Xh-XhoI, Xb-XbaI, E-EcoRI, Sm-SmaI, Bs-BstEII, C-ClaI. Solid boxes are exon 1 (E1) or exon 2 (E2). Open boxes are introns. Lines below are fragments subcloned for sequencing or isolated for use as probes.

The nucleotide sequence data are in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers U72251 (Gns5), U72252 (Gns6), U72253 (Gns7), U72254 (Gns8), and U72255 (Gns9). The DNA sequences obtained for Gns5, Gns6, Gns7, Gns8, and Gns9 revealed the presence of two exons, signal peptide and mature peptide, separated by a single intron in each of genes (Fig. 1). Exon 1 encodes the majority of the 29-37 amino acid signal peptide. The intron position is identical to that of the other cereal endo-1,3,-β-glucanase genes. Exon 2 encodes that last 3 amino acids of the signal peptide and all of the mature peptide (Table 1). The mature peptide has a core structure which ranges from 303-313 amino acids in Gns2-Gns8. Gns9, and certain other β-glucanases have N-terminal and/or C-terminal extension added onto that basic structure. Gns9 isozyme includes a C-terminal extension 25 aa longer than the other rice glucanases (Table 1). The isozymes encoded by Gns5- Gns8 are predicted to possess acidic pKIs, while Gns9 has a basic pKI. The endo-β-glucanases are a diverse gene family, having 60-90% sequence similarity to each other and considerable variation in diagnostic amino acids (Table 2).

3.2 Size of the gene family

Genomic Southern hybridization was done to match the Gns5- Gns9 clones with the corresponding fragments of genomic DNA (Fig. 2). Blots containing EcoRI/BamHI fragments of the Gns1 to Gns9 gene clones were hybridized with the glucanase oligo 716 probe. Genomic bands corresponding to the Gns1-Gns4 clones were identified previously (Romero et al., 1997). These nine clones correspond to five bands in the genomic DNA hybridization, leaving three of the

Table 1. Structural properties of rice β-glucanases

	Size of Mature Peptide (aa)	MW (kDa)	pKI	Size of Signal Peptide (aa)	Size of C terminal extension (aa)
Gns 1	306	31.9	6.2	28	none
Gns 2	308	32.6	4.6	37	none
Gns 3	306	32.2	4.9	29	none
Gns 4	303	32.8	9.6	29	none
Gns 5	304	31.6	4.2	27	none
Gns 6	307	32.3	4.6	25	none
Gns 7	311	32.5	5.3	25	none
Gns 8	313	32.5	4.8	26	none
Gns 9	322a	34.5	9.9	26	25

[a]Length without 25 aa-long C-terminal extension.

Table 2. Protein sequence similarity between Gns5, Gns6, Gns7, Gns8, and Gns9 and other cereal β-glucanase genes, and amino acids at activity-specific positions.

	Percent Sequence Similarity[a]					Residues at Diagnostic Positions[b]			
	Gns5	Gns6	Gns7	Gns8	Gns9	34	130	136	209
β1,3-glucanase									
Barley GI	74.01	77.12	69.97	68.32	64.72	F	F	T	M
Barley GII	76.64	79.02	71.95	69.74	66.01	F	F	S	M
Barley GIII	70.20	73.84	67.00	66.67	61.31	E	F	T	M
Barley GIV	73.03	74.10	71.15	70.39	63.16	S	F	S	M
Barley GV	73.51	74.10	69.51	68.42	63.31	N	F	S	M
Barley GVII	76.90	80.33	70.39	69.08	63.64	A	F	T	M
Maize Glu	82.51	73.93	67.55	68.54	62.95	F	Q	G	L
Rice Gns2	73.00	70.20	69.23	67.00	60.85	S	M	S	M
Rice Gns3	73.27	74.75	66.89	66.67	62.29	L	L	T	M
Rice Gns4	74.00	78.33	69.33	68.33	66.34	Y	F	S	M
Rice Gns5	100.00	77.96	70.76	69.54	63.49	F	Q	G	I
Rice Gns6	77.96	100.00	70.30	69.97	64.71	F	Q	S	M
β1, 3; 1,4-glucanase									
Rice Gnsl	73.60	70.30	67.43	68.20	63.07	A	Q	Y	T
Barley EI	74.26	70.30	67.10	70.49	65.36	A	Q	Y	T
Barley EII	74.59	69.97	69.08	71.15	65.36	A	Q	F	T
Oat G11	75.58	70.96	68.42	71.15	65.36	A	Q	Y	T
Wheat Glub	36.68	32.07	44.96	42.04	65.36	A	Q	Y	T
Subgroup 3									
Barley GVI	70.43	69.54	89.97	86.36	64.33	H	T	S	I
Barley Hv34	69.64	68.20	84.24	87.50	62.02	H	T	S	I
Rice Gns7	70.76	70.30	100.00	87.66	64.72	H	T	S	I
Rice Gns8	69.54	69.97	87.66	100.00	63.90	H	T	S	I
Subgroup 4									
Wheat Tagel1	57.00	60.26	61.31	59.09	61.99	D	L	S	Q
Rice Gns9	63.49	64.71	64.72	63.90	100.00	D	L	S	Q

strongly hybridizing genomic Southern bands not represented in the genomic clone collection.

In the EST database (http://www.ncbi.nlm.nih.gov/dbEST/index.html) we have identified cDNA clones representing five more endo-β-glucanase genes of rice. Preliminary analysis of partial DNA sequences suggest that Gns10 is in the 1,3-β-glucanase Subfamily 1, while Gns11-Gns14 are Subfamily r (data not shown). These five genes may correspond to the three unassigned bands on the genomic Southern hybridization (Fig. 2). Further genomic Southern analysis, including use of additional restriction enzymes, may show that the cloning of this gene family in rice is complete or nearly complete. Thus, there are at least fourteen β-glucanase genes in the rice genome.

3.3 Glucanase gene expression analysis

Patterns of glucanase gene expression were studied by northern hybridization analysis. Gene-specific probes isolated from the 3' flanking sequence of Gns5 to Gns7 and Gns9. The Gns8 probe was a 222 bp BstEII fragment isolated from within exon 2 (Fig. 1). Southern hybridization of these probes with the glucanase gene clones demonstrated very little cross-hybridization (Fig. 2). The specificity of each probe was further demonstrated by genomic Southern analysis, showing hybridization of each probe to only a single band (data not shown). Total RNA

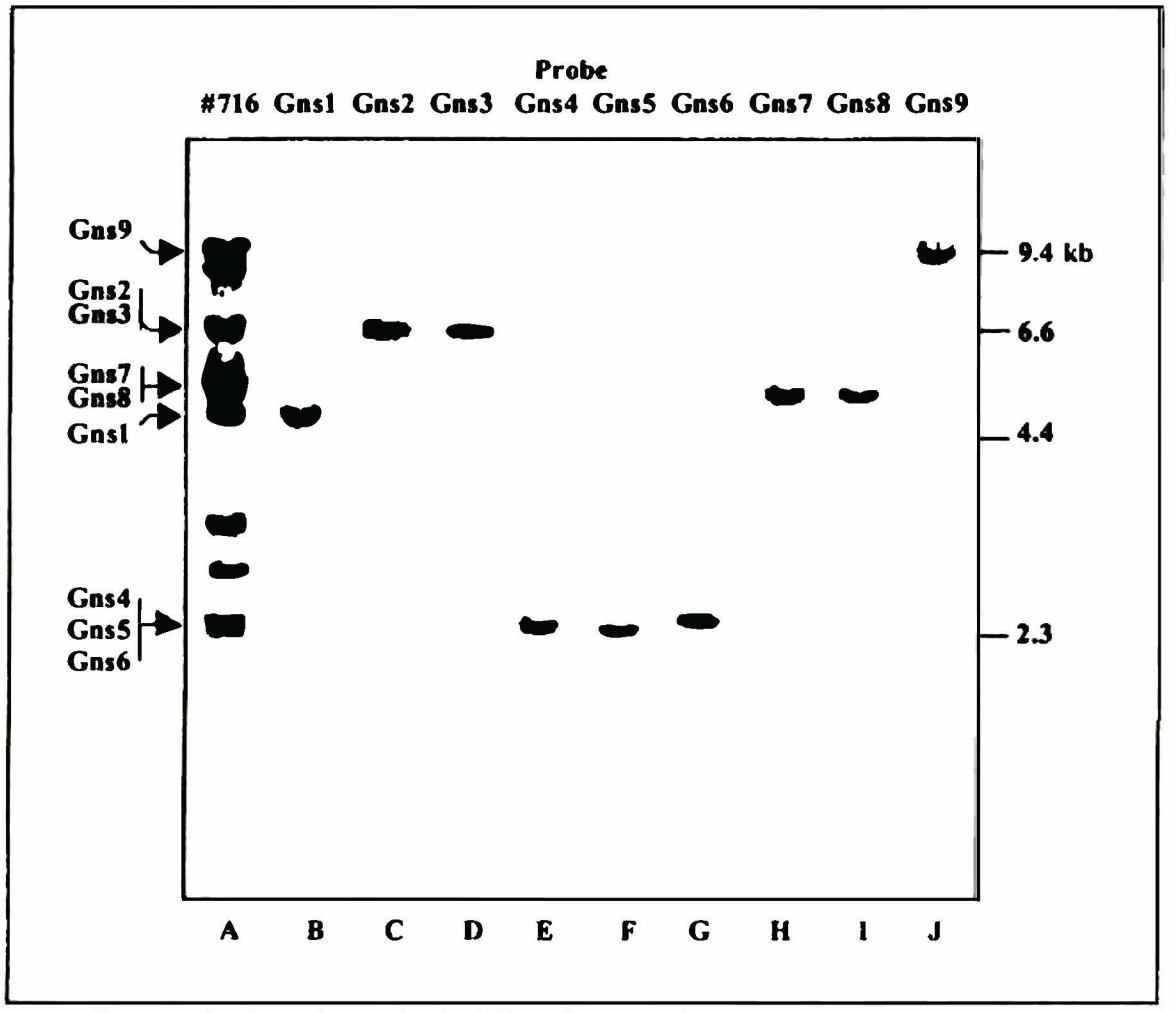

Figure 2. Genomic Southern hybridization probes.

was isolated from various plant organs and young shoots treated with hormones or stress-related treatments. RNA quality was demonstrated by gel electrophoresis and northern hybridization (Romero et al., 1997), while quantitation of absolute levels of RNA from each glucanase gene was done using northern slot blots. Absolute RNA levels were determined relative to a glucanase DNA standard curve, so quantitative comparisons of expression levels between different genes are meaningful.

Each of the genes showed differential expression across tissues and treatment (Table 3). Very little expression was detected for Gns5 and Gns6. The greatest expression of Gns7 was detected in germinating seed and root. Gns8

Table 3. β-glucanase gene expression patterns (pg per 10 μg total RNA).

	Gns1	Gns2	Gns3	Gns4	Gns5	Gns6	Gns7	Gns8	Gns9
Calli	29	0	0	0	0	0	4	2	51
Immature Seed	25	0	0	27	1	0	0	0	38
Germinated Seed	7	1	10	194	2	2	25	6	0
Root	64	0	4	9	2	2	15	15	30
Coleoptile	0	0	0	2	0	0	0	0	52
Young Etiolated Leaf	6	0	2	0	1	1	7	9	35
Mature Leaf	12	0	10	150	20	10	6	80	0
Young Green Leaf (Control)	108	0	0	0	0	1	1	0	0
Gibberellic Acid	32	0	0	74	1	3	5	8	5
Auxin	30	0	0	45	0	3	6	8	5
Ethylene	52	0	0	0	1	4	7	7	6
Abscisic Acid	0	0	0	87	0	0	7	9	6
Fungal Elicitor	117	0	0	0	1	30	4	3	4
Salicylic Acid	140	0	0	93	2	5	6	0	1
Wounding	183	0	0	0	0	6	5	4	3

expression was lower than that of Gns7 in all RNA samples tested, with maximal expression in root. Gns9 was highly expressed in callus, coleoptile, immature seed, etiolated shoot, and root.

4. DISCUSSION

Analysis of protein sequence similarities and diagnostic amino acid residues allow the cereal glucanase genes (Høj et al., 1995; Rodriguez et al., 1997; Lai et al., 1993; Cruz-Ortega et al., 1995; Romero et al., 1997; Simmons et al., 1992) to be classified into four Subfamilies. The 14 β-glucanase genes in rice are similar in number to those in some other plant species. Based on the number of genes cloned and characterized, the β-glucanase gene family consists of at least 10 members in barley (Li et al., 1996; Rodriguez et al., 1997), and at least 14 in tobacco (Meins et al., 1992), but the exact number of glucanase genes in the large genomes of these organisms has never been determined. The size of the gene family in other plant species was estimated through either medium-stringency hybridization or gene reconstruction experiments. The β-glunacase gene copy number is one in bean (Chang et al., 1992), at least ten in potato (Beehues and Kombrink, 1994), at least two in *Nicotiana plumbaginifolia* (De Loose et al., 1988), and at least six in alfalfa (Maher et al., 1993).

4.1 1,3-β-glucanase Subfamily 1

Glucanase Subfamily 1 was defined based on gene dendrogram analysis (Simmons, 1994) and substrate specificity of the encoded isozymes (Hrmova et al., 1993). These isozymes hydrolyze 1,3-β-glucan linkages, but differ widely in their tolerance or requirements for 1,6-β-glucan branch linkages nearby on the polymer chain. Polymers of 1,3-β-glucan are found in both plants and fungi, but polymers of 1,3; 1,6-β-glucan are found only in fungi. Thus, β-glucanases specific for hydrolysis of 1,3; 6-β-glucans are likely to be defense-related isozymes, but isozymes that hydrolyze only 1,3-β-glucans might function in plant development and/or in plant defense. Two rice β-glucanase isozymes from Subfamily 1 have recently been characterized. The rice Gns4 isozyme was purified from rice bran (Akiyama et al., 1996); Romero et al., 1997). This isozyme is specific for hydrolysis of 1,3; 1,6-β-glucans and may therefore be a defense-related isozyme. A second β-glucanase purified from rice bran (Akiyama et al., 1997) matched the deduced protein sequence of Gns5 in 56 of 57 amino acid residues sequenced, with the only possible mismatch coming in a region of ambiguity in the protein sequence. This presumed Gns5 isozyme has much greater activity against linear 1,3-β-glucans that does the Gns4 isozyme, but the substantial activity for hydrolysis of 1,3;6-β-glucans also suggests a defense-related role for the Gns5 isozyme.

Oligosaccharides of chitin (Kaku et al., 1997) or glucan (Mueller et al., 1993), produced via breakdown of the fungal cell wall, elicit expression of 1,3-β-glucanases and other plant defense genes via the octadecanoid pathway. A second

plant defense signalling pathway mediated via salicylic acid can also induce expression of certain defense-related 1,3-β-glucanase genes (Durner et al., 1997). Gns4 was expressed in germinating seedling and was strongly induced in shoots by treatments with salicylic acid (Table 3). We detected little or no expression for Gns2, Gns3, Gns5, and Gns6. The treatment types, treatment intensities, and incubation times were not optimized in this gene expression study, so there is likely to be considered expression of these genes that remains to be detected via more detailed studies.

The genes of Subfamily 1 are highly divergent both in overall sequence similarity (averaging 70-80% similar) and in their diagnostic amino acids (Table 2). Defense-related 1,3-β-glucanases are found in both monocots and dicots, suggesting an ancient subfamily that evolved prior to the divergence of these lineages. All of the genes characterized to date in Subfamily 1 of rice are found in gene clusters that have no obvious homologue in barley (Li et al., 1996), suggesting gene loss or gene duplication events that are unique to each species. In a dendrogram analysis all of the defense-related glucanases of dicots clustered separately from those of monocots (Simmons, 1994), even though it seems most likely that they all evolved from one ancient event. Fungal cell wall glucans differ widely in their branching patterns (Wessels and Seitsma, 1981), perhaps explaining in part why plants need to have multiple isozymes of β-glucanase. Fungi also make proteins that inhibit certain isozymes of β-glucanase (Ham et al., 1997), so the plant glucanases must also be under natural selection for the ability to avoid this inhibition. The fungal cell wall at the interface between host and pathogen is critical for plant strategies of recognition and defense against infection. Thus, rapid devolution of β-glucanase Subfamily 1 may be needed as the plant co-evolves with a wide array of potential fungal pathogens.

Different glucanase isozymes are localized specifically in the vacuole or are secreted into the apoplast. All β-glucanase genes encode a hydrophobic N-terminal signal peptide that directs the nascent polypeptide into the endoplasmic reticulum. Most of these proteins are secreted into the apoplast. A glycosylated C-terminal propeptide extension directs certain dicot β-glucanase isozymes having a basic isoelectric point (pKI) to become localized in the vacuole (Neuhaus et al., 1996; Linthorst, 1991). The propeptide is cleaved during transport so the vacuolar glucanase is similar in size to the secreted glucanases. Cell lysis at the site of an infection may release these vacuolar isozymes into the intercellular space where they can help defend the plant against fungi. The GIV gene of barley appears to encode a vacuolar isozyme, having a basic pKI (Xu et al., 1992) and an N-glycosylation site (Shinshi et al., 1988) within the C-terminal propeptide extension. None of the rice glucanase genes characterized to date in Subfamily 1 have this type of C-terminal peptide extension. Barley and rice express a number of β-glucanase isozymes which have a basic pKI, but lack the glycosylated C-terminal propetide (Xu et al., 1992; Romero et al., 1997); these isozymes are presumably secreted into the apoplast. The biochemical and physiological significance of

the isozyme pKI is not known, but this trait is apparently independent of the vacuolar localization mechanism.

4.2. 1,3; 1,4-β-glucanase Subfamily 2

The 1,3; 1,4-β-glucanases of Subfamily 2 have 80-90% sequence similarities to each other (Simmons, 1994) and diagnostic amino acid residues A, Q, Y, and T. The Gns1 gene has been classified in Subfamily 2 based on homology to the EI and EII genes of barley (Simmons et al., 1992). Genes of Subfamily 2 have high % protein sequence similarity across species (80-90%) (Simmons, 1994) and high conservation in diagnostic amino acid (Table 2). This homogeneity is consistent with the recent evolution of these genes within the grass family lineage and the uniformity of the 1,3; 1,4-β-glucan substrate that their encoded isozymes are responsible for hydrolyzing. The substrate is found only in grass plant cell walls, so secretion of these glucanase isozymes likely has a function in plant growth and development. Distinct regulatory properties show that the genes have diverged somewhat to function in different stages of plant development (Slakeski and Fincher, 1992).

Gns1 of rice is moderately expressed in roots, young shoots, callus, and immature seed, but there is very little expression in seedling caryopsis or coleoptile (Fig. 3). Considering the higher specificity of the hybridization probe used here, these expression results supersede those reported previously (Simmons et al., 1992). It has been widely assumed that this subfamily is responsible for all breakdown of 1,3; 1,4-β-glucan that occurs during cereal seed germination and coleoptile elongation. Its patterns of gene expression suggests that Gns1 does not serve this role. It is possible that another gene from Subfamily 2 remains to be isolated in rice, similar to the two barley genes of Subfamily 2. Even in barley, these genes of Subfamily 2 cannot easily account for the 1,3; 1,4-β-glucanase activity observed in the elongating coleoptile (Slakeski and Fincher, 1992). Alternative, 1,3; 1,4-β-glucan breakdown may be catalyzed in part by isozymes encoded by other glucanase gene Subfamilies.

4.3. β-glucanase Subfamily 3

Glucanase Subfamily 3 is defined by its distinct set of diagnostic amino acids (H, T, S, and I), the high percent similarity (84-90%) of the protein sequences that the genes encode (Table 2) (Simmons, 1994). Similar to Subfamily 2, this suggests relatively recent evolution of this Subfamily 1 and/or hydrolysis of a uniform and slowly- evolving substrate. Clustering of the Subfamily 3 gene GVI of barley with the Subfamily 2 genes EI and EII in a dendrogram analysis (Høj and Fincher, 1995) suggests the possibility of a growth-related role for Subfamily 3. There are no data on the substrate specificity of the encoded isozymes. Genes in this subfamily were not induced in shoots by any defense-related treat-

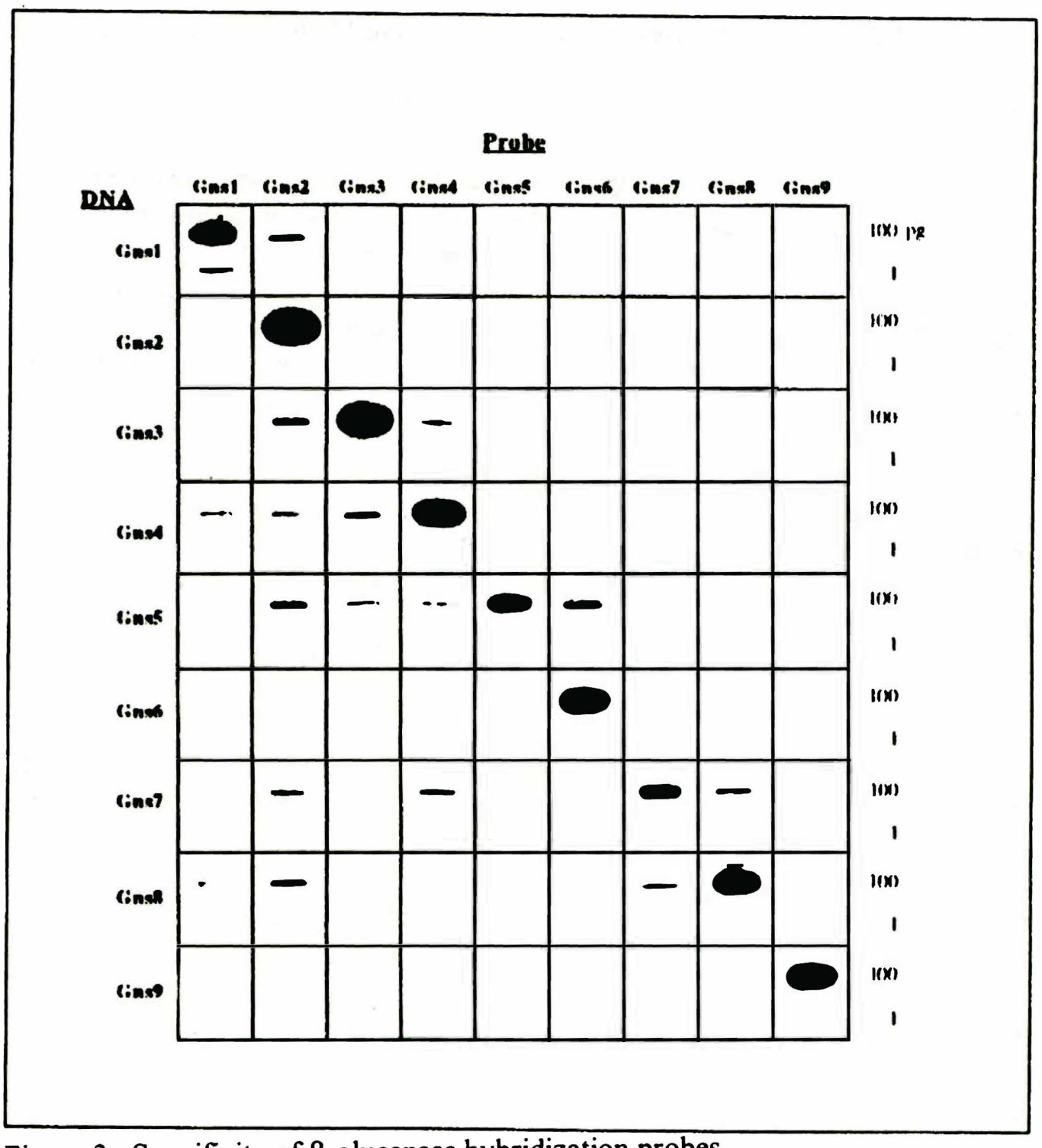

Figure 3. Specificity of β-glucanase hybridization probes.

ment (fungal elicitor, salicylate, wounding) (Table 3). Genes of Subfamily 3 had their highest expression levels in growing tissues such as germinating seedlings, roots, and etiolated shoots, but very little expression of these genes was detected in coleoptile.

4.4. β-glucanase Subfamily 4

Glucanase genes were classified into Subfamily 4 based on their low % sequence similarity to the other Subfamilies, a unique set of diagnostic amino acids (D, L, S, and Q) (Table 2) and their C-terminal peptide extensions. High-level expression of Gns9 in coleoptile, etiolated shoot, callus, developing seed and root, with no induction in response to defense-related treatments (Table 3) suggests a growth-related role for Gns9. Very little is known about the expression of the wheat glucanase gene in Subfamily 4 (Cruz-Ortega et al., 1995). The monocot glucanase genes of Subfamily 4 may be related to the A6 genes of *Brassica* and *Arabidopsis* (Hird et al., 1993). The wheat, *Arabidopsis,* and *Brassica,* glucanases all have C-terminal extensions 115 bases in length. These dicot genes also have diagnostic amino acids (amino acids, D, M, S, M) similar to those of the monocot genes in Subfamily 4 (amino acids D, L, S, Q). There are no data on the substrate specificity of the isozymes encoded by Subfamily 4, but the expression pattern of the A6 gene is consistent with the time of callase enzyme production during microsporogenesis (Hird et al., 1993). Such a fundamental role for glucanase in the reproductive development of the plant is likely to be an ancient trait, predating the divergence of the monocot and dicot lineages. The low % similarity among the genes (62%) (Table 2) is consistent with a long evolutionary history for the subfamily.

Processing and localization of the Subfamily 4 glucanase isozymes are not known. By analogy to Subfamily 1, the C-terminal extension on these isozymes suggest vacuolar localization. Unexpectedly, antiserum raised against A6 anther-specific glucanase from *Arabidopsis* binds to a 60 kDA band on western blots (Hird et al., 1993). This could represent a novel type of β-glucanase in which the C-terminal extension remains as part of an unusually large mature protein. A lack of C-terminal processing could also suggest that the A6 isozyme is secreted rather than localized in the vacuole. On the other hand, the callase endo-glucanase enzyme of the monocot *Lilium* has a molecular weight of 32 kDa (Stieglitz, 1977), typical of β-glucanases from Subfamilies 1 and 2. Perhaps the anther-specific callase in monocots is represented by Gns9 and related genes. The 27 base C-terminal extension of the rice Gns9 isozyme is considerably shorter than that of the A6 isozyme, so the mature protein molecular weight of Gns9 will be close to 32 kDa regardless of whether the C-terminal extension is removed or not. Further work will be needed to define the processing and subcellular localization of Gns9 and other isozymes of Subfamily 4.

The structural and functional complexity with the β-glucanase gene family makes its evolution interesting. It has been proposed that the growth-related genes of Subfamily 2 evolved from defense-related genes of Subfamily 1 (Hϕj and Fincher, 1995). It seems unlikely that defense-related glucanase genes evolved de novo to serve originally in a defense-related role. We propose an alternate model in which growth-related 1,3-β-glucanases were the ancestral members of the gene family. Both the defense-related 1,3-β-glucanase Subfamily 1 and 1,3;1,4-β-glucanase Subfamily 2 may have been recruited from among these ancestral β-glucanases. Evolution of the defense-related Subfamily 1 must have occurred prior to the divergence of the dicots and monocots. Subfamily 2 evolved more recently, after divergence of the grasses from the other monocots. Growth-related glucanases in plants most likely coevolve in concert with the structure of the plant cell wall polysaccharides that they hydrolyze. While it is possible for defense-related β-glucanases to have been recruited back into a growth-related role to create the 1,3; 4-β-glucanases of Subfamily 2, it seems simpler to propose that the growth-related β-glucanases of Subfamily 2 evolved from preexisting growth-related β-glucanases.

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ADDENDA*

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